Journal of Innovations in Pharmaceutical and Biological Sciences (JIPBS)
ISSN: 2349-2759
Available online at www.jipbs.com

Research article

Inhibitory effect of ethanolic extract of *Physalis minima* L. (Ciplukan) leaves on acetylcholine muscarinic-3 receptors induced on isolated guinea pig tracheal

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Key words: *Physalis minima* L, Ciplukan, Ethanolic extract, Acetylcholine-muscarinic-3 receptors, in vitro.

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Abstract

Objective: The study was aimed to investigate the inhibitory effect of ethanolic extract of *Physalis minima* L. leaves (EEPML) against acetylcholine (ACh)-induced contraction of the ACh-muscarinic-3 receptor. **Materials and methods**: The study of the inhibitory effect of the ethanolic extract of *physalis minima* L. on the contraction by ACh concentration series ($1x10^{-8}$-$3x10^{-3}$ M) was conducted in vitro using isolated guinea pig tracheal organ in the Krebs solution. **Results:** EEPML (0.5 - 4 mg/ml) concentration has relaxation effect on the trachea smooth muscle contraction induced by acetylcholine 1.43 x 10^{-4} M ($r=0.982$; $p<0.05$). EEPML has concentration of 4mg/ml, that is not different with atropine sulfate 1 x 10^{-6} M in reduced the contraction of guinea pig's trachea smooth muscle induced by acetylcholine 1.43 x 10^{-4} M ($p>0.05$). EEPML at 0.5 - 4 mg/ml concentration has relaxation effect, EEPML 3.2 M has no difference in terms of ability as atropine sulfate 1 x 10^{-6} M on the trachea smooth muscle contraction induced by ACh 1.43 x 10^{-4} M. It showed that the possibility of the mechanism of the relaxation effect of EEDC mediated through inhibition muscarinic receptors **Conclusion:** The EEPML has relaxation effect on the trachea smooth muscle contraction induced by ACh And showed antagonist effect on ACh-muscarinic-3 receptor.

Introduction

Physalis minima L. in Indonesia known as ciplukan is one of the plants of the family Solanaceae. Rural communities use the leaves, fruit and root as diuretics, immunomodulators, antidiabetes, acute toxicity, anticancer, antimicrobial, antiasthma, antimalarial, analgesic, antirheumatic, and antiinflammation [1]. Previous research suggests that these plants contain alkaloids [2-3], glycosides, flavonoids, saponins, tannins and steroids /triterpenoids [2]. *Physalis minima* L. leaves contains atropin and scopolamin which possess anticholinergic effect. Cholinergic agents has constriction effect to the smooth muscle of respiratory tract. In asthma bronchiale there is on airway constriction.of contains has been studied have a pharmacological activity as Antioxidant [4], Antibacterial [2], Anticancer [5], Antidiabetes [6], immunoglobulin activity M [7], Antiinflammation [8].

Epidemiology study showed almost 20% people in the world suffer from diseases associated with allergy and asthma [9]. Almost 300 million people suffer from asthma, especially asthma which related to allergy. The main risk factors of asthma in developed and poor countries is caused by allergies and respiratory tract irritation induced by allergens and particles in the air [10]. The respiratory diseases such as asthma, chronic bronchitis, and emphysema were the fourth reason of death in Indonesia [11].

Important nervous system associated with human lung is the cholinergic nervous system. Cholinergic or parasympathetic nervous system was largely expressed in the proximal airways and decreases toward the periphery. Acetylcholine (Ach) released by the parasympathetic system plays an important role in controlling the airways and mucus release from submucosal glands, as well as goblet cells in the airway epithelium [12]. In human airway muscarinic receptors over expressed in M_1, M_2, and M_3 types. Excessive activation on the cholinergic nerve can cause of respiratory tract disorders [14].

Indonesia has great biodiversity that potential for the discovery of new drugs. Therefore, it is possible to find a new alternative treatment for asthma from natural resources. Although C. fel-terrae has been used traditionally by the community as a medicine such as for asthma, the scientific data associated with pharmacological activity reports still lack.

Based on this reason, researchers are interested in evaluating the ethanolic extract of *Physalis minima* L. leaves (EEPML) in the respiratory tract primarily on the contraction induced by ACh on ACh receptors muscarinic-3.

Materials and methods

Materials

Drugs and chemicals used in this study were Ach (Sigma-Aldrich), dimethyl sulfoxide (Sigma-Aldrich, USA), and ethanol 96% (Merck), atropin sulfat (Sigma-Aldrich). Instrument used in this experiment was organ bath PowerLab (ML0146/50, PanLab, ADInstruments, New Zealand).

