

The Effect of Plantain Active Ingredient Aucubin on Isolated Rat Smooth Muscle Tissue and Primary Cell Lines

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Abstract: This study aims to assess the pharmacodynamics of aucubin, the active ingredient in plantain (*Plantago*), *in vitro* rat bladder and trachea tissues, and explore its cell protective effects on primary lung and kidney cell lines. The study was carried out by repeated applications of acetylcholine, atropine, verapamil and oxybutynin, alongside Ca⁺⁺ in a calcium-free environment, on urinary bladder tissue, and repeated applications of acetylcholine, atropine, carbachol and mecamlamine on trachea tissue. At the same time, cell viability and catalase and superoxide dismutase activities was measured in primary cell lines. The results indicated that aucubin had a relaxant effect on urinary bladder and trachea tissues. It was conceived that aucubin acted as a cholinergic antagonist through different subreceptors (muscarinic-3 receptors). The results also indicated that aucubin caused a statistically insignificant increase in remaining at the level of the control group in cell viability in the primary lung and kidney cell lines at increasing concentrations (1-10 µM), while causing a significant reduce in cell viability at 20 and 50 µM concentrations in the same cell lines. In conclusion, it is suggested that plantain, which is used in folk medicine, and its active ingredient aucubin might have beneficial effects on bronchoconstriction and other respiratory conditions, as well as on pulmonary and renal diseases, urinary incontinence, etc.

Keywords: *Plantain, Aucubin, Urinary bladder, Trachea tissue, Smooth muscle, Primary cell line.*

Sinir Otu Etkin Maddesi Aucubinin İzole Rat Düz Kasları ve Primer Hücre Hatları Üzerine Etkisi

Özet: Bu çalışmada, sinir otu (*Plantago*) etkin maddesi aucubinin *in vitro* rat idrar kesesi ve trakea dokusunda farmakodinamik ve primer akciğer ve böbrek hücre hatları üzerine hücre koruyucu etkilerinin incelenmesi amaçlandı. Çalışma, idrar kesesi dokusuna tekrarlı olarak asetilkolin, atropin, verapamil ve oksibutin ile kalsiyumsuz ortamda Ca⁺⁺ verilerek, trakea dokusuna ise tekrarlı asetilkolin, atropin, karbakol ve mekamilamin uygulamalarıyla yapıldı. Aynı zamanda, primer hücre hatları üzerinde hücre canlılığı, katalaz ve süperoksit dismutaz enzim aktivitelerinin ölçümü gerçekleştirildi. Çalışma sonucunda, aucubinin idrar kesesi ve trakea dokusunda gevşetici etkisi belirlendi. Aucubinin kolinerjik antagonist olduğu, ancak bu etkisini farklı subreseptörler (muskarinik-3 reseptörler) üzerinden gösterdiği düşünüldü. Ayrıca, artan konsantrasyonlarda (1-10 µM) primer akciğer ve böbrek hücre hatlarında hücre canlılığında kontrol grubu seviyesinde kalan istatistiksel olarak önemsiz bir artışa neden olurken, aynı hücre hatlarında 20 ve 50 µM konsantrasyonlarda hücre canlılığında önemli bir düşüşe neden olduğu belirlendi. Sonuç olarak, halk arasında tedavi amacıyla kullanılan sinir otunun ve etkin maddesi aucubinin solunum yolları daralması ve hastalıkları, akciğer ve böbrek hastalıkları, üriner inkontinans vb. durumlarda yararlı olabileceği kanaatine varıldı.

Anahtar Kelimeler: *Sinir otu, Aucubin, İdrar kesesi, Trakea dokusu, Düz kas, Primer hücre hattı*

Introduction

Plantain is a group of plants in the *Plantago* genus of the *Plantaginaceae* family, known with the names of fleaworth, ribleaf, ribworth, white man's foot, narrowleaf or broadleaf plantain (Yıldırım and Ekim, 2003; Deniz et al., 2010). Leaves, seeds, infusion and paste of plantain are used for medicinal purposes (Ezer and Avci, 2004; Deniz et al., 2010; Akan and Bakır Sade, 2015).

