Prolongation of AV nodal refractoriness by *Ruta graveolens* in isolated rat hearts

Potential role as an anti-arrhythmic agent

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ABSTRACT

الأهداف: الهدف الرئيسي من هذه الدراسة هو من أجل تقييم آثار اعتماد– التركيز المستخلص الكامل لروتا جرافيلونس والجزء القلوي المنقى منها على الخواص الأساسية العقدية والوظيفية.

الطريقة: في دراسة تجريبية حاضرة، استخدمنا طريقة لانقودوف للتسريب لقلب الجرذ المعزول من اجل تحديد آثار التركيزات المختلفة لمستخلص فيجن الإستقلابي
(1.25×10^{-6}% weight per volume percent [W/V]; 2.5×10^{-6} % W/V; 3.75×10^{-6} % W/V) and total alkaloid of Rue (0.25×10^{-6} % W/V; 0.5×10^{-6} % W/V)
على الخواص الكهربائية الفسيولوجية لنسيج القلب. تم استعمال إجراءات التحفيز الاختياري لقياس استقلالية التغطية العقدية الوريدية الشريانية والتسهيل والإجهاد. استعملنا ثلاث مجموعات (N=24) للإعدادات العقدية الوريدية الشريانية لقلب الجرذ المعزول من اجل دخول التأثير للمستخلص فيجن. أجريت الدراسة في أكتوبر 2006م بمعمل الفسيولوجية الكهربائية للجهاز القلبي الوعائي ومركز الأبحاث بجامعة جولستان للعلوم الصحية.

النتائج: أظهرت نتائجنا أن لدى كلا من مستخلص النبتة الكامل والجزء القلوي لروتا جرافيلونس تفاعلات متشابهة على وقت الترشيد العقدي والتفاعل. المزيد من ذلك قمنا بمراقبة (83±4to 108±5 msec) (157.6±3to 163.7±4 msec) عند التركيز الأقصى ل
3.75×10^{-6} % W/V

خاتمة: تشير النتائج أعلاه الى أثر مضاد انتظام ضربات القلب المثالي لروتا جرافيلونس في علاج فوق تسرع ضربات القلب اللانظامي البطيني.

Objectives: To evaluate concentration-dependent effects of total extract of *Ruta graveolens* and its purified alkaloid fraction on the nodal basic and functional properties.

Methods: In the present experimental study, we used the Langendorff model for perfusion of isolated rat hearts to determine the effects of various concentrations of methanolic extract of Rue (1.25×10^{-6} % weight per volume percent [W/V]; 2.5×10^{-6} % W/V; 3.75×10^{-6} % W/V) and total alkaloid of Rue (0.25×10^{-6} % W/V; 0.5×10^{-6} % W/V) on electrophysiological properties of cardiac tissue. Selective stimulation protocols were used to independently quantify atrioventricular (AV) nodal recovery, facilitation, and fatigue. We used 3 groups (N=24) of isolated perfused rat AV nodal preparations to assess the effect of Rue extracts. The study was carried out in October 2006 in the electrophysiology laboratory of the Cardiovascular Research Center of Golestan University of Medical Sciences, Golestan, Gorgan, Iran.

Results: Our results showed that both the total plant extract and the alkaloid fraction of *Ruta graveolens* had a similar trend of action on nodal conduction time and refractoriness. Furthermore, we observed increased atrioventricular conduction time (83±4 to 108±5 msec) and functional refractory period (157.6±3 to 163.7±4 msec) at a maximum concentration of 3.75×10^{-6} % W/V.

Conclusion: The above results indicated a potential antiarrhythmic effect of *Ruta graveolens* in treating supra ventricular tachyarrhythmia.