Preparation of extract

Physalis minima L. (Ciplukan) was collected from Tiga Juhar, Deli Serdang District, Sumatera Utara and identified by Herbarium medanense University of Sumatera Utara Indonesian. The leaves were washed and dried at 30-35°C, then grinded until dried powder was obtained. The dried powder was percolated using ethanol 96% then the obtained percolate was evaporated and freeze-dried.

Tissue preparation

Male guinea pig weighing 300-500 g (3-4 months) were housed in a room with controlled temperature and lighting and allowed free access to chow and water. The animals were sacrificed by cervix dislocation. Trachea was dissected out and the connective tissue was gently removed. Subsequently, the rings were cut with a length of 8-9 rings and both sides cartilage was bound with which connected to the transducer MLT0201 (PanLab, ADInstrument) connected with Power Lab T15- 0676 (PanLab, ADInstrument) [15-17]. The experimental protocol was approved by the Animal Reseach Ethics Committee Universitas Sumatera Utara (292/KEPH-FMIPA/2017).

Experiment of series contractions ACh concentrations in smooth muscle trachea

ACh testing was performed to measure the maximum extent of guinea-pig tracheal contraction to obtain EC_{80}. Guinea-pig tracheal in the Krebs solution was contracted gradually with series concentration of ACh ($1x\ 10^{-8} – 3\ x\ 10^{-3}$ M) to the tissue bath as a control concentration-response curve until maximum contraction was achieved [18-19].

Experiment of relaxation effects of EEPML on tracheal smooth muscle contracted by ACh

EEPML relaxation tests were performed as follows; guinea-pig tracheal is conditioned with Krebs solution in tissue bath connected to the transducer. Trachea was contracted with EC80 ACh in a submaximum concentration. After obtaining a stable contraction then given cumulative EEPML concentration (0.5 – 4 mg/ml) [18-19].

Experiment of relaxation effects of Atropine sulfate on tracheal smooth muscle contracted by ACh

Atropine sulfate relaxation tests were performed as follows; guinea-pig tracheal is conditioned with Krebs solution in tissue bath connected to the transducer. Trachea was contracted with EC_{80} ACh in a maximum submaxion of contractions. After obtaining a stable contraction then given cumulative atropine sulfate concentration ($1x\ 10^{-8} – 3\ x\ 10^{-5}$ M) [18-20].

Inhibitory effect of EEPML to the contraction induced by agonist ACh-muscarinic-3 receptor

After equilibration, guinea-pig tracheal was contracted gradually with series concentration of ACh ($1x10^{-8}$-$3x10^{-3}$ M) to the tissue bath as a control concentration-response curve until maximum contraction was achieved. Subsequently, ACh was washed out of the bath and complete relaxation of tracheal allowed. The ability of EEPML to challenge Ach induced tracheal contraction was tested using cumulative addition of ACh after 20 minutes preincubation of the tracheal with extract (3.2 M). Then compared with The ability of atropine sulfate to challenge Ach induced tracheal contraction was tested using cumulative addition of ACh after 20 minutes preincubation of the tracheal with atropine sulfate ($1\ x\ 10^{-6}$ M) or with control. All the experiment conducted using Krebs buffer with gas flowing O_2 : CO_2 (95%: 5%) [18-20].

Statistical analysis

Analysis of all results was performed using SPSS 22 Independent sample T Test. P values for significance were set at 0.05. All data are presented as mean ± standard error of the mean.

Results and discussion

Experiment of smooth muscle contraction of guinea-pig tracheal were isolated by addition of ACh concentrations from $1\ x\ 10^{-8}$ to $3\ x\ 10^{-3}$ M to obtain EC_{80} used to test the relaxation effect of EEPML. The percentage of maximal contraction of smooth muscle of guinea-pig tracheal was obtained at ACh concentration $1\ x\ 10^{-3}$ M, and submaximal contraction (EC_{80}) at concentration $1.43\ x\ 10^{-4}$ M showed in Figure 1.

ACh administrations have increased tracheal smooth muscle contraction through ACh-M_3 receptor stimulation. ACh-M_3 receptors expressed in various types of cells and this receptor cellular signal is mediated by ACh. These receptors play an important role in controlling the physiological response of the central and peripheral nerve activity.

