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Ajuga iva water extract antihypertensive effect on stroke-prone spontaneously hypertensive rats, vasorelaxant effects *ex vivo* and *in vitro* activity of fractions

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ABSTRACT

Ethnopharmacological relevance: *Ajuga iva* (L.) Schreb. (Labiatae) (AI) is used in folk medicine for a variety of ailments, including diabetes mellitus and hypertension.
Aim of the study: In this work, we aimed to investigate the antihypertensive and vasorelaxant effects of AI aqueous extract in stroke prone spontaneously hypertensive rats (SHR-SP).
Material and methods: Male SHR-SP rats were orally force-fed AI aqueous extract (500 mg/kg body weight) daily for one week. Systolic blood pressure and urine output were recorded *in vivo* by non-invasive methods. AI vasoactive effects on noradrenaline contractile response and acetylcholine-evoked relaxation were assessed ex *vivo* on aorta rings of treated and untreated SHR-SP rats. AI extract was then subjected to bio-guided fractionation using solvents of increasing polarity. For each fraction, *in vitro* vasorelaxation assay was performed on noradrenaline-precontracted aorta of Wistar rats, in the absence/presence of N-nitro-L-arginine (L-NNA). HPLC analysis of AI total extract, and the most *in vitro* active AI residual aqueous extract fraction (A1) was performed using naringin, naringenin, apigenin, apigenin 7-O-glucoside as marker compounds.
Results: AI aqueous extract (500 mg/kg) significantly ($P < 0.05$) decreased systolic blood pressure (SBP) in SHR-SP rats, while not affecting the urine output. In *ex vivo* experiments, the total extract decreased contractile response to noradrenaline of aortic rings isolated from AI-treated SHR-SP rats with or without addition of N-nitro-L-arginine, but endothelium dependent relaxation evoked by acetylcholine in noradrenaline-contracted aortic rings was not affected by the extract treatment. *In vitro* experiments on AI aqueous extract fractions showed that its polar fraction was the only one affecting *in vitro* noradrenaline induced contractions, but only in an endothelium dependent manner. This fraction was shown by HPLC-UV to contain flavonoid glycosides among other polar compounds whose activity and mode of action may be modified *in vivo* by metabolization.
Conclusion: These results support the use of AI as antihypertensive treatment in folk medicine. The systolic blood pressure decrease may be attributed at least in part to vasorelaxant glycosylated/polar phenolic compounds as flavonoids and/or their metabolites.

Abbreviations: Ach, acethylcholine; AI, *Ajuga iva*; BW, body weight; Emax, Maximum Effect; eNOS, endothelial NO synthase; L-NNA, NO-synthase inhibitor N-nitro-L-arginine; Nad, noradrenaline; NO, nitric oxide; EC50, concentration producing half-maximal effect; SBP, systolic blood pressure; SHR-SP, spontaneously hypertensive stroke-prone rat.

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1. Introduction

Hypertension is considered as a global public health problem. It is frequently an asymptomatic chronic disease, and still represents a key risk factor for myocardial infarction, heart failure and stroke. High blood pressure concerns one billion patients worldwide and kills nine million people per year. This makes hypertension one of the world's leading causes of premature morbidity and mortality (Androulakis et al., 2009; Sudharsanan and Geldsetzer, 2019).

Most developing countries are still facing major challenges to deal with hypertension complications. This drives people to use medicinal plants as an alternative medicine for the prophylaxis and treatment of cardiovascular diseases. Because of this trend, many reports have evaluated the effects of several medicinal plants and their constituents on the cardiovascular system, aiming to provide a scientific basis for their therapeutic use (Xiong et al., 2013).

Stroke-prone spontaneously hypertensive rat (SHR-SP) is a convenient animal model of systemic hypertension (Papazzo et al., 2013). It has been used to investigate preventive strategies for both stroke and cardiac hypertrophy due to severe hypertension (Kim et al., 1996). The animal model develops all features of human cardiovascular disease such as (i) the endothelial dysfunction caused by increased NO inactivation and/or decreased NO production by endothelial NO synthase (eNOS) (Duarte et al., 2001), (ii) changes in mechanics and functionality of arterial vessels, particularly in terms of myogenic tone and vascular compliance, (iii) myogenic active tone as a result of increased Ca^{2+} permeability and impaired endothelial function (Arribas et al., 1999; Henning et al., 2010), and (iv) high levels of angiotensin II, which is implicated in vascular damage and hypertensive complications (Kouno et al., 2005).