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Herbal remedies are suitable alternatives for synthetic drugs due to their availability, minimal side effects, and lower price.[1] Several botanicals, including *Crataegus oxyacantha* and *Digitalis purpura*, have proven therapeutically beneficial for the treatment of cardiovascular disease. Herbal treatments have been used in patients with congestive heart failure, systolic hypertension, angina pectoris, atherosclerosis, cerebral insufficiency, venous insufficiency, and arrhythmia.[2] *Ruta graveolens* L. (Rutaceae), a plant well known as (Rue) "syn", is a sturdy shrub between 30-80 cm in height with a woody root and a crooked, branched rhizome. The leaves of this plant are 4-11 cm long and 3-7 cm wide. The plant is native to Northern Asia, the Southern Alps and France.[3] The most important compounds of *R. graveolens* are rutin (Flavonoids), acridine, quinazoline, quinoline (alkaloids), hydroxycoumarine, lignans, and pyranocoumarins, and the methanolic extract of Rue is known to possess water-soluble glycosides Cnidioside.[3-5] Previous reports have demonstrated that *Ruta graveolens (R. graveolens)* has positive chronotropic and inotropic effects on the isolated right atria of normotensive rats, however, the substances responsible for the observed effect on the cardiovascular system have not been identified.[6] Other reports have investigated the effects of *R. graveolens* on ionic currents and potassium (K^+) depolarization in intact myelinated nerve fibers. Results of these experiments showed that the plant extract was able to block K^+-current. The plant was also able to block Na^+- current, although to a lesser extent.[7] Additionally, lignans of *R. graveolens* have previously been shown to possess calcium (Ca^{2+}) channel blocking activity.[8] In the present study, we investigated the effect of the total extract of *R. graveolens* and its purified alkaloid fraction on AV nodal conduction time and refractoriness.

Methods. The study was carried out in October 2006 in the electrophysiology laboratory of the Cardiovascular Research Center of Golestan University of Medical Sciences, Golestan, Gorgan, Iran. The aerial parts of *R. graveolens* were collected from a botanical garden in Kandelos, a region in the Mazandaran province of Iran. The plant materials were taxonomically identified and authenticated by the University of Mazandaran, Iran. A voucher specimen (3-67-3) was deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Mazandaran University of Medical Sciences. Air-dried powder (700 g) from leaves of *R. graveolens* was extracted by percolation at room temperature with 70% ethanol. The extract was concentrated at 40°C in a rotary vacuum evaporator to yield a gelatinous material. The extract was dissolved in distilled water and filtered. The filtrate was then evaporated to dry. The dried mass weighed 182 g (26%) and was diluted with Tyrode's solution on the day of the experiments. Total alkaloids are largely extractable with chloroform from an alkaline solution. Briefly, the extracted fluid was acidified with 2N hydrochloric acid (HCl) and heated in a water bath. After cooling, aqueous ammonia was added to bring the extract to pH 10, at which point an alkaline reaction was initiated and a chloroform extraction was performed. The organic solvent was evaporated over a water bath, and the residue was absorbed with 20 ml of 2N HCl. The dried extract was first reconstituted in 70% ethanol and then tested by standard phytochemical methods[9] for the presence of alkaloids, flavonoids, saponin, tannin, and glycosides. Chemical screening was then performed to elucidate the composition of the extract. Dragendorff's reagent (potassium bismuth iodide) was used to screen for alkaloids, magnesium (Mg^{2+}) and HCl for flavonoids, and the ability to produce foam for saponins. The presence of tannin and glycosides in the extract was also determined using ferric chloride and Ninhydrin reagent. Verapamil was purchased from Sigma (St. Louis, Missouri, United States of America [USA]). Tyrode's materials and reagents for phytochemical analysis were purchased from E. Merck (Darmstadt, Germany). Sodium heparin was purchased from B. Braun Medical S.A. (Spain). All drugs were first dissolved in distilled water at the time of the experiments and then diluted in the Tyrode's solution at the final concentration described in the text.

