Kütahya Dumlupınar University
Institute of Graduate Studies

Journal of Scientific
Reports-A
E-ISSN: 2687-6167
Number 44, June 2020

RESEARCH ARTICLE

INVESTIGATION OF THE EFFECTION MECHANISM OF CINNAMIC ACID ON CONTRACTION AND RELAXATION OF SMOOTH MUSCLES OF ILEUM AND BLADDER OF RATS

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Recieve Date:09.05.2019 *Accepted Date: 10.04.2020*

ABSTRACT

Cinnamic Acid is a phenolic acid derivative commonly found in *Cinnamomi cortex* (Cinnamon) plant, and it is named after cinnamon plant. It have Antimicrobial, Antitumoral, Anticancer and Antifungal properties and beyond that its vasodilator effect were also detected.

This study was performed separately for ileum and bladder in 7 different groups, namely atropine, phentolamine, propranolol, nifedipine, tetraethyl ammonium and atropine + phentolamine + propranolol applied groups. In the study, the contraction-relaxation responses to cinnamic acid of the relevant tissues were examined after application of different antagonists or channel blockers to KCl or carbachol pre-contracted ileum / bladder tissues.

Cinnamic acid caused constraction and relaxation of the ileum and bladder as dosage dependent manner. Atropine did not change the relaxation response while further contracting the ileum. It increased relaxation while inhibiting contraction responses in bladder. The contraction and relaxation responses of phentolamine on ileum and bladder was not changed. The propranolol inhibited the contraction responses of ileum, but did not change the relaxation responses. In bladder, the contraction responses increased, but relaxation responses did not change. Nifedipine did not alter the relaxation responses while inhibiting the response of contraction to cinnamic acid in ileum, and increasing relaxation responses without affecting the contraction responses in the bladder. TEA increased relaxation responses while it did not affect the contraction responses in bladder, but did not change contraction and relaxation responses in ileum. At the same time, the adrenergic and cholinergic receptor blockade performed at the same time altered the contraction and relaxation responses in the ileum and bladder, but did not significantly affect them.
In conclusion, it is thought that cinnamic acid applied on rat ileum and bladder smooth muscles, is also influenced by different receptors or pathways other than receptor antagonists and channel blockers preferred by us.

Keywords: *Atropine, Bladder, Cinnamic Acid, Ileum, Nifedipine, Phentolamine, Propranolol, Tetraethyl Ammonium.*

1. INTRODUCTION

Phenolic compounds in plant-based foods are related to human health with particularly epidemiological consequences that reduce the incidence of cancer. Many researchers' reports show that flavonoids are mutation and cancer-inhibiting. Hydroxycinnamic acids and hydroxybenzoic acids are bounded by OH and OCH_3 groups to form phenolic acids. The most important of these compounds are Hydroxycinnamic acids [2]. Hydroxycinnamic acids have different properties depending on the number and place of hydroxyl groups attached to the phenylpropane ring. Cinnamic Acid, Ferulic Acid, Caffeic Acid, O-kumaric Acid and P-Kumaric Acid are the known important acids. Hydroxycinnamic acids are free in trace amounts and usually found in the form of acid derivatives. Esters of hydroxycinnamic acids are commonly used in foods. Hydroxycinnamic acid glycosides and amides also occur in many plants. In cinnamic acid structures, there are 3 different free radical bonds. They are mostly compounds that come into play on the C_6-C skeleton [2]. Cinnamic Acid (CA) is a phenolic acid derivative commonly found in *Cinnamomi cortex* (Cinnamon) plant. Antimicrobial, Antitumoral, Anticarcinogenic and Antifungal properties and vasodilator effect were determined [38], [63], [48], [25] and [41].

Former studies found that CA have anticancerogen [38] antifungal [63] effects. In addition, it has also the antitumoral effect in human tumor cells [63]. The role of Ca in spontaneous, ACh-stimulated and KCl-induced contractions of rabbit small intestine longitudinal and circular smooth muscles was investigated and found that atropine decrease the amplitude and tonus of contraction in both types of muscles and also reduces the frequency of contraction in the circular muscles and nifedipine reduced ACh and KCl-induced contractions and the role of extracellular Ca in spontaneous contractions in rabbit ileus and extracellular and intracellular Ca involvement in ACh and KCl-induced contractions were concluded [20]. The vasodilator action and mechanism of CA in the rat thoracic aorta were investigated and consequently endothelium-dependent vasodilatation through the nitric oxide-cGMP-PKG mediated pathway in the rat thoracic aorta was observed [25]. The effects of *Achillea millefolium* extract on rat ileum contractions were investigated. Contracted ileum created by applying KCl and ACh showed the relaxant effects of A. millefolium extract. It is proved that propranolol, a β-adrenoceptor antigonist, does not inhibit the relaxant effect of A. millefolium. It may be due to the fact that the relaxant effect of extract blocks the voltage-gated Ca channels [46]. The effect and possible mechanisms of Rosa damascena hydroalcoholic extract on rat ileum smooth muscle contractions heve been investigated. The cumulative administration of R. damascena extract reduced the KCl-induced ileal contractions by dose-dependent manner. Propranolol has been found to reduce the inhibitory effect of the extract. β-adrenoceptors may play a role in ileal movement reducing activity of extract [59]. A study of CA on vascular smooth muscle cells and platelet-derived growth factors resulted that CA suppresses early signal transduction and downregulates cell cycle-positive regulatory proteins [41]. The ex-vivo study investigated the interactions of cholinergic, serotonergic and adrenergic receptor systems with Aegle marmelos leaf extract in the ileum, stomach and trachea. Acetylcholine (10^{-9}-10^{-4} M), atropine (10^{-7} M) and Aegle marmelos extract (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) were used in the ileum. 5-HT (10^{-9}-10^{-5} M), ketanserin (10^{-6} M) and Aegle marmelos extract (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) were used in stomach. Isoprenaline (10^{-6} M), propranolol (1 ng/ml) were used in the trachea, and doses of Aegle marmelos extracts were selected by starting from a dose of 1 µg/ml and titrating various concentrations by increasing 10 times in each step. As a result, Aegle marmelos aqueous extract has agonistic effects on cholinergic, serotonergic and adrenergic receptors in isolated rat ileum, stomach and tracheal tissues [32].

