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Anticonvulsant Activity of Tetanus Leaves (L*eea aequata* L.)Ethanolic Extract on Guinea Pig (*Cavia cobaya*) Isolated Ileum By *IN VITRO* Method

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Abstract : Tetanus plant (*Leea aequata* L.) is a plant of Leaceae family, which used a traditional medicine in Tanah Karo, North Sumatera province as a cure wounds and antitetanus drugs. One of the major signs of tetanus are muscle spasms or seizures in part of or the entire body. This study aimed to determine the effects of anticonvulsants in the form of a relaxation of the ethanol extract of tetanus leaves against contracting guinea pig ileum isolated induced by acetylcholine chloride.

This study was performed *in vitro* using an *organ bath*. Stages of the research are the preparation of materials and testing separate ileum relaxation effect. The parameters measured in this study are relaxation of isolated ileum smooth muscle. Before testing, guinea pig ileum isolated were equilibrated for 45 minutes to obtain a stable condition in Tyrode solution with a temperature 37^{o} C aerated with karbogen gas (O_2:CO_2) 95%:5%.The tests of relaxing effect of guinea pig ileum isolated is after induced by acetylcholine chloride, then each ileum is given cumulative concentration of ethanol extract of tetanus leaves and atropine sulfate. The concentration of acetylcholine chloride that required to increase the contraction of guinea pig ileum isolated is 1.889 x 10^{-4} M. The cumulative concentration of ethanol extract of tetanus leaves that given is 0.5 – 4 mg/ml and cumulative concentration of atropine sulfate given is $6.95 \times 10^{-6} – 2.08 \times 10^{-2}$ mg/ml.

The test results showed that ethanol extract of tetanus leaves has a relaxing effect. Statistical analysis showed that ethanol extract of tetanus leaves at a concentration of 2.5 mg / ml (105.4203± 2.9151) has the ability not dissimilar to atropine sulfate 6.95 x 10^{-3}mg/ml (113.9796 ± 4.5825) in reducing the smooth muscle of ileum contraction induced by acetylcholine chloride 1.889 x 10^{-4} M ($p > 0.005$). In conclusion that ethanol extract of tetanus leaves was relaxing effect on the smooth muscle of ileum isolated with the ability not dissimilar to atropine sulfate.

Keywords : tetanus leaves, ileum isolated, guinea pig, relaxation, in vitro.

Introduction

Drugs from the natural materials or synthetic has many benefits. Medicines from natural materials there are efficacious as antidiabetic [1], antimalarial [2], anticancer [2-4], antibacterial [5,6] and antiaging [7,8]. In developing countries such as Indonesia a lot of factors precipitating cause of the disease, the main cause is the lack of public awareness of the importance of healthy living and economic factors that led to the poor

community can not meet the need to live in place that is clean and decent place to live, as well as the inability people to buy drugs in line with the rise of modern medicine [9].

One of the diseases with high case rate in developing countries is tetanus. Tetanus is one cause potentially fatal characterized by increased stiffness and spasms in skeletal muscle. One manifestation of seizures is ongoing contraction of the muscles in part of or the entire body. Tetanus is caused by changes in the form of bacterial spores of Clostridium tetani. The first muscle stiffness usually involves the jaw (lockjaw) and neck and then into the whole body [10].

Mechanism of action of tetanus is by inhibiting the release of inhibitory transmitter that glycine at the synapse so that the excitatory transmitter will dominate at the synapse, the high excitatory transmitter at synapses is what will increase continuously until the contractions lead to seizures [11]. The balance between inhibitory and excitatory transmitter transmitter on synaptic transmission is essential to maintain the normal function of the nervous system. The imbalance between inhibitory and excitatory transmitter transmitter at synapses will lead to problems in the body. Examples are inhibitory transmitter GABA, glycine, nitric oxide and excitatory transmitter is acetylcholine, histamine, norepinephrine.