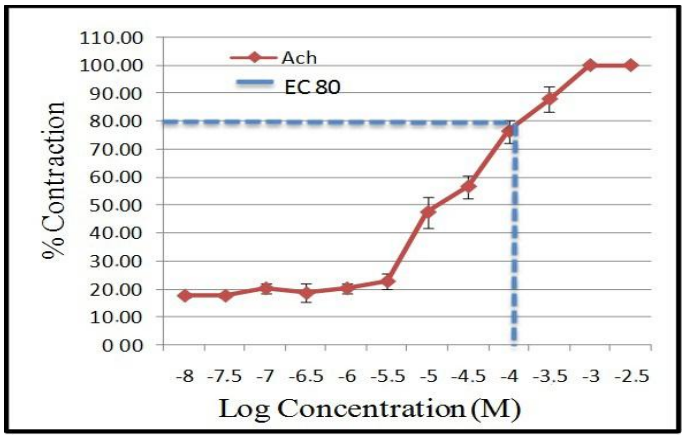

Figure 1. Percentage of smooth muscle contraction of isolated guinea-pig tracheal contracted with ACh concentration series.

ACh-M_3 receptors stimulation could activate phospholipase C enzyme, and enhance the formation of inositol 1,4,5-triphosphate (IP_3) and diacylgycerol (DAG). Both of these compounds are the second messenger that plays an important role in increasing Ca^{2+} intracellular concentration. IP_3 play a role in the increased [Ca^{2+}] through IP_3 receptor activation on sarcoplasmic reticulum so that stimulating the release of Ca^{2+} deposits to the cytosol. DAG activate influx of calcium via opening calcium channels in cell membranes. Increasing levels [Ca^{2+}] modulates calcium bond with calmodulin which will activating myiosin light chain kinase (MLCK). MLCK activation results are cross-linking between actin and myosin. The bond formation between myosin and actin and this may lead to contraction of the smooth muscle [21-26]. Stimulation of ACh-M3 receptor on the smooth muscle of the airways can cause airway disorders such as bronchoconstriction and increased mucus production from the submucosal glands [27-29]. It is known that human muscarinic receptor is predominantly expressed in smooth muscle cells, epithelial cells, and fibroblasts [30]. Inhibition of ACh-M reseptor induced contraction by extract indicated that the chemical compounds contained in the extracts possess work at these receptors. Several compounds have been reported posses relaxation effect on smooth muscle muscarinic mediated-receptor such as alkaloids. *Physalis minima* L. leaves contains alkaloid atropin and scopolamin which possess anti cholinergic. Atropine causes the occurrence of resistance to cholinergic receptors by occupation M_3 receptors will occur resistance of M_3 agonists with their receptors.

EEPML relaxation tests on isolated trachea smooth muscle were performed by contracting of smooth muscle of guinea pig's trachea isolated with ACh 1.43 x 10^{-4} M, followed by concentration series of EEPML 0.5 - 4 mg/ml. Relaxation effects were observed by increased percentage of relaxation on tracheal showed in Figure 2.

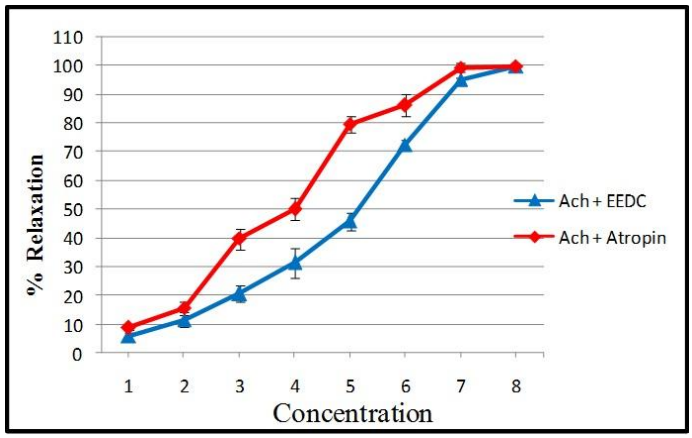

Figure 2: Effect of EEPML (1 = 0.5; 2 = 1; 3 = 1.5; 4 = 2; 5 = 2.5; 6 = 3; 7 = 3.5; 8 = 4 mg/ml) and atropine sulfate (1 = 1 x 10^{-8}; 2 = 3 x 10^{-8}; 3 = 1 x 10^{-7}; 4 = 3 x 10^{-7}; 5 = 1 x 10^{-6}; 6 = 3 x 10^{-6}; 7 = 1 x 10^{-5}; 8 = 3 x 10^{-5} mg/ml) on ACh-induced contractile response in isolated guinea-pig tracheal. Data presented as mean ± standard error of mean from n=4, *p<0.05

Testing of action mechanisms by inhibition of muscarinic receptors was done by comparing the strength of ACh contraction (1.43 × 10^{-4} M) which was incubated with EEPML at concentrations of 3.2 M with ACh contraction strength (1.43 × 10^{-4} M) without incubation and the strength of ACh-induced contraction incubated with atropine sulfate (1 x 10^{-6} M) showed in Figure 3.