Some organs show a response to the electrical stimulation of autonomic nerves and are not affected by the pharmacological inhibition of these systems and the existence of the residual response puts forth the presence of adrenergic or noncholinergic nerve axons in the nerves [61] and [26]. This third system in the neurochemical classification of the sympathetic and/or parasympathetic nervous system is described as non-adrenergic non-cholinergic nervous system (NANK) [27]. The first finding of the NANK system is the pelvic nerve stimulation by atropine-resistant excitation in the mesentery [35]. In the next few decades; Mc Swiney and Robson (1929), Ambache (1951) and Paton and Vane (1963) stated that ganglion stimulating nicotine induces intestinal relaxation after inhibition of contraction by atropine [62]. The atropine and adrenergic neuron blocker bretylium formed large hyperpolarizations by stimulation of the intrinsic nerves of the intestine; however, these hyperpolarizations were inhibited by tetrodotoxin. It may be inhibitory junction potentials in response to stimulation of NANK nerves conducted on the cat, adrenergic nerve inhibiting agents were found to be ineffective in the presence of atropine in the relaxation created by vagal nerve stimulation [62]. The NANK system has been expressed in the urogenital, pulmonary and cardiovascular systems as well as the gastrointestinal system of all vertebrates. Nine different neuron types were identified morphologically in the enteric plexuses and ATP, VIP, tachykinins, GABA and nitric oxide may have possibility to be neurotransmitters of the non-adrenergic non-cholinergic system [62]. Nitric oxide inhibits the secretion of acetylcholine from intrinsic cholinergic nerve endings in the gastric fundus of rats, dogs and rabbits [37]. The NANK system regulates loosening in the guinea pig colon and the contraction of the guinea pig with adrenergic, cholinergic and NANK nerves were also produced. Electrical stimulations of NANK transmission may produce relaxation or contraction in longitudinal and circular smooth muscles in the ileum [62].

The transient receptor potential (TRP) channels [9] are divided into 7 subgroups: TRPC (classical or canonical, TRPC1-7), TRPM (melastatin, TRPM1-8), TRPV (vanilloid receptor, TRPV1-6), TRPA (ankyrin-rich protein, TRPA1), TRPP (polysistin), TRPML (mucolipin), TRPN (NOMPC, "no mechanoreceptor potential" C) [9], [43], [10], [12], [44] and [45]. Most of these channels conduct Na^{+} and Ca^{+2} as cotransport. The TRPA1 group of channels has been proven to be present in the ileum [23], [50] and [47]. TRPA1 agonists stimulate contractions in the guinea pig ileum and colon [50] and [47]. TRPA1 [4], TRPV1 [33],[40] group channels present in the bladder. The TRPA1 group [4] and the TRPV1 group channels [33] contract bladder. TRPV1 antagonists have reduced or prevented bladder contractions [4]. There is evidence that cinnamic acid and its derivatives use TRPA1 [4], [21] and TRPV1 group channels.

Hydroxycinnamic acids and hydroxybenzoic acids are linked by -OH and -OCH3 groups to form important phenolic acid derivatives. The most important of these compounds are Hydroxycinnamic acids [2]. Cinnamic Acid is a phenolic acid derivative commonly found in Cinnamomi cortex (Cinnamon) plant, and its name is derived from cinnamon. Antimicrobial, Antitumoral, Anticarcinogenic and Antifungal properties and vasodilator effect were determined [38], [63], [48], [25] and [41].

Atropine is a mixture of D and L hyoscyamine in equal proportions. It is obtained synthetically as well as by extraction from Solanaceae. Atropine is a non-selective muscarinic receptor antagonist. The affinities of the muscarinic receptor antagonists to receptors can be overridden among different subtypes. This is due to the fact that antagonists can not selectively discriminate receptor subtypes [3]. The selectivity of these compounds results from the level of receptor expression in the relevant tissue or cell and from the affinity constant of the receptor antagonists. The affinity of atropine for binding to M1 receptors was 9.0-9.7, while for M2 receptor 8.7-9.3, for M3 8.9-9.2, for M4 8.9-9.1 and for M5

8.9-9.7 and they are found to be very high [11]. Atropine has the following effects [3]; 1) It has psychological excitation causing euphoric effect on some regions of the central nervous system with little doses. 2) It has direct parasympatholytic effect in the autonomic nervous system. This effect follows an indirect sympathomimetic effect. 3) Atropine reduces muscarinic effect of acetylcholine by paralyzed action on smooth muscle fibers. Atropine causes relaxation of the bladder, urine accumulation and urine drainage difficulty [18].

Fentolamine is an imidazoline derivative non-selective α-adrenergic receptor antagonist. Fentolamine has equal binding sites on both the α-1 and α-2 receptors. The effects of phenolamine are nonselective [7]. Phentolamine, an alpha-adrenergic receptor antagonist, has direct smooth muscle relaxant, cholinomimetic, histaminic and sympathomimetic activity [24]. In addition to blocking the alpha-adrenergic receptor, it also inhibits the effects of 5-HT. The phenolamine may induce an agonistic effect on muscarinic receptor, histamine H1 and H2 receptors [53].

Beta-blockers belong to the class of antagonists that block the effect of the adrenergic neurotransmitter on beta adrenoceptors. The most important of these are timolol, pindol, metaprolol and propranolol [36]. Beta receptors are examined in two groups, β-1 and β-2. Propranolol is a nonselective β-adrenoceptor antagonist that inhibits the function of both β-1 receptors and β-2 receptors [15]. Propranolol, β-adrenergic receptor antagonist, increase inhibitory effects of hydroalcoholic extract of Allium ampeloprasum plant extract on potassium chloride caused contractions in ileum[58]. They concluded that the hydroalcoholic extract of Allium ampeloprasum leaf should affect beta-adrenergic receptors and voltage-dependent calcium channels in order to influence rat ileum motor activity [48]. There was a significant difference between the effects of incubation with the Ileum β-adrenergic receptor antagonist propranolol and R. damascena isolate on propranolol presence and absence of ileum contraction caused by KCl [59]. The active substances in the extract are likely to induce inherent inhibitory activity by affecting the β-adrenergic receptor which the presence and inhibitory effects are determined [6]. Here, contraction in the KCl-induced depolarized smooth muscle is due to the presence of calcium in the environment [65].

Nifedipine's effect is displayed by blocking voltage-dependent calcium channels on the cell membrane [30]. Dihydropyridine-derived calcium channel blockers show antihypertensive effects in two pathways. One of these pathways is the direct relaxant effect due to the inhibition of calcium entry from smooth muscle L-type calcium channels [14].

