CHOLINERGIC BLOCKING EFFECT OF QUERCETIN

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Quercetin extracted from natural sources such as the leaves of Psidium guajava (Myrtaceae), possessed the cholinergic blocking effect. A pharmacological study of quercetin was made in both rat sciatic nerve-gastrocnemius in in vivo preparation and rat phrenic nerve-hemidiaphragm preparation; in in vitro system. From this study it was found that quercetin caused a decrease in neurally-evoked twitch. Quercetin in high dose produced a complete neuromuscular blockade. It was therefore suggested that quercetin produced a synergistic effect on succinyl cholinergic neuromuscular blocking action. However, tetraethylammonium (TEA), neostigmine and Ca^{2+} could not antagonize the neuromuscular blocking effect of quercetin. Further investigation dealing with the post-tetanic potentiation (PTP) was not abolished by quercetin. In addition, quercetin does not have a synergistic effect with hemicholinium (HC_3). Quercetin in high dose could suppress the twitch amplitude of ACh contracture in rat denervated gastrocnemius preparation. Therefore, the results suggested that quercetin exerts its effect on the neuromuscular junction due to depolarized blocking action at the postsynaptic site and/or a decrease sensitivity of motor endplate to ACh. Therefore, the results suggested that quercetin exerts its cholinergic properties at nicotinic site of neuromuscular junction.

KEY WORDS : Quercetin, Cholinergic blocking effect, nicotinic site.

INTRODUCTION

Compounds affect cholinergic transmission may be classified into several groups. Chemical constituents extracted from plants have been claimed to exert biological activity on cholinergic synapse. The group of flavonoids is one of the chemical constituents which produces pharmacological activity on cholinergic synapse. One such flavonoid is quercetin, a chemical compound which can be extracted from a natural sources. Lutterodt and Maleque (1988) reported that quercetin was found in the methanol extract of the dried leaves of *Psidium guajava*.[1]

Quercetin is a phenolic constituent of plants; it has a bright yellow colour on paper in UV light and it is clearly separated from other constituents and other flavonoids by Forestal chromatograms (R_f=41).[2] Identfication can be confirmed by UV spectroscopy and microdegradation with alkali. The melting point of quercetin is rather high (between 280 and 357°C).

Quercetin used in this investigation is a yellow powder and insoluble in water. The structure of quercetin is 3,3,4,5,7-pentahydroxy-flavone, and the general formula is $C_{15}H_{10}O_7.2H_2O$. The formula molecular weight of quercetin is 338.3. Bhatt (1991) reported that a piscicidal flavonoid, quercetin glycoside, obtained from *Engelhardtia colebrookiana* (Lindle) affected neuro-architecture in medulla oblongata of freshwater fish, *Barilius bendelisis* (Ham.), after 32 days exposure by the neurolysis in hind brain of fish.[3] The pharmacological action of quercetin on the neuromuscular system has not yet been reported. The purpose of this study is to investigate the pharmacological effect of quercetin on neuromuscular junction of cholinergic system in in vitro and in vivo preparations.

MATERIALS AND METHODS

Both sexes of adult albino rats; the Sprauge Dawley strain, weighing 200-300 grams were used in this investigation.

Isolation of rat phrenic nerve-hemidiaphragm for recording neurally-evoked twitch.

The method was based on the modification of the techniques described by Bulbring (1946) and Chantaratham (1974).[4,5] A rat was put down by decapitation. The triangular-shape sections of left and right hemidiaphragm were dissected and excised with their accompanying phrenic nerves. The preparation was then mounted in a tissue bath containing 50 ml of Krebs' solution aerated with oxygen and the temperature was maintained at 30°C throughout each experiment by a thermoregulator. The thread loop at the base of the preparation was attached to a glass rod in the glass tissue bath while the thread at the apex was connected to a force-displacement transducer. The phrenic nerve was drawn through the loop of a stainless bipolar stimulating electrode which was connected to a Grass stimulator. The skeletal muscle contractile responses were recorded by a polygraph. The neurally-evoked twitch was recorded by stimulation of electrical pulses of supramaximal voltage at a frequency of 0.4 Hz and duration of 0.6 msec throughout all experiments.

