

Anti-Contraction Effects of Euscaphic Acid Isolated from *Crataegus azarolus* var. *aronia* L on Rat's Aortic Smooth Muscle

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Abstract

The current study represents the first attempt to investigate the effect of the Euscaphic acid (EA) on Rats isolated thoracic aortic smooth muscle cells. Isolated aorta was used to test the anti-contraction effects and the possible mode of action(s) of the EA (1×10^{-7} M) and (3×10^{-7} M) isolated from *Crataegus azarolus* var. *aronia* L. Euscaphic acid showed high anti-contraction effects on norepinephrin (NE), (1×10^{-9} - 10^{-4} M) induced contraction in aortic smooth muscle cells in endothelium-intact, endothelium-denuded, and aortic rings pre-incubated with potassium (K^+)-channels blocker (tetraethylammonium, TEA), prostaglandin I₂ (PGI₂) inhibitor (indomethacin) and cyclic guanosine monophosphate (cGMP) inhibitor (methylene blue). On the other hand, other K^+ channels subtype blockers glibenclamide (GLIB); barium chloride (BaCl₂) and 4-aminopyridine (4-AP) demonstrated that adenosine triphosphate sensitive K^+ (K_{ATP}), inwardly rectifying K^+ (K_{ir}) and voltage-dependent K^+ (K_V) channels played no role in anti-contraction induced by EA. Furthermore, the role of L-types calcium (Ca^{++}) channels in EA anti-contraction effects on aortic smooth muscle cells was proved, by using the Ca^{++} -channel blocker verapamil, as indicated by the production of a potent anti-contraction effect. The results of the current study indicate that the anti-contraction effects of EA may be due to the activation of calcium dependent, K^+ (K_{Ca}) channels and blocking of L-type Ca^{++} channels. Thus, from these results it can be concluded that both K^+ and Ca^{++} channels play an important role in anti-contraction effects of EA, which are mediated possibly through opening of K_{Ca} channels and blockade of voltage-dependent calcium channels, which may justify the use of medicinal plant *C. azarolus* in cardiovascular disease.

Keywords: *Crataegus azarolus* var. *aronia*, Euscaphic acid, smooth muscle cells, K^+ -channels blockers, Ca^{++} -channels blocker.

1- Introduction

Medicinal herbs form an important part of folk medicine in most countries with a vital importance in treatment procedures (Rezaei *et al.*, 2014). The use of alternative medicines is well documented in patients with chronic diseases such as hypertension, acute coronary syndrome, coronary heart disease, heart failure, peripheral arterial disease, and stroke (Charoonratana *et al.*, 2014).

Medicinal plants represent good sources for new, safe, biodegradable and renewable drugs and according to the World Health Organization (WHO) report (1993), about 65-80% of the developing countries populations depend essentially on plants and plant derived compounds for their primary healthcare needs (Roja *et al.*, 2014). In modern pharmacy, about 50% of drugs are natural products derived from plants (Dhami, 2013). The newest drug discovery projects adoption based on traditional medicinal plant strategy to assure their safety uses (Dhami, 2013; Mishra and Tiwari, 2011). In folk medicine, hawthorn leaves, flowers and fruits are important parts of the plant used as coronary vasodilator, cardiogenic, and hypotensive remedies (Keser *et al.*, 2014), and their phytochemical and pharmaceutical importance are reflected by the presence of bioactive compounds such as phenols, flavonoids, alkaloids, steroids, terpenoids and tannins in medicinal herbs and plants (Mirzaei and Mirzaei, 2013).

The positive effects of *Crataegus azarolus* on the cardiovascular system have recently received a great scientific attention in phytotherapy (Caliskan *et al.*, 2012). Bioactive compounds present in hawthorn include aromatic amines, essential oils, phenolic acids, flavonoids, proanthocyanidins (Keser *et al.*, 2014) and triterpenes (Hu *et al.*, 2014). *In vitro* and *in vivo* pharmacological investigations revealed that Euscaphic acid has a variety of biological activities, such as inhibitory effect against protein tyrosine phosphatase 1B (Li *et al.*, 2014), enzymes involved in DNA replication (Jung *et al.*, 2005), and lipid peroxidation (Marzouk, 2009). Furthermore, it can inhibit atherosclerosis and xanthoma (Zhang *et al.*, 2006), and decrease intracellular melanin content (Song *et al.*, 2013). In addition, Euscaphic acid has diuretic, hepatoprotective (Lee *et al.*, 2009), anti-hyperglycemic, and antitumor-promoting (Kim *et al.*, 2012), and antinociceptive properties (Jovel *et al.*, 2007). However, since EA has been isolated for the first time from *C. azarolus* and no attempt has been made so far to study its effect on smooth muscle physiology, the present study aimed to investigate the anti-contraction effects of EA on a rat's aortic smooth muscle cells with emphases on the role of endothelium-derived relaxing factors, Ca^{++} and K^+ channels in its anti-contraction effects.

2- Materials And Methods

Euscaphic acid from *Crataegus aronia*

Euscaphic acid (Jacaranoic acid) was isolated for the first time from *C. azarolus* ethyl acetate fraction, purified and identified after several analytical processes, including TLC (direct and reverse phase), column chromatography, ^1H and ^{13}C NMR spectra, ESI-MS spectrum etc. Detailed purification and identification procedures are fully described by Mahmud *et al.*, (2015).

Albino Rats

Adult male albino rats, *Rattus norvegicus* weighing 200 – 300 gs, used in the current study were bred in the Animal House, Department of Biology, Faculty of Science / University of Zakho and maintained in plastic cages (460 x 30 x 20 cm). They were kept under standard laboratory conditions at 22 ± 2 °C and exposed to a photoperiod of 12 hrs. light followed by 12 hrs. of darkness, using an automated light-switching devise. The rats were fed on standard rat pellets with free access to dechlorinated tap water *ad libitum*.

Aorta Preparation and Experimental Protocols

The rats were injected intraperitoneally (IP) with heparin, (1500 units/ kg body weight) and left for 30 min to avoid blood clotting and possible damage of endothelial layer of the aorta, and then anesthetized with ketamine (40 mg /kg) and Xylazine (10 mg/Kg) intraperitoneally. The chest cavity was opened, aorta was isolated and transferred into a petri-jar containing Krebs or calcium free Krebs solution, aerated with carbogen (95% O₂ and 5% CO₂) and maintained in a water bath at 37 °C. The aorta cut into rings of about 2-4 mm long and the first four rings of the aorta distal to aortic arch were taken to avoid the difference in the amplitude of contraction.

The procedure described by Al-Habib and Shekha, (2010) was followed with a slight modifications to study the vascular reactivity in isolated aorta. Two stainless steel wires were carefully passed through the lumen of the aortic rings, one of them was anchored to the base of glass organ bath chamber (Panlab/ Harvard, Model LE01046) and the other wires was attached to a force transducer (ADInstruments, Australia, Model MLT0420), connected to a transbridge amplifier (Quad Bridge Amplifier, Model FE 224, ADInstruments Pty Ltd., Australia), PowerLab Data Acquisition System (Model PL 3508, ADInstruments Pty Ltd., Australia) with computer running chart software (Version 7) (Model MLS060/7, ADInstrument, Australia) used for isometric tension measurement.