Animals. Male Sprague-Dawley rats (250-350g) from the Institute Pasteur of Iran were injected intraperitoneally with 2500 units of sodium heparin and then anesthetized with sodium pentobarbital (40 mg/kg). The animals were housed in standard cages with free access to food and water. The vivarium temperature was maintained at 23±3°C with a 12 hour light/dark cycle (light on from 06:00 to 18:00 hours). All trials were conducted in accordance with ethical guidelines for the investigation of experimental animals of Golestan University of Medical Sciences. Rat chest cavities were opened with a midsternal incision. The heart was quickly removed and placed in cold perfusion fluid under artificial respiration (REPIRATOR NARCO BIOSYSTEM, USA). The aorta was cannulated and retrogradely perfused with Tyrode's solution of the following composition (g/l): sodium chloride (NaCl), 8.0, potassium chloride (KCl), 0.075, calcium chloride ($CaCl_2$) 0.1, sodium bisulfite $NaHO_3$,

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0.05, and dextrose, 1.0. The perfusion had a pH of 7.35 when equilibrated with a 95% O_2, 5% CO_2 gas mixture and was maintained at 37.5°C. The perfusion was controlled by maintaining a constant perfusion pressure of 68±2 mm Hg, resulting in a coronary flow of 9-12 ml/min. Local electrograms were obtained using bipolar, Teflon coated silver electrodes (diameter 0.25 mm, interelectrode distance 0.25 mm (A-M Systems, US). They were placed near the sinus node and on the right ventricle. Preparations were stimulated through bipolar platinum–iridium electrodes (A-M Systems, US), placed on the right atrium close to the sinus node region. Stimulation protocols were executed using custom-made software running on a computer interfaced with an analog to digital converter. The heart was stimulated with isolated constant-current square wave pulses, 0.4 ms duration, with the stimulus current adjusted to twice diastolic threshold (WPI, A365, USA). Amplification and readouts of the electrograms were provided by polygraph (Power Lab, ML870, Australia) with a bandwidth of 30 Hz-3 KHz. Verapamil was used as a standard negative dromotropic drug to assess the relative efficacy of the plant. The extract and verapamil were first dissolved in distilled water and then diluted in the Tyrode's solution at the final concentrations described in the text. Three groups of experiments were performed as follows: Experiment A (n=8) evaluated the effect of the methanolic extract of Rue (1.25, 2.5, 3.8×10^{-6}% W/V) on electrophysiological properties of AV-node. Experiment B (n=8) assessed the effect of different concentrations of the alkaloids fraction of the extract (0.25, 0.5 mg% W/V) on electrophysiological properties of the AV-node. Experiment C (n=8) investigated the role of the Ca^{2+} channel blocker, verapamil (0.1µM), on the electrophysiological properties of AN-node. The stimulation protocols were carried out during control conditions (no intervention) and in the presence of various concentrations of extract. Control measurements were collected in the presence of Tyrode's solution. Different concentrations of Rue extract were then added to the superfusion solutions and the same stimulation protocols were repeated after 15 minutes. Preparations were allowed to equilibrate in Tyrode's solution for 30 minutes and were stimulated at basic cycle length (BCL) before the protocols were initiated. Only those preparations that maintained stable AV nodal conduction and Wenckebach cycle length (WBCL) during this time were used. The spontaneous mean sinus cycle length at the start of the experiments was 220±30 ms. The BCL was 20% shorter than sinus cycle length, and the mean basic conduction time (AVCT) was 67±5 ms. Specific stimulation protocols were used to quantify recovery and atrioventricular conduction time, as previously described.[10] The WBCL (Wenckebach) was determined under control conditions by decreasing the atrial pacing cycle length by 10 ms decrements every 10 beats until a second degree AV block occurred. This protocol was performed before and after each experimental protocol to ensure the stability of WBCL. The AVCT was measured from the first rapid atrial activation in the atrial electrogram until the second deflection of the ventricle electrogram. The functional and effective refractory period of the AV node (FRP and ERP) were measured using an extrastimulus technique. The FRP was defined as the shortest V1V2 output interval resulting from premature atrial stimulation and ERP was defined as the longest A1A2 interval activating the atrial septum close to the AV node but failing to propagate through the ventricle. Specific stimulation protocols were used as previously described.[10] Briefly, AV-nodal recovery refers to the slow and progressive recovery of excitability that causes the nodal conduction time (NCT) to increase prematurely. To construct the basic recovery curve, a signal premature of delayed stimulus (S2) was introduced after every 10 basic stimuli (S1). The relationship between the conduction time of the test beat (A2V2) and the preceding recovery time (V1A2) was established and fitted to an exponential function as previously described.[10]

