In vivo hemodynamic and electrocardiographic changes following *Crataegus aronia syn. Azarolus (L)* administration to normotensive Wistar rats

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ABSTRACT

الأهداف: دراسة تأثير الخلاصة المائية لكامل نبات الزعرور على الوظائف الميكانيكية لعمل القلب (سرعة انقباض القلب والضغط الشرياني) وعلى فترات التخطيط القلبي الكهربائي في الجرذان.

الطريقة: أجريت هذه الدراسة في معامل أبحاث قسم علم وظائف الأعضاء بكلية الطب، جامعة الملك خالد، أبها، المملكة العربية السعودية خلال الفترة من فبراير حتى يونيو 2012م، وقد تم تقسيم البحث إلى مرحلتين في المرحلة الأولى تمت دراسة تأثير هذه الخلاصة على ضغط الدم الشرياني وسرعة انقباض القلب وتخطيط القلب الكهربائي وفي المرحلة الثانية تمت دراسة هذه الآلية التي من خلالها يؤثر هذا النبات على ضغط الدم الدم الشرياني ونبض القلب.

النتائج: أظهرت نتائج المرحلة الاولى أن الحقن الوريدي للخلاصة المائية لنبات الزعرور (بتركيز 0.05– 20 ميكروغرام/كغم) تؤدي إلى نقصان ملحوظ في سرعة انقباض القلب وضغط الدم الشرياني وهذا النقصان يتناسب طردياً مع زيادة الجرعة المعطاة ولكن الجرعات العالية (تركيز 15– 20 ميكروغرام/كغم) أدت إلى نقص شديد في سرعة انقباض القلب وضغط الدم الشرياني وأظهر تخطيط القلب وجود انغلاق متدرج في انتقال السيال الكهربائي ما بين الأذين والبطين مع هذه الجرعات العالية فقط بينما لم يظهر أي تغير على فترات موجات تخطيط القلب الكهربائي مع جميع الجرعات الأخرى المستخدمة. كما أظهرت نتائج المرحلة الثانية أن آلية عمل هذه الخلاصة لتخفيف سرعة انتقباض القلب تتم عبر تحفيز المستقبلات المسكرينيكية (نوع م2) وعلى تثبيط مستقبلات بيتا الموجودة في النسيج القلبي بينما تقوم هذه الخلاصة بزيادة بناء وافراز اوكسيد النيتريت لتخفيف ضغط الدم الشرياني.

خاتمة: الحقن الوريدي للخلاصة المائية لنبات الزعرور في الجرذان يؤدي إلى تخفيض سرعات انقباض القلب وضغط الدم الشرياني من دون حدوث أي تغير على فترات موجات تخطيط القلب الكهربائي.

Objectives: To evaluate the effects of the whole plant aqueous extract of *Crataegus aronia (C. aronia) syn. Azarolus (L)* on the hemodynamic and electrocardiographic intervals in albino rats.

Methods: This study was carried out in 2 stages at the Research Laboratory, Physiology Department, Medical College of King Khalid University, Abha, Kingdom of Saudi Arabia between February and June 2012. First, the effects of *C. aronia syn. Azarolus (L)* on the hemodynamics and electrocardiograph in 54 Wistar male rats were assessed, then the mechanisms underlying the hemodynamic and electrocardiographic changes observed in the first stage were evaluated in 48 rats of the same species.

Results: The *C. aronia* administered at escalating doses (0.05-20 µg/kg) produced a dose-time-dependent decrease in heart rate (HR) and mean arterial pressure (MAP). Higher doses (15 and 20 µg/kg) produced the most significant reduction in both HR and MAP, and induced sinus node suppression and progressive atrio-ventricular blockade. The underlying mechanism of the induced bradyarrhythmia appeared to be due to the direct stimulation of the muscarinic receptor M2 and possible blockade of beta-receptors, while the hypotension was caused by enhanced nitric oxide release. No significant alterations in the electrocardiogram (ECG) components were observed.

Conclusion: The administration of the *C. aronia syn. Azarolus* extract induced bradyarrhythmia and hypotension, without alteration in the ECG components.

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Cardiovascular diseases comprise the major cause of morbidity and mortality in the world, with an overall prevalence of approximately 34% in the USA.[1] Identifying novel drugs to prevent and/or treat cardiovascular disorders is therefore of critical importance for improving general health and longevity. New drug discovery has increased tremendously in the past decades, and many drugs are approved for clinical use annually.[1] Extensive efforts are ongoing to develop novel cardiotonic agents that would replace currently available cardiovascular drugs that are associated with serious adverse effects.[1] Over the years, plants have been an indispensable component in the research and development of new cardiovascular drugs.[2] Hawthorn (genus *Crataegus*) plant extracts have been used by many cultures for a variety of therapeutic purposes for many centuries, and are considered amongst the most potentially valuable remedies for cardiovascular diseases found in the plant kingdom.[3] Although more than 200 species of hawthorn are found worldwide, only a few have been tested and used medically for treating cardiovascular diseases, including *Crataegus (C) oxycantha, C. laevigata, C. monogyna, C. orientalis,* and *C. pinnatifida*.[4] Apart from the reported potent antioxidant effects of various hawthorn species, several studies have demonstrated that extracts from some of these species increase myocardial contraction and dilate peripheral and coronary blood vessels; these findings indicate the possibility of the use of these plant extracts in the treatment of systemic hypertension, early stages of heart failure, and angina pectoris.[5-7] Furthermore, the potential anti-arrhythmic effects of various hawthorn species have been reported.[4,8,9] However, through our search in literature, we could not find any single study evaluating the effects of hawthorn on electrocardiographic (ECG) components with a focus on QT intervals, which often limit the use of several known anti-arrhythmic agents.[10]

The *C aronia syn. Azarolus (L)*, the species predominantly found in the mountains of the Mediterranean basin, has not been scientifically examined in detail.[11,12] In Arabic traditional medicine, Jordanians and Palestinians use *C. aronia* to treat cardiovascular diseases, cancer, diabetes, hyperlipidemia, and sexual weakness.[11-13] In a recent report by our laboratory, oral administration of aqueous extract of *C. aronia syn. Azarolus (L)* to albino rats was demonstrated to have an anti-coagulant effect, and prolongs the bleeding time via the inhibition of thromboxane B2 synthesis. In this study, there was no acute or subacute toxic effects on blood counts, liver, or kidney biochemical function tests.[14,15] The *C. aronia* has been claimed to be useful in various pathological conditions with extensive unsupervised use. Indeed, *C. aronia* is available in the herbal market in the Kingdom of Saudi Arabia (KSA), and used over the counter without restrictions; therefore, it is essential to study the possible cardiotoxic effects of this plant, which have thus far not been assessed. In the cardiotoxicity assessment of an unknown drug, the first step comprises the assessment of the effect of the drug on cardiac electrophysiology, including all physiological properties such as chronotropy (heart rate [HR]), dromotropy [P, PR, and QRS duration]), QTc interval, and irritability (spontaneous or provoked ectopia).[16,17] Therefore, this study was designed and conducted in 2 stages as follows: Stage 1 - to study the electrocardiographic and hemodynamic effects induced by intravenous (IV) administration of different doses of *C. aronia* in albino rats; and Stage 2 - to study the possible mechanisms underlying these effects in an anesthetized rat model.