Isolation of the rat hemidiaphragm for recording directly-evoked twitch.

The preparation was set up as previously mentioned as for that of isolation of hemidiaphragm. One end of a stainless steel bipolar stimulating electrode was attached to the base of the hemidiaphragm, and another stainless steel needle was inserted into the muscle below the roots of the phrenic nerve. The preparation was transferred into the glass tissue bath containing 50 ml of Krebs' solution, aerated with oxygen while the temperature was maintained at 30°C throughout each experiment by a thermoregulator. The directly-evoked twitch was recorded by electrical stimulation of supramaximal voltage at a frequency of 0.4 Hz and for a duration of 0.6 msec. The preparation was completely blocked by adding 0.005 mM of pancuronium in order to have a complete block neuromuscular transmission.

Preparation of rat sciatic nerve-gastrocnemius muscle for recording neurally-evoked twitch, in situ.

The preparation of rat sciatic nerve-gastrocnemius muscle for recording contractile response was based on Mc. Load *et al* (1970).[6] The left sciatic nerve was exposed by making a skin incision at a mid portion of the thigh. A pair of threads were tied tightly to the main sciatic nerve, about 2 mm apart, the nerve was cut between the threads in order to avoid any central connection. A pair of stainless steel bipolar stimulating electrodes were placed to contact with the sciatic nerve. The electrode was then connected to stimulator. The left achillis tendon was dissected and tied with a strong thread, attached to a force displacement transducer then it was connected to polygraph. The neurally-evoked twitch was obtained by stimulation of supramaximal voltage at a frequency of 0.3 Hz and duration of 0.5 msec.

Preparation of denervated gastrocnemius muscle for recording acetylcholine-contracture.

This preparation was performed to investigate the effect of quercetin on postsynaptic site of myoneural junction.[7] The denervation was done for 14-21 days in order to induce supersensitivity at motor endplate.[8] The right femoral artery was cannulated for closed intra-arterial injection. The left achillis tendon was dissected and tied to a strong thread attached to a force displacement transducer. The preparation was performed in the similar way to that for recording the neurally-evoked contractile response, in situ, but no electrical stimulation was applied. ACh (100 µg/mg body weight) was injected intra-arterially to produce ACh contracture.

STATISTICAL ANALYSIS

The twitch response amplitude was expressed as a percent change from control. Average data were expressed as mean ± standard error of mean (mean ± S.E.), n=8. The significant difference of means were determined by student's paired "t" test.

RESULTS

Part 1 Effect of quercetin on the neurally-evoked twitch.

1. Study of the effect of quercetin in the isolated rat phrenic nerve-hemi-diaphragm preparation.

Quercetin in doses of 3.0×10^{-4}, 6.0×10^{-4}, 1.2×10^{-3} and 2.4×10^{-3} M were added into the tissue bath in order to observe the drug effect on the contractile response. It was found that every dose tested 20 minutes after adding quercetin into the tissue bath produced a decrease in the muscle twitch amplitude of 11.2 ± 3.I, 18.8 ± 3.0, 28.3 ± 3.3 and 81.3 ± 8.0 percent respectively, and these effects were dose-dependent. The relationship between various doses of quercetin and the percent decrease of neurally-evoked twitch 20 minutes after adding the drug into the tissue bath, in vitro, was expressed as a regression line, the correlation coefficient (r) was 0.9808, n=8 (Fig. 1).

2. Study of the effect of quercetin in rat sciatic nerve-gastrocnemius muscle preparation.

Quercetin in the dose range of 2, 4, 8 and 16 mg/kg body weight was injected into the anesthetized rat in order to study the effect on contractile response. It was found that the various doses of quercetin produced a decrease in muscle twitch amplitude. These effects were dose-dependent and time-related. Quercetin in the dose of I6 mg/kg produced a complete neuromuscular blockade within 30 minutes. But the respiratory rate was not reduced. The relationship between various doses of quercetin and the percent decreases of neurally-evoked twitch 30 minutes after intra-aterial injection, in vivo, was expressed as a regression line, the correlation coefficient (r) was 0.9655, n=6 (Fig. 2).