Results. *Phytochemical analysis.* Preliminary phytochemical analysis of the methanolic extract of *R.*

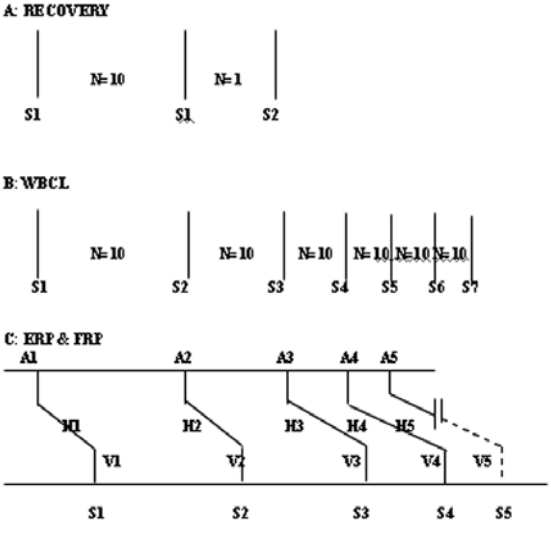

Figure 1 - Stimulation protocols used to quantify atrioventricular (AV) nodal recovery, wenckebach cycle length (WBCL), effective and functional refractory period (ERP and FRP). S1 - represents basic stimuli; Sn (2,3,4,5,…) test stimuli used to assess the AV behavior, A - atrial, H - his bundle, V - ventricular conduction. Effective refractory period; Minimum AA during continuous atrial stimulation (A4A3), Functional refractory period; Minimum VV during continuous atrial stimulation. Block conduction.

graveolens leaves revealed the presence of alkaloids (+++), flavonoids (+), and saponins (+). In contrast, tannins and glycosides were absent from the methanolic extract (**Figures1 & 4**).

Electrophysiological effects of total and alkaloid fraction of methanolic extract of R. graveolens on isolated heart. The addition of the extract to the experimental system increased WBCL, AV-conduction time, and effective and functional refractory periods in a concentration-dependent manner (**Figure 2**). **Figure 3** illustrates an example of a basic nodal recovery curve for one rats isolated heart preparation. The extract causes the basic recovery curve to shift upward in a dose-dependent fashion. At concentrations of 1.25×10^{-5} (w/v) and 2.5×10^{-6} (w/v) the extract increased the time constant of recovery from 40.8±5 ms (control)