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Methods. This study was performed between February and June 2012 at the Research Laboratory, Physiology Department, Medical College of King Khalid University, Abha, KSA. All procedures were approved by the Ethical Committee of the College of Medicine, King Khalid University (REC-2011-05-01), and were performed in agreement with the Principles of Laboratory Animal Care, advocated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.[18]

Chemicals and drugs. Urethane, heparin, hexamethonium bromide, atropine sulphate, adrenaline, ephedrine and N-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co., (St. Louis, MO, USA). All drugs were prepared freshly before use in double-distilled de-ionised (DDD) water according to these concentrations: urethane - 1.5 g/kg; heparin - 50 U/mL; atropine sulphate - 2 mg/kg; hexamethonium bromide - 20 mg/kg, ephedrine - (one mg/kg); and L-NAME - 20 mg/kg. All these drugs were administered intravenously except for urethane, which was administered intraperitoneally.

Preparation of the extract. Fresh *C. aronia* whole plant (stems, leaves, and flowers) was purchased from a

local market in Jordan. The plant was identified, dried, and extracted in the Department of Pharmacognosy, College of Pharmacy, King Khalid University, KSA. The dried plant material was ground to a powder, and extracted by maceration using distilled water (one kg/L, w/v) for 3 days at 37°C. The extract was filtered and evaporated under reduced pressure in a rotary evaporator. The resulting residue (40 g) was then stored at 4°C. The residue was re-constituted in DDD water, and filtered through 0.2-μM filters to obtain a stock solution of a strength of one mg/mL, which was diluted further to obtain the concentrations required in our study.[15]

Animals. A total of 102 male, 7-weeks-old Wistar albino rats, weighing 200-250 gm were used in the 2 stages of this study. All animals were housed in polypropylene cages in a room maintained at 28-32°C. A 12-hour light-dark cycle was maintained, and the animals were fed with normal rat feed, with water given ad libitum.

Experimental procedure. Stage 1. Hemodynamics and electrocardiographic changes with different doses of C. aronia. A total of 54 rats were used in this study. The rats were divided into 9 groups, with one control (n=6), and 8 experimental groups (n=6 each) treated with different doses of *C. aronia* aqueous extract. For all groups of rats, invasive right carotid blood pressure and surface electrocardiogram (ECG) were recorded before, and after IV administration of DDD water as vehicle (control group), or IV administration of a single dose of extract (0.05, 0.1, 1, 3, 6, 12, 15 and 20 μg/kg). The final volume of the vehicle or extract administered to rats was 0.2 mL. In brief, the rats were anesthetized with urethane (1.5 g/kg, ip), and an intra-tracheal probe coupled to an artificial ventilator (Harvard Rodent Ventilator 683, Harvard Apparatus, South Natick, MA, USA) was placed, and the respiratory volume and rate were subsequently adjusted to keep blood gases and pH within the normal range. Subsequently, the right carotid artery was located, prepared, and cannulated using a polyethylene catheter, and attached to a fluid-filled pressure transducer (MLT0670, AD Instruments, Sydney, Australia) connected to a pre-calibrated bridge amplifier (FE117 BP Amp, AD Instruments, Sydney, Australia). The arterial line was pre-filled with heparin (50 U/mL). The time required for the surgical procedure and preparing the carotid artery for cannulation was approximately 5 minutes. Simultaneously, the ECG was recorded using 3 touch electrodes (MLA1214, AD Instruments, Sydney, Australia) connected to an animal bio-amplifier (FE136 Animal Bio Amp, AD Instruments, Australia) attached to the skin of the animals in the standard 3 positions. The rat's tail was also cannulated for IV administration of the vehicle or extract. The rats' body temperature was maintained at 38±1°C throughout the procedure using an electrical heater blanket. After stabilization of the cardiovascular parameters, the systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), mean arterial blood pressure (MAP), heart rate (HR), and ECG were recorded for 10 minutes before the experiment (baseline values, 0.0 reading), and then for 60 minutes consecutively after IV administration of the selected dose of *C. aronia* extract (*C. aronia*-treated groups) using the PowerLab data acquisition system (PL3516/P PowerLab 16/35, AD Instruments, Sydney, Australia). Data were analyzed by Labchart Pro 7.2 software (AD Instruments, Sydney, Australia). Similar recordings were obtained separately after IV administration of the DDD water (control group).The data were sampled at a frequency of 500 Hz. Later, the recorded ECG was used to calculate HR, QRS, PR, and QT intervals with the help of the same software. Bazett's equation was selected to calculate the QTc intervals.

Stage 2. Analysis of the mechanism of action of C. aronia underlying the changes in MBP and HR. The following series of experiments were performed to assess the possible in vivo mechanisms underlying the hemodynamic changes (MAP and HR) observed in the previous experiment (Stage 1). The experimental procedure and the in vivo recording of HR and MAP were conducted as previously described using rats from the same bred colony in our animal house, and with a similar weight as previously used in stage 1. To assess the possible roles of the cholinergic and adrenergic pathways (autonomic nervous system) and nitric oxide (NO) in the genesis of bradycardia and hypotension induced by *C. aronia*, changes in MAP and HR were recorded in 8 groups of rats (n=6 each; 48 rats in total) treated with *C. aronia* at its most effective and quickest acting dose (20 μg/kg) using the following protocols: Group 1 (Control group) - administered *C. aronia* (20 μg/kg) alone; Group 2 - administered atropine sulphate (2 mg/kg) alone;[19] Group 3 - administered atropine sulphate (2 mg/kg) followed by *C. aronia* (20 μg/kg); Group 4 - administered hexamethonium bromide (20 mg/kg) alone;[19] Group 5 - administered hexamethonium bromide (20 mg/kg) followed by *C. aronia* (20 μg/kg); Group 6 - administered a combined dose of hexamethonium bromide (20 mg/kg) with L-NAME (20 mg/kg),[19] followed by *C. aronia* (20 μg/kg); Group 7 - administered atropine sulphate (2 mg/kg) with *C. aronia* (20 μg/kg) followed by adrenaline (250 μg/kg);[20] and Group 8 - administered

atropine sulphate (2 mg/kg,) with *C. aronia* (20 µg/kg) followed by ephedrine (one mg/kg). Each treatment was administered intravenously through the rat's tail in a final total volume of 0.2 mL. The *C. aronia* was administered to the experimental groups after ensuring that the effect of the antagonist/blocking agent was achieved by monitoring the hemodynamics. In all the experimental groups, the changes in MAP and HR after *C. aronia* administration were recorded for 30 minutes consecutively.