Ajuga iva (L.) Schreb. (AI) is used as decoction to treat diabetes, hypertension, ulcers, inflammation, dysuria and other disorders in North Africa (Bellakhdar et al., 1991; El-Hilaly et al., 2003), renal diseases in Asia (Aliotta and Pollio, 1994), fever, toothache, dysentery and hypertension in East Africa (Kokwaro, 2009).

Plants of the genus *Ajuga* possess multiple pharmacological properties. These include antidiabetic (Boudjelal et al., 2015; Wang et al., 2017), antibacterial and antifungal (Medjeldi et al., 2018), anti-tumoral (Takasaki et al., 1999), antifeedant (Jannet et al., 2000), hypocholesterolemic (Taleb-Senouci et al., 2012), and vasorelaxant activities (El-Hilaly et al., 2004a).

We have previously explored the effect of AI aqueous extract on blood pressure and vasoactivity in normotensive rats. Our findings have shown that the plant is devoid of hypotensive activity on normotensive Wistar rats: 4 days of gavage with AI total extract did not modify the systolic blood pressure in AI-treated Wistar rats when compared to untreated Wistar rats receiving distilled water. Nevertheless, it was shown to exhibit potent *ex vivo* and *in vitro* vasorelaxant effects (El-Hilaly et al., 2004a). Furthermore, we showed previously that, although AI extract exhibits toxicity when given intraperitoneally, the daily oral administration of 600 mg/kg during 3 months and a single dose of up to 14000 mg/kg did not show any toxicity (El Hilaly et al., 2004b).

Although many pharmacological activities of AI have been demonstrated, there is still a lack of information concerning its *in vivo* antihypertensive effect. In this context, it was important to ascertain the ethnopharmacological antihypertensive property attributed to AI and to determine whether AI treatment could affect SBP and vascular function of hypertensive rats.

To this aim, we conducted *in vivo,* and *ex vivo* experiments using SHR-SP rats to assess the antihypertensive and vasorelaxant effect of AI aqueous extract in hypertensive rats, and analyzed the composition of its active vasorelaxant fraction.

2. Materials and methods

2.1. Preparation of the whole extract

Mature whole *Ajuga iva* (L.) Schreb. plants were harvested in mountains of Taounate province (GPS position: 34° 41' 41.041''; W5° 4' 45.804"), in north Morocco. Authentic samples were identified by the department of botany, Scientific National Institute (Rabat, Morocco), where a voucher specimen was deposited (H63). Plant name was verified using http://www.theplantlist.org site, last accessed on March 21, 2019. The Plant List database showed *Ajuga iva* (L.) Schreb. name as accepted with high confidence level.

The whole plant was washed and dried in shade and airy conditions. Coarsely powdered dried plant material was extracted with bi-distilled water (10%, m/v). The mixture was heated and boiled under reflux for 30 min. The decoction obtained was centrifuged, filtered, frozen at −20 °C and then lyophilized (FreeZone® Dry 4.5, USA), yielding after lyophilization, 22% of dry extract.

2.2. Fractionation of AI aqueous extracts

The fractionation process is summarized in Fig. 1. For each fraction, *in vitro* vasorelaxation assay was performed. 50 g of AI aqueous extract (10%, m/v) were sequentially extracted 3 times with each immiscible solvent of increasing polarity, using a separatory funnel; the order being hexane < dichloromethane < ethyl acetate < butanol. Then, the remaining aqueous phase was precipitated by acetone (v = v). The resulting precipitate (A2) and supernatant (A1) were lyophilized.

2.2.1. HPLC conditions

HPLC (Accela, Thermo Fisher Scientific) analysis was performed using a reverse phase column (LiChrospher 100 RP-18 ec, 250 × 4 mm, 5 µm particle size, Merck). Both the total AI aqueous extract and residual