The flow of calcium ions into the muscle fibers through the slow calcium-sodium channels via the KCl effect allows the formation of contractions [52]. Calcium channels are the main channels that cause muscle contraction [31]. Increase of the how cytosolic concentration of Ca^{2+} ions has been questioned because of the how L-type Ca^{2+} channel blockers significantly reduces CCh-induced contractions [39], [66] and PLC inhibitors are not able to prevent CCh-induced contractions [57]. Experiments with verapamil, an L-type Ca^{2+} channel blocker, and confirmed that Ca^{2+} entry via L-type Ca^{2+} channels is an important contributor to CCh-induced contraction[28]. The formation of carbachol and inositol phosphates is insensitive to nifedipine concentrations and therefore nifedipine normally blocks intracellular Ca^{2+} uptake via voltage-gated channels [56]. Hodgkin and Huxley described voltage-dependent K^+ channels in their work as delayed rectifiers. The channel activation is rather slow compared to the sodium channels, current in one direction is easier to pass than the flow in the other direction. There is no inactivation as long as there is a stimulation in these currents [19], [64], [13] and [49]. In addition to these channels, K^+ currents activated with depolarization but rapid inactivation were also observed. These are referred to as K^+ currents or A currents that are rapidly

inactived. The operation of delayed rectifier type potassium channels is inhibited by tetraethylammonium (TEA). Vasoactive agents such as ACh, bradykinin, ATP, adenosine and histamine lead to extracellular flow through hyperpolarization in endothelial cells. The K^+ channels activated by Ca^{+2} are of two types: potassium channels with high and small conductance. The activation of these channels depends on intracellular ion concentration [17]. Activation of these potassium channels in endothelial cells changes the cell membrane potential through synthesis and secretion of endothelium-derived factors by intracellular Ca^{+2} ion concentration. Hyperpolarization and relapse associated with activation of K^+ channels are lost in the presence of TEA, which is a high K^+ or non-selective K^+ channel blocker [7].
In this study, the CA contraction and relaxation responses on the rat's bladder and ileum were investigated, and the aim was to find out the mechanisms it reacted.

2. METHODS AND TOOLS

2.1. Animals Used in Experiments

Male Spraque-Dawley rats weighing 200-300 g 8 weeks old were obtained from Dumlupınar University-Animal Application and Research Center and used in experiments. Prior to the commencement of the study, the approval is received from Dumlupınar University Medical School-Local Ethics Committee of Animal Experiments (DPÜ HADYEK dated 26.10.2016 and decision no. 2016.10.03). Experimental animals were housed in Dumlupınar University-Experimental Animal Application and Research Laboratory with a 12 hour dark, 12 hour light cycle, 40-60% humidity and a constant room temperature of 22±1 °C.

2.2. Methods

2.2.1. Isolated organ bath experiments

Male Spraque-Dawley rats were subjected to cervical dislocation followed by longitudinal abdominal and thoracic incision to open the abdomen and chest cavity [33]. Preparations were taken from the ileum and bladder organs and placed in a petri dish containing cold Krebs-Henseleit physiological solution. The composition of the Krebs-Henseleit physiological solution was prepared to be 119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2 PO_4 and 11.1 mM Glucose. The organs taken from the experimental animals were ripped off from the surrounding fat and other tissues and then transferred to an isolated organ chamber aerated with 95% O_2 and 5% CO_2 at 37 °C. The one tip of the tissue sample is then ligated to isolated organ and the other tip to transducer. During the experiment, 1 gr tension was applied to ileum and bladder. Ileum and bladder responses were detected with an isotonic transducer and recorded via a recorder.

2.2.2. Protocol studies

A total of 56 Sprague-Dawley male rats were used. Two different protocols were applied in each arranged group study as shown in Table 1. Animals were divided into 7 equal groups as shown in Table 2.

Table 1. Protocols. In the protocol 1, antagonist or channel blocker substance administration after each wash was performed and the 20 min incubation time was applied as shown. Unlike the groups, two protocols were applied in the control group study, but no any receptor antagonist administration was performed.

Protocol Number	Protocol Procedure
1	**The organs (viable with KCI solution) washed and rested for 20 min** ↓ **the receptor antagonist administered** ↓ **Resting 45 min** ↓ **10^{-6} M CA was applied and washed** ↓ **waiting 20 min** ↓ **10^{-5} M CA applied and washed** ↓ **waiting 20 min** ↓ **10^{-4} M CA was applied and washed** ↓ **waiting 20 min** ↓ **10^{-3} M CA applied** ↓ **protocol 1 termination**
2	**The organs (KCI determined vitality) were left to rest for 20 min** ↓ **The receptor antagonist was applied** ↓ **Rested for 60 min** ↓ **Carbachol-contracted organs were cumulatively administered in order of 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M with CA** ↓ **Protocol 2 termination**

Table 2. Protocols. In the protocol 1, antagonist or channel blocker substance administration after each wash was performed and the 20 min incubation time was applied as shown. Unlike the groups, two protocols were applied in the control group study, but no any receptor antagonist administration was performed.

Groups	Group Procedures
1	**In the Group 1 study, which was accepted as the control group, merely CA's effects on the organs (contraction-relaxation responses) were observed.** ↓ **Two different protocols were applied in the control groups.** ↓ **Two different pieces were taken surgically from all applied organs.** ↓ **Each piece of organ was applied separately in both protocols.** ↓ **A total of 8 animals were used in the group 1 study.**
2	**α-adrenoceptors were blocked with a non-selective α-adrenoceptor antagonist, fentolamine (10^{-5} M), and the response of organs was determined by administering each organ to CA.** ↓ **Two different pieces were taken from all applied organs.** ↓ **Each piece of organ was applied separately in two protocols.** ↓ **Two different protocols were applied in the group 2 study. (8 animals)**
3	**The β-adrenoceptors were blocked by propranolol (10^{-6} M), a non-selective β-adrenoceptor antagonist, and the response of organs was determined by administering CA to each organ.** ↓ **Two different pieces were taken from all applied organs.** ↓ **Each piece of organ was applied separately in two protocols.** ↓ **Two different protocols were applied in the group 3 study. (8 animals)**
4	**Cholinergic receptors were blocked by a non-selective cholinergic receptor antagonist atropine (10^{-6} M), and the response of organs was determined by applying each organ with CA.** ↓ **Two different pieces were taken from all applied organs.** ↓ **Each piece of organ was applied separately in two protocols.** ↓ **Two different protocols were applied in the group 4 study. (8 animals)**
5	**The L-type Ca channel blocker, tetraethylammonium (10^{-3} M), blocked the L-type Ca channels and the response of the organs was determined by applying CA to each organ.** ↓ **Two different pieces were taken from all applied organs.**

	↓ **Each piece of organ was applied separately in two protocols.** ↓ **Two different protocols were applied in the group 5 study. (8 animals)**
6	**The K-channel blocker, nifedipine (10-6 M), blocked the K channel and the response of the organs was determined by applying CA to each organ.** ↓ **Two different pieces were taken from all applied organs.** ↓ **Each piece of organ was applied separately in two protocols.** ↓ **Two different protocols were applied in the group 6 study. (8 animals)**
7	**In this group, non-selective α-adrenoceptor antagonist fentolamine (10^{-5} M), β-adrenoceptor antagonist propranolol (10^{-6} M) and cholinergic receptor antagonist atropine (10^{-6} M) were administered together to block both the adrenergic receptor and cholinergic receptor and the responses of the organs were determined by applying CA to each organ.** ↓ **Two different pieces were taken from all applied organs.** ↓ **Each piece of organ was applied separately in two protocols.** ↓ **Two different protocols were applied in the group 7 study. (8 animals)**

2.3. Statistical Analysis

The contraction responses to CA in the presence or absence of trachea and mesenteric antagonists and blockers in the experimental groups were calculated as a percentage of 80 mM KCl-induced contraction responses. Relaxation responses to CA were expressed as a percentage of carbachol contractions. One-way ANOVA and Dunnett's post hoc test were used for statistical analysis. All evaluations were performed in computer environment with GraphPad Prism program. The data were expressed as mean±standard error and $p<0.05$ was considered meaningfully significant.