Calculations. The percentage of changes in MAP, HR, PR, QRS and QTc at any given time interval after administration of the extract in both stages 1 and 2 were calculated using the following equation: (mean value of a parameter at given interval - mean value of the same parameter at baseline)/(mean value of the same parameter at baseline)

Statistical analysis. For stage 1 of the study, the mean percentage changes in MAP, HR, PR, QRS, and QTc at 10-minutes intervals in the experimental groups were compared with each other within each group and with the corresponding time intervals of other groups. For stage II of the study, the mean percentage changes in MAP and HR in the different experimental groups recorded within 30 minutes were compared with each other and with the control group. Plotting of the graph and comparisons were performed using a 2-way ANOVA analysis for data of stage one, and by one-way ANOVA test using GraphPad Prism (version 5) followed by Post Hock Tukey's t-test to determine the statistical significance. Data are expressed as mean ± SEM, and statistical significance was assigned at the $p \leq 0.05$ level (95% of confidence interval)

Results. All the study data are depicted in **Figures 1-6**. Changes in the MAP and HR in the control and experimental groups treated with selected dose of the aqueous extract of *C. aronia* are shown in **Figure 1** & **Figure 2**. The ECG analysis and plots for mean values of PR, QRS, and QTc versus time (minutes) for all groups are shown in **Figure 1** and **Figure 3**. A dose of *C. aronia* at 20 µg/kg was lethal after 3-5 minutes, and also a dose of 15 µg/kg after 15-20 minutes. Therefore, the data for the rats that received 20 µg/kg of *C. aronia* are shown only in **Figures 5** and **6**.

Blood pressure and ECG alterations in DDD water-treated rats. The IV administration of DDD water did not affect the MAP, HR, PR, QRS or QTc intervals during any of the time intervals of the study (**Figures 1-3**). The baseline readings of the parameters studied among the rats administered different doses of *C. aronia* were not compared with the rats administered with DDD water due to baseline variations for each parameter measured among the rats.

Stage 1 (Hemodynamic and electrocardiographic changes). Effect of C. aronia on MAP. **Figure 1 (A-H)** shows the influence of the administration of the aqueous extract of *C. aronia* on the MAP of all groups of rats. **Figure 2B** shows the corresponding calculated percentage changes in MAP. Escalating doses of the extract, ranging from 0.05-20 µg/kg, were used in identical experimental conditions. Administration of the extract had no effect on MAP at the lowest dose (0.05 µg/kg) at all minutes interval ($p>0.9999$) of the study (**Figures 1B and 2B**). The graphs shows that the administration of *C. aronia* at doses ranging from 0.1-12 µg/kg produced a dose-time-dependent decreases in MAP without resulting in the death of any tested animal for the study period (60 minutes) (**Figures 1C-E and Figure 2B**). However, the hypotensive responses to the higher doses of *C. aronia* (12 µg/kg) at all time intervals were significantly greater ($p<0.05$) than those recorded for all the previous doses (58.1±3.87), with the highest reduction occurring at minute 60 ($p<0.0032$) (66±2.2%) (**Figure 1G and Figure 2B**). High doses of the extract (15 and 20 µg/mL) produced the most significant ($p=0.0012$) and fastest decline in MAP as compared to the previous lower doses with subsequent death of all the animals after 15-20 and 3-5 minutes (**Figure 1H and Figure 2B**).

Effect of C. aronia on HR. A low dose of *C. aronia* (0.05 µg/kg) did not cause any significant changes in HR at any of the time intervals as compared to the baseline reading ($p>0.9999$), or the control group ($p>0.9999$) (**Figure 2A**). The HR decreased progressively and significantly in a dose-time-dependent manner among most time intervals with all the other doses (0.1, 1, 3, 12, and 15, except for the dose of 6 µg/kg; the 6 µg/kg dose caused a significant decrease in HR only at minute 60, being significantly different as compared to the same time interval in the control group ($p=0.0019$) but not significantly different when compared to the lower doses (0.1 [p=0.1798], 1 [p=0.5877], and 3 µg/kg [p=0.9321]) at the same minute interval. In contrast, when compared to the baseline values, a significant dose-dependent decrease in HR was observed after 20 ($p=0.0499$), 30 ($p=0.0343$), 40 ($p=0.0313$), 50 ($p=0.031$), and 60 minutes ($p=0.010$) for the dose of 0.1 µg/kg; for 1 µg/kg (20 [$p=0.0421$], 30 [$p=0.0443$], 40 [$p=0.0413$], 50 [$p=0.0302$], and 60 minutes [$p=0.009$]); and 3 µg/kg (20 [$p=0.0371$], 30 [$p=0.0343$], 40 [$p=0.0306$], 50 [$p=0.0202$], and 60 [$p=0.0031$]) with the maximum decrease observed at a dose of 3 µg/kg (**Figure 2A**). As compared to the controls, the percentage changes for these doses were