In this study, to obtain an increase in muscle contraction is by giving excitatory transmitter is acetylcholine chloride. The method used is the method of isolated organs and organ used is the ileum smooth muscle of guinea pig. Acetylcholine receptor chloride role in smooth muscle contraction is a muscarinic ileum. Muscarinic receptors are known to have five receptor subtypes, namely M1 - M5 although the precise location and functionalities not yet known. Therefore, it is important to know about the different subtypes of muscarinic receptors. In the digestive tract are the fifth intestinal receptor subtype [12].

Increased intestinal motility resulting in intestinal smooth muscle contraction that occurs is the result of stimulation of acetylcholine activates muscarinic receptors (M1 and M3) [13]. reports that data from the study on rodents (rodentia) and dogs showed that the most prominent M3 muscarinic receptor to increase intestinal motility, although the ratio of M2 and M4 receptors is 4: 1 [14, 15].

Indonesia is famous as a country that has a number of medicinal plant diversity. Medicinal plants have been known since long as a medicinal herb [16]. One example of this medicinal plant is the leaves tetanus (Leea aequata L.). These plants are used as traditional medicine for wounds and tetanus in Tanah Karo, North Sumatra Province. Part of the plant used as a tetanus tetanus Karo area is the leaves. Khare (2007) said stems and roots are used as an astringent, antelmentik, indigestion, jaundice, chronic fever and malaria. Leaves and branches are used as an antiseptic and to treat wounds [17].

How to use traditional medicine leaves tetanus in Tanah Karo is to include ± 2 grams of leaves powder into a bottle of alcoholic beverage that is 20% white samsu 250 ml. Its use is to take one teaspoon of extract of leaves and put the mouth of the patient seizure tetanus. When exposed to blunt force injury due to accidents, the leaves extract can also be applied around the injured area.

Based on the results of phytochemical screening, Leea aequata plant secondary metabolites that L contains alkaloids, glycosides, steroid / terpenoids, flavonoids and tannins. The ethanol extract of leaves tetanus has an antibacterial effect against Staphylococcus aureus, Staphylococcus epidermidis and pseudomonas aeruginosa were performed using agar diffusion method using paper pencadang [18]. According Raihan, et al., (2011) that Leea indica that has the same family, namely leaceae memiliiki strong sedative effects in mice [19].

Tetanus leaves pharmacological activity in reducing the contraction of smooth muscles has not been tested scientifically and by the use of tetanus leaves traditionally as an anticonvulsant hereditary as well as previous studies of antibacterial characterization and testing. Researchers are interested to test the anticonvulsant activity of the ethanol extract of leaves of tetanus to separate guinea pig ileum in vitro organ bath use traditional tools.

Materials and Methods

The study was conducted at the Laboratory of Pharmacology and Laboratory of Pharmacognosy Faculty of Pharmacy, University of Sumatera Utara.The method used in this research is the experimental method.

Research tools

The tools used in this study includes devices laboratory glassware, analytical balance (Boeco Germany), animals balance (Presica Geniweigher), a set of preparation organ tools (Germany), vortex (Boeco Germany), stirrer (Dell), four set the volume of 50.0 ml organ bath (ML0146/50, Panlab magnet, BelArt Products), isometric transducer (MLT0201, Panlab, ADInstruments, Spain), computers(ADInstruments, Spain), micro volume pipette (Socorex, Switzerland), heating and magnetic stirrer (Velp Scientifica, Europe), thermostat (ML0146/50, Panlab, ADInstruments, Spain), PowerLab 15T (serial T150676, ADInstruments, Australia), Quad Bridge Amplifier (serial 2240448, ADInstruments, Australia).

ResearchMaterials

The sample used in this study is the leaves tetanus (*Leea aequata* L.), a chemical used is tirode solution (consisting of NaCl, KCl, MgCl2, NaH2PO4, CaCl2, NaHCO3, and D-Glucose) (Merck), containing carbogen gas 95% oxygen and 5% carbon dioxide (Tri Gases, Medan, Indonesia), acetylcholine chloride (Sigma, Switzerland), atropine sulfate (Sigma, USA), dimethyl sulfoxide (DMSO) (Merck) and distilled water.