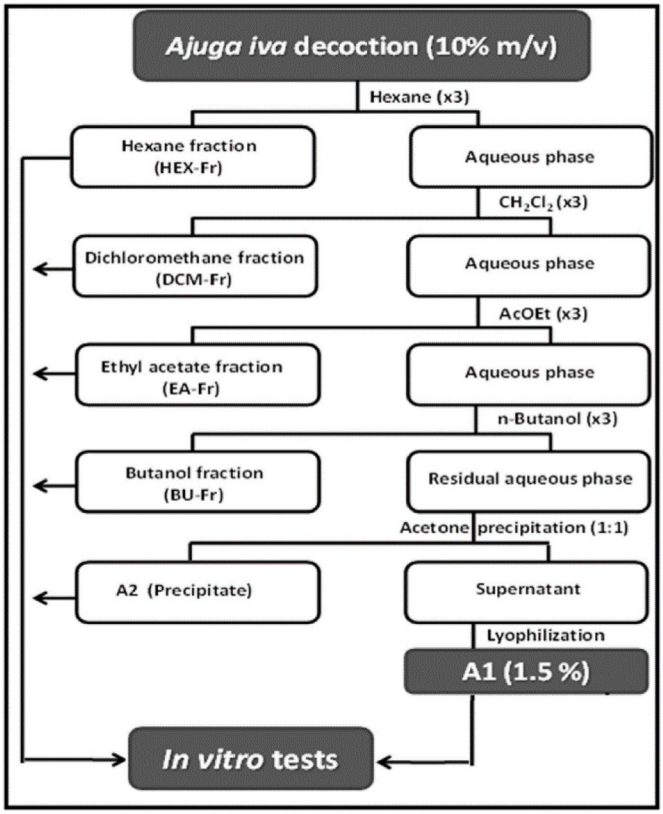

Fig. 1. Procedure for fractionation of AI crude extract.

aqueous extract (A1) were analyzed using as mobile phase a gradient of two systems (Boudjelal et al., 2015): a solvent system B (2.5% formic acid in acetonitrile) and solvent system A (2.5% formic acid in water) with the following gradient: 0 min: 5% B; 10 min: 15% B; 30 min: 25% B; 35 min: 30% B; 50 min: 90% B; then kept until 70 min at 100% B. The solvent flow rate was 1 ml/min, the temperature was kept at 25 °C by using a Merck-Hitachi T6300 column heater, and the injector volume selected was 20 µl. The solvents (acetonitrile (MeCN), formic acid) were purchased from VWR Prolab (Leuven, Belgium) in HPLC grade. The eluate was monitored with a photo-diode array detector (DAD) set at 330 and 280 nm.

The peaks of flavonoids in the samples were identified by comparing the retention time (RT) and UV spectra with standards analyzed under the same conditions. The spiked solution was prepared with 500 µl of solution of the extract in water-ACN (95–5) (30 mg/ml) and 100 µl of solution containing the controls (25 µl of each control at 1 mg/ml in MeOH): naringin, naringenin, apigenin, apigenin 7-O-glucoside (structures: see Fig. 2).

2.3. Experimental animals

Male SHR-SP and Wistar rats, aged 14–15 weeks and weighing 250–280 g were used. They were imported from IFFA CREDO (France) and supplied with a health certificate on reception. All rats were maintained at a constant temperature (24 ± 1 °C), with a 12 h dark–light cycle and on standard chow. The care and handling of the animals were in accordance with the internationally accepted principles for laboratory animal use and care as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

2.4. In vivo experiments

SHR-SP rats were randomly assigned to 2 groups: (i) a first group received distilled water (control, n = 6 rats), (ii) a second group received AI extract suspended in water (500 mg/kg BW, n = 6 rats). Since *in vivo* methods are costly in terms of ethical, laboratory animals, labor and economic, the *in vivo* dose was chosen based on our previous studies. These showed that AI extract causes a dose-dependent *in vitro* vaso-relaxant effect without affecting SBP of normotensive rats (El-Hilaly et al., 2004a), and is lower than the NOAEL of 600 mg/kg/day in normal rats (El Hilaly et al., 2004b).

An adaptation period for vehicle administration, metabolic cages and blood pressure measurement was allowed before the initiation of the experimental protocols. Rats were trained to force-feeding during this period. Then all rats were treated orally and daily by force feeding for 1 week. During the experimental period, animals were kept in metabolic cages to collect urine. They were also weighted every day before administration of the treatment. Systolic blood pressure and heart rate were measured by tail-cuff plethysmography (Physiograph Narco, Houston, Tx, USA). SBP was expressed in mm Hg. Each value is the average of three consistent readings.

2.5. Ex vivo experiments

Vascular effects of AI were tested *ex vivo* on aorta isolated from SHR-SP rats that had or not received an oral daily dose of AI (500 mg/kg) for 8 days. Rats were killed by decapitation using small animal guillotine. Thoracic aorta was isolated and placed immediately into a cold (4 °C) physiological solution of the following composition (mM): NaCl 122, KCl 5.9, $NaHCO_3$ 15, $MgCl_2$ 1.25, $CaCl_2$ 1.25 and glucose 11, supplemented with indomethacin (10 µM) and aerated with 95% O_2 and 5% CO_2. Aortas were cleaned of adherent connective tissues and cut into rings of ±2 mm length. Aortic rings were suspended between two stainless steel hooks in 12.5 ml jacketed organ baths containing physiological solution at 37 °C. One hook was fixed to the chamber and the other was connected to an isometric force-displacement transducer as previously described by Morel and Godfraind (1994). Aortic rings were stretched to 20 mN of resting tension.