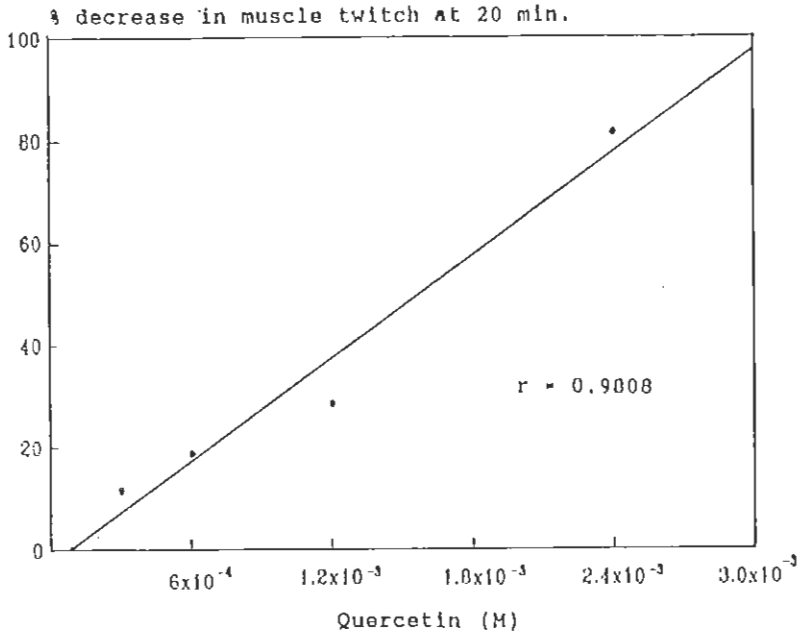

Fig. 1 The dose-response regression line of quercetin in producing the decrease of neurally-evoked twitch in the isolated rat phrenic nerve-hemidiaphragm preparation.

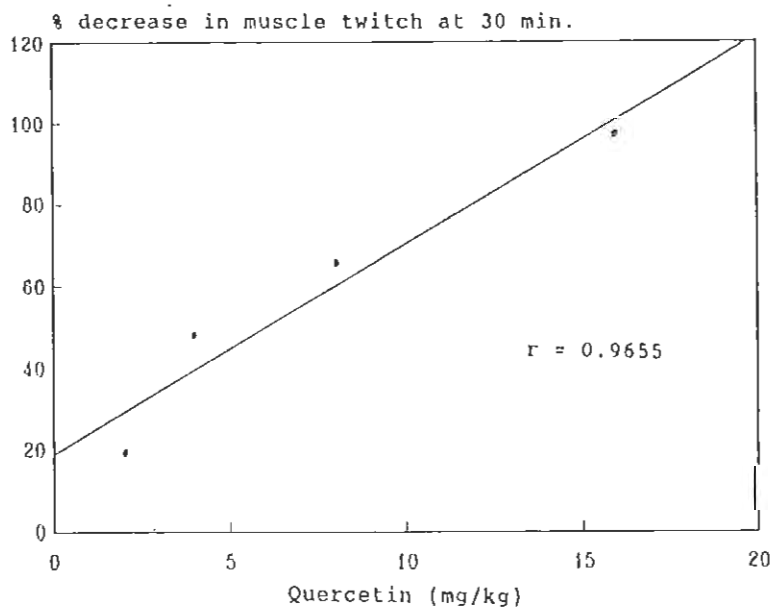

Fig. 2 The dose-response regression line of quercetin in producing the decrease of neurally-evoked twitch in the rat sciatic nerve-gastrocnemius preparation.

Part 2 Interaction of quercetin and standard drugs.