3. FINDINGS

Experiments were performed separately for ileum and bladder in 7 different groups applied as control, atropine, phentolamine, propranolol, nifedipine, tetraethyl ammonium and atropine+phentolamine+propranolol. Two different protocols were applied in this study. The contraction-relaxation responses of related tissues to cinnamic acid after administration of different

antagonists or channel blockers to KCl or carbachol pre-contracted ileum/bladder tissues were examined. We preferred the logarithmic data conversion process for its normalizing effect as it was shown in all figures.

3.1. The Effects of Cinnamic Acid on Ileum

Cinnamic acid produced carboxylazole-induced relaxation response in dose dependent manner (Fig. 2), while it produced rat ileum smooth muscle contractions in dose dependent manner (Fig. 1).

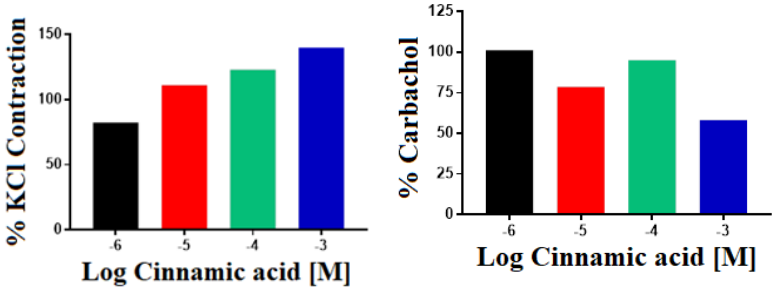

Figure 1(left). The effects of cinnamic acid on ileum

Figure 2(right). The effects of cinnamic acid on ileum pre-contracted by carbachol.

3.1.1. The effects of non-selective muscarinic receptor antagonist atropine on cinnamic acid contraction-relaxation responses

The non-selective muscarinic receptor antagonist atropine-induced contraction responses to cinnamic acid were increased in dose dependent manner (Figure 3). There was no statistically significant difference between the atropine applied group and the control group contraction responses ($p > 0.05$). The non-selective muscarinic receptor antagonist atropine did not alter cinnamic acid responses of charbacol pre-contracted ileum smooth muscle ($p>0.05$) (Fig. 4).

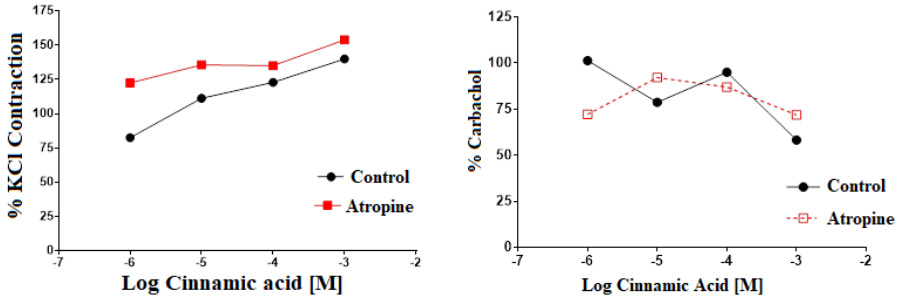

Figure 3(left). The effects of cinnamic acid on ileum with or without atropine

Figure 4(right). The effects of cinnamic acid on ileum pre-contracted by carbachol with or without atropine.

3.1.2. The effects of non-selective α-adrenergic receptor antagonist phentolamine on cinnamic acid contraction-relaxation responses

Contraction responses of cinnamic acid in a dosage dependent manner were not changed by the non-selective α-adrenergic receptor antagonist phentolamine ($p>0.05$) (Figure 5). The non-selective α-adrenergic receptor antagonist phentolamine did not change cinnamic acid responses on carbachol-precontracted ileum smooth muscle relaxation ($p>0.05$) (Fig.. 6).

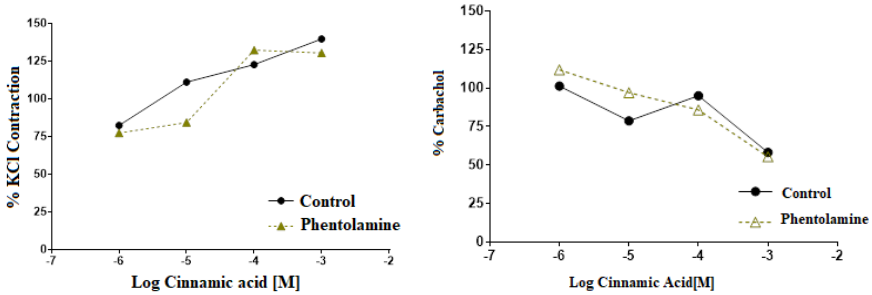

Figure 5(left). The effects of cinnamic acid on ileum with and without phentolamine.
Figure 6(right). The effects of cinnamic acid on ileum pre-contracted by carbachol with or without phentolamine.

3.1.3. The effects of non-selective β-adrenergic receptor antagonist propranolol on cinnamic acid contraction-relaxation responses

The non-selective β-adrenergic receptor antagonist propranolol significantly inhibited the contraction responses of cinnamic acid in a dosage dependent manner ($p<0.05$) (Figure 7). The non-selective β-adrenergic receptor antagonist propranolol did not alter carbachol pre-contracted ileum smooth muscle cinnamic acid responses ($p>0.05$) (Fig. 8).

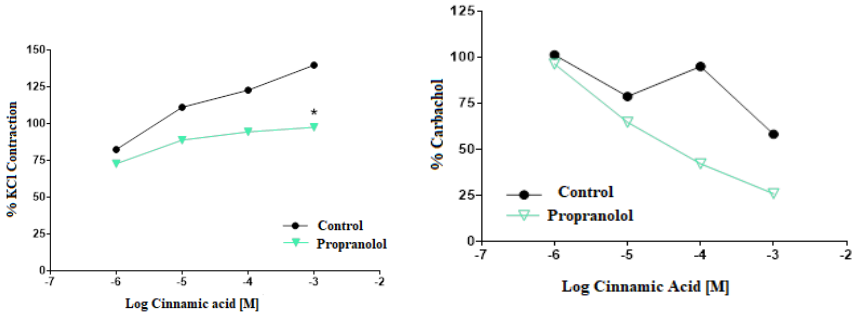

Figure 7(left). The effects of cinnamic acid on ileum with or without propranolol,
Figure 8(right). The effects of cinnamic acid on carbachol pre-contracted ileum with or without propranolol.