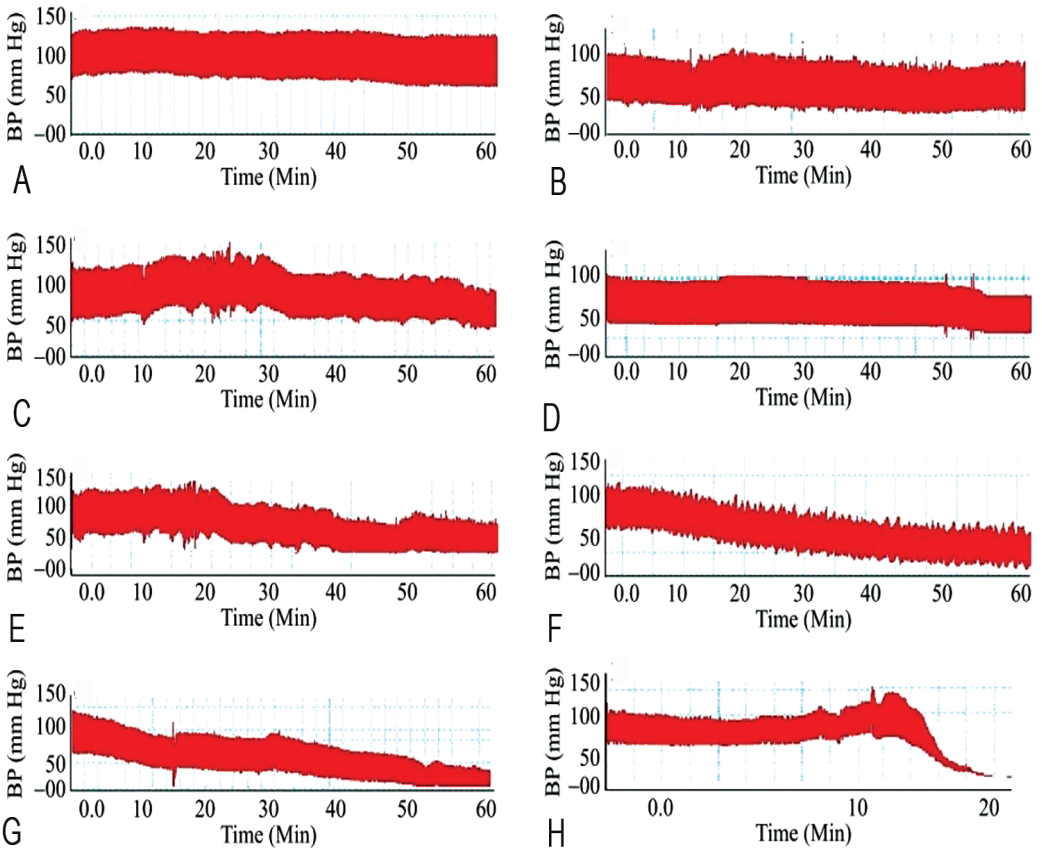

Figure 1 - Arterial blood pressure recording from the control (**A**) and *Crataegus aronia (C. aronia)*-treated groups (**B-H**). The signals from the blood pressure bridge amplifier were filtered and amplified and sent to an analogue-to-digital converter (Power Lab data acquisition and analysis system (AD Instruments, Sydney, Australia). Data were recorded and analyzed using Labchart Pro 7 software (AD Instruments, Sydney, Australia). B - 0.05, C - 0.1, D - 1.0, E - 3, F - 6, G - 12, and H - 15 µg/kg of *C. aronia* aqueous extract.

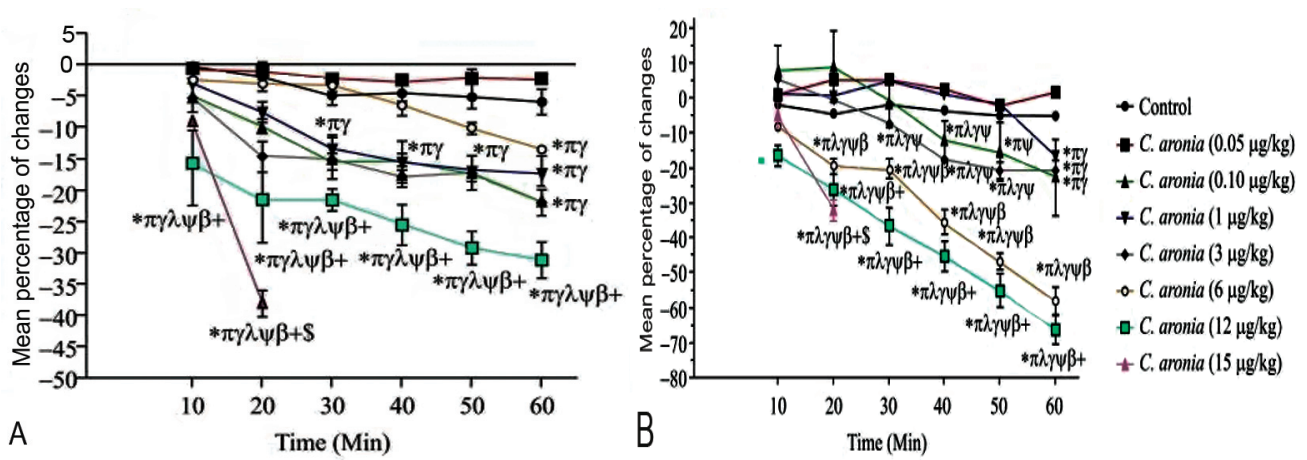

Figure 2 - Mean percentage of change in the heart rate (**A**) and mean arterial pressure (**B**) in the control and all experimental groups of rats. Values are expressed as mean ± SEM for 6 rats in each group. Analysis was performed using one-way ANOVA and Tukey's t-test. Values were considered statistically significant at $p<0.05$. *Significantly different when compared to the baseline value. [π]Significantly different when compared to the control group at the same time interval. [γ]Significantly different when compared to the 0.05 µg/kg dose group at the same time interval. [λ]Significantly different when compared to the 0.1 µg/kg dose group at the same time interval. [ψ]Significantly different when compared to the 1 µg/kg dose group at the same time interval. [β]Significantly different when compared to the 3 time interval. [+]Significantly different when compared to the 6 µg/kg dose group at the same time interval. [$]Significantly different when compared to the 12 µg/kg dose group at the same time interval.