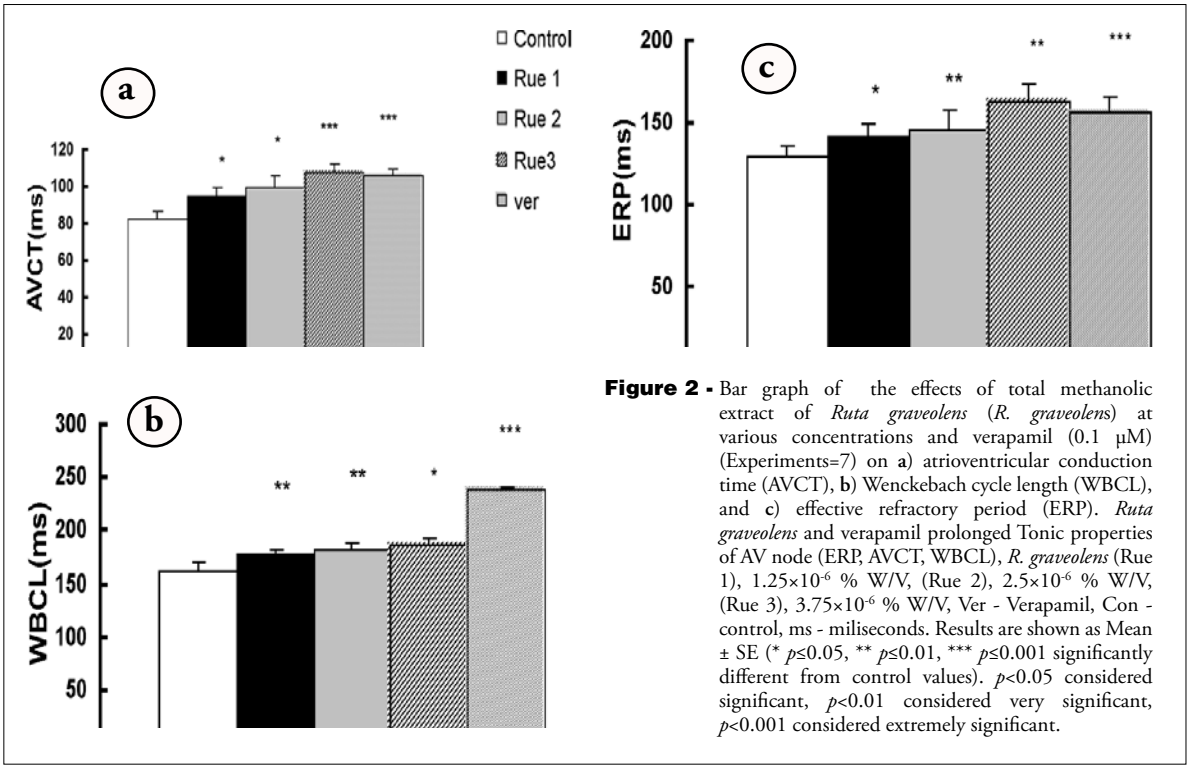

Figure 2 - Bar graph of the effects of total methanolic extract of *Ruta graveolens* (*R. graveolens*) at various concentrations and verapamil (0.1 µM) (Experiments=7) on **a**) atrioventricular conduction time (AVCT), **b**) Wenckebach cycle length (WBCL), and **c**) effective refractory period (ERP). *Ruta graveolens* and verapamil prolonged Tonic properties of AV node (ERP, AVCT, WBCL), *R. graveolens* (Rue 1), 1.25×10^{-6} % W/V, (Rue 2), 2.5×10^{-6} % W/V, (Rue 3), 3.75×10^{-6} % W/V, Ver - Verapamil, Con - control, ms - miliseconds. Results are shown as Mean ± SE (* $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$ significantly different from control values). $p<0.05$ considered significant, $p<0.01$ considered very significant, $p<0.001$ considered extremely significant.