Plantain is reported to have a beneficial effect on wound healing, digestive and circulatory system

organs and skin conditions alongside its various other anti-inflammatory, antibacterial, anti-cancer and immunomodulator effects. The plant contains several active ingredients, primarily iridoid glycosides (aucubin, aucubigenin, catalpol, melittoside) as well as caffeic acid derivatives (plantamajoside and acteoside), alkaloids and flavonoids (Rønsted et al., 2000; Samuelsen, 2000). An important iridoid glycoside, aucubin is noted for its antimicrobial, hepatoprotective,

neuroprotective, anti-inflammatory and antitumoral effects (Rakotondramasy et al., 2010; Xu et al., 2012; Xue et al., 2012; Kim et al., 2014).

There are a limited number of studies that explore the pharmacodynamics of aucubin (Atta and Mouneir, 2005; Fleer and Verspohl, 2007). At the same time, cell culture studies are mainly focused on the anticancer effects of aucubin (Hung et al., 2008; Rakotondramasy et al., 2010; Xu et al., 2012; Xue et al., 2012; Kim et al., 2015). This study aims to assess the pharmacodynamics of aucubin, the active ingredient in plantain, on *in vitro* rat bladder and trachea tissues, and explore its cell protective effects on primary lung and kidney cell lines.

Materials and Methods

Chemicals:The active ingredient used in the study, aucubin (55561) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, US) and used to prepare a 1 mg/ml stock solution in ultrapure water. The remainder of the agents, namely acetylcholine (A6625), verapamil (V4629), oxybutynin (O2881), carbachol (C4382), PBS (Dulbecco's Phosphate Buffered Saline, D5652), RPMI-1640 Medium (R8758) and thiazolyl blue tetrazolium bromide (M5655) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, US); calcium chloride (CaCl₂, 328757) from Carlo Erba Reagents S.A.S. (Italy); penicillin/streptomycin/amphotericin B (450-115 EL) from Wisent Inc. (Canada); atropine (226680100) from Acros Organics (Belgium); mecamlamine (M202600) from Toronto Research Chemicals Inc. (Ontario, Canada); trypsin (25200056) Gibco, Thermo Fisher Scientific (Massachusetts, US); and FBS (Fetal Bovine Serum, S181G) Biowest (Nuaille, France). All agents used in the study were analytical grade.

Animals: In this study, 36 adult Wistar Albino male rats of 6 to 9 months of age, each weighing 250±20 g, were used and the animals were raised in Laboratory Animals Department of Sivas Cumhuriyet University. The study was conducted by the approval of Sivas Cumhuriyet University Animal Experiments Local Ethics Board (65202830-050.04.04-49 dated April 6, 2016).

Pharmacodynamics Studies:The tissues used in the study were extracted and hanged in the isolated organ bath in line with the methods specified by Çelebi and Doğan (2000) and Baydan et al. (2014) for urinary bladder, and by Estrade-Soto et al. (2012) for trachea tissue. Each isolated urinary bladder was placed in Krebs solution (in mM: NaCl 118; KCl 4.6; NaHCO₃ 25; MgSO₄ 1.2; KH₂PO₄ 1.2; CaCl₂ 2.5; glucose 10; EDTA 0.025; pH 7.4). Then, tissue samples were prepared in strip form and

placed in an isolated organ bath containing 5 ml Krebs solution at 37°C temperature and this mixture was constantly ventilated with a 95% O₂-5% CO₂ gas mixture (Çelebi and Doğan, 2000; Baydan et al., 2014). Each isolated trachea tissue was placed in Krebs solution (in mM: NaCl 118; KCl 4.6; NaHCO₃ 25; MgSO₄ 1.2; KH₂PO₄ 1.2; CaCl₂ 2.5; glucose 10; pH 7.4). The trachea rings were then hung in the isolated organ bath at 37°C with the help of stainless steel rings and in a continuously ventilated 5 ml krebs solution with 95% O₂-5% CO₂ gas mixture (Estrade-Soto et al., 2012). Isometric smooth muscle movements of the tissues were monitored and recorded using a "force transducer" (Force Displacement Transducer-FDT 05, Commat İletişim Ltd., Turkey) and an "acquisition system" (MP150 Biopac System, Commat İletişim Ltd., Turkey). The tissue samples were initially applied with a tension of 1,000 mg, and they were allowed to get used to the environment provided that they change the solution of krebs every 15 minutes for at least 1 hour. Once they reached equilibrium, the bladder and trachea tissues were stimulated using acetylcholine at EC₅₀ values (10⁻⁶ M, 10⁻⁵ M, respectively). After the contraction response, krebs solution in the baths was replaced and the protocols stated below were followed (Yıldırım, 2005; Estrada-Soto et al., 2012; Baydan et al., 2014; Vasconcelos et al., 2016).