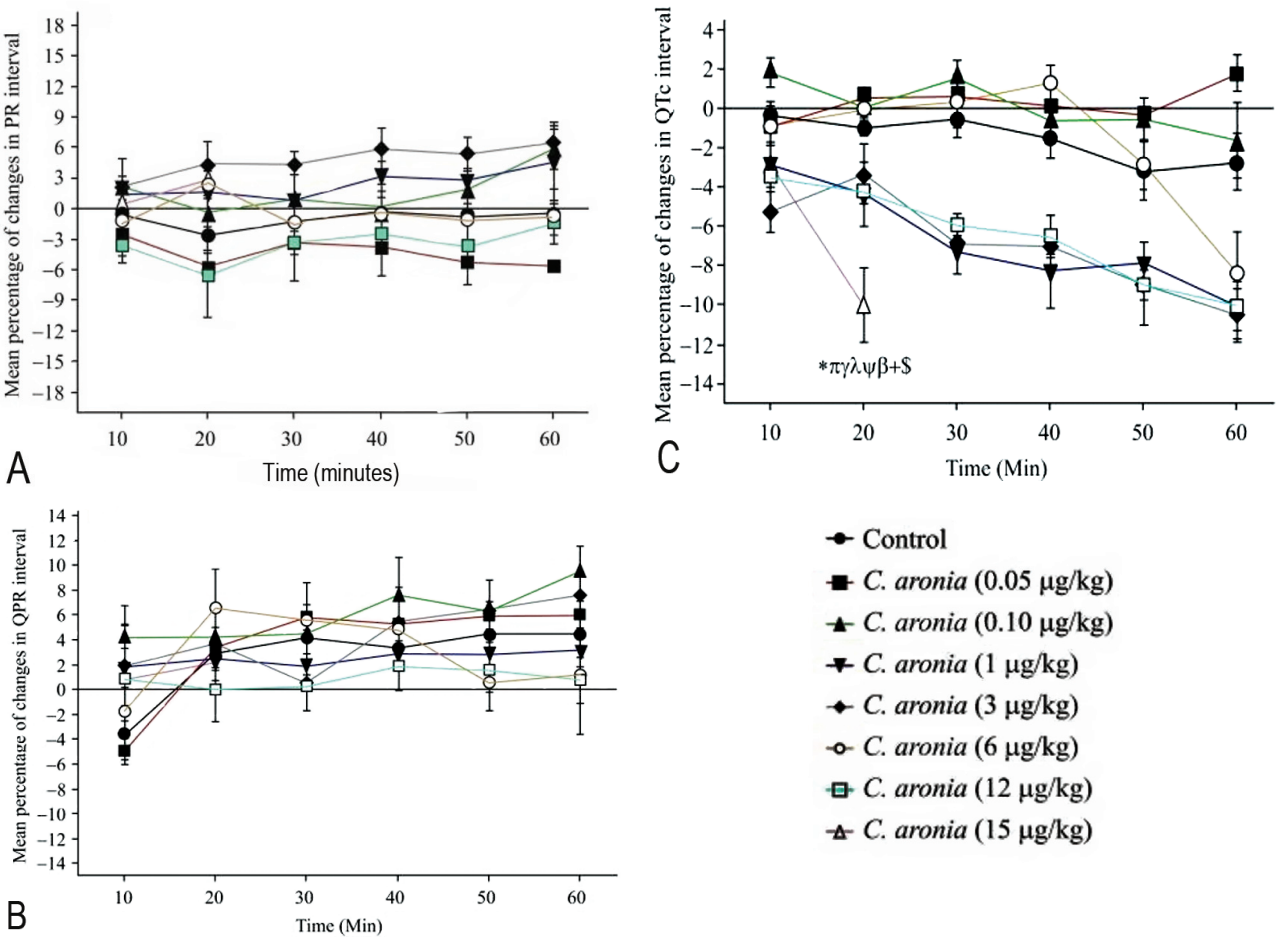

Figure 3 - Mean percent of changes in PR (**A**), QRS (**B**) and QTc (**C**) intervals in the control and all experimental groups of rats. Values are expressed as mean ± SEM for 6 rats in each group. Analysis by one-way ANOVA and Tukey's t-test. Values were considered significantly different at $p<0.05$. *Significantly different when compared to the baseline value. [π]Significantly different when compared to the control group at the same time interval. [γ]Significantly different when compared to the 0.05 µg/kg dose group at the same time interval. [λ]Significantly different when compared to the 0.1 µg/kg dose group at the same time interval. [Ψ]Significantly different when compared to one µg/kg dose group at the same time interval. [β]Significantly different when compared to the 3 µg/kg dose group at the same time interval. [+]Significantly different when compared to the 6 µg/kg dose group at the same time interval. [$]Significantly different when compared to the 12 µg/kg dose group at the same time interval. [&]Significantly different when compared to the 15 µg/kg dose group at the same time interval. *Crataegus aronia - C. aronia*

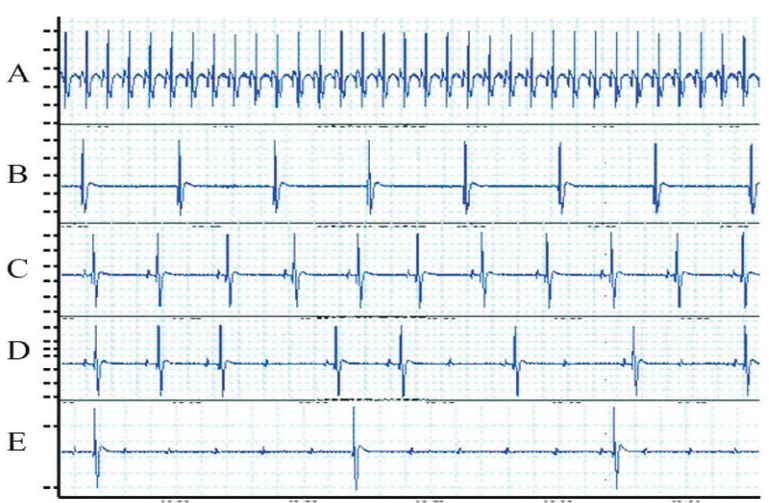

Figure 4 - A representational ECG recording after the administration of *Crataegus aronia (C. aronia)* 15 µg/kg: **A**) Baseline recording with heart rate (HR) of 357.5 bpm and mean PR: 47.5 ms; **B**) Recording with junctional rhythm; **C**) Recording during first-degree block, mean HR: 121.9 bpm and PR: 73.6 ms; **D**) Recording with type 1 second-degree block followed by type 2 second-degree block; **E**) Recording of third-degree block.