Prior to the experiment, the organ bath temperature was set at 37 °C for at least one hour, followed by the addition 10 ml of Krebs's with or calcium free solution to the tissue glass chamber. The preparation was aerated continuously with carbogen. Aortic rings were connected to the base of the chamber from one end and to the force transducer from the other end. The initial tension was set at 2 g weight and left for 60-90 min with changing the solution every 15 min. The aortic rings were initially exposed to 1 μM (1×10^{-6} M) norepinephrin (NE) or 100 μM (1×10^{-4} M) CaCl₂ to test their functional integrity and 10 μM (1×10^{-5} M) acetylcholine (ACh) to test endothelium integrity. This was followed by changing the bath solution several times until a stable resting tone was recorded and then the experiments were started. The anti-contraction effects of two doses of EA (1×10^{-7} M and 3×10^{-7} M) on aortic rings post-contracted with different doses of NE (1×10^{-9} - 10^{-4} M) or CaCl₂ (1×10^{-5} , 3×10^{-5} , 1×10^{-4} , 3×10^{-4} , 1×10^{-3} , 3×10^{-3} and 1×10^{-2} M) following an incubation period of 30 min.

The present study included the following groups of experiments.

Group I

To study the anti-contraction effects of EA (1×10^{-7} M and 3×10^{-7} M) on endothelium-intact (+E) aortic rings, post-contracted with different doses of NE (1×10^{-9} - 10^{-4} M), after pre-incubation of EA for 30 min.

Group II

To investigate the role of endothelial cells in anti-contraction effects of EA (1×10^{-7} M) and (3×10^{-7} M) on endothelium-denuded (-E) aortic rings, post-contracted with different doses of NE (1×10^{-9} - 10^{-4} M), by gentle removal of endothelium. The removal of the endothelium was confirmed by the absence of relaxation induced by ACh (1×10^{-5} M) following NE (1×10^{-6} M) pre-contraction.

Group III

The role of endothelial nitric oxide (NO), cGMP and PGI₂ in association with anti-contraction effects of EA (1×10^{-7} M and 3×10^{-7} M) were evaluated, following pre-incubation of endothelium-intact aortic rings separately with each of NO synthase inhibitor, N-nitro-L-arginine methyl ester (L-NAME, 3×10^{-4} M), cGMP inhibitor, methylene blue (1×10^{-5} M) and PGI₂ inhibitor, indomethacin (3×10^{-5} M) in combination with EA (1×10^{-7} M and 3×10^{-7} M) and then post-contracted with different doses of NE (1×10^{-9} - 10^{-4} M).

Group IV

To study the role of K⁺ channels in the development of the anti-contraction effects of EA (1×10^{-7} M and 3×10^{-7} M) on endothelium-intact aortic rings, the aortic rings were pre-incubated separately with each of the following K⁺ channels subtype blockers, K_{Ca} channels blocker, (TEA, 1 mM), K_{ATP} channels blocker, (GLIB, 1×10^{-5} M), K_{ir} channels blocker, (BaCl₂, 1mM) and K_V channels blocker, (4-AP, 1 mM) in combination with EA (1×10^{-7} M

and 3×10^{-7} M) post-contracted with different doses of NE (1×10^{-9} - 10^{-4} M).

Group V

To elucidate the role of Ca^{++} channels in anti-contraction effects induced by EA (1×10^{-7} M and 3×10^{-7} M) in endothelium-intact aortic rings pre-incubated with L-type Ca^{++} channels blocker (verapamil 10nM (1×10^{-8} M), in combination with EA and then post-contracted with different doses of $CaCl_2$ (1×10^{-5} , 3×10^{-5} , 1×10^{-4} , 3×10^{-4} , 1×10^{-3} , 3×10^{-3} and 1×10^{-2} M) was studied.

Statistical Analysis

All data were expressed as means \pm SEM and the median effective concentrations (EC_{50}) values are given as geometric mean with 95% confidence intervals (CI). The statistical analysis was performed using two-way analysis of variance (ANOVA) supported by Bonferroni test when carrying out a pairwise comparison between the same dose of different groups using Graphpad prism program (GraphPad Software, USA). P-values less than 0.05 were considered as statistically significant. In all figures, the symbols *, ** and *** indicate that the differences between means are significant at 0.05, 0.01 and 0.001 levels, respectively.

3- Results

Anti-contraction Effect of Euscaphic Acid on Endothelium-intact Aorta

Dose response-curves for EA anti-contraction effects on NE induced contraction in aortic rings are shown in Figure (1). Euscaphic acid at doses 1×10^{-7} M and 3×10^{-7} M caused a highly significant ($P < 0.001$) anti-contraction effects on NE induced dose-dependent contraction at doses between 1×10^{-7} - 10^{-4} M in aortic rings as compared to the control rings. The Log EC_{50} , (Log EC_{50} of CI 95%) and the % of contraction are shown in Table (1). Euscaphic acid at doses 1×10^{-7} M and 3×10^{-7} M produced a potent dose-dependent anti-contraction effect on NE induced contractions, with a Log EC_{50} -5.400 mg/mL (Log EC_{50} of CI 95% between -5.594 to -5.205) and -4.791 mg/mL (Log EC_{50} of CI 95% between -5.477 to -4.105), respectively, where as in absence of EA, it was -5.964 mg/mL (Log EC_{50} of CI 95% between -6.092 to -5.835). The amplitude of contraction in NE induced contraction in aortic rings was reduced from 100 ± 0.002 %, in aortic rings pre-incubated with EA at concentration 1×10^{-7} M and 3×10^{-7} M to 83.993 ± 0.003 % and 75.527 ± 0.003 %, respectively.