1. Single and cumulative concentration of aucubin applications to urinary bladder and trachea tissues
2. Acetylcholine application to urinary bladder and trachea tissues after incubation in aucubin
3. Aucubin application to urinary bladder after incubation in atropine, verapamil and oxybutynin
4. Aucubin application to trachea tissue after incubation in atropine, carbachol and mecamlamine
5. 1mM CaCl₂ application to urinary bladder smooth muscle in non-calcium Krebs solution after incubation in aucubin and verapamil

Cell Culture Study:The study was carried out on primary cell lines, which were obtained from the lung and kidney tissues taken from the rats used in the study in accordance with the method reported by Freshney (2010). Using the stock solution, five different concentrations of aucubin were prepared (1, 5, 10, 20 and 50 µM) and applied to the cell lines (Hung et al., 2008). The aucubin-applied cells were left to be incubated for 24 hours. After incubation, cell viability and antioxidant enzyme activities were measured. Cell viability was established using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity analysis

(Noureini and Esmaili, 2014). The test kit for the measurement of antioxidant, catalase (CAT) and superoxide dismutase (SOD) enzyme activities was used in line with the protocol recommended by the kit's producer (Cayman Chemical Company, US).

Statistical Analyses

The results of the study are presented as mean and standard error of the mean (SEM). Contraction responses are expressed as affinity (pD_2), with the pD_2 value given as the negative logarithm of the molar agonist concentration that produces half of the maximum acetylcholine response ($pD_2 = -\log EC_{50}$). The maximum response to the active ingredient was calculated as the percentage of the maximum response produced by the acetylcholine (E_{max}). Mann-Whitney U test was utilized to assess the difference between the tissue samples. Statistical significance was set at $P < 0.05$. pD_2 , EC_{50} , E_{max} and IC_{50} values were calculated using GraphPad Prism (Version 8.2.0), while CAT and SOD results analyses and other statistical analyses were made using SPSS (Version 23).

Results

Pharmacodynamics Results

Single and cumulative concentration aucubin applications to urinary bladder and trachea tissues: Aucubin was observed to have under the baseline a relaxant effect on urinary bladder and trachea tissues in single and cumulative concentrations. In cumulative concentration applications to urinary bladder and trachea tissues, pD_2 and E_{max} values were 4.634 ± 0.03 and 102.8 ± 0.36 ; 5.118 ± 0.10 and 102.2 ± 2.33 , respectively. Based on the EC_{50} values obtained from single and cumulative concentration applications to the urinary bladder, the aucubin concentration 5×10^{-5} M was selected as the study concentration for the subsequent protocols as it produced a better response (Figure 1A). For trachea tissues, the study focused on cumulative concentration applications as these were revealed to produce a more pronounced results than those of single concentration.

Acetylcholine application to urinary bladder and trachea tissues after incubation in aucubin: The response produced by the aucubin concentration of 5×10^{-5} M (36.35%) was observed to increase (87.18%) with the application of acetylcholine ($P < 0.01$) (Figure 1B). Application of cumulative concentration (10^{-7} - 10^{-3} M) of aucubin to trachea tissues followed by acetylcholine (10^{-5} M) resulted in an increase in contraction response;

however, no correlation was observed between the concentration and response.

Aucubin application to urinary bladder after incubation in atropine, verapamil and oxybutynin:

The contraction created by aucubin (29.17%) on bladder showed a reduction in the presence of atropine (10^{-6} M) (17.88%), verapamil (10^{-7} M) (13.18%) and oxybutynin (10^{-8} M) (19.06%) ($P < 0.05$). Responses to acetylcholine (10^{-6} M) in the presence of the same antagonists showed a decline ($P < 0.01$) (Figure 1C).