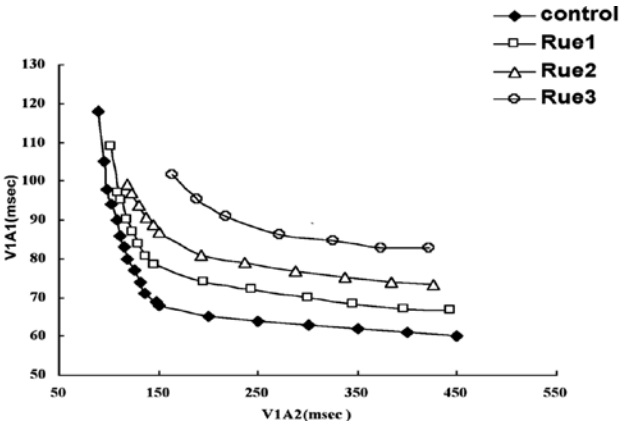

Figure 3 - Plots of effects of different concentration of total methanolic extract of *Ruta graveolens* (*R. graveolens*) on recovery curve, in one rat heart preparation. *R. graveolens* shifted recovery curve upward in a dose-dependent fashion. Three different concentration of *R. graveolens* (Rue): Rue 1 - 1.25×10^{-6} % W/V, Rue 2 - 2.5×10^{-6} % W/V, Rue 3 - 3.75×10^{-6} % W/V, Con - control, A2V2 - conduction time, V1A2 - recovery time, msec - miliseconds.

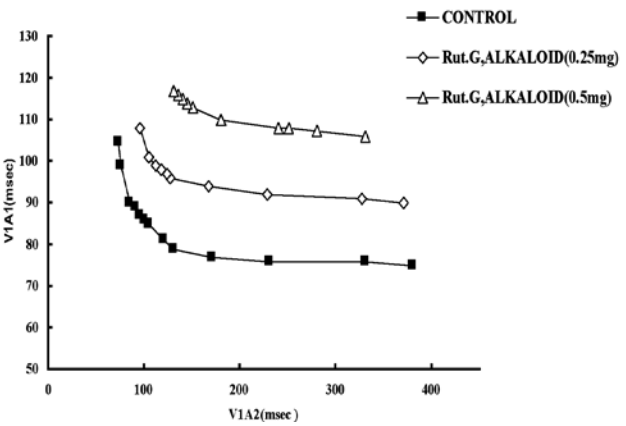

Figure 4 - Plots of the effects of different concentration of total alkaloid extract of *Ruta graveolens* (*R. graveolens*) on recovery curve in a one rat heart preparation. *R. graveolens* shifted recovery curve upward in a dose dependent fashion. *R. graveolens* (Rue G, Alk 0.25×10^{-6} % W/V), (Rue G, Alk 0.5×10^{-6}%W/V), A2V2 - conduction time, V1A2 - recovery time, msec - miliseconds.

Table 1 - Values of constants characterizing recovery in the presence of total extract of *Ruta graveolens (R. graveolens).*

Variables	Mean±SE	*P*-value
τ *(ms)*		
Con	40.8±5	
Rue 1	48.9±10.4	0.1
Rue 2	38±6	0.2
Rue 3	40.2±4.2	0.1
β *(ms)*		
Con	240.4±30.5	
Rue 1	310±28	0.05
Rue 2	377.7±66	0.01
Rue 3	974.4±90	0.05

The effects of various concentration of methanolic extract of *R. graveolens* on recovery parameters (τ and β are constants of recovery protocol). Rue - *R. graveolens:* Rue 1 - 1.25×10^{-6} % W/V, Rue 2 - 2.5×10^{-6} % W/V, Rue 3 - 3.75×10^{-6} % W/V, ms - miliseconds, Con - control. Results are shown as Mean ± SE (Experiments = 8). τ - time constant of the slop of recovery curve, β - maximum atrioventricular conduction time, $p<0.05$ considered significant as compared with control, $p<0.01$ considered very significant as compared with control, $p<0.005$ considered extremely significant as compared with control

Table 2 - Values of constants characteri*zing* basic properties of hearts in the presence of *Ruta graveolens (R. graveolens)* alkaloids.