3.1.4. The effects of L-type Ca^{2+}channel blocker nifedipine on cinnamic acid contraction-relaxation responses

The L-type Ca^{2+} channel blocker nifedipine significantly inhibited the contraction responses of cinnamic acid in a dosage dependent manner ($p<0.05$) (Figure 9). The L-type Ca^{2+} channel blocker nifedipine did not change the carbachol pre-conracted ileum smooth muscle relaxation responses with cinnamic acid ($p>0.05$) (Figure 10).

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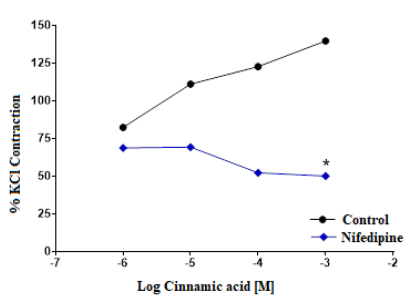


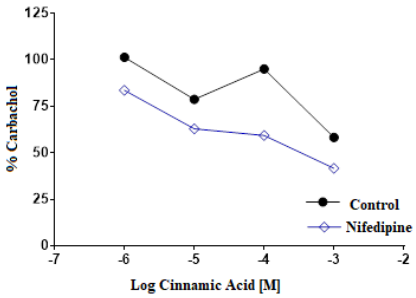
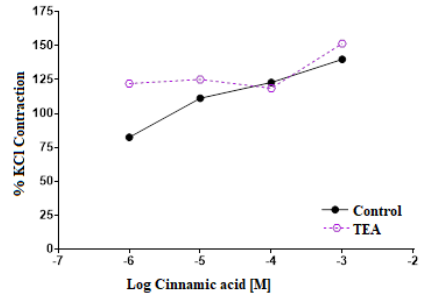


Figure 9(left). The effects of cinnamic acid on ileum with or without nifedipine,
Figure 10(right). The effects of cinnamic acid on carbachol pre-contracted ileum with or without nifedipine.

3.1.5. The effects of K^+ channel blocker tetraethylammonium on cinnamic acid contraction relaxation responses

Contraction responses of the cinnamic acid in dosage dependent manner were not changed by the K^+ channel blocker tetraethyl ammonium ($p>0.05$) (Figure 11). In the smooth muscle relaxation responses to cinnamic acid in carbachol-pre-contracted ileum, the K^+ channel blocker tetraethyl ammonium provided contraction but no significant difference was observed between the groups ($p>0.05$) (Figure 12).



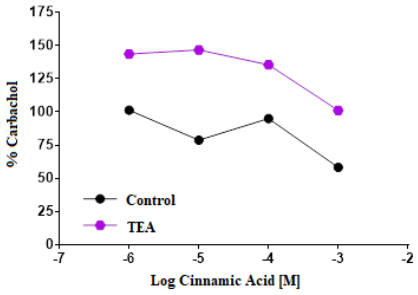
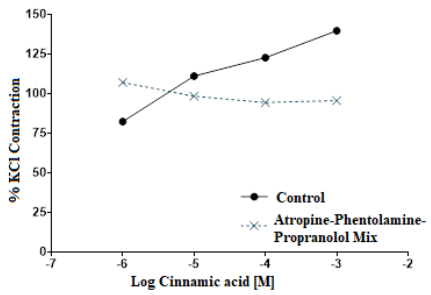


Figure 11(left). The effects of cinnamic acid on ileum with and without TEA,
Figure 12(right). The effects of cinnamic acid on carbachol pre-contracted ileum with and without TEA.

3.1.6. The effects of atropine, phentolamine and propranolol mix on cinnamic acid contraction-relaxation responses

Contraction responses of cinnamic acid in a dosage dependent manner decreased by the non-selective muscarinic receptor antagonist atropine, non-selective α-adrenergic receptor antagonist phentolamine and non-selective β-adrenergic receptor antagonist propranolol, but no statistically significant difference was found between the groups ($p>0.05$) (Figure 13). A mixture of non-selective muscarinic receptor antagonist atropine, non-selective α-adrenergic receptor antagonist phentolamine, and non-selective β-adrenergic receptor antagonist propranolol resulted in a contraction of pre-contracted smooth muscle of ileum relaxation response to carbachol with cinnamic acid, but no statistically significant difference was found between the groups ($p>0.05$) (Figure 14).



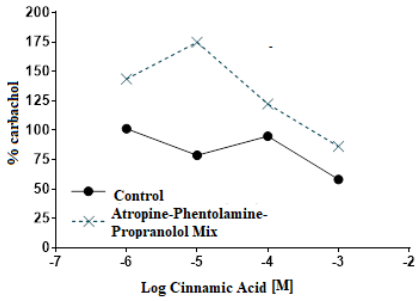
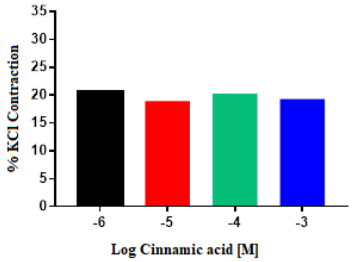


Figure 13(left). The effects of cinnamic acid on ileum with or without atropine- phentolamine-propranolol mix.
Figure 14(right). The effects of cinnamic acid on carbachol pre-contracted ileum with or without atropine-phentolamine-propranolol mix.

3.2. The Effects of Cinnamic Acid on Bladder

Sinnacic acid responded as contractions in rat bladder smooth muscles in a dose dependent maner (Fig. 15) but formed relaxation responses against carbachol in a dose dependent manner (Fig. 16).



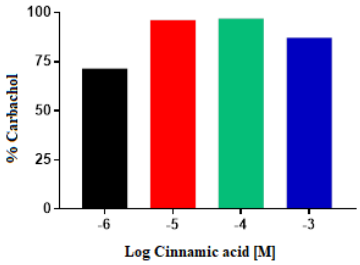
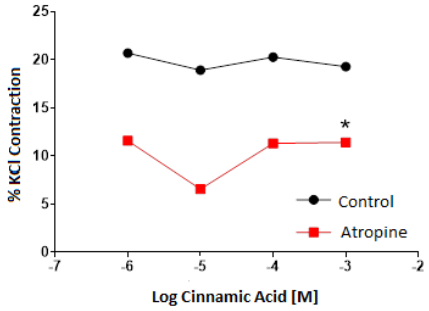


Figure 15(left). The effects of cinnamic acid on bladder,
Figure 16(right). The effects of cinnamic acid on carbachol pre-contracted bladder.

3.2.1. The effects of non-selective muscarinic receptor antagonist atropine on cinnamic acid contraction-relaxation responses

The non-selective muscarinic receptor antagonist atropine significantly inhibited the contraction responses of cinnamic acid in a dosage dependent manner ($p<0.05$) (Figure 17). Carbachol-induced pre-contracted bladder smooth muscle relaxation responses by cinnamic acid were increased by non-selective muscarinic receptor antagonist atropine, but no significant difference between the groups was not observed ($p>0.05$) (Figure 18).