Aucubin application to trachea tissue after incubation in atropine, carbachol and mecamlamine:

The contraction created on tracheal tissue (28.41%) by cumulative concentrations of aucubin (10^{-7} - 10^{-3} M) changed in the presence of atropine (10^{-6} M), carbachol (10^{-6} M) and mecamlamine (10^{-5} M) (responses: 27.36%, 31.16% and 27.65%, respectively) ($P > 0.05$). A statistically significant difference was observed between the responses to acetylcholine (10^{-5} M) in the presence of the same agents and aucubin responses ($P < 0.01$) (Figure 1D).

1mM CaCl₂ application to urinary bladder smooth muscle in non-calcium Krebs solution after incubation in aucubin and verapamil:

Aucubin response (tension difference: 36.5) was observed to increase (tension difference: 97.66) upon application of 1mM CaCl₂ following aucubin (5×10^{-5} M) and verapamil (10^{-7} M) incubation in non-calcium Krebs solution, with the increase taking the form of a stronger response ($P < 0.01$) (Figure 1E).

Cell Culture Results

MTT Results: Cell viability was shown to be at a maximum in cells treated with 10 μ M of aucubin, with further increases in concentration reducing cell viability (Figure 2A). Aucubin application revealed an IC_{50} value of 18.80 μ M in primary lung cells, and 17.63 μ M in primary kidney cells.

CAT and SOD Results: CAT levels showed a statistically significant increase as a result of application of aucubin in concentrations of 5, 10 μ M to primary lung cells ($P < 0.05$) and in concentrations of 1, 5, 10 μ M to primary kidney cells ($P < 0.05$, $P < 0.01$, $P < 0.05$) (Figure 2B); likewise, concentrations of 10 μ M in primary lung and kidney cells ($P < 0.05$) increased SOD levels significantly compared to control (Figure 2C).

Discussion and Conclusions

Since the earliest times, humans have been using plants as food as well as medicine for a variety of diseases and conditions. It is necessary to identify the local names, active ingredients, action mechanisms and usage indications of the plants and