Animal Experiments

Experimental animals used were male guinea pigs (*Cavia cobaya*), weighing between 300-450 grams, 3-4 months of age with a healthy condition. These animals are acclimatized for a week with the aim to unify the food and life with homogeneous conditions that are considered eligible for the study.

Plant Material Preparation

The samples are tetanus leaves are still fresh green (not too old and not too young), taken from the village of Suka Nalu, District Barus Ginger, Karo District, North Sumatra Province. Sample collection is done purposively without comparing with plants from other regions.

Plant Identification

Identification of plants is done by Bogoriense Herbarium, Department of Botany, Biology Center for Research and Development, Indonesian Institute of Sciences (LIPI), Cibinong, Bogor.

Ethanolic Extract of Tetanus Leaves by Maceration

A total of 500 g tetanus leaves powder was added to the dark glass container dark, pouring 75 parts ethanol 96%, closed, left for 5 days and protected from light and stirring often, squeezed, and added ethanol 96% to obtain 100 parts. Move into the closed vessel, leave it in a cool, protected from light for 2days and filtered. The filtrate was evaporated using a rotary evaporator at a temperature of 40°C to obtain a tetanus leaves ethanolic extract.

Stages Experimental

Tirode Physiological Solution

Buffer solution used is a tirode solution. Materials (NaCl, KCl, $MgCl_2$, NaH_2PO_4, $CaCl_2$) were dissolved separately with distilled water until dissolve.$NaHCO_3$ and D-glucose was added last after all the ingredients mixed. After all the ingredients mixed, the solution is aerated with karbogen (95% O_2, 5% CO_2) in order to prevent precipitation of calcium salts that are marked with turbidity. Furthermore, the solution was adjusted to pH 7.4. Tirode solution can used for 24 hours [20].

Acetylcholine Solution

In this study, cholinergic agonists are acetylcholine chloride is used as an inducer. These compounds can cause contraction of smooth muscle of the ileum. Solution made by dissolving acetylcholine into distilled water to obtain a concentration of 2×10^{-1} M. Then made a dilute solution to 2×10^{-6}M.

Tetanus Leaves EthanolicExtract Solution

An 800 mg of tetanusleaves ethanol extract (TLEE) was dissolved in 1 ml of DMSO (Dimethyl Sulfoxide), and then dilute with a tirode solution up to 5 ml (160 mg / ml) as stock solution. DMSO (Dimethyl Sulfoxide) is a solvent that is inert, non-toxic, and can dissolve almost all compounds and is a solvent that semipolar, but can still be mixed with media tirode [21-23]. Limit the amount of the solvent DMSO were added to the organ bath was 1% v/v 40mL of 400 mL[24].

Atropine Sulfate Solution

In this study, atropine sulfate is used as cholinergic. Antagonist can inhibit smooth muscle contraction in the ileum. Parent solution made by dissolving atropine sulfate into distilled water so that the concentration obtained 138.968 mg/ml. Then made a dilute solution to 0.00139 mg/ml.

Preparation of Guinea Pig Organ

Guinea pig weighed and then sacrificed by spinal dislocation head (cervix). Abdominal surgery, the skin of the abdomen is cut using scissor. Ileum cleaned of protected layers. Mesenteric tissue is relaxed, cut the lower bowel segment is approaching the cecum along 2-3 cm. By using the needle both ends of the intestine tied with string in the reverse direction.Lower intestine tied to the rod holder and thread the upper intestine. Transduceris connected to the small intestine was added to the organ bath containing a trode solution, the solution temperature is maintained 37ºC and continuously aerated with karbogen. Tissue which has been isolated incubated for 30 minutes with broth changes tirode every 10 menit. Dibiarkan while until conditions are optimal rhythmic [25].