The effect of quercetin on neuromuscular drugs:

In isolated rat phrenic nerve-hemi-diaphragm preparation, the neuromuscular blocking action of quercetin in the presence of neuromuscular blocking drugs, pancuronium, succinylcholine (SCh) and hemicholinium (HC_3) was compared to that of quercetin on its own. It was found that the percent decrease in muscle twitch amplitude produced by quercetin (3.0×10^{-4} M) in the presence pancuronium (4.3×10^{-7} M) and HC_3 (7.0×10^{-5} M) produced a slight decrease in muscle twitch amplitude, no different from that of quercetin alone but quercetin produced a decrease in muscle twitch amplitude when present in SCh more than that of quercetin alone (Fig. 3).

The effect of an anticholinesterase agent, neostigmine on neuromuscular blockade produced by quercetin was also studied. Quercetin in the dose of 2.4×10^{-3} M produced a neuromuscular blockade, but this effect was not reversed by neostigmine (Fig. 4).

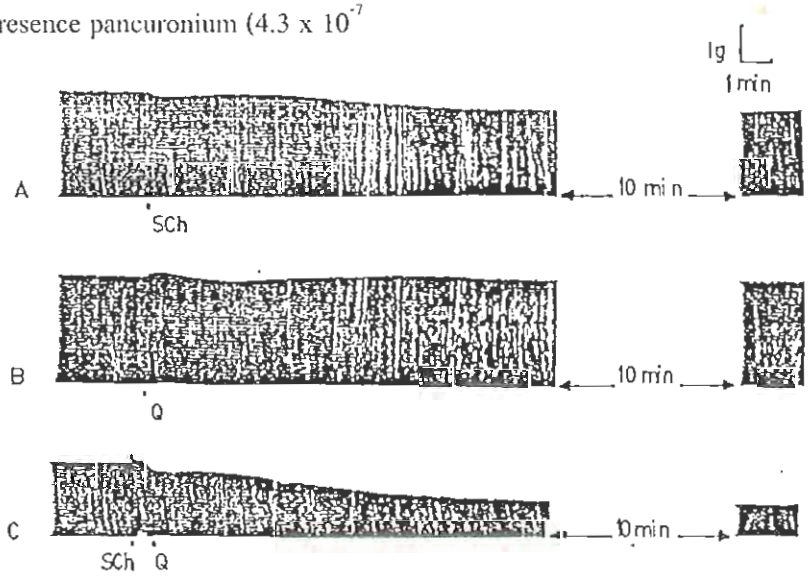

Fig. 3 The effect of Q (quercetin) in presence of SCh (succinylcholine) in the isolated rat phrenic nerve-hemidiaphragm preparation. **A** : The effect of SCh 4.4×10^{-6} M. **B** : A slight decrease in the muscle twitch amplitude produced by Q 3.0×10^{-4} M. **C** : Q 3.0×10^{-4} M produced a synergistic effect on the SCh blocking action.