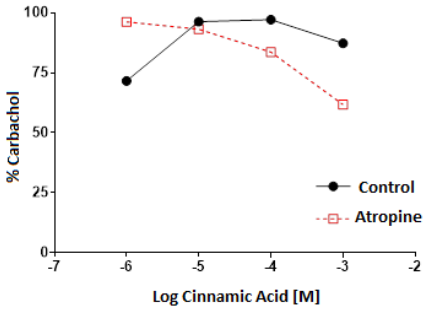
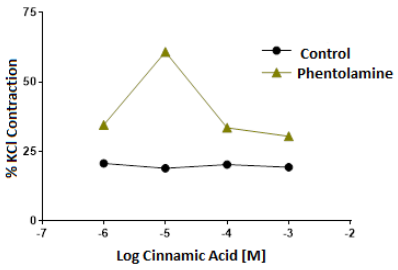


Figure 17(left). The effects of cinnamic acid on bladder with and without atropine.
Figure 18(right). The effects of cinnamic acid on pre-contracted bladder with or without atropine.

3.2.2. The effects of non-selective α-adrenergic receptor antagonist phentolamine on cinnamic acid contraction-relaxation responses

Contraction responses of cinnamic acid in a dosage dependent manner were increased by non-selective α-adrenergic receptor antagonist phentolamine, but no significant difference was observed between the groups ($p>0.05$) (Figure 19). Non-selective α-adrenergic receptor antagonist phenolamine did not change carbachol-preadministered with cinnamic acid pre-contracted bladder smooth muscle relaxation responses ($p>0.05$) (Figure 20).



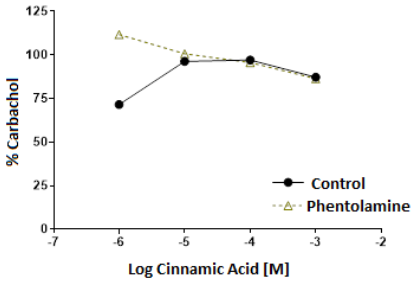


Figure 19(left). The effects of cinnamic acid on bladder with and without phentolamine.
Figure 20(right). The effects of cinnamic acid on carbachol pre-contracted bladder with and without phentolamine.

3.2.3. The effects of non-selective β-adrenergic receptor antagonist propranolol on cinnamic acid contraction-relaxation responses

The non-selective β-adrenergic receptor antagonist propranolol significantly increased the contraction responses of cinnamic acid in a dosage dependent manner ($p<0.05$) (Figure 21). The non-selective β-adrenergic receptor antagonist propranolol did not change carbachol-preadministered with cinnamic acid bladder relaxation responses ($p>0.05$) (Fig. 22).

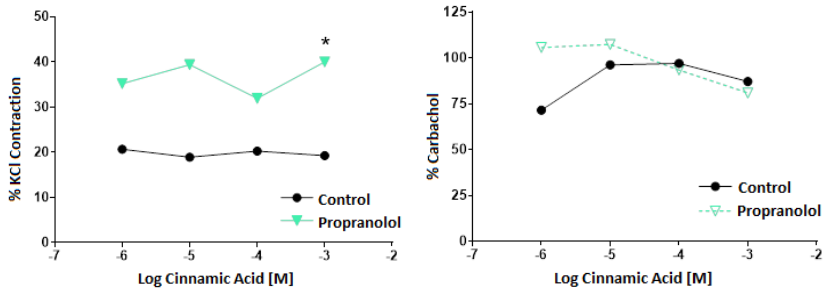


Figure 21(left). The effects of cinnamic acid on bladder with and without propranolol.
Figure 22(right). The effects of cinnamic acid on carbachol pre-contracted bladder with and without propranolol.

3.2.4. The effects of L-type Ca^{2+}channel blocker nifedipine on cinnamic acid contraction-relaxation responses

The L-type Ca^{2+} channel blocker nifedipine did not change the contraction responses of the cinnamic acid in a dosage dependent manner ($p>0.05$) (Figure 23). The L-type Ca^{2+} channel blocker nifedipine significantly increased the carbachol-preadministered pre-contracted with cinnamic acid bladder smooth muscle relaxation responses ($p<0.05$) (Fig. 24).

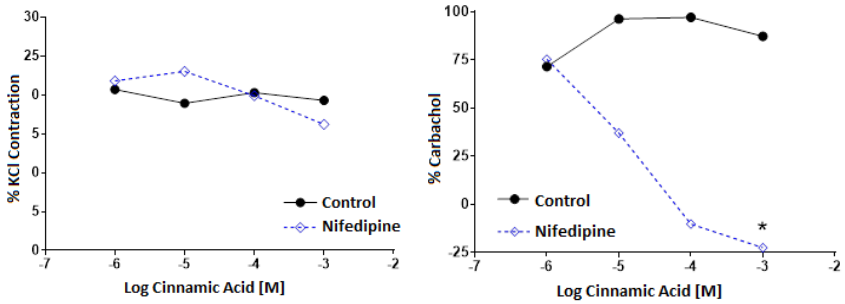
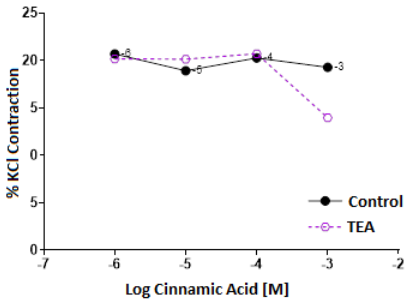


Figure 23(left). The effects of cinnamic acid on the bladder with and without nifedipine**.**
Figure 24(right). The effects of cinnamic acid on carbachol pre-contracted bladder with and without nifedipine.

3.2.5. The effects of K^+ channel blocker tetraethylammonium on cinnamic acid contraction-relaxation responses

Contraction responses of the cinnamic acid in a dosage dependent manner were decreased by the K^+ channel blocker tetraethylammonium but no significant difference was observed between the groups ($p>0.05$) (Figure 25). Carbachol-precontracted bladder smooth muscle with cinnamic acid relaxation responses were significantly increased by K^+ channel blocker tetraethyl ammonium ($p<0.05$) (Figure 26).



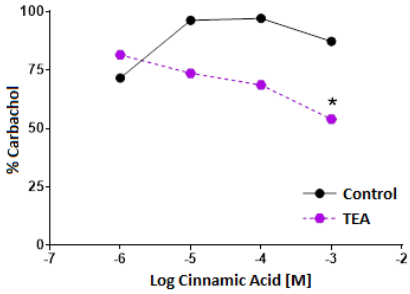
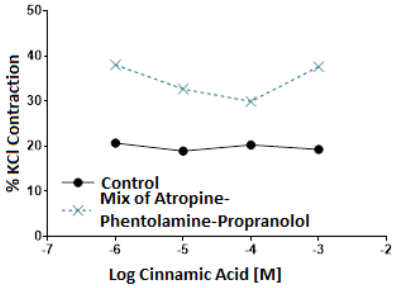


Figure 25(left). The effects of cinnamic acid on the bladder with and without TEA.
Figure 26(right). The effects of cinnamic acid on carbachol pre-contracted bladder with and without TEA.