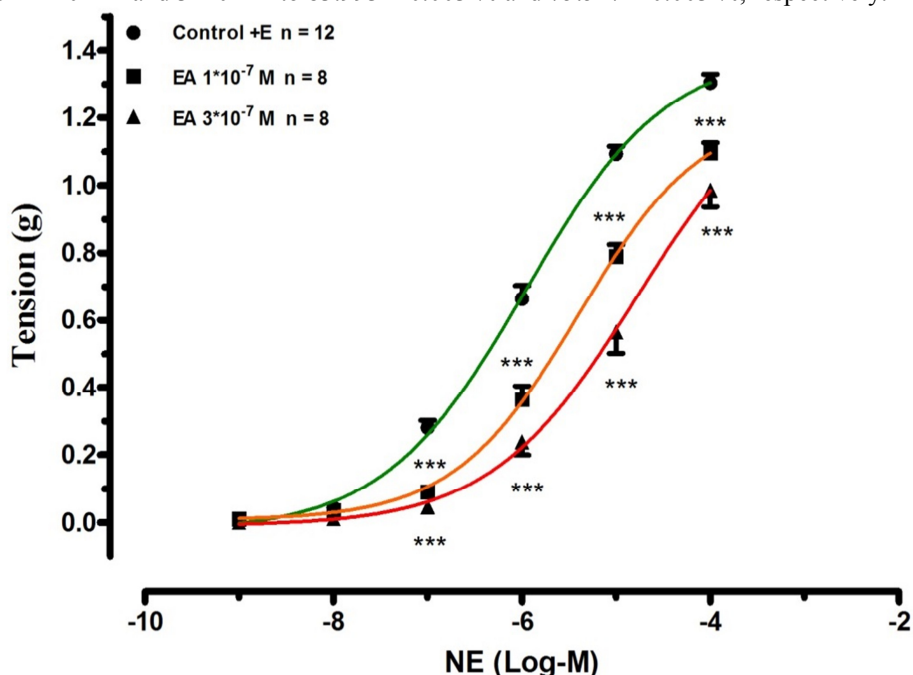


Fig. 1. Cumulative dose-response curves for the anti-contraction effects of EA on NE induced contraction in the rat's endothelium-intact aortic rings.

Table 1. The LogEC₅₀ (LogEC₅₀ of CI 95%) and contraction percent for the effects of pre-incubation with EA on NE post-contracted endothelium-intact aortic rings.

Vasoconstrictor	NE		
	Control	Euscaphic Acid 1X10 ⁻⁷ M	Euscaphic Acid 3X10 ⁻⁷ M
Treatments			
LogEC ₅₀	-5.964	-5.400	-4.791
LogEC ₅₀ of CI 95%	-6.092 to -5.835	-5.594 to -5.205	-5.477 to -4.105
Contraction (%) ± SEM	100 ± 0.002	83.993 ± 0.003	75.527 ± 0.003

Anti-contraction Effect of Euscaphic Acid on Endothelium-denuded Aorta

Dose response-curves for the EA anti-contraction effects on NE induced contraction in endothelium-denuded aortic rings are shown in Figure (2). Both EA doses (1*10⁻⁷ M and 3*10⁻⁷ M) caused a highly significant (P<0.001) anti-contraction effects on NE induced dose-dependent contraction (between 1*10⁻⁷- 10⁻⁴ M) in the aortic rings as compared to the control aortic rings. Euscaphic acid at a concentration of 1*10⁻⁷ M produced a potent effect on NE induced contractions, with a LogEC₅₀ -5.493 mg/mL (LogEC₅₀ of CI 95% between -5.676 to -5.311), whereas EA at a concentration 3*10⁻⁷ M produced a further, but limited anti-contraction effect with LogEC₅₀ -5.336 mg/mL (LogEC₅₀ of CI 95% between -5.496 to -5.175), as compared with that of the control (absence of EA) in which the LogEC₅₀ was -6.240 mg/mL (LogEC₅₀ of CI 95% between -6.424 to -6.056) (Figure 2 and Table 2). Also EA at a concentration of 1*10⁻⁷ M showed much of its anti-contraction effect on aortic rings since the amplitude of contraction was reduced from 100.00 to 84.999 %, whereas at a higher EA dose (3*10⁻⁷ M), it was further reduced, but to a lesser extent, to 80.086% (Table 2).

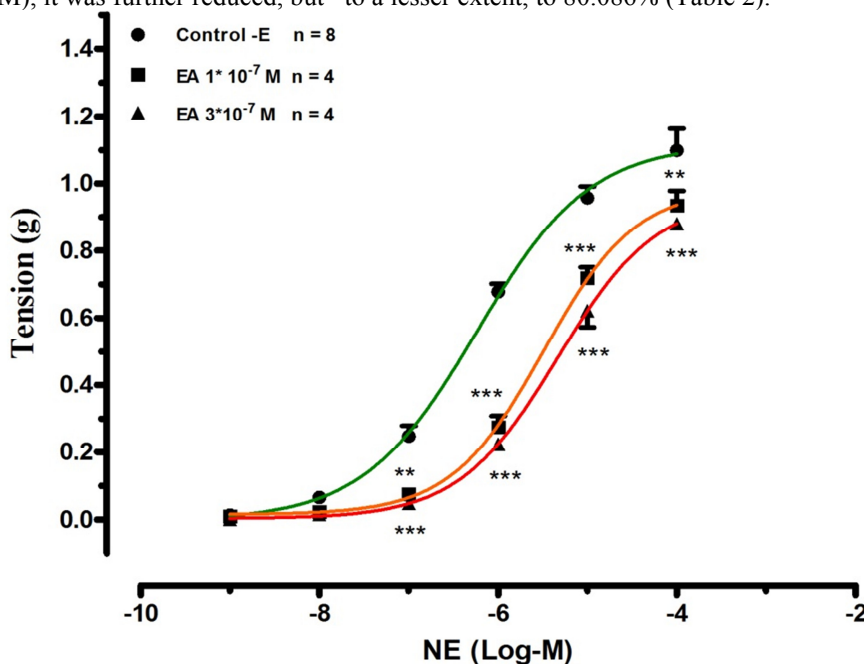


Fig. 2. Cumulative dose-response curves for the anti-contraction effects of EA on NE induced contraction in rat endothelium-denuded aortic rings.

Table 2. The LogEC₅₀ (LogEC₅₀ of CI 95%) and % of contraction for the effects of pre-incubation with EA on NE post-contracted endothelium-denuded aortic rings.

Vasoconstrictor	NE		
	Control	Euscaphic Acid 1X10 ⁻⁷ M	Euscaphic Acid 3X10 ⁻⁷ M
Treatments			
LogEC ₅₀	-6.240	-5.493	-5.336
LogEC ₅₀ of CI 95%	-6.424 to -6.056	-5.676 to -5.311	-5.496 to -5.175
Contraction (%) ± SEM	100 ± 0.004	84.999 ± 0.006	80.086 ± 0.001

Anti-contraction Effect of EA on Aortic Rings Pre-incubated individually with L-NAME (NO Synthase Inhibitor), Indomethacin (PGI₂ Inhibitor) and Methylene blue (cGMP Inhibitor):

Anti-contraction Effect of EA on Aortic Rings Pre-incubated with L-NAME (NO Synthase Inhibitor):

Dose response-curves for EA anti-contraction effects on NE induced contraction in aortic rings pre-incubated

with L-NAME (3×10^{-4} M) are shown in Figure (3). Euscaphic Acid at both doses 1×10^{-7} M and 3×10^{-7} M didn't showed any significant effect on aortic rings pre-incubated with L-NAME, (the inhibitor of NO) and post-contracted with different doses of NE.