Variable	Mean±SE	*P*-value
WBCL (ms)		
Con	153.3±2.1	
Alk_1	168.2±6.2	≤0.05
Alk_2	180.4±4.3	≤0.01
AVCT (ms)		
Con	64.2±5.7	
Alk_1	76.6±5.1	≤0.01
Alk_2	92.3±6.5	≤0.01
ERP (ms)		
Con	120.8±6.5	
Alk_1	140.8±6.0	≤0.05
Alk_2	150.0±4.0	≤0.05
FRP (ms)		
Con	154.6±8.4	
Alk_1	175.0±9.0	≤0.05
Alk_2	205.5±11.2	≤0.01

The effects of total alkaloid of *R. graveolens* at various concentrations (Experiments=8) on effective and functional refractory period (ERP and FRP), AVCT - atrioventricular conduction time, WBCL - Wenckebach cycle length, *R. graveolens* prolonged tonic properties of AV node. Results are shown as Mean±SE: Alk_1 (0.25×10^{-6} % W/V), Alk_2 (0.5×10^{-6} % W/V), Con - control, Alk - alkaloids of *R. graveolens*, ms - miliseconds, $p<0.05$ considered significant from control values, $p<0.01$ considered very significant from control values

Table 3 - Values of constants characteri*z*ing recovery in the presence of *Ruta graveolens* (*R. graveolens)* alkaloids.

Variable	Mean±SE	*P*-value
τ		
Con	30.3±5.0	
Alk_1	30.0±1.1	≤1.1
Alk_2	32.2±9.5	≤0.2
β		
Con	339.0±114.1	
Alk_1	429.2±120.6	≤0.01
Alk_2	550.1±-90.1	≤0.05

The effects of different concentrations of total alkaloid (Alk) extract of *R. graveolens* on recovery parameters (τ and β are constants of recovery protocol). Results are shown as Mean±SE (Experiments=8). $P<0.05$ considered significant, *R. graveolens* Alk_1 (0.25×10^{-6} % W/V), *R. graveolens* Alk_2, 0.5×10^{-6}% W/V, Con - control, τ - time constant of the slop of recovery curve, β - maximum AV conduction time

to 48.9±10.4 ms and 38±6 ms (**Table 1**). The effects of the alkaloid fraction on the basic (AVCT, Wenckebach, ERP, FRP) and functional (recovery protocol) characteristics of perfused hearts are demonstrated in **Table 2**. The effects of this fraction on the recovery curve were similar to those observed with the total extract. However, low concentrations (0.25 mg W/V %) of the alkaloid fraction also induced a nonsignificant prolongation of the recovery curve parameters. These parameters were increased to significant levels following exposure to high concentrations (0.5 mg W/V %) of the alkaloid fraction (**Table 3**). Addition of the positive control, verapamil (0.1μM), to the perfusion solution increased Wenckebach, EFP, FRP, and AVCT (**Figure 2**). With the exception of the prolonged recovery curve parameters, verapamil exerted similar effects on basic and rate dependent properties of the AV node to those observed following the addition of the total extract and the alkaloid fraction.

Discussion. The present study was designed to evaluate the effects of *R. graveolens* (Rue) on the electrophysiological properties of isolated heart, with particularly attention to its effect on the rate dependent properties of the AV-node. Our study showed that extract isolated from *R. graveolens* depressed the basic and functional properties of isolated heart and that these actions were ascribed to the alkaloid fraction. The wide variety of delays that the AV-node can generate in response to an increased rate are explained by dynamic interactions among the 3 intrinsic properties of recovery, facilitation, and fatigue.[11] To date, numerous studies have examined the effects of autonomic, purinergic,