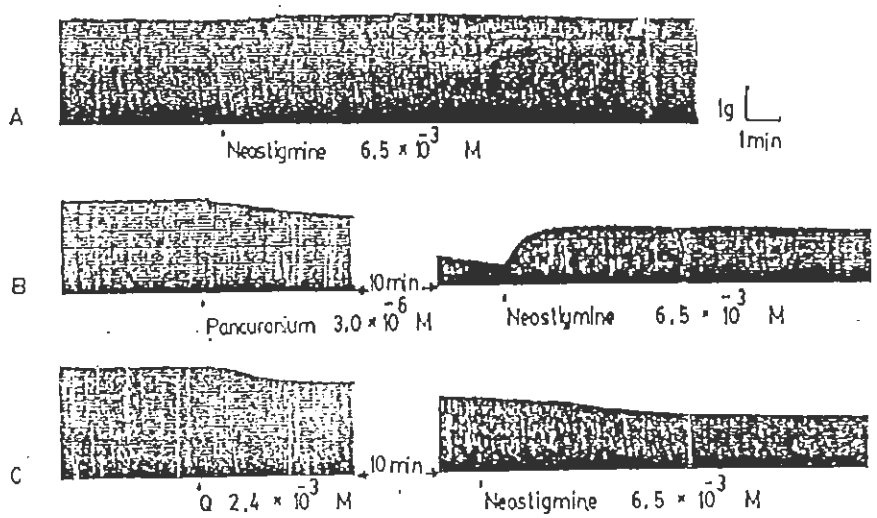

Fig. 4 The effect of neostigmine on the quercetin (Q)-induced neuromuscular blockade in the isolated rat phrenic nerve-hemidiaphragm preparation. A : The effect of neostigmine (6.5×10^{-3} M). B : The effect of neostigmine (6.5×10^{-3} M) on the pancuronium (3.0×10^{-4} M)-induced neuromuscular blockade. C : The effect of neostigmine (6.5×10^{-3} M) on the quercetin (2.4×10^{-3} M)-induced neuromuscular blockade.

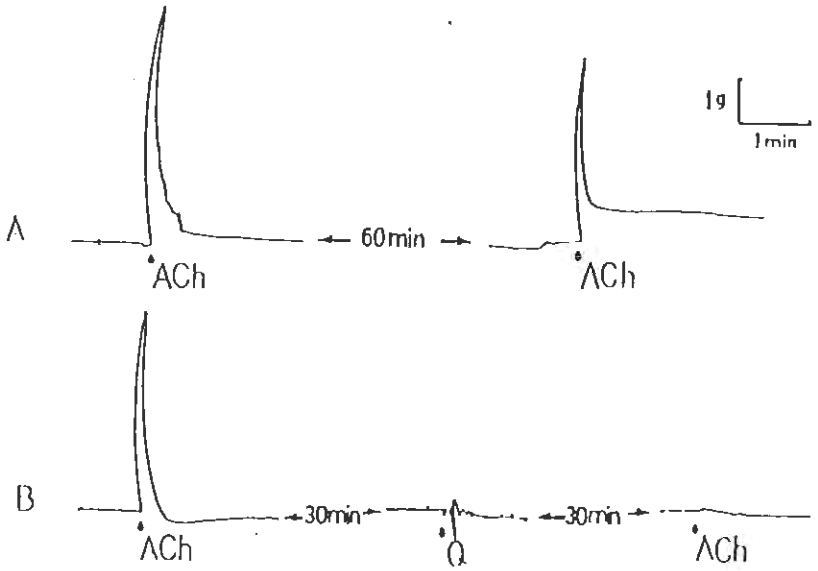

Fig. 5 Interaction of Q (quercetin 16 mg/kg body weight) on Ach (acetylcholine 100 μg/kg body weight) contracture in rat denervated gastrocnemius muscle preparation. A : Control, Ach-contracture. B : Quercetin completely inhibited Ach contracture.

ACh in the dose of 100 μg/kg body weight could produce muscle contraction in denervated muscle. Quercetin in the dose of 16 mg/kg body weight could suppress the muscle twitch amplitude of ACh contracture (Fig. 5). Quercetin was investigated in order to observe the effect of Ca^{2+} on the neuromuscular blockade produced by quercetin in the isolated rat phrenic nerve-hemidiaphragm preparation. It is known that Ca^{2+} plays an important role in ACh release from the motor nerve terminal. It was found that $Ca^{2+}(CaCl_2)$ could antagonize neuromuscular blockade induced by Ca^{2+} deficiency (Krebs' solution without calcium chloride).

The neuromuscular blockade produced by quercetin in the dose of 2.4×10^{-3} M could not be antagonized by Ca^{2+}. Tetraethylammonium (TEA) is a drug acting on several excitable tissues.[9,10,11] The twitch potentiating effect of TEA is due to an enhancement of ACh liberation at the neuro-muscular junction.[5,12]

The isolated rat phrenic nerve-hemidiaphragm preparation experiment was performed in order to study the antagonistic effect of TEA on neuromuscular blockade produced by quercetin (2.4×10^{-3}M). For a 50 percent twitch depression produced by quercetin, TEA could increase the twitch amplitude but it was not significantly greater than TEA alone ($p > 0.05$).