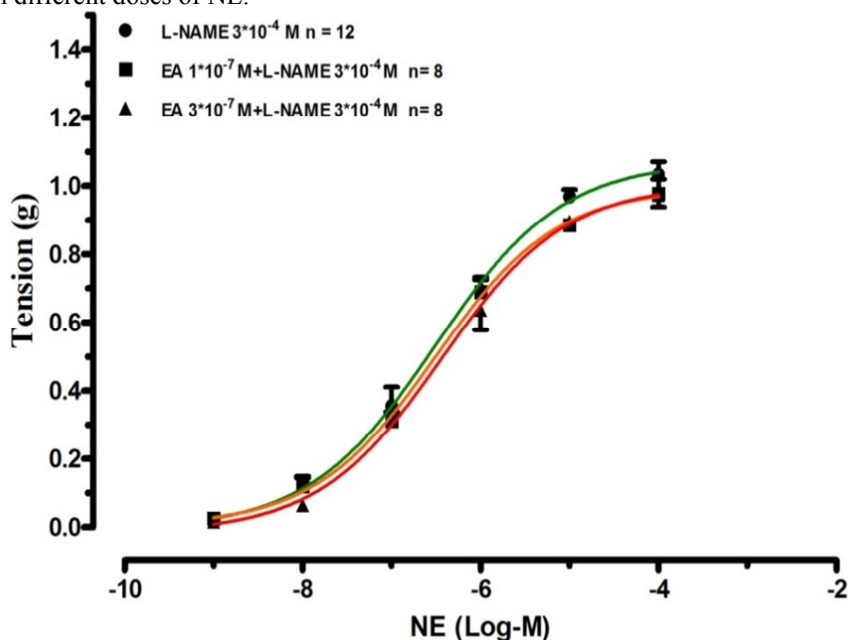


Fig. 3. Cumulative dose-response curves for the anti-contraction effects of EA on NE induced contraction in rat aortic rings pre-incubated with L-NAME (3×10^{-4} M).

Anti-contraction Effect of EA on Endothelium-intact Aortic Rings Pre-incubated with Indomethacin (PGI_2 Inhibitor)

Dose response-curves for the anti-contraction effects of EA on NE induced contraction in aortic rings pre-incubated with indomethacin (3×10^{-5} M) are shown in Figure (4). Euscaphic acid doses 1×10^{-7} M and 3×10^{-7} M produced a highly significant ($P < 0.001$) anti-contraction effects on NE induced dose-dependent contraction (at doses between 1×10^{-6} – 10^{-4} M) in aortic rings pre-incubated with indomethacin. The LogEC_{50} , (LogEC_{50} of CI 95%) and the % of contraction are shown in Table (3). Euscaphic acid at a concentration 1×10^{-7} M had more potent anti-contraction effect on NE induced contraction in aortic rings pre-incubated with indomethacin with LogEC_{50} -5.440 mg/ml (LogEC_{50} of CI 95% between -5.668 to -5.211). Furthermore, EA at a concentration of 3×10^{-7} M, also showed a further anti-contraction effect, but to a lesser extent, on NE post-contracted aortic rings pre-incubated with indomethacin, with LogEC_{50} -5.250 mg/ml (LogEC_{50} of CI 95% between -5.705 to -4.796), while the LogEC_{50} was -5.692 mg/ml (LogEC_{50} of CI 95% between -5.871 to -5.513) in NE post-contracted aortic rings pre-incubated with indomethacin in the absence of EA. Euscaphic acid at a low concentration (1×10^{-7} M) produced most of its anti-contraction effect in which the amplitude of contraction was reduced from 100.00 to 81.122%, whereas at a higher concentration of EA (1×10^{-7} M), the amplitude of contraction was further reduced, but at a slower rate, to 76.468%.

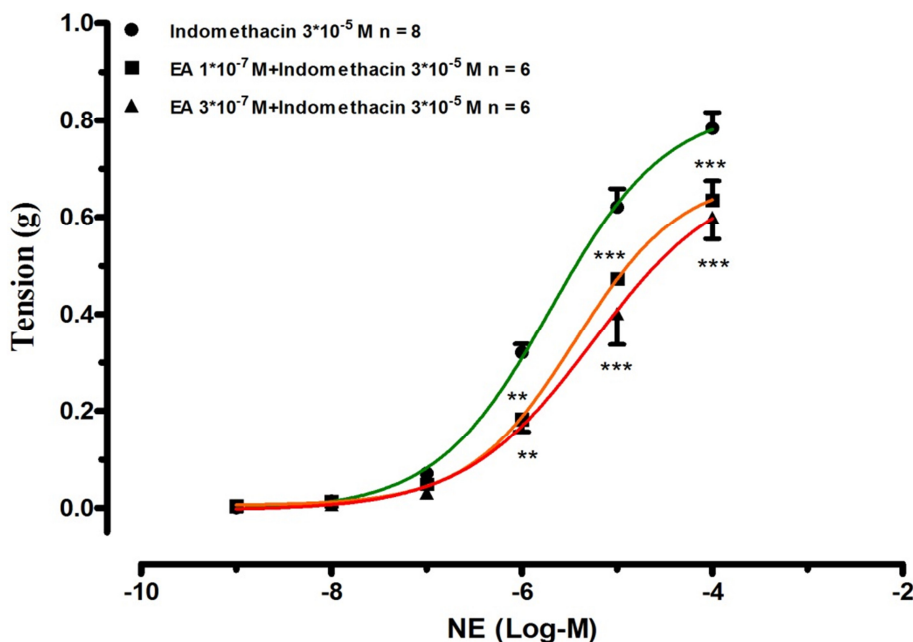


Fig. 4. Cumulative dose-response curves for the anti-contraction effects of EA on NE induced contraction in rat aortic rings pre-incubated with indomethacin (3×10^{-5} M).

Table 3. The LogEC_{50} (LogEC_{50} of CI 95%) and % of contraction for the effects of pre-incubation EA on NE post-contracted aortic rings pre-incubated with indomethacin (3×10^{-5} M).

Vasoconstrictor	NE		
	Control Indomethacin 3×10^{-5} M	Euscaphic Acid 1×10^{-7} M + Indomethacin 3×10^{-5} M	Euscaphic Acid 3×10^{-7} M + Indomethacin 3×10^{-5} M
LogEC_{50}	-5.692	-5.440	-5.250
LogEC_{50} of CI 95%	-5.871 to -5.513	-5.668 to -5.211	-5.705 to -4.796
Contraction (%) \pm SEM	100 \pm 0.0005	81.122 \pm 0.0006	76.468 \pm 0.001

Anti-contraction effect of Euscaphic Acid on endothelium-intact aortic rings pre-incubated with methylene blue (cGMP Inhibitor)

Dose response-curves for EA anti-contraction effects on NE induced contraction in aortic rings pre-incubated with methylene blue (1×10^{-5} M) are shown in Figure (5). Euscaphic acid doses between 1×10^{-7} M and 3×10^{-7} M caused highly significant ($P < 0.01 - < 0.001$) anti-contraction effects on NE induced contraction in rats aortic rings at NE concentrations between 1×10^{-7} M and 1×10^{-8} to 10^{-4} M. The LogEC_{50} (LogEC_{50} of CI 95%) and the % of contraction are shown in Table (4). Euscaphic acid at a concentration of 1×10^{-7} M produced a moderate anti-contraction effect on NE induced contractions, with a LogEC_{50} -7.331 mg/ml (LogEC_{50} of CI 95% between -7.583 to -7.078), whereas EA at a higher concentration (3×10^{-7} M), produced a more potent anti-contraction effect with LogEC_{50} -7.147 mg/ml (LogEC_{50} of CI 95% between -7.396 to -6.898), as compared with the control experiment, in which the LogEC_{50} was -7.847 mg/ml (LogEC_{50} of CI 95% between -8.146 to -7.548) in absent of EA. Furthermore, also EA at concentrations 1×10^{-7} M and 3×10^{-7} M, produced potent anti-contraction effects on aortic rings since the amplitude of contraction was reduced from 100.00 (in the absence of EA) to 76.984 and 57.089%, respectively.