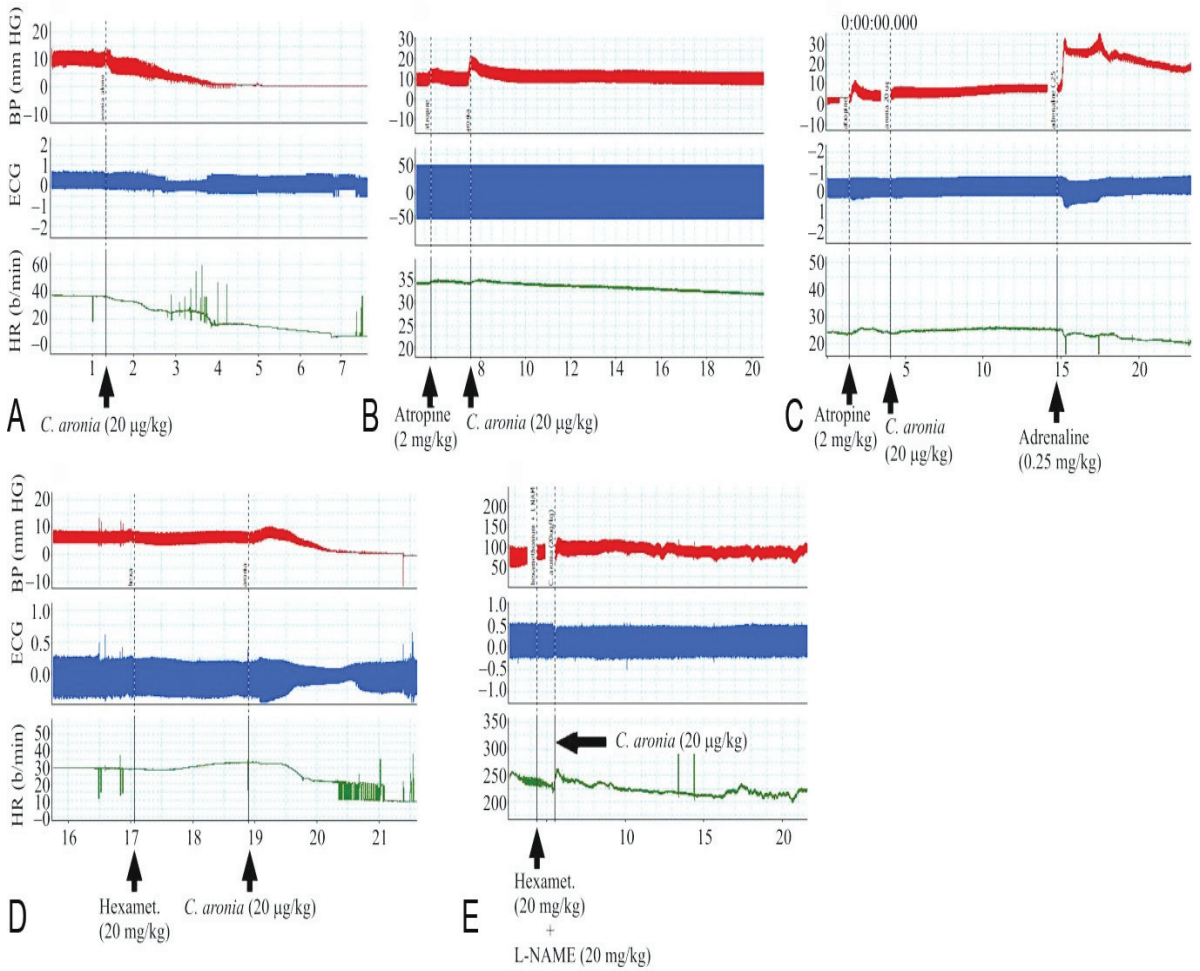

Figure 5 - Blood pressure (BP), electrocardiograph (ECG), and heart rate (HR) recordings for the mechanism Stage II study. The signals from the blood pressure bridge amplifier were filtered and amplified and sent to an analogue-to-digital converter (Power Lab data acquisition and analysis system [AD Instruments, Sydney, Australia]). Data were recorded and analyzed by Labchart Pro 7 software (AD Instruments, Sydney, Australia): **A**) *Crataegus aronia (C. aronia)* alone (20 µg/kg); **B**) *C. aronia* (20 µg/kg) and Atropine (2 mg/kg); **C**) *C. aronia* (20 µg/kg) then Atropine (2 mg/kg) and then Adrenaline (0.25 mg/kg); **D**) hexamethonium (20 mg/kg) then *C. aronia* (20 µg/kg); and **E**) hexamethonium (20 mg/kg) N-nitro-L-arginine methyl ester (L-NAME) (20 mg/kg) then *C. aronia* (20 µg/kg).

significantly different only at 40, 50, and 60 minutes after administration of the extract (for 0.1 µg/kg dose: at 40 (p=0.0081); 50 (p=0.0076) and 60 minutes (p=0.0034); for 1 µg/kg dose: at 40 [p=0.029]; 50 [p=0.0031]; and 60 minutes [p=0.0019]; and for 3 µg/kg: 40 [p=0.0029], 50 [p=0.0029]; and 60 [p=0.0013]). The ANOVA test revealed no significant differences in the percentage of decrease in the HR between these doses at the corresponding time intervals. However, the maximum percentage of decrease in HR at all time intervals of the study was observed at a dose of 12 µg/kg as compared with other doses and controls. The highest dose (15 µg/kg) resulted in a maximum decrease in the HR at 20 minutes after extract administration with a percentage decrease of 38±2.1% (**Figure 2A**).

Effect of C. aronia on ECG indices. During all periods of the study and for all the doses tested except 15 µg/kg, the QTc, QRS, and PR intervals were not significantly different within each group administered *C. aronia* at any dose (from the baseline reading), or between the groups (as percentage of change) (p>0.05) (**Figure 3**). Moreover, the percentages of changes in these parameters during all time intervals of the experiment were similar for inter-group comparisons (**Figure 3**). The QTc was significantly shortened (p=0.0498) only at minute 20 (just before death) after administration of *C. aronia* at a dose of 15 µg/kg, with a percentage decrease of 10.2±1.34% (**Figure 3**). Analysis of the ECG recordings from the anesthetized rats revealed that the reduction in HR at all doses, except for the 0.05 µg/