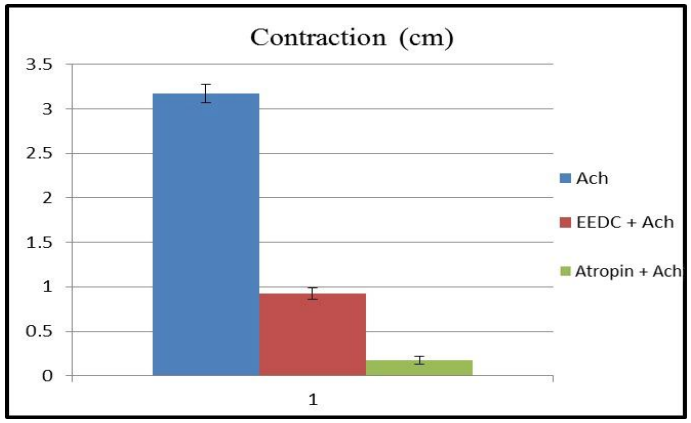

Figure 3: The contraction effect (cm) of ACh 1.43 x 10^{-4} M, incubation EEPML concentration 3.2 M and atropine sulfate 1 x 10^{-6} M after contracted with acetylcholine 1.43 x 10^{-4} (mean ± SEM, n = 4).

The contraction effect of acetylcholine incubated with EEPML compared with acetylcholine without incubation showed statistically significant different results ($p < 0.05$). The statistical comparison of contraction of acetylcholine (1.43 x 10^{-4} M) incubated EEPML concentration of 3.2 M with acetylcholine (1.43 x 10^{-4} M) incubated atropine sulphate (1 x 10^{-6} M) also showed results which differ significantly ($p < 0.05$). EEPML and atropine sulphate both have a relaxant effect on acetylcholine-induced contractions, but the strength of contractions incubated

with atropine sulfate is less, since atropine sulphate is used as a pure compound, whereas in EEPML it is not a pure compound but a chemical compound of plants in it there are still some other plant chemical compounds.

Based on statistical data it can be indicated that EEPML relaxes the smooth muscle of tracheal marmots by inhibiting muscarinic receptors, this can be proven by looking at statistically statistically non-incubated acetylcholine and incubated EEPML acetylcholine which show significantly different contraction results between the two ($p < 0.05$). EEPML inhibits contraction increases by acetylcholine which has been described above that acetylcholine acts to stimulate muscarinic receptors that increase smooth muscle contraction. The increase in contraction by acetylcholine decreases because the muscarinic receptor is largely inhibited by EEPML so that muscarinic receptors do not all interact with acetylcholine to produce contractions. Inhibition of acetylcholine release results in reduced muscarinic receptor activity that mediates smooth muscle contraction [31]. Ciplukan leaf extract contains alkaloids of atropine and scopolamine. It is known that atropine as anticholinergic may relax the smooth airway muscle which is one of therapy in asthma [32].

Acetylcholine is a neurotrasmitter acting as an agonist on M_3 receptors; activation of M_3 receptor by acetylcholine will induce contraction resulting in bronchocontriction. Atropine sulphate is a muscarinic antagonist whose action selectively inhibits parasympathetic nerve activity, so it is called parasympatolytic. The effect of a muscarinic antagonist drug is opposite to the muscarinic agonist effect. The muscarinic antagonist effect on the respiratory tract is bronchodilation.

Atropine blocks the action of cholinomimetics on reversible muscarinic receptors (depending on the number) it indicates competition for fighting places of bondage. The bonding results of muscarinic receptors are to prevent actions such as IP_3 release and adenylyl cyclase inhibition caused by acetylcholine or other muscarinic antagonists [33].

Conclusion

The EEPML has relaxation effect on the trachea smooth muscle contraction induced by ACh And showed antagonist effect on ACh-muscarinic-3 receptor.

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