The interaction of quercetin on post-tetanic potentiation:

Post-tetanic potentiation (PTP) is a synaptic phenomenon produced by repetitive electrical stimulation. The mechanism of PTP in cat muscle has been postulated to be due to hyperpolalization of the motor nerve terminals by repetitive nerve impulses.[13] In the present study, the PTP produced by motor nerve stimulation at the frequency of 20 Hz and a duration of 10 seconds in the rat sciatic nerve-gastrocnemius preparation is primarity neurogenic nature[14] and the PTP is proposed to be due to ACh release at the presynaptic site of neuromuscular junction. In this investigation, the percent peak of PTP in the presence of quercetin (16 mg/kg body weight) was determined in

comparison to that of control PTP and the standard drugs such as succinylcholine (300 μg/kg body weight) and d-tubocurarine (50 μg/kg body weight). It was found that the percent peak of PTP in the presence of quercetin was not significantly different from control PTP and SCh but significantly different from d-tubocurarine ($p < 0.05$).

The direct effect of quercetin on skeletal muscle:

In this part of the study, It was found that quercetin produced a decrease in muscle twitch amplitude. However, quercetin in every dose produced a decrease in muscle twitch in the neurally-evoked twitch more than in the directly-evoked twitch.

DISCUSSION

The results obtained from this study showed that quercetin exerted the action on the cholinergic system. For the rat phrenic nerve-hemidiaphragm preparation (in vitro) and the rat sciatic nerve-gastrocnemius preparation (in vivo) the results indicated that quercetin produced a dose-dependent decrease in the muscle twitch amplitude on the neurally-evoked twitch in both systems. In vivo preparation, a high dose of quercetin produced a complete neuromuscular blockade and this effect was irreversible.

The physiology of neuromuscular transmission shows that acetylcholine (ACh) is released from the nerve terminals by nerve impulses, and it acts on the post junctional membrane of the motor endplate to set in action the chain of events that leads to muscle contraction.[15] From the results of both in vitro and in vivo preparations, it was shown that quercetin may have an effect on the neuromuscular junction. The mechanism of action of quercetin was also postulated by studying the interaction of quercetin on some neuromuscular agents: pancuronium, a nondepolarized neuromuscular blocking drug, and succinylcholine (SCh), a depolarized neuromuscular blocking drug. It was found that quercetin in the dose of 3.0×10^{-4} M produced a synergistic effect

on the SCh blocking action at the neuromuscular junction but not pancuronium.

SCh is the depolarizing neuromuscular blocking drug used as an anesthetic adjuvant. It reacts with the nicotinic cholinergic receptor to open the channel and cause depolarization of the endplate.[16] From this result, the effect of quercetin may similar with depolarizing neuromuscular blocking drug.

Neostigmine, an anticholinesterase agent, exerted its action by inhibition of enzymes acetylcholinesterase at the cholinergic synapse, leading to an accumulation of ACh there by producing the twitch potentiation, fasciculation due to its anticholinesterase properties.[16,17] The antagonistic effect of neostigmine on the neuromuscular blocking effect of pancuronium[16] was also observed in this study. The result shows that neostigmine cannot antagonize the neuromuscular blockade produced by quercetin. Thus the blockade of quercetin may not be nondepolarized type.

In 1959, Axelsson and Thesleff reported that the sensitivity of the motor endplate of the denervated muscle was increased to ACh in chronically denervated muscle in cats.[7] In this study the rat denervated gastrocnemius preparation was performed to investigate the effect of quercetin on postsynaptic site of the neuromuscular junction. The result shows that quercetin could suppress the muscle twitch amplitude of ACh contracture in chronically denervated muscle in rats. This finding suggests that the neuromuscular blockade produced by quercetin is probably due to the postsynaptic site of the neuromuscular junction.