3.2.6. The effects of atropine, phentolamine and propranolol mix on cinnamic acid contraction-relaxation responses

Contraction responses of cinnamic acid in a dosage dependent manner was increased by the mix of non-selective muscarinic receptor antagonist atropine, non-selective α-adrenergic receptor antagonist fentolamine and non-selective β-adrenergic receptor antagonist propranolol, but no statistically significant difference was observed between the groups ($p>0.05$) (Figure 27). Cinnamic acid enhanced carbachol pre-contracted bladder smooth muscle relaxation responses were increased by a mixture of non-selective muscarinic receptor antagonist atropine, non-selective α-adrenergic receptor antagonist fentolamine and non-selective β-adrenergic receptor antagonist propranolol, but no statistically significant difference was found between the groups ($p> 0.05$) (Figure 28).



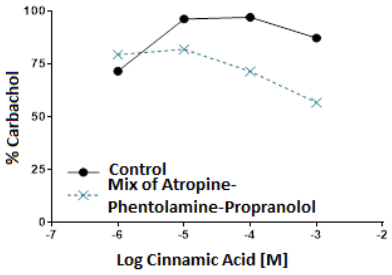


Figure 27(left). The effects of cinnamic acid on bladder with and without the mix of atropine, phentolamine and propranolol.
Figure 28(right). The effects of cinnamic acid on carbachol pre-contracted bladder with and without the mix of atropine, phentolamine and propranolol.

4. DISCUSSIONS

Cinnamic Acid's effect on aortic smooth muscles and vasodilator effect on rat aortic rings was determined [25]. However its vasodilator effect has not been investigated on the ileum and bladder smooth muscles and this fact has attracted our attention and led us to work on this topic. Thus, the mechanisms of action of CA on the ileum and bladder smooth muscles have been investigated at *in*

vitro conditions. In the study, pharmacological agents such as phentolamine, propronolol, atropine, nifedipine, tetraethylammonium (TEA) were used and the responses given to these agents were observed. The study was planned on the implementation of two different protocols. In both protocols, receptor antagonists were administered to the organs left to rest for 20 minutes and CA administration was performed at different doses. Next to that, in the second protocol, CA was applied to carbachol-contracted organs. Moreover, a total of 56 Spraque-Dawley rats were used in the control group. As a result of the study, the effects of CA on the bladder and tracheal organs and the mechanism or mechanisms by which this effect was carried out are intended to contribute to the production of this substance and other substances of similar activity.

In our study, 7 different groups applied as control, atropine, phentolamine, propranolol, nifedipine, tetraethyl ammonium and atropine+phentolamine+propranolol was performed separately for ileum and bladder. Two different protocols were applied in this study. The contraction-relaxation responses of related tissues to cinnamic acid after application of different antagonists or channel blockers to KCl or carbachol pre-contracted ileum/bladder tissues were examined. Cinnamic acid caused contractions and relaxations in ileum and bladder in a dose dependent manner.

Atropine did not change the relaxation response of the group more contracted by the treatment of cinnamic acid. It increases relaxation while inhibiting contraction responses in bladder. Cholinergic receptors have created a mechanism that causes contraction of bladder [1], [42]. The inhibition of contraction by the result of atropine administration is expected. Many researchers have mentioned atropine-resistant excitation in smooth muscles [55], [34]. We consider that the reason for more contractions of ileum by cinnamic acid is caused by the noncholinergic system even though atropine is applied, and we think that muscarinic receptors are not used for the cinnamic acid caused ileum contraction responses.

It is reported that TRPA1 channels increase contraction in the ileum [50], [47] and cinnamic acid and its derivatives use these channels [54]. We also think that the cause of contractions in the ileum may be the channels with transient receptor potentials. Atropine causes relaxation of bladder to occur [18]. It is observed that the bladder responses were normal responses to atropine. The bladder response is consistent with the general information given in Goth's book.

Contraction and relaxation responses of ileum and bladder were not changed in the phentolamine-treated group. Phentolamine is a non-selective α-adrenergic receptor antagonist [24]. The α-adrenoceptors exist in the neck of the bladder and exist in trigone more than other parts of the bladder [1], [42]. α_1-adrenoceptors are thought to be effective not only on the contraction of the bladder but also on the secretion of neurotransmitters [42]. Findings makes a point that α_2-adrenoceptor agonists reduce bladder capacity [22]. They pointed out that α_1-adrenoceptor agonists increase the frequency of bladder contraction [16] and [29]. It has been guessed that α_1-adrenoceptor agonists inhibit the contractile responses of ileum but the α_2-adrenoceptor agonists have no effect on the mechanism of contractility of the ileum [59]. In our study, the remained unchanging of response of ileum and bladder to cinnamic acid in the groups treated with phentolamine suggesting that the mechanism of action of cinnamic acid is via nonadrenergic pathways.

In propranolol administration, the inhibited relaxation responses of the contraction responses of the ileum were not changed. In the bladder, relaxation responses were not changed while the contraction responses increased. Propranolol is a non-selective β-adrenergic receptor antagonist that blocks both β_1 receptors and β_2 receptors [36], [51]. β-adrenoceptors regulate relaxation in the bladder smooth

muscles [1], [42]. In our study, an increase in cinnamic acid responses in the presence of propranolol in the bladder is an expected normality. It has been shown that β_1-adrenoceptor agonists have no effect on the mechanism of contraction of the ileum but β_2-adrenoceptor agonists inhibit contraction responses [60]. The results of our studies of this group are incompatible with previous studies. Increased contraction responses are expected due to the antagonist usage. The inhibition of responses suggests that cinnamic acid is effective through nonadrenergic pathways.

Nifedipine did not alter relaxation responses while inhibiting contraction responses of cinnamic acid in ileum, and increased relaxation responses without affecting contraction responses in the bladder. Nifedipine is a calcium antagonist that blocks voltage-dependent L-type calcium channels in smooth muscle cell membranes [30]. The activation of the L-type Ca channels allows the smooth muscle to contract. Their inactivation inhibits contraction. In our study, the inhibition of cinnamic acid responses in ileum in the presence of nifedipine suggests that cinnamic acid uses this pathway. The fact that cinnamic acid relaxation responses of bladder to carbachol increased in the presence of nifedipine suggests that cinnamic acid uses this pathway. However, the relaxation responses to carbachol in the ileum and the unchanged contraction responses in the bladder suggests that cinnamic acid in the presence of nifedipine may have used transient receptor potential channels (TRPs) permeable to different cations beside the L-type Ca channels.