Contraction Effects of Acetylcholine

Tests on muscarinic agonists is to measure the maximum size that can be demonstrated on the guinea pig ileum contraction, in order to obtain a concentration submaximum or the Effective Concentration (EC80) of acetylcholine. Measurements carried out in stages with a cumulative provision of acetylcholine in order to obtain the concentration in the organ bath 1×10^{-8}M to 3×10^{-3} M

Concentration Acetylcholine Solution (M)	Volume Added to Organ Bath (µl)	Concentation of Acetylcholine in Organ Bath (M)
2×10^{-6}	200	1×10^{-8}
2×10^{-6}	400	3×10^{-8}
2×10^{-5}	140	1×10^{-7}
2×10^{-5}	400	3×10^{-7}
2×10^{-4}	140	1×10^{-6}
2×10^{-4}	400	3×10^{-6}
2×10^{-3}	140	1×10^{-5}
2×10^{-3}	400	3×10^{-5}
2×10^{-2}	140	1×10^{-4}
2×10^{-2}	400	3×10^{-4}
2×10^{-1}	140	1×10^{-3}
2×10^{-1}	400	3×10^{-3}

Relaxation Effects of Tetanus Leaves EthanolicExtract

Testing the activity of the tetanus leaves ethanolic extract to the increase contractions in rat ileum performed with the addition of ethanol extract of leaves tetanus successive concentration is by giving 1 mg/ml - 4 mg/ml tetanus leaves ethanolic extract in the organ bath. Ileum of guinea pigs has equilibrated for 45 minutes (with tirode broth changes every 15 minutes) given ethanol extract of leaves of tetanus in the organ bath.

Concentration of Tetanus Leaves Ethanolic Extract Solution(mg/ml)	Volume Added to Organ Bath (µl)	Concentration of Tetanus Leaves Ethanolic Extract in Organ Bath (mg/ml)
160	125	0.5
160	125	1.0
160	125	1.5
160	125	2.0
160	125	2.5
160	125	3.0
160	125	3.5
160	125	4.0

Relaxation Effects of Atropine Sulfate

Ileum contracted by administering 38 mL solution of acetylcholine chloride 2×10^{-1} M thus obtained the maximum concentration of acetylcholine chloride sub $1{,}889\times10^{-4}$ M in the organ bath. Having obtained the maximum contraction stable condition later be giving atropine sulfate concentration.

Concentration of Atropine Sulfate Solution(mg/ml)	Volume Added to Organ Bath (µl)	Concentation of Atropine Sulfate in Organ Bath (mg/ml)
0.0013896	200	6.95×10^{-6}
0.0013896	400	2.08×10^{-5}
0.013896	140	6.95×10^{-5}
0.013896	400	2.08×10^{-4}
0.13896	140	6.95×10^{-4}
0.13896	400	2.08×10^{-3}
1.3896	140	6.95×10^{-3}
1.3896	400	2.08×10^{-2}

Data Analysis

The data obtained in this study is data ileum smooth muscle contraction on the computer (the computer program LabChart® 702). Data obtained as a percentage (%) in response to the maximum response is achieved. Furthermore, the relationship between the concentration against% response.EC80 value (the agonist concentration to produce a response of 80% of the maximum response) receptor agonist, is calculated based on the graph of concentration against% response. Furthermore, the data presented in tabular form and the average value ± SEM (Standard Error Mean) [24]. Data% contraction were statistically analyzed by using test Independent-Samples T Test. Before the first test conducted Kolmogorov-Smirnov test for normality.

Results and Discussions

Characterization test results of tetanus leaves simplicia powder can be seen in Table 1.

Table 1. Water Content, Content Of Water Soluble Extract, Sea Levels Ethanol Extract, Total Ash and Acid Insoluble Ash Content Tetanus Leaves Simplicia Powder

No.	Parameter	Percentage (%)
1.	Water Content	4.00
2.	Water Soluble Extract Content	8.11
3.	Ethanol Soluble Extract Content	9.61
4.	Total Ash	7.58
5.	Acid Insoluble Ash Content	0.65

[18]

Terms levels of soluble extract in water, the levels of soluble extract in ethanol, total ash content and ash content of acid insoluble in general to each of the bulbs are not the same. In this examination, characterization simplicia not listed in Materia Medika Indonesia (MMI). The results of the assay of water from leaves simplicia tetanus which is 4% which indicates that the water content simplicia meet the requirements shall not exceed 10% [26].