Acetylcholine, the neuromuscular transmitter which acts on the muscarinic and nicotinic cholinergic receptors, is known to decrease of releasing in the condition of Ca^{2+} deficiency, following by the result of neuromuscular blocking effect.[18] The evidence of the present study indicated Ca^{2+} could not antagonize the neuromuscular blocking effect of quercetin. Therefore, it might be indicated that neuromuscular blocking effect of quercetin is probably not due to a decrease of ACh release from the nerve terminal.

Tetraethylammonium (TEA) is a ganglionic blocking drug, and competes with ACh at the cholinergic receptor in autonomic ganglion.[16] Besides this action, it also acts on several excitable tissues.[9,10,11] The twitch potentiating effect of TEA is due to enhancement of ACh liberation at the neuromuscular junction.[5,12] Kensler (1950) and Apisariyakul (1975) reported that TEA could antagonize d-tubocurarine and pancuronium-induced blocking effect.[19,20] It is suggested that TEA may increase ACh release from the nerve terminal causing an increase in muscle twitch amplitude in the presence of nondepolarized neuromuscular blocking drugs.[12,20] Thus, TEA is an anticurare agent.[5,10,12,20,21,22] In this study, TEA does not produce an antagonistic effect on the neuromuscular blockade caused by quercetin. Thus the neuromuscular effect of quercetin is probably not due to presynaptic action.

The effect of quercetin and some standard drugs on post-tetanic potentiation (PTP) which is a synaptic phenomenon produced by repetitive electrical stimulation was also studied in order to postulate the mechanism of action of quercetin.[14] The PTP was proposed to be due to ACh release at presynaptic site of the neuromuscular junction. In this investigation, it was found that the percent peak of PTP in the presence of quercetin was not significantly different from that of PTP in the presence of SCh. But the peak of PTP in the presence of quercetin is significantly different from that of d-tubocurarine ($p<0.05$). Some non-depolarized neuromuscular blocking drugs in the dose which produced neuromuscular blockade could decrease ACh release from the nerve terminal.[23,24] On the other hand, SCh clearly acts at the motor endplate but only slightly affects ACh release from the nerve terminal.[16] Thus, The action of quercetin on the neuromuscular blockade was different from that of nondepolarized neuromuscular blocking drugs. It's action is probably not similar to that of non-depolarized neuromuscular blocking drugs, but probably is similar to that of depolarized neuromuscular blocking drugs, such as SCh which was used in this study.

In the study of the direct effect of quercctin on skeletal muscle, quercetin was investigated to observe the contractile response in isolated rat hemidiaphragm curarized preparation. The results showed that quercetin caused a decrease in muscle twitch amplitude on the directly-evoked twitch and a relatively high dosc of quercetin produced a decrease in the amplitude of the neurally-evoked twitch which was morc than that for the directly-evoked muscle twitch. Thus quercetin may has a direct effect on skeletal muscle.

The result in this study, showed that quercetin has no synergistic effect on HC_3. So it is suggested that quercctin has no prejunctional effect in blocking neurotransmitter release.

It could be concluded that quercetin exerts its effect on the neuromuscular junction. The neuromuscular depression of quercetin may be due to its effect at the postjunctional membrane and/or produce a decrease sensitivity of motor endplate to ACh and probably have the direct effect on skeletal muscle but it has not effect at the presynaptic site. However, the neuromuscular blocking effect of quercetin was similar to that of SCh but this effect is irreversible which different from SCh which the neuromuscular blocking effect is reversible.