TEA administration increased relaxation responses without affecting contraction responses in the absence of changes in contraction and relaxation responses of the ileum. TEA is a non-selective potassium ion channel blocker. Hyperpolarization and relaxation associated with activation of K^+ channels are lost in the presence of TEA, which is a high K^+ or non-selective K^+ channel blocker [7]. In our study, the unchanged contraction responses of cinnamic acid in the ileum and the bladder in the presence of TEA and the increase in relaxation responses of the bladder suggests that cinnamic acid did not use this pathway. We think that in the presence of TEA, cinnamic acid may have used transient receptor potential channels permeable to different cations beside K^+ channels. Adrenergic and cholinergic receptor blockade made at the same time altered the contraction and relaxation responses in the ileum and bladder, but it did not affect significantly. The fact of the response of this group being not different from the response of the control group suggests that the mechanism of action of cinnamic acid in both organs is through non-adrenergic-non cholinergic pathways. In the study made in the rat thoracic aorta suggests that cinnamic acid acts through the NO-cGMP-protein kinase G pathway [25]. We support the idea that cinnamic acid uses the same pathway in ileum and bladder. In addition, TRP channels have been shown to increase contraction activity in the ileum and bladder [50], [47], [4], [33] and usage of these channels by cinnamic acid and its derivatives [4], [21] and [54]. We also assume that the reason for unchanged cinnamic acid responses to contraction-relaxation during cholinergic and adrenergic blockade compared to the control group's response experimented at the same time may be the transient receptor potential channels.

Based on our results, we can say that cinnamic acid also uses cholinergic, adrenergic, non-adrenergic non-cholinergic and TRP channels in the ileum and bladder smooth muscles. We believe that the future works to be performed using NANK inhibitors and TRP antagonists will explain these mechanisms much better.

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ATTACHMENTS

APPENDIX 1. Experimental Animals Local Ethics Committee Approval Certificate

T.C.
DUMLUPINAR ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
ARAŞTIRMA BAŞVURUSU ONAYI

BAŞVURU BİLGİLERİ	ARAŞTIRMANIN ADI	**Sıçan İleumu, Mesanesi ve Trakeası Düz Kası Kasılma ve Gevşeme Cevapları Üzerine Sinnamik Asit'in Etki Mekanizmasının Araştırılması**
	ARAŞTIRMA YÜRÜTÜCÜSÜ KURUMU	Doç.Dr.M.Kasım ÇAYCI Dumlupınar Üniversitesi Fen Edbiyat Fakültesi Genel Biyoloji ABD
	PROJE YÜRÜTÜCÜSÜ KURUMU	Doç.Dr.M.Kasım ÇAYCI Dumlupınar Üniversitesi Fen Edbiyat Fakültesi Genel Biyoloji ABD
	YARDIMCI ARAŞTIRICILAR	Doç.Dr.M.Kasım ÇAYCI — Yrd.Doç.Dr.Yasemin TEKŞEN YL.Ögr. Emre KUNDAKÇI — Öğr.Gör.Sinan DARCAN Dr.Öğr.Fatih ALAN — Prof.Dr.Hayri DAYIOĞLU
	ARAŞTIRMANIN TAHMİNİ SÜRESİ	12 Ay
	KULLANILACAK HAYVAN TÜRÜ VE SAYISI	Sprague Dawley (E) – 56 Adet
	DESTEKLEYİCİ KURULUŞ	-

	Belge Adı	Tarihi
DEĞERLENDİRİLEN İLGİLİ BELGELER	DİLEKÇE	**17.10.2016**

KARAR BİLGİLERİ

Karar No : 2016.10.03 **Tarih : 26.10.2016**

Yukarıda başvuru bilgileri verilen dilekçe gerekçe, amaç ve yöntemler dikkate alınarak görüşüldü ve ilgili belgeler incelendi. 2016.03.06 karar nolu çalışmada hayvan soyunun değişmesi, gruplardaki hayvan sayısının azaltılması, destekleyici kuruluşlardan DPÜBAP'in çıkarılması, çalışmanın diploma derecesine yönelik kısmına Doktora derecesi eklenmesi, çalışmaya Dr.Öğr.Fatih ALAN ve Prof.Dr.Hayri DAYIOĞLU'nun eklenmesi ile ilgili dilekçenin etik açıdan uygun olduğuna,çalışmanın aşağıdaki hususlar dikkate alınarak yürütülmesine ve sorumlu araştırıcıya iletilmesine **OY BİRLİĞİ** ile karar verildi.

1) Projede herhangi bir değişiklik gerektiğinde kurulumuzdan onay alınması,
2) Projede çalışacağı bildirilen araştırıcılarda değişiklik olduğunda kurulumuzdan onay alınması,
3) Deney hayvanları üzerinde yapılacak girişimin başlangıç ve bitiş tarihinin bildirilmesi,
4) Çalışma süresinde tamamlanamaz ise ek süre talebinde bulunulması,
5) Çalışma tamamlandığında sonuç raporunun gönderilmesi.

ETİK KURUL BİLGİLERİ

ÜYELER

Unvanı / Adı / Soyadı EK Üyeliği	Uzmanlık Dalı	Kurumu	İlişki (*)	İmza
Doç.Dr. Aynur GÜLCAN Başkan	Mikrobiyoloji ve Klinik Mikrobiyoloji Anabilim Dalı	Tıp Fakültesi	☐ E ■ H	
Doç.Dr.M.Kasım ÇAYCI Üye	Genel Biyoloji Anabilim Dalı	Fen-Edebiyat Fakültesi	■ E ☐ H	
Doç.Dr.Sermet İNAL Üye	Ortopedi ve Travmatoloji Anabilim Dalı	Tıp Fakültesi	☐ E ■ H	
Yrd.Doç.Dr.Yasemin TEKŞEN Üye	Farmakoloji Anabilim Dalı	Tıp Fakültesi	■ E ☐ H	
Yrd.Doç.Dr.Sezer AKÇER Üye	Anatomi Anabilim Dalı	Tıp Fakültesi	☐ E ■ H	
Yrd.Doç.Dr.Hasan METİNEREN Üye	Ortopedi ve Travmatoloji Anabilim Dalı	Tıp Fakültesi	☐ E ■ H	
Yrd.Doç.Dr.Ahmet Haris Selçuk ÖZEN Üye	Zooloji Anabilim Dalı	Tıp Fakültesi	☐ E ■ H	
Yrd. Doç. Dr. Hasan Emre AYDIN Üye	Beyin ve Sinir Cerrahisi Anabilim Dalı	Tıp Fakültesi	☐ E ■ H	
Vet. HEKİM Aydın AKCILAR Üye	Veteriner HEKİM	Tıp Fakültesi DEHYUAM	☐ E ■ H	
Biyolog Mehmet ÖNCEBE Üye			☐ E ■ H	

* Araştırma ile İlişkisi