Levels of water soluble extract of leaves tetanus simplicia 8.11% and the content of ethanol soluble extract of leaves tetanus simplicia 9.61%. Determination of ash content in leaves simplicia tetanus showed total ash content of 7.58% and ash content of acid insoluble at 0.65%.

Extraction Results

500 g powder extraction simplicia by maceration using ethanol 96%, aiming to extract the compound contained in the leaves simplicia tetanus, both polar and non-polar, acquired tetanus leaves ethanol extract as much as 67 g.

Identification of Plants Results

The plant is identified determined by Bogoriense Herbarium, Department of Botany Research Center for Biology LIPI Bogor is tetanus by the name of species *Leea aequata*L.

Contraction of Acetylcholine Results

Contraction induced by acetylcholine can be observed through the observation of changes in% response of isolated ileum smooth muscle contraction to increase the concentration of acetylcholine series (1×10^{-8} Mto 3×10^{-3} M) in the ileum organ. The percentage of maximum contraction of smooth muscle of ileum was obtained at a concentration of 3×10^{-3} M acetylcholine and submaximal concentrations of acetylcholine at a concentration of 1.889×10^{-4} M terraced with acetylcholine performed to obtain submaximal concentration or the Effective Concentration (EC80) acetylcholine, which would then be used to assess the effect relaxation ethanol extract of leaves of tetanus. Results can be seen in Figure 1.

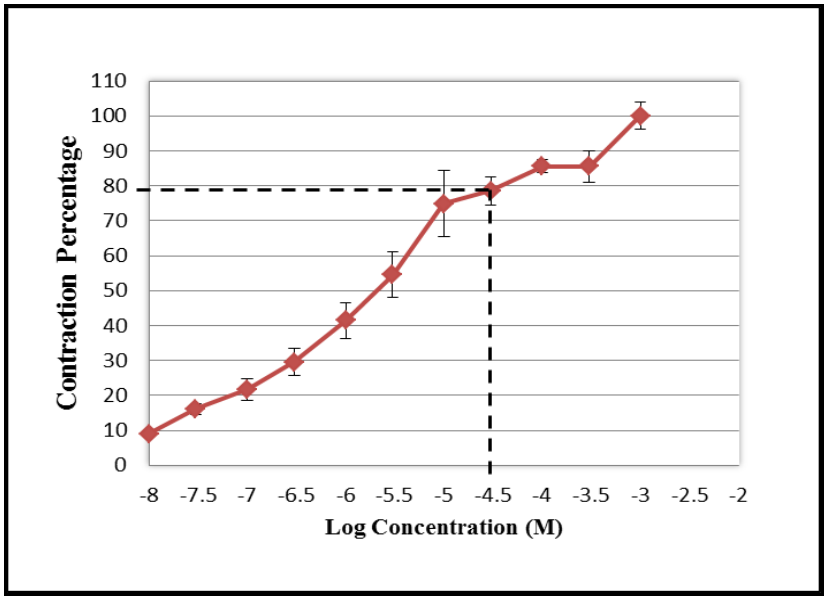

Figure 1. Graph % concentration of isolated ileum smooth muscle organ that contracted with the provision of a concentration series of acetylcholine (-8,0=10^{-8}; -7,5=3x10^{-8}; -7,0=10^{-7}; -6,5=3x10^{-7}; -6,0=10^{-6}; -5,5=3x10^{-6}; -5,0=10^{-5}; -4,5=3x10^{-5}; -4,0=10^{-4}; -3,5=3x10^{-4}; -3,0=10^{-3}; -2,5=3x10^{-3} M). Data is presented with average value ± SEM, n=3.