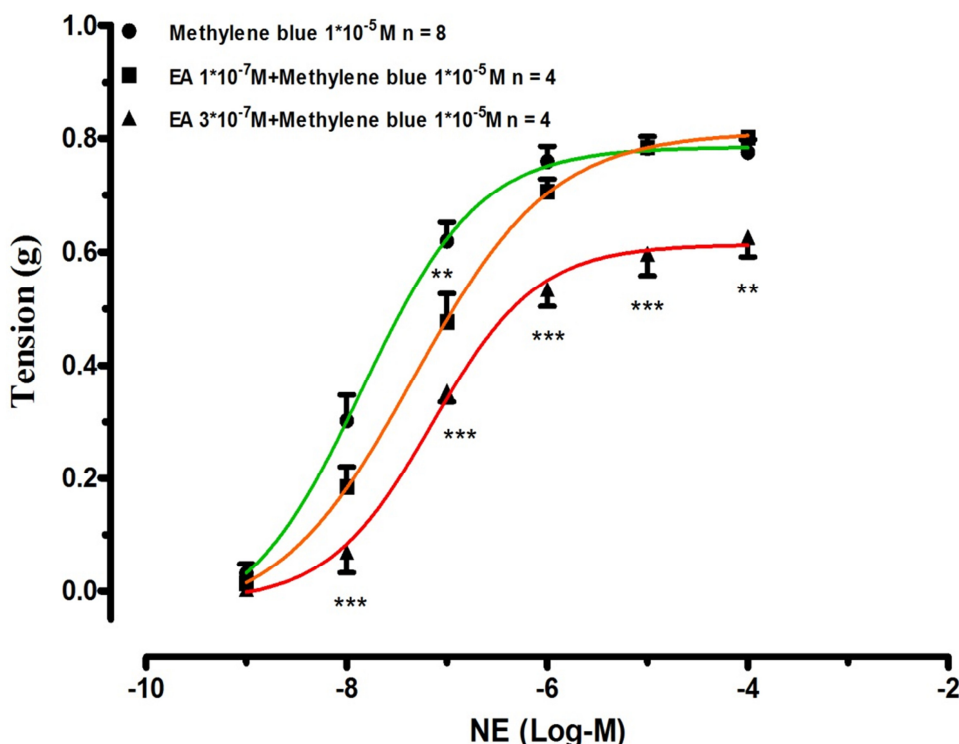


Fig. 5. Cumulative dose-response curves for the anti-contraction effects of EA on NE induced contraction in rat aortic rings pre-incubated with methylene blue (1×10^{-5} M).

Table 4. The LogEC_{50} (LogEC_{50} of CI 95%) and contraction (%) \pm SEM for the effects of pre-incubation EA on NE post-contracted aortic rings pre-incubated with methylene blue (1×10^{-5} M).

Vasoconstrictor	NE		
	Control Methylene blue 1×10^{-5} M	Euscaphic Acid 1×10^{-7} M + Methylene blue 1×10^{-5} M	Euscaphic Acid 3×10^{-7} M + Methylene blue 1×10^{-5} M
LogEC₅₀	-7.847	-7.331	-7.147
LogEC₅₀ of CI 95%	-8.146 to -7.548	-7.583 to -7.078	-7.396 to -6.898
Contraction (%) \pm SEM	100 \pm 0.01	76.984 \pm 0.008	57.089 \pm 0.008

Anti-contraction Effect of Euscaphic Acid on K^+ Channels Subtype in Endothelium-intact Aorta Rings: Anti-contraction Effect of Euscaphic Acid on K_{Ca} Channel in Endothelium-intact Aortic Rings

Dose response-curves for the EA anti-contraction effects on NE induced contraction in aortic rings pre-incubated with K_{Ca} channel blocker, TEA (1×10^{-3} M) are shown in Figure (6). Both EA doses, 1×10^{-7} M and 3×10^{-7} M, caused a significant ($P < 0.01$) anti-contraction effect on NE induced dose-dependent contractions at doses between 1×10^{-6} - 10^{-4} M and 1×10^{-7} - 10^{-4} M, respectively. In aortic rings pre-incubated with TEA, EA at a concentration of 1×10^{-7} M produced a weak anti-contraction effect in NE induced contractions, with a LogEC_{50} -5.578 mg/ml (LogEC_{50} of CI 95% between -5.723 to -5.434), whereas at a higher EA concentration (3×10^{-7} M), a more potent anti-contraction effect was produced with LogEC_{50} -5.420 mg/ml (LogEC_{50} of CI 95% between -5.554 to -5.286), as compared with that of the control in which the LogEC_{50} was -5.725 mg/ml (LogEC_{50} of CI 95% between -5.901 to -5.549). Furthermore, EA at a concentration (1×10^{-7} M) showed a moderate anti-contraction effect on NE induced contraction in aortic rings pre-incubated with TEA since the amplitude of contraction was reduced from 100 ± 0.002 % to 91.479 ± 0.002 %, whereas at a higher EA dose (3×10^{-7} M), the anti-contraction effect on NE induced contraction in aortic rings pre-incubated with TEA was further enhanced, and reduced to 87.01 as compared with the control.