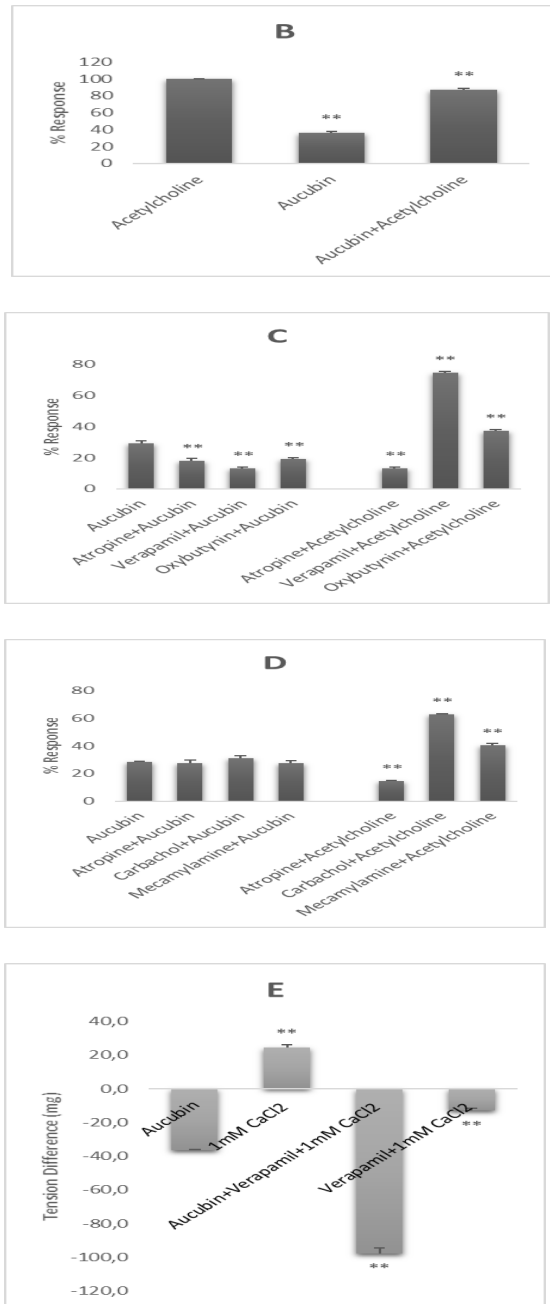


Figure 1. Single concentration relaxing responses to aucubin in bladder (A), acetylcholine (10^{-6}) relaxing response on single-concentration application of aucubin to bladder (B), agonist/antagonist aucubin, agonist/antagonist acetylcholine relaxing responses in bladder (C) and in trachea tissue (D), 1 mM $CaCl_2$ relaxing responses in bladder after aucubin and verapamil (10^{-7} M) incubation in non-calcium krebs solution (E) (** $P<0.01$).

herbs used in traditional medicine to ensure their proper, efficient and sufficient use (Akan and Bakir Sade, 2015).

This study aims to explore the pharmacodynamics of aucubin, the active ingredient in plantain, an herb used in folk medicine, on *in vitro* rat bladder and trachea tissues as well as its cell protective effects on primary lung

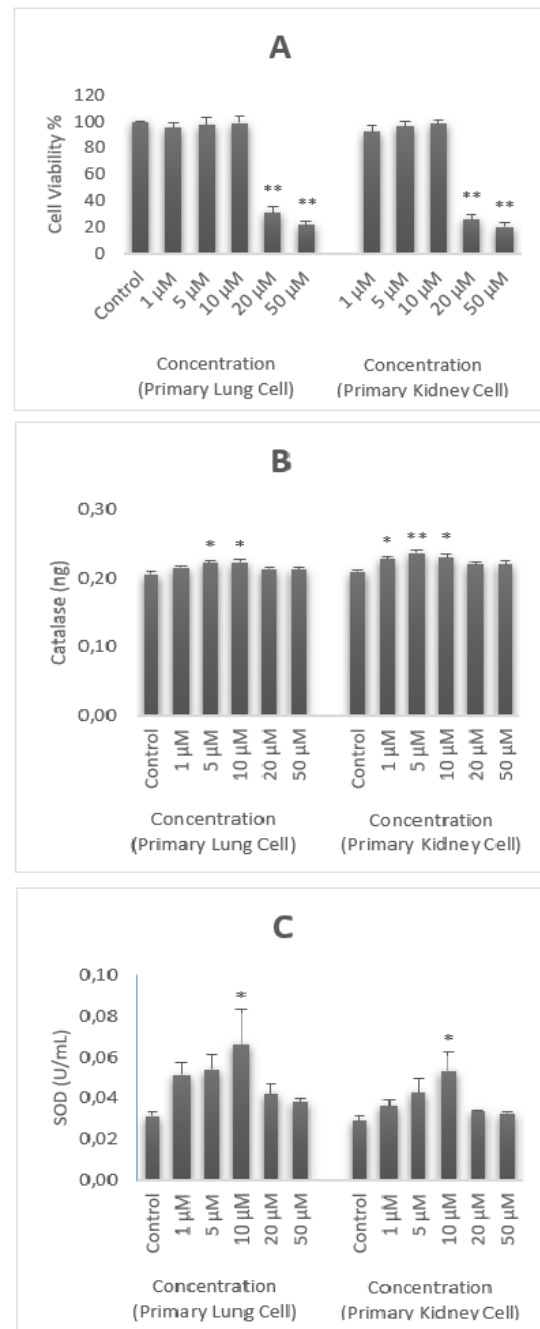


Figure 2. Cell viability % values of primary lung and kidney cells treated with aucubin (A), catalase (B) and SOD values of primary lung and kidney cells treated with aucubin (C) (* $P<0.05$; ** $P<0.01$).