Relaxation Effects of Tetanus Leaves Ethanolic Extract (TLEE)

Testing the relaxing effect of tetanus leaves ethanolic extract on isolated ileum smooth muscle is done by contracting the smooth muscle of the ileumwith acetylcholine 1,889 ×10^{-4} M , followed by administration of the extract concentration series of 0.5 to 4 mg / ml . Extract relaxation effects are observed through the observation of changes in % a relaxing effect on the organ extract ileum. Giving a series of concentration of tetanus leaves ethanolic extract produces a relaxing effect on the contraction induced by acetylcholine 1,889 ×10^{-4} M

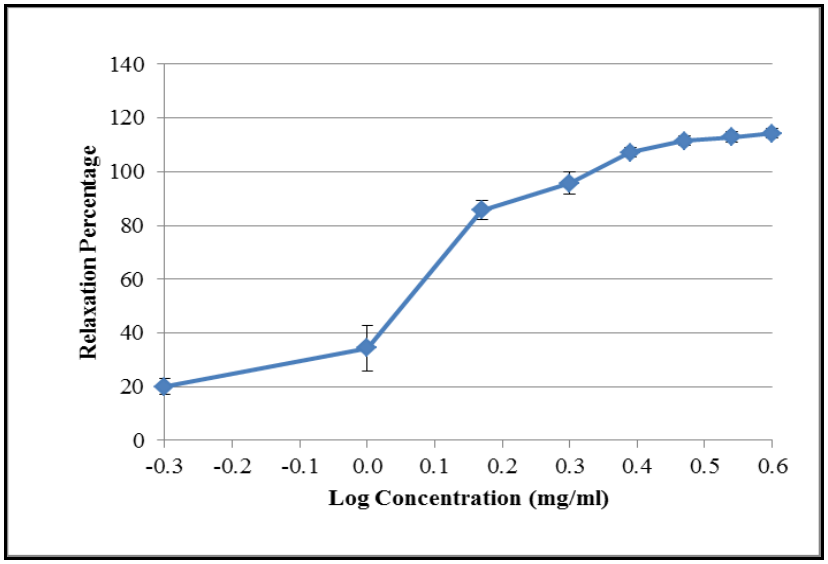

Figure 2. Graph % relaxation after administration of tetanus leaves ethanolic extract concentration series (-3 = 0.5 ; 0 = 1.0 ; 0:17 = 1.5 ; 0.3 = 2.0 ; 0:39 = 2.5 ; 0:47 = 3.0 ; 0:54 = 3.5 ; 0.6 = 4.0 mg / ml) on isolated ileum smooth muscle that is contracted with acetylcholine 1,889x10-4 M. the data presented is the average value ± SEM , n = 6 .

The correlation to the percentage of the relaxation effect at a concentration of tetanus leaves ethanolic extract is a positive correlation with 0.891 correlation value (the correlation is close to 1) and the value of R Square (R^2) of 0.794. It can be stated that as many as 79.4% increase in the percentage of relaxation effect is influenced by the increased concentration of the extract. Under these conditions, the percentage of extract relaxing effect on smooth muscle of ileum increased with increase in concentration.

Relaxation Effects of Atropine Sulfate

Testing the effect of atropine sulfate to relaxation of smooth muscle isolated ileum is done by contracting the smooth muscle of the ileum to acetylcholine $1{,}889\times10^{-4}$ M, followed by administration of atropine sulfate concentration series $6{,}95\times10^{-6}$mg/ml to $2{,}08\times10^{-2}$ mg/ml. Giving series atropine sulfate concentration produces a relaxing effect on the contraction induced by acetylcholine $1{,}889\times10^{-4}$ M.

The relaxant effect of atropine sulfate is observed through the observation of changes in% relaxing effect on the administration of atropine sulfate concentration series $6{,}95\text{x}10^{-6}$mg/ml to $2{,}08\times10^{-2}$ mg/ml in ileum organ. Figure 3 showed administration of atropine sulfate concentration series produces a relaxing effect on the contraction induced by acetylcholine 1.889×10^{-4} M. The percentage of atropine sulfate relaxing effect on smooth muscle of ileum increased with increase in concentration.