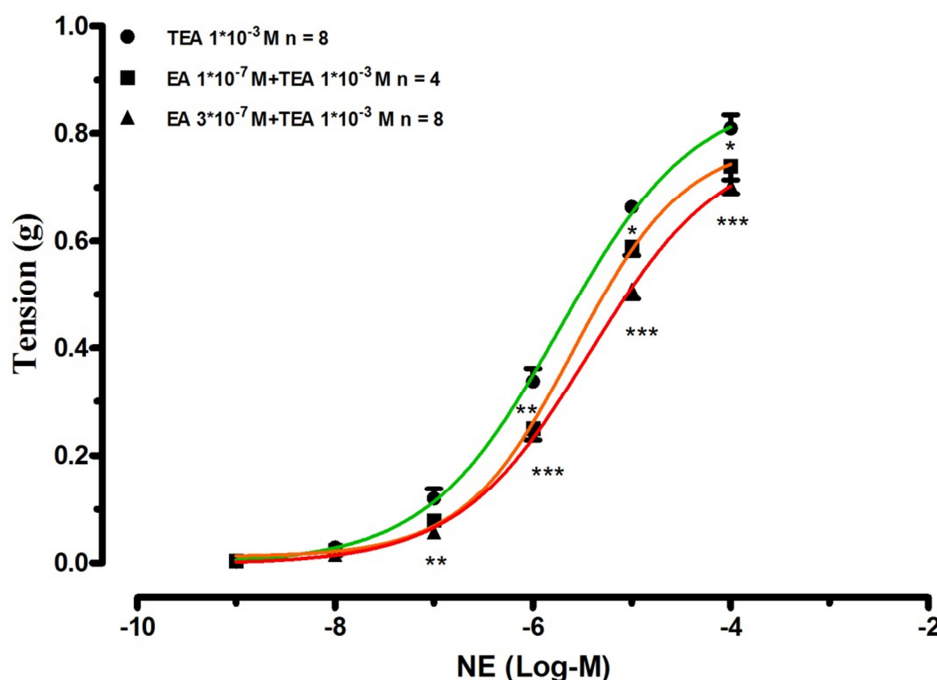


Fig. 6. Cumulative dose-response curves for the anti-contraction effects of EA on NE induced contraction in rat aortic rings pre-incubated with TEA (1×10^{-3} M).

Table 5. The LogEC_{50} (LogEC_{50} of CI 95%) and contraction (%) \pm SEM for the effects of pre-incubation with EA on NE post-contracted aortic rings pre-incubated with TEA (1×10^{-3} M).

Vasoconstrictor	NE		
	Control TEA 1×10^{-3} M	Euscaphic Acid 1×10^{-7} M + TEA 1×10^{-3} M	Euscaphic Acid 3×10^{-7} M + TEA 1×10^{-3} M
Log EC_{50}	-5.725	-5.578	-5.420
Log EC_{50} of CI 95%	-5.901 to -5.549	-5.723 to -5.434	-5.554 to -5.286
Contraction (%) \pm SEM	100.00 \pm 0.002	91.479 \pm 0.002	87.008 \pm 0.001

Anti-contraction Effect of Euscaphic Acid on Endothelium-intact Aortic Rings Pre-incubated with GLIB (K_{ATP} Channel Blocker)

Dose response-curves for the anti-contraction effects of EA on NE induced contraction in aortic rings pre-incubated with GLIB (1×10^{-5} M) are shown in Figures (7). Both EA doses, 1×10^{-7} M and 3×10^{-7} M used didn't showed any significant effects on aortic rings pre-incubated with GLIB and post-contracted with different doses of NE.

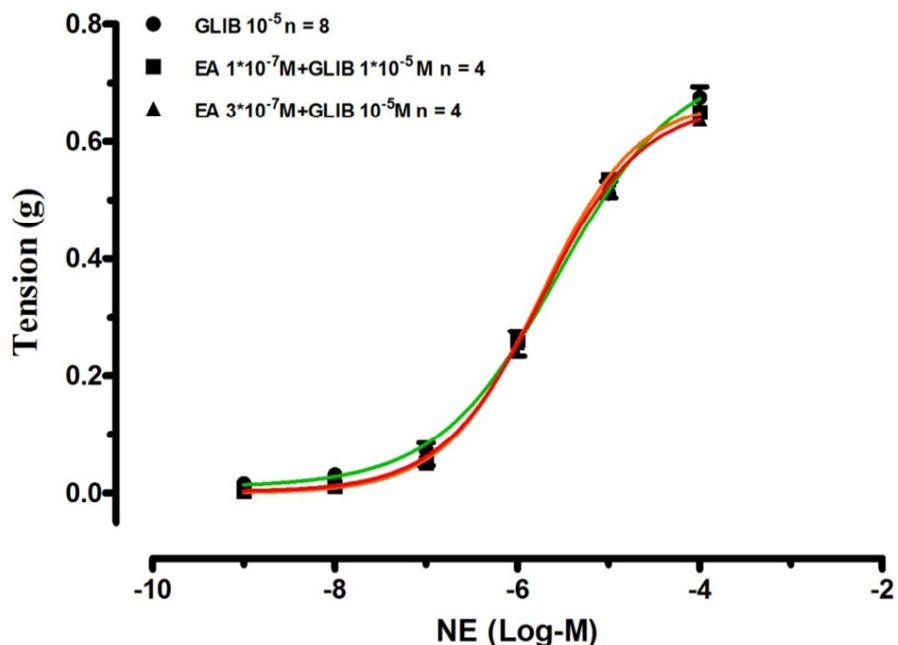


Fig. 7. Cumulative dose-response curves for the effects of EA on NE induced contraction in rat aortic rings pre-incubated with GLIB (1×10^{-5} M).

Anti-contraction Effect of Euscaphic Acid on Endothelium-intact Aorta Rings Pre-incubated with BaCl₂ (K_{ir} Channel Blocker)

Dose response-curves for anti-contraction effects of EA on NE induced contraction in aortic rings pre-incubated with BaCl₂ (1×10^{-3} M) are shown in Figure (8). Both EA doses 1×10^{-7} M and 3×10^{-7} M also didn't showed any significant anti-contraction effect on aortic rings pre-incubated with BaCl₂ and induced contraction with different doses of NE.

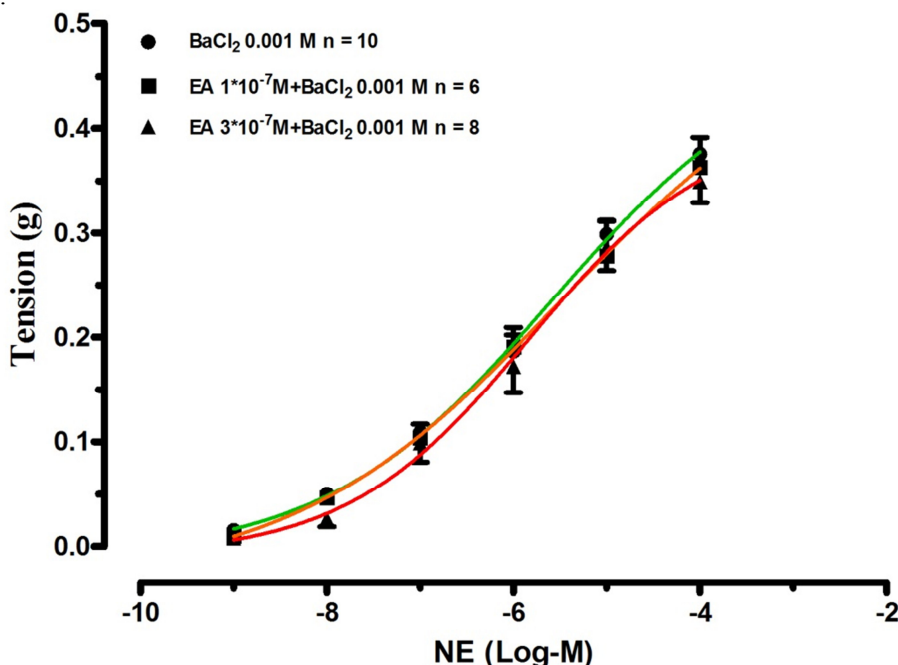


Fig. 8. Cumulative dose-response curves for the anti-contraction effects of EA on NE induced contraction in rat aortic rings pre-incubated with BaCl₂ (1×10^{-3} M).