and some pharmacological interventions on nodal functional properties in detail.[11,12,13] *Ruta graveolens* caused a dose-dependent increase in atrioventricular conduction time, Wenckebach, and the functional and effective nodal refractory periods. These results on the electrophysiological parameters of isolated rat heart preparation were similar to those observed after the addition of verapamil. The Ca^{2+}-blockers have been shown to suppress basic and functional properties of AV- nodal cells due to a selective inhibition of the slow inward Ca^{2+} current.[14] Our results showed that *R. graveolens* also suppressed nodal properties with an efficacy smaller to that of verapamil. A previous study also demonstrated the Ca^{2+}-inhibitory activity of *R. graveolens,* therefore, compounds found in this plant may be assumed to modulate the slow inward Ca^{2+}-current for nodal cells.[8] The mechanisms underlying rate-dependent AV nodal properties have not yet been fully elucidated.[15] It is possible that the time dependent recovery of Ca^{2+} channels from in activation plays a major role in determining the AV-node curve.[16] Hashino[13] proposed that at least I_K and I_{CaL} are involved in the rate-dependent recovery of excitability of AV nodal cells. When nodal function is represented with a recovery curve (premature Atrial-His interval versus cycle length), the fast pathway (based on compact node) accounts for atrial-His intervals observed at long and intermediate cycle lengths. During anterograde conduction, the premature impulses travel through fast and slow pathways in the flat and steep portions of the nodal curve. Ablation of the posterior extension (slow pathway) removes the left steep rise of the anterograde curve, thus prolonging effective nodal refractory period and shortening nodal conduction time maximum without changing the curve baseline.[17] The present findings indicated that both the flat and steep rising portions of the nodal recovery curve are affected by *R. graveolens*. An upward shift in the baseline of the nodal recovery curve may therefore reflect the presence of depressed slow and fast pathways and support a potential role for *R. graveolens* in both pathway and in re-entry.

We demonstrated that verapamil had more pronounced effects on nodal conduction time and refractory period compared with *R. graveolens*. Nodal refractoriness determines the minimum cycle length, which ventricles may be activated, and can thereby determine cardiac function in the presence of supraventricular tachyarrhythmia. Three indices of nodal refractoriness have been developed: effective, functional refractory periods, and Wenckebach. Our results showed that *R. graveolens* significantly prolonged effective and functional refractory periods in a rate dependent manner. Billette[18] has shown that ERP and FRP originated in the proximal and distal nodes. However, the degree of refractoriness can be modulated by the contribution of I_{Na}, I_f, or I_{CaT}. This may be expected to contribute to a variable extent among the different cell types in the AV node.[15] Therefore, agents that increases AV nodal refractoriness, such as *R. graveolens*, would be expected to modulate the ionic currents responsible for AV nodal refractoriness (I_{Na}, I_f or I_{CaT}). Previous studies have shown that *R. graveolens* possessed K^+ and Na^+ inhibitory activities, thus confirming the depressant effects of this plant on refractoriness.[7]

Results from our preliminary phytochemical analysis shows the presence of alkaloids, flavonoids, and saponins in the leaf extract. Many plant-derived compounds are known to affect calcium channels.[19] For example, several alkaloids were shown to possess Ca^{2+}, Na^+ and K^+ blocker activities.[20,21] Previous studies have identified several quinoline, quinazoline, and acridine alkaloids in the *R. graveolens* extract.[3] The results of the present study showed that the alkaloid extract of *R. graveolens* had a similar activity to the total leaf extract. Therefore, it seems that depressant effects of this plant on functional and basic properties of the heart are likely to be ascribed to these components present in the leaves. The Ca^{2+}-antagonists, such as verapamil, are highly effective in the treatment of proximal supraventricular tachycardia.[22] Therefore, *R. graveolens* may be beneficial in the treatment of proximal supraventricular tachycardia.

In the present study, although there are significant changes in the basic and rate-dependent properties of the AV-node by this plant, we never considered the potential anti-arrhythmic role of the *R. graveolens* in an in-vivo experimental animal model, also the exact cellular mechanism of the plant on the ionic channels of the transitional and compact nodal cells of the AV-node have not been determined. Further studies need to be carried out to clarify these mechanisms.

In conclusion, we have shown that *R. graveolens* alters the functional and basic properties of the AV-node in isolated heart preparations. These changes result in slowed AV-node conduction and prolongation of nodal refractoriness. Furthermore, our results indicate that the alkaloid fraction is likely to be responsible for these actions.

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