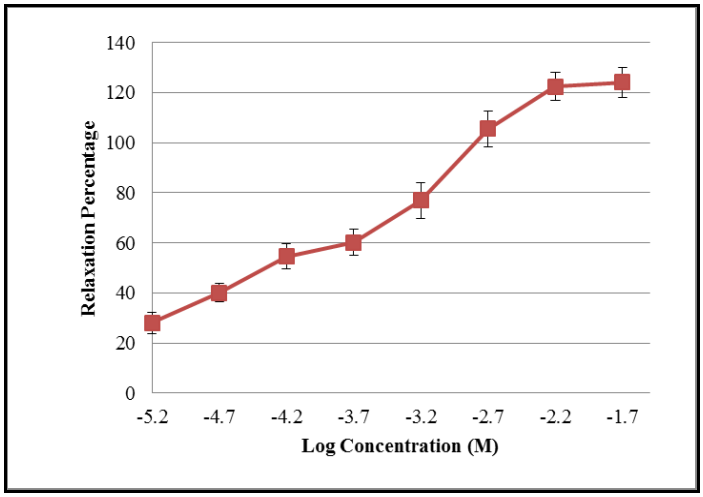

Figure 3. Graph % relaxation after administration of atropine sulfate concentration series ($-5.2 = 6.95\text{x}10^{-6}$; $-4.7 = 2.08\text{x}10^{-5}$; $-4.2 = 6.95\text{x}10^{-5}$; $-3.7 = 2.08\text{x}10^{-4}$; $-3.2 = 6.95\text{x}10^{-4}$; $-2.7 = 2.08\text{x}10^{-3}$; $-2.2 = 6.95\text{x}10^{-3}$; $-1.7 = 2.08\text{x}10^{-2}$ mg / ml) on isolated ileum smooth muscle that is contracted with acetylcholine 1,889x10-4 M. the data presented is the value mean ± SEM , n = 6 .

Comparison % Relaxation of Atropine Sulfate and Tetanus Leaves Ethanolic Extract (TLEE)

Testing the effect of atropine sulfate to relaxation of smooth muscle isolated ileum is done by contracting the smooth muscle of the ileum to acetylcholine 1.889×10^{-4} M, followed by administration of atropine sulfate concentration series $6{,}95\times10^{-6}$ - $2{,}08\times10^{-2}$ mg / ml.

The relaxant effect of atropine sulfate is observed through the observation of changes in % a relaxing effect on the organ extract ileum. Giving series of atropine sulfate concentration produces a relaxing effect on the contraction induced by acetylcholine 1.889×10^{-4} M. The percentage of atropine sulfate relaxing effect on smooth muscle of ileum increased with increase in concentration. TLEE also has a relaxing effect similar pattern with atropine sulfate. Charts comparison of % relaxation of tetanus leaves ethanolic extract and atropine sulfate can be seen in Figure 4.