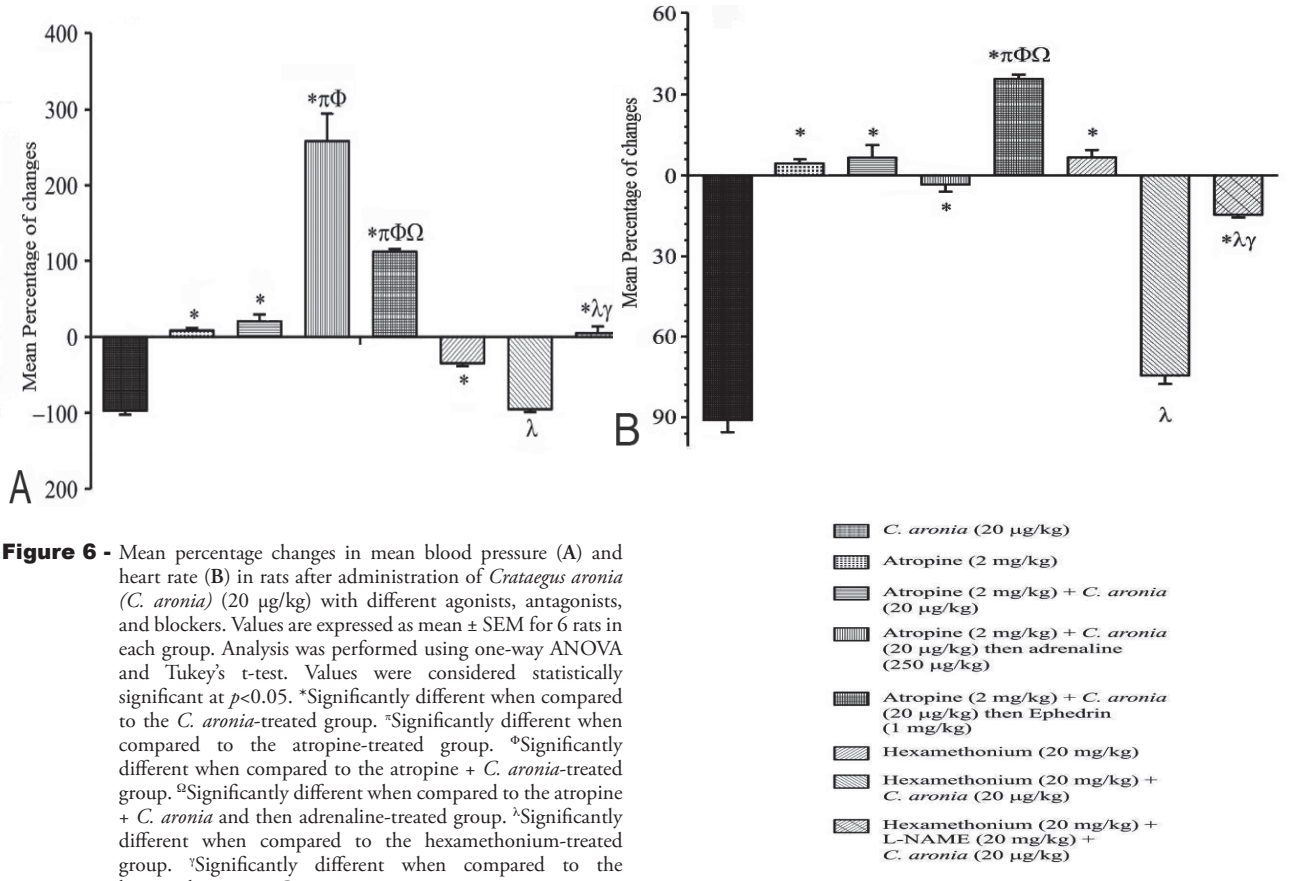

Figure 6 - Mean percentage changes in mean blood pressure (**A**) and heart rate (**B**) in rats after administration of *Crataegus aronia (C. aronia)* (20 µg/kg) with different agonists, antagonists, and blockers. Values are expressed as mean ± SEM for 6 rats in each group. Analysis was performed using one-way ANOVA and Tukey's t-test. Values were considered statistically significant at $p<0.05$. *Significantly different when compared to the *C. aronia*-treated group. $^{\pi}$Significantly different when compared to the atropine-treated group. $^{\Phi}$Significantly different when compared to the atropine + *C. aronia*-treated group. $^{\Omega}$Significantly different when compared to the atropine + *C. aronia* and then adrenaline-treated group. $^{\lambda}$Significantly different when compared to the hexamethonium-treated group. $^{\gamma}$Significantly different when compared to the hexamethonium + *C. aronia* group.

kg dose, were due to sinus bradycardia; the dose of 15 µg/kg initially caused sinus bradycardia followed by junctional escape rhythm, followed by an increase in PR intervals (first-degree atrioventricular block (AVB), which progressed to a higher degree of AVB: from type I second-degree AVB to type II second-degree AVB, and finally to a complete heart block, eventually leading to death (**Figure** 4). Similar ECG changes were observed with the highest dose used (20 µg/kg), however, the progression was more dramatic, with early death of all the studied rats (3-5 minutes).

Stage 2 (Mechanism of action). Intravenous administration of *C. aronia* alone at its highest dose (20 µg/kg) produced significant rapid lethal decreases ($p<0.001$) in both MAP (-96.4±2.34%) and HR (-90.2 ± 4.23%) (**Figure 5A** and **Figures 6A & 6B**). The administration of atropine sulphate alone (2 mg/kg, IV) resulted in a significant increase in the baseline MAP (+7.97±0.75%, $p=0.0423$), and HR (+3.83±1.47%, $p=0.0478$) (**Figure 5B** and **Figures 6A & 6B**). As expected, the administration of hexamethonium bromide alone (20 mg/kg, IV) induced significant ($p=0.0093$) decreases in the baseline MAP (-33.26±1.78%), and a significant increase in HR ($p=0.0378$) (+6.55±2.17%) (**Figure 5D** and **Figures 6A & 6B**) as compared to baseline readings. Pre-treatment with hexamethonium bromide neither significantly modified the dose-dependent decreases in MAP or HR elicited by *C. aronia* ($p>0.05$, **Figure 5D**) nor protected the animal from death. When *C. aronia* was administered after hexamethonium pre-treatment, the percentage of decreases in HR ($p=0.991$) and MAP ($p>0.0999$) remained high and non-significantly different as compared with the group administered *C. aronia* alone (-73.7±2.8% versus -90.2±4.23% and -92.6±0.75% versus -96.4±2.34%) (**Figure 5D** and **Figures 6A & 6B**). In contrast, pre-treatment with atropine sulphate (2 mg/kg) completely abolished the *C. aronia*-induced hypotensive and bradyarrhythmic responses, and protected the rats from death during the whole study period. Indeed, atropine pre-treatment before *C. aronia* administration resulted in a significant increase ($p=0.0001$) in MAP (+6.5±2.6% versus -96.4±2.34%), and HR (+21.1±5.1% versus -90.2±4.23%) when compared with *C. aronia*

administration alone (**Figure 5B** and **Figures 6A & 6B**). Furthermore, pre-treatment with a combined dose of hexamethonium bromide and L-NAME (20 mg/kg) significantly modified the dose-dependent decreases in MAP and HR elicited by *C. aronia* (**Figure 5E** & **Figure 6**), resulting in a significant increase (p=0.0007) in MAP (+3.95±0.9%) and decrease (p=0.0001) in HR (-14±0.411%) compared to the *C. aronia*-only group (-96.4±2.34% and -90.2±4.23%), which was sufficient for keeping the animals alive. Pre-treatment with atropine sulphate (2 mg/kg) and *C. aronia* (20 µg/kg) followed by administration of adrenaline resulted in a significant increase (p=0.0008) in MAP (+258±36%) with no significant decrease (p=0.934) in HR (-2.63±2.6%) (**Figures 5C** and **Figures 6A & 6B**) when compared to the group administered atropine alone. Similarly, the administration of ephedrine (1 mg/kg) to a group of rats pre-treated with atropine sulphate (2 mg/kg) and *C. aronia* (20 µg/kg) resulted in significant increases (p=0.0001 and p=0.0012) in both MAP and HR (112.2±3.7% and 35.5±1.2%) (**Figures 6A & 6B**).

Discussion. In this study, we measured the HR and MAP, and analyzed the ECG changes in anesthetized normotensive rats after IV administration of different doses of *C. aronia* in order to determine the mechanisms underlying the possible cardiotoxic effects of *C. aronia*, which is widely used in traditional medicine. The most important finding of this study was the dose-time-dependent reduction in both HR and MAP, which was rapid in onset and lethal at the highest doses of 15 and 20 µg/kg, while lower doses ranging up to 12 µg/kg were safe and produced smooth reduction in both HR and MAP.

At high doses (15 and 20 µg/kg), *C. aronia* caused a complete suppression of the sino-atrial node (SAN); this was manifested by sinus bradycardia followed by junctional escape rhythm (**Figure 4B**) and a variable degree of AVB, ranging from first-degree AVB to complete AVB (**Figures 4C-E**). However, no significant alteration was observed in the ECG components in the entire study period (60 minutes), apart from the changes observed in the PR intervals due to heart blocks occurring immediately before the death of the animal (high doses: 15 and 20 µg/kg). Of particular importance was the assessment of the effect of *C. aronia* on the QT interval because of the association of prolongation of QT interval with the ventricular arrhythmias and sudden death due to torsades de pointes (TdP).[21] The QT prolongation can either be congenital, or more commonly, due to electrolyte imbalance and/or drugs, including certain anti-arrhythmic agents.[22] At the cellular level, QT interval represents the repolarization phase of myocytes, which is driven predominantly by the outward movement of potassium ions. There are 2 important K+ currents participating in ventricular repolarization, which are the subtypes of the delayed rectifier current, that is, IKr (rapid) and IKs (slow). Blockade of either of these outward potassium currents may prolong the action potential, of particular importance is IKr, which is prone to drug influences, and can result in proarrhythmia.[23] Previous reports have shown that hawthorn protects against reperfusion arrhythmia, including ventricular fibrillation.[9,24] The reported mechanism of the effect of hawthorn as an anti-arrhythmic agent is not yet clear. However, it is considered to be due to an increase in the refractory period and inhibition of the inward K+ current, similar to the action of class III anti-arrhythmic agents; therefore, proarrhythmia due to hawthorn remains a major concern.[25] Although no significant changes were observed in the QRS duration or QTc interval, even at high doses of *C. aronia* in the present study, extreme caution must be exercised when interpreting the effects of any new drug on the QT interval due to the inherent influence of HR on QT interval measurement, and the lack of an ideal method for correcting the resultant QT intervals. In the current study, we used the commonly used method, the Bazett's equation to correct the QT intervals for HR, however, Bazett's equation tends to underestimate the QT intervals during extreme bradycardia, and overestimate them for extreme tachycardia.[26-28]