REFERENCES

1. Lutterodt GD and Malegue A. Effects on locomotor activity in mice of a narcotic-like principle from Psidunm guajava leaves. *J Ethnopharmacol* 1988 ; 24 : 219-231.
2. Harbone JB. Comparative Biochemistry of Flavonoids. Phytochemical Unit, The Hartley Botanical Laboratories. The University of Liverpool, England, 1967.
3. Bhatt JP. Neurolytic manifestation of piscicidal flavonoid of plant, *Engelhardtia colebrookiana* (Lindle) in fish. Indian. *J Exp Biol* 1991 ; 29(6) : 588-590.
4. Bulbring E. Observation on the Isolated Phrenic Nerve-Diaphragm Preparation of the Rat. *Br J Pharmacol* 1946; 1(38).

5. Chantaratham A. "A Comparison of the Effects of Two Ethonium Ions on Neuromuscular Transmission in the Rat." Ph.D. Thesis, Faculty of Science Mahidol University, 1974.
6. Mc. loed LJ. Pharmacological Experiments in Intact Preparation. Edinburgh E & S Livingstone Ltd., 1970.
7. Axelsson J and Thesleff S. A study of supersensitivity in denervated skeletal muscle. *J Physiol* 1959 ; 149 : 178-193.
8. Colquhoun D. Mechanism of Drug Action at the Voluntary Muscle Endplate. *Ann Rev Pharmacol* 1975 ; 15 : 307-325.
9. Hajiwara S and Watanabe A. The effect of tetraethylammonium chloride on the muscle membrane examined with an intracellular microelectrode. *J Physiol* 1955 ; 129 : 513.
10. Kuperman AS and Okamoto M. A comparison of the effects of some ethonium ions and their structural analogues on neuromuscular transmission in the cat. *Br J Pharmacol* 1965 ; 24 : 223.
11. Kuperman AS and Okamoto M. A comparison between the effects of tetraethylammonium and triethyl-(3-hydroxyphynyl) ammonium on frog neuromuscular transmission. *Br J Pharmacol* 1966 ; 26 : 218.
12. Collier B and Exley KA. Mechanism of antagonism by tetraethylammonium of neuromuscular block due to d-tubocurarine or calcium deficiency. *Nature* 1963 ; 149 : 702-703.
13. Standaert FG. Post-tetanic repetitive activity in the cat soleus nerve. *J Gen Physiol* 1963 ; 47 : 53.
14. Nasode B and Apisariyakul A. Study of the mechanism of actions of streptomycin on neuromuscular transmission. *Chiang Mai Med Bull* 1977 ; 16 : 113.
15. Ganong WF. Review of Medical Physiology. 11th Ed. Califonia; Lang Medical Publications, 1983.
16. Goodman LS and Gilman A. The pharmacological Basis of Therapeutics. 7th Ed., New York, Macmillan Publishing Co., 1985.

17. Riker WFJr and Wescoe WC. The direct action of prostigmine on skeletal muscle; its relationship to the choline esters. *J Pharmacol Exp Ther* 1946 ; 88 : 58.

18. Landau EM. The interaction of presynaptic polarization with calcium and magnesium in modifying spontaneous transmitter release from mammalian motor nerve terminals. *J Physiol* 1969 ; 203 : 281-299.

19. Kensler CJ. The anticurare activity of tetraethylammonium ion in the cat. *Br J Pharmacol* 1950 ; 5 : 204-209.

20. Apisariyakul A. The anticurare effect of tetraethylammonium (TEA) and 3-OH phenyltriethylammonium (3-OH PTEA) on the isolated rat phrenic nerve-diaphragm preparation. *Chiang Mai Med Bull* 1975 ; 14 : 209.

21. Koketsu K. Action of tetraethyl-ammonium chloride on neuromuscular transmission in frogs. *Am J Physiol* 1958 ; 193 : 213.

22. Stovner J. The anticurare activity of TEA. *Acta Pharmacol Tox* 1958 ; 14 : 317.

23. Gergis SD, Long JP, Dretchen KL and Sokoll MD. The actions of pancuronium on the motor nerve terminal. *Proc Soc Exp Biol Med* 1972 ; 139 : 74-76.

24. Blaber LC. The prejunctional actions of some non-depolarizing blocking drugs. *Br J Pharmacol* 1973 ; 47 : 109-116.