Anti-contraction Effect of Euscaphic Acid on Endothelium-intact Aorta Rings Pre-incubated with 4-AP (K_v Channel Blocker)

Dose response-curves for anti-contraction effects of EA on NE induced contraction in aortic rings pre-incubated with 4-AP (1×10^{-3} M) are shown in Figure (9). Euscaphic acid doses 1×10^{-7} M and 3×10^{-7} M didn't produced any significant anti-contraction effect on aortic rings pre-incubated with 4-AP and induced contraction with different doses of NE.

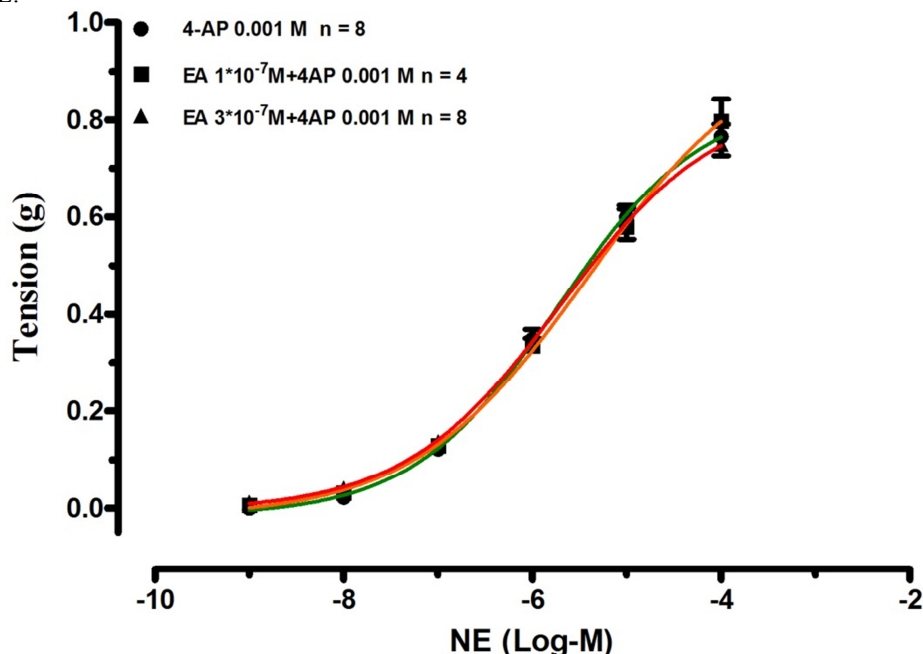


Fig. 9. Cumulative dose-response curves for the anti-contraction effects of EA on NE induced contraction in rat aortic rings pre-incubated with 4-AP (1×10^{-3} M).

Anti-contraction Effect of Euscaphic Acid on Endothelium-intact Aorta Rings Pre-incubated with Verapamil (L-type Ca⁺⁺ Channel Blocker)

Dose response-curves for anti-contraction effects of EA on CaCl₂ induced contraction in aortic rings pre-incubated with verapamil (1×10^{-8} M) are shown in Figure (10). Euscaphic acid doses 1×10^{-7} M and 3×10^{-7} M produced a highly significant ($P < 0.001$) anti-contraction effect on CaCl₂ induced contraction at concentrations between 1×10^{-4} and 1×10^{-2} M in aortic rings pre-incubated with verapamil. The LogEC₅₀, (LogEC₅₀ of CI 95%) and the amplitude of contraction are shown in Table (6). Euscaphic acid at doses 1×10^{-7} M and 3×10^{-7} M produced significant ($P < 0.05$ to 0.001) effects on CaCl₂ induced contraction in aortic rings pre-incubated with verapamil with LogEC₅₀ -3.740 mg/ml (LogEC₅₀ of CI 95% between -3.946 to -3.533) and -3.795 mg/ml (Log EC₅₀ of CI 95% between -3.961 to -3.628), respectively, as compared with the control experiments (in absent of EA), in which the LogEC₅₀ was -3.992 mg/ml (LogEC₅₀ of CI 95% between -4.088 to -3.897). Furthermore, EA at doses 1×10^{-7} M and 3×10^{-7} M produced strong anti-contraction effects on aortic rings pre-incubated with verapamil in which the amplitude of contraction was reduced from 100 ± 0.002 % (in the absence of EA) to 86.572 ± 0.001 % and 62.351 ± 0.0007 %, respectively.

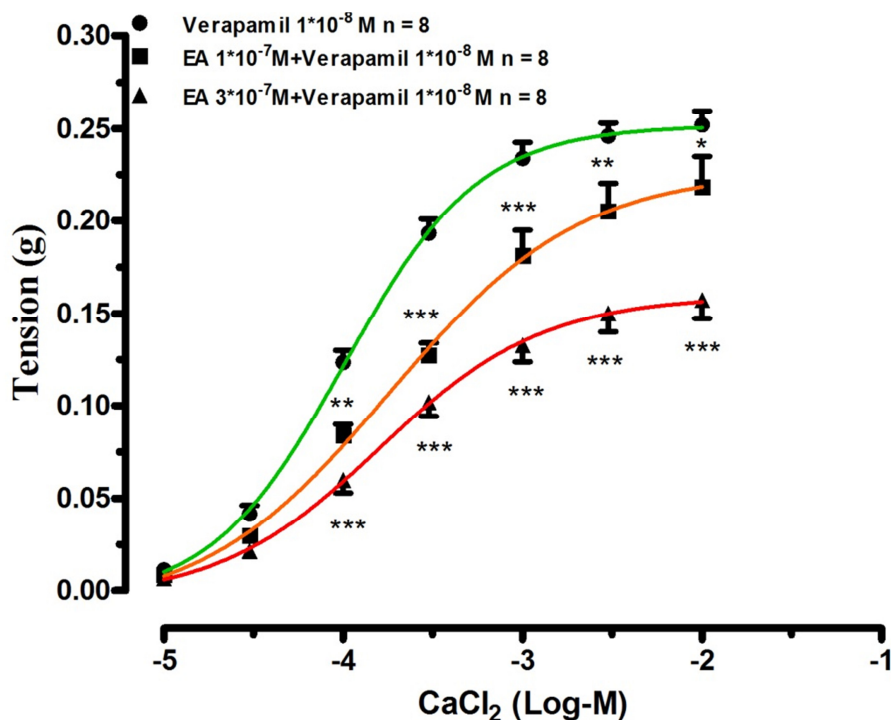


Fig. 10. Cumulative dose-response curves for the anti-contraction effects of EA on CaCl_2 induced contraction in rat aortic rings pre-incubated with verapamil (1×10^{-8} M).