In the current study, *C. aronia* aqueous extract caused a decrease in both the MAP and HR of the anesthetized rats in a dose-dependent manner. The hypotensive effect of this extract observed herein was consistent, and in agreement with many published reports involving both animal models and humans, using other hawthorn species, as well as different modes of administration.[29-32] However, different results for the effect of hawthorn and its different ingredients on HR have been reported.[29,32] These variations could be due to differences in plant species, duration of recording the HR, and/or the differences in plant extraction methodology.

Blood pressure (BP) and HR are closely interrelated. Blood pressure is the product of cardiac output (CO) and peripheral vascular resistance,[33] while CO is the product of HR and stroke volume (SV).[34] Therefore, it is expected that significant changes in HR would contribute to changes in BP.[35] Furthermore, HR and BP are also under the control of the autonomic nervous system (parasympathetic and sympathetic nervous

systems), being the main controllers of the SA and AV nodes' functions-through muscarinic (cholinergic M2 subtype) and beta-1 adrenergic receptors, and regulating the rate of depolarization, propagation, and conduction of these nodes.[36-39] Taking into consideration the hemodynamic and ECG changes observed following *C. aronia* administration, the possible contribution of the cholinergic and/or adrenergic pathways on the generation of bradyarrhythmia and hypotension induced by *C. aronia* was evaluated. In order to understand the mechanisms of the action of *C. aronia*, the lethal dose (20 μg/kg) was used for 2 reasons: first, this dose produced its maximum hypotensive and bradycardiac effects within 3-5 minutes, which coincide with the time of the maximum effects of the used agonistic and antagonistic agents. Second, prevention of hypotension, bradycardia, and subsequently the death of rats with the use of agonistic and antagonistic agents, will certainly assure the exact mechanism of action of the plant extract. Hence, we initially evaluated the bradyarrhythmic and hypotensive effects of this plant after the administration of atropine sulphate, a non-selective muscarinic receptor antagonist,[40] before the administration of *C. aronia*. Under these experimental conditions, the expected hypotension and bradycardia induced by *C. aronia* were completely abolished and were not significantly different in order of magnitude as those measured after the addition of atropine sulphate alone (**Figure 5B**). Furthermore, no heart blocks occurred, and the animals survived throughout the study period. These findings support that *C. aronia* had a cholinergic effect. In order to evaluate whether *C. aronia* exerted the cholinergic effect via the direct stimulation of muscarinic receptors, or via enhancement of vagal tone, we performed another experiment wherein the rats were pre-treated with hexamethonium, a ganglionic blocker.[19] Subsequent administration of *C. aronia* led to decreased MAP and HR even when the autonomic nerve drive, which contributes to the maintenance of HR and BP was eliminated by hexamethonium. The animals developed heart blocks and died within the expected time, similar to that observed with *C. aronia* alone (**Figures 5A & 5D**). This finding suggested that *C. aronia* acted as a direct muscarinic receptor agonist. Salehi et al[41] reported identical findings, with a decreased contraction frequency following hawthorn extract exposure in neonatal cultured murine cardiomyocytes, observing that the effect was mediated via muscarinic receptor activation.

In the present study, pre-treatment with atropine not only prevented bradycardia but also completely abolished the hypotensive effect of *C. aronia*. It is known that in vascular beds, the stimulation of muscarinic (M3 subtype) receptors produces intense vascular dilation despite the lack of apparent cholinergic innervation of most blood vessels.[36,42,43] The muscarinic receptors responsible for vascular relaxation are located on the endothelial cells of the vessels and when they are stimulated, the endothelial cells release endothelium-derived relaxing factors,[43] mainly nitric oxide (NO), which diffuses to the adjacent smooth muscle cells, causing them to relax with subsequent hypotension).[39,40] We therefore tested this possibility by pre-treating the rats with hexamethonium as the ganglionic blocker, and L-NAME, an inhibitor of NO-synthase,[44] which led to complete elimination of the hypotension induced by *C. aronia*, while the HR continued to decrease progressively (**Figure 5E** & **Figure 6**). This finding clearly supports the contribution of NO production in the generation of hypotension induced by *C. aronia*. Loizzo et al[45] reported the hypotensive effects of different species of hawthorn through the inhibition of angiotensin-converting enzyme (ACEi), therefore, NO production may not be the only mechanism underlying the hypotension induced by *C. aronia*.

To evaluate the role of adrenergic receptors in the bradycardia and hypotension induced by *C. aronia*, we performed several experiments where adrenaline (beta and alpha receptor agonists) was used. Adrenaline administration generally results in an increase in the HR and BP.[42] The rats were pre-treated with atropine sulphate and *C. aronia* to maintain the HR and BP, followed by the administration of adrenaline (**Figure 5C**). The expected increase in HR with adrenaline was blunted, in fact, the HR continued to decrease slowly, while the MAP increased significantly. This finding suggests that *C. aronia* probably had a beta-blocking activity, and the increase in BP was due to the effect of adrenaline on the unblocked alpha receptors. We also performed a similar experiment where ephedrine (an alpha receptor agonist) was administered instead of adrenaline, and this resulted in an increase in both HR and MAP,[46] supporting the hypothesis that *C. aronia* is not an alpha receptor blocker.

Although the aqueous extract of the whole plant of *C. aronia syn. Azarolus (L)* produced a serious hemodynamic and electrocardiographic effects at high doses (15 and 20 μg/kg), the lower doses are of potential use as antihypertensive, as well as antianginal therapy. For future work, it would be of great interest to isolate and purify the different active ingredients in the aqueous extract of *C. aronia*, which is a limitation of this study, and investigate the effects of

individual component on the electrocardiography, and cardiovascular hemodynamics to reach a potentially safe agent for the possible use in cardiovascular diseases.

In conclusion, the present study demonstrated that the aqueous extract of the whole plant of *C. aronia syn. Azarolus (L)* induced dose-time-dependent bradyarrhythmic and hypotensive effects, possibly by blocking beta-adrenergic receptors and through the direct stimulation of muscarinic receptors, with subsequent production of NO. Hence, it should be avoided in patients with inadequate BP and/or HR, and should be used cautiously in combination with well-known SAN and AV nodal blocking agents, such as beta and calcium channel blocker.

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