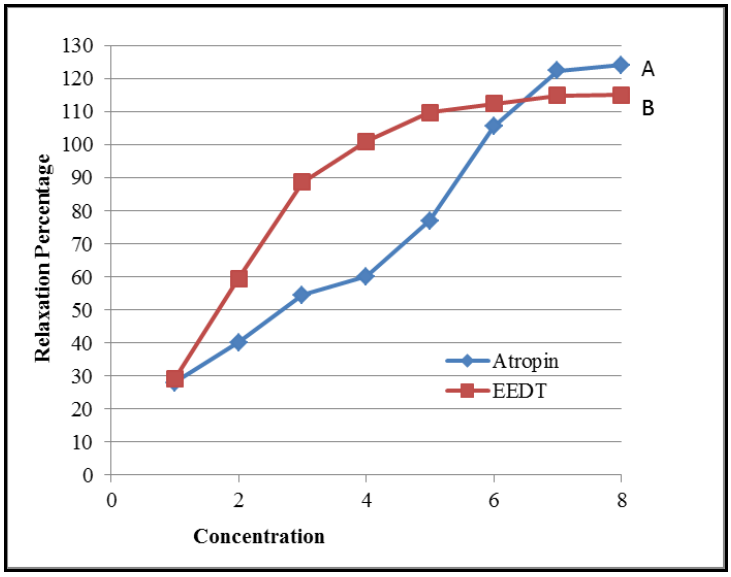

Figure 4. Percentage relaxation after administration of concentration series (A) atropine (1 = 6.95x10^{-6} ; 2 = 2.08x10^{-5} ; 3 = 6.95x10^{-5} ; 4 = 2.08x10^{-4} ; 5 = 6.95x10^{-4} ; 6 = 2.08x10^{-3} ; 7 = 6.95x10^{-3} ; 8 = 2.08x10^{-2} mg/ml) and (B) of ethanol extract of leaves Tetanus (TLEE) (1 = 0,5 ; 2 = 1.0 ; 3 = 1.5 ; 4 = 2.0 ; 5 = 2.5 ; 6 = 3.0 ; 7 = 3.5 ; 8 = 4.0 mg/ml) on isolated ileum smooth muscle that is contracted with acetylcholine 1.889 × 10^{-4} M.Data presented is the average value mean ± SEM , n = 6 .

Comparison between the relaxing effect of atropine sulfate at a concentration of 6.95x10-3 mg / ml (± 113.9796 4.5825) with TLEE the extract of 2.5 mg / ml (± 105.4203 2.9151) against the ileum contractions induced by setilkolin shows that the relaxation percentage difference between the two did not differ significantly (p > 0.05) . In Figure 3.7 indicates that TLEE concentration of 2.5 mg / ml have capabilities that are not much different with atropine sulfate concentration 6.95x10-3 mg / ml in reducing the contraction induced by acetylcholine 1,889x10-4 M. presence capabilities relaxing effect of the ethanol extract of the leaves tetanus is possible for their secondary metabolites that play a role . Leea aequata secondary metabolites are alkaloids , glycosides , steroid / terpenoids , flavonoids and tannins [18]

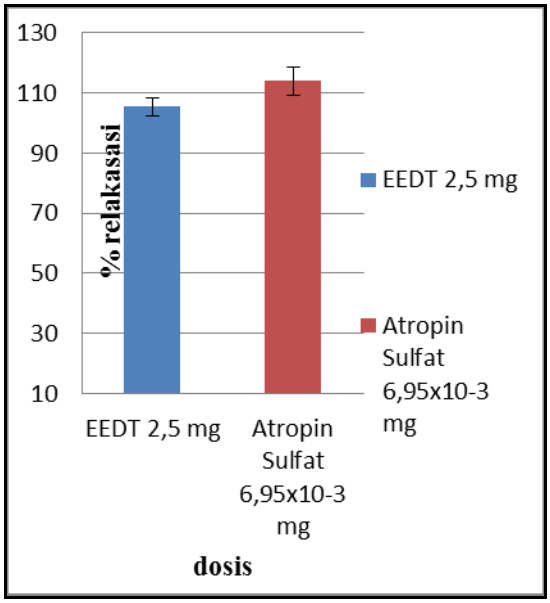

Figure 5. Percentage relaxation after administration of tetanus leaves ethanolic extract 2.5 mg / ml and atropine sulfate 6.95 x 10^{-3} mg / ml after acetylcholine contracted with 1.889 × 10^{-4} M. The data presented is the average value ± SEM , n = 6 .

Tarannita (2013) suspect that the alkaloid can relax the small intestine via the M3 receptor antagonists. M3 receptor is the receptor that is spreading most in the small intestine smooth muscle. [27] reported that traditional herbs are rich in flavonoids and phenolic groups can be used as vasorelaxation. In addition to the alkaloids and flavonoids, and Gilani Khan (2008) suggested that the tannins and sterols play a role in lowering blood pressure and relaxation of the heart muscle is isolated [28].

It is not certain how the ethanol extract of the leaves of this tetanus. There are many paths to be merelaksikan ileal smooth muscle. This study seeks only to see if the ethanol extract of leaves tetanus can relax the isolated guinea pig ileum induced by acetylcholine.

Conclusions

The tetanus leaves ethanolic extract (*Leea aequata* L.) has a relaxing effect on smooth muscle contraction isolated guinea pig ileum induced by acetylcholine. The ethanol extract of leaves tetanus (*Leea aequata* L.) concentration of 2.5 mg/ml has capabilities that are not much different with atropine sulfate $1x10^{-5}$M in reducing the contraction of guinea pigs smooth muscles isolated ileum induced by acetylcholine 1,889×10-4 M (p> 0 , 05).

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