and kidney cell lines. The results of this study showed that aucubin had a relaxant effect on urinary bladder and trachea tissues (Figure 1A). In a study, Fler and Verspohl (2007) assayed the antispasmodic effects of *P. lanceolata* L. and certain compounds isolated from its extract (aucubin, luteolin, acteoside) on guinea pig ileum and trachea tissues. According to their results, the extract reduces tracheal contractions induced by barium

ions and has an antispasmodic effect on ileum contractions induced by agonists such as acetylcholine, histamine, potassium and barium ions, with aucubin suppressing the acetylcholine-induced impulse. This study revealed that aucubin produces a lower response than acetylcholine. But, after aucubin treatment, percentage of response (%) increased with the application of acetylcholine (Figure 1B). These results were consistent with previous studies. Plantain's antispasmodic effects have been reported in other studies, with recent ones also documenting a bronchiolytic activity (Fleer and Versphol, 2007). Studies associated these effects with the tannins, flavonoids, unsaturated sterols/triterpenes, carbohydrates, lactones and proteins/aminoacids contained plantain (Atta and Mouneir, 2005). This study has revealed that atropine, verapamil and oxybutynin increased the relaxant effect of aucubin on urinary bladder ($P<0.05$) (Figure 1C). On the other hand, it was shown that the relaxant effect produced on trachea tissue by cumulative concentrations of aucubin (10^{-7} - 10^{-3} M) increased slightly in the presence of atropine and mecamlamine, but decreased in the presence of carbachol (Figure 1D). Aucubin response was observed to increase upon application of 1mM $CaCl_2$ following verapamil incubation in non-calcium krebs solution, with the increase taking the form of a stronger response (Figure 1E). In a study by Atta and Mouneir (2005) who explored the effect of extracts of *P. major* L. and seven other herbs on isolated rabbit duodenum tissue, it was reported that *P. major* L. extract inhibited duodenum movement after a brief impulse, and that it had a partial anti-diarrheal impact.

There are several studies that evaluate the effect of aucubin on various cancer cell lines, compared to a relatively limited number of studies that assess its effect of primary cell lines. Aucubin is reported to contribute to antiproliferative activity, alter antioxidative capacity and have a beneficial effect on pathological changes brought about by reactive oxygen species (Hung et al., 2008; Xue et al., 2011; Xue et al., 2012). In a study assessed the effects of aucubin on apoptosis induced by H_2O_2 on rat pheochromocytoma (PC12) cell lines by regulating endogenous oxidant-antioxidant balance, results indicated aucubin's potential use as a protective agent in the treatment of oxidative stress-caused neurodegenerative diseases (Xue et al., 2012). This study revealed that aucubin application in different concentrations to primary lung and kidney cells produced a concentration-based change in CAT and SOD levels in comparison to the control group (Figure 2B, 2C).

Kim et al. (2014) reported that aucubin facilitated neural differentiation and neurite growth in neural stem cell culture obtained from rat primary embryonic hippocampus, as well as axonal outgrowth in peripheral nervous system injuries. As a result, the study noted aucubin's potential therapeutic use for neural regeneration in the treatment of various neural injuries. In this study, cell viability was at its maximum in primary lung and kidney cells treated with 10 μ M of aucubin (99.09% and 98.38%, respectively) (Figure 2A). However, further increases in concentration are reported to reduce cell viability and increase cytotoxic effect.

The results of this study revealed that aucubin, an active ingredient in plantain, had a relaxant effect on urinary bladder and trachea tissues among others. Aucubin was also shown to reduce the effect of acetylcholine, and to be affected in varying degrees by all antagonists. This study also conceives that aucubin acts as a cholinergic antagonist through different subreceptors (muscarinic-3 receptors) due to its interaction with oxybutynin. In addition, the study also revealed that aucubin caused a statistically insignificant increase in remaining at the level of the control group in cell viability in the primary lung and kidney cell lines at increasing concentrations (1-10 μ M) ($P>0.05$), while causing a significant decrease in cell viability at 20 and 50 μ M concentrations in the same cell lines ($P<0.01$) (Figure 2A). In conclusion, it is suggested that plantain, an herb used in folk medicine, and its active ingredient aucubin might have beneficial effects on bronchoconstriction and other respiratory conditions as well as pulmonary and renal diseases, urinary incontinence, etc.

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