Table 6. The LogEC_{50} (LogEC_{50} of CI 95%) and contraction (%) \pm SEM for the effects of pre-incubation with EA on CaCl_2 post-contracted aortic rings pre- incubated with verapamil (1×10^{-8} M).

Vasoconstrictor	NE		
	Control Verapamil 1×10^{-8} M	Euscaphic Acid 1×10^{-7} M + Verapamil 1×10^{-8} M	Euscaphic Acid 3×10^{-7} M + Verapamil 1×10^{-8} M
LogEC₅₀	-3.992	-3.740	-3.795
LogEC₅₀ of CI 95%	-4.088 to -3.897	-3.946 to -3.533	-3.961 to -3.628
Contraction (%) \pm SEM	100 \pm 0.002	86.572 \pm 0.001	62.351 \pm 0.0007

4- Discussion

The current study was performed to investigate the possible anti-contraction effects of the novel triterpenoid EA isolated from *Crataegus azarolus* var. *aronia* on rat aortic smooth muscle at doses 1×10^{-7} M and 3×10^{-7} M. Euscaphic acid produced anti-contraction effects on aortic smooth muscle cells in association with endothelium-derived relaxing factors, K^+ and L-type Ca^{++} channels. The results of the current study represents the first report on the anti-contraction effect of EA on rat aortic rings smooth muscle, after its isolation for the first time from *C. azarolus*.

The results clearly indicated that the inhibitory effect of EA on the contraction of aortic smooth muscle in association with production of PGI_2 , since the use of indomethacin (a nonspecific cyclooxygenase inhibitor) showed that PGI_2 plays an important role in EA vasodilator effect. Since no data are available on the effect of Euscaphic acid-induced relaxant effect on aortic smooth muscle, it is difficult to compare the results. However, Aguirre-Crespo *et al.*, (2005) showed that the vasodilator effect of other triterpenoids isolated from *L. caulescens* was produced via cyclooxygenase synthase pathway. Wongsawatkul *et al.*, (2008), demonstrated that the relaxant effect of *Spilanthes acmella* extract containing phenolic and triterpenoids is produced partially via endothelium induced PGI_2 production. Recently, Dood *et al.*, (2013), suggested that the hawthorn induced the production of prostacyclin in rat smooth muscle cells (SMCs) and since EA is one of the active constituent of hawthorn, it may acts via the production of prostacyclin.

The mechanism by which PGI_2 inhibits smooth muscle contraction includes the activation of adenylyl cyclase via the specific cell surface IP₂ receptor-coupled guanine nucleotide regulatory protein, Gs, which in turn elevates intracellular adenosine 3':5'-cyclic monophosphate (cAMP) levels (Yamaki *et al.*, 2001). Increased intracellular concentration of cAMP inhibits the sensitivity of the contractile proteins to Ca^{++} ions (Clyman, 2006).

The results of the current study showed that cGMP has an important role on the anti-contraction effect of EA on aortic smooth muscle since the use of methylene blue (cGMP inhibitor) demonstrated clearly the role of cGMP in EA anti-contraction effects. According to our data, EA induce the production cGMP in endothelial cells, which in turn decreases the contraction of smooth muscle cells. This conclusion is supported by the observation that the guanylate cyclase inhibitor methylene blue reduced the relaxant effect of EA. Nitric oxide once released from the endothelium, diffuses into the arterial smooth muscle fibers to activate guanylate cyclase and thus increases cytoplasmic cGMP levels, leading to vasorelaxation in rat isolated mesenteric arteries (Chen *et al.*, 1998). It has been reported that triterpenoids have significant endothelium-dependent vasorelaxant effect in rat aortic SMC through NO release (Rios *et al.*, 2012), and since EA is triterpenoids, it may possibly act via inducing NO, which activate guanylate cyclase and in turn increases cGMP level and leading to vasorelaxation.

The mechanism of cGMP action in vasodilation activity was explained by Gadelha de Cerqueira *et al.*, (2012), which includes that cGMP activates intracellular effectors, such as protein kinase G (PKG), which causes diminishing of intracellular Ca^{++} ions and disassociation of actin and myosin filaments and ultimately leading to relaxation of the smooth muscle cells. Furthermore, they also explained the interaction of cGMP-cAMP cell signaling systems in the relaxation of smooth muscle. In other words, cGMP activates PKG, and then mediates vascular relaxation through phosphorylation of various targets (Hildebrand *et al.*, 2013).

The results compiled from the effect of TEA (K_{Ca} channels blocker) on anti-contraction effect of EA on aortic smooth muscle cells indicate that K_{Ca} channels play a prominent role in increasing its anti-contraction effect and revealed that EA activate the K_{Ca} channels, but not other K^+ channels subtypes such as K_{ATP} , K_{ir} and K_V channels. These observations are in partial agreement with those reported by Chen *et al.*, (1998), since they found that K_{Ca} channels, using a potent K_{Ca} channels inhibitor Iberitoxin, plays a minor role in relaxant action hawthorn extract. Furthermore, they added that GLIB fail to change the vasorelaxation action of *Crataegus* extract in rat arterial SMCs.

The role of L-type Ca^{++} channel in smooth muscle contraction has been demonstrated by Fransen *et al.*, (2012), and they showed that it is responsible for regulating the influx of Ca^{++} into muscle cells, which in turn stimulates smooth muscle contraction. Euscaphic acid used in the present study produced a sharp anti-contraction effect on aortic rings pre-incubated with verapamil (L-type Ca^{++} channel blocker). This proves that EA blocks the activity of L-type Ca^{++} channels which in turn leads to aortic vasorelaxation. Al-Surchi, (2010) working on the inhibitory effect of *C. azarolus* extract on rat's aortic smooth muscle claimed that this effect may be due to the interference of active ingredient present in the extract such as triterpenoids either with Ca^{++} release from SR or with Ca^{++} influx through voltage gated L-type Ca^{++} channels located in the plasma membrane of smooth muscle cells. Furthermore, Chen *et al.*, (1998) reported that hawthorn extract may have a direct inhibitory effect on Ca^{++} entry through voltage-sensitive Ca^{++} channels in smooth muscle cell membrane.

5- Conclusions

From the results of the current study it has been concluded that EA has anti-contraction and cardioprotective effects which are mediated possibly through enhancement of the production of endothelium-derived relaxing factors (particularly cGMP and PGI_2). Furthermore, EA also block Ca^{++} channels and ultimately Ca^{++} release from intracellular stores house and increase the K^+ conduction via increasing K_{Ca} channels activity. These observations might be justifying the medicinal use of EA which isolated from *C. azarolus* in hypertension and other cardiovascular diseases. However, more studies are required to establish the safety, efficacy and activity of this compound.

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