At the end of the equilibration period (60 min), each preparation was contracted by cumulatively increasing the concentration of noradrenaline (Nad) in the physiological solution. The endothelial integrity of aorta rings was assessed with acetylcholine-induced relaxation (10^{-6} M). Rings not showing maximal relaxation of the Nad-induced contractions were discarded. The presence of viable endothelium occurred

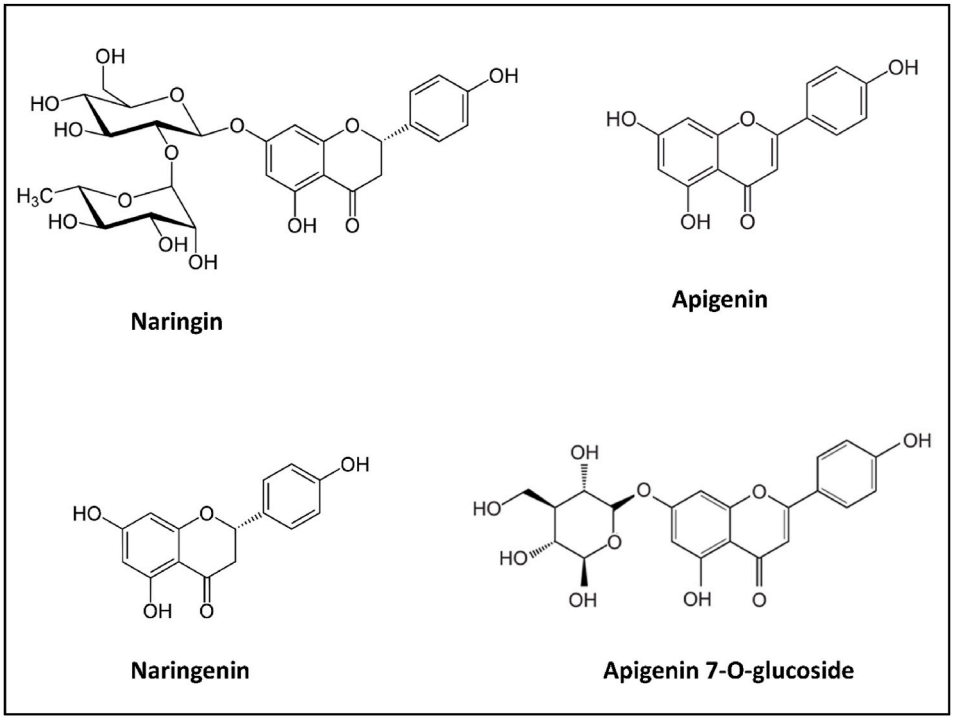

Fig. 2. Chemical structures of naringin, apigenin, naringenin, and apigenin 7-O-glucoside.

when ACh evoked relaxations exceeded 80% of precontraction. After a recuperation period (60 min), the second Nad contraction was conducted. Relaxation to cumulative acetylcholine concentration was measured in aortic rings precontracted with Nad (3 μM). When required, aortic rings were incubated with nitro-L-arginine (L-NNA- 100 μM) for 30 min before contraction to Nad was evoked. At the end of the experiment, lasting 3–4 h, the endothelial integrity of some aorta rings was reexamined, then the tissues were blotted between filter paper and weighed. Contractile tension was expressed in mN per mg of wet weight.

2.6. In vitro experiments

This study was used for investigating the vascular activity of fractions isolated from AI total extract. Aortic rings were prepared as previously described in *ex vivo* tests. However, in these tests, the aorta was isolated from untreated Wistar rats, and AI fractions were added into the bathing solution during the plateau phase of the contraction to noradrenaline (0.3 μM) in rat aortic rings in the presence or in the absence of L NNA. Maximum response is expressed as a percentage of the maximum contraction obtained in the first stimulation to noradrenaline.

2.7. Statistical analysis

Statistical analysis was performed by parametric tests using GraphPad Prism7, since the data fit homoscedastic normal distributions (Shapiro-Wilk test and Levene's test). Data are expressed as mean ± standard error of the mean (SEM) and p-values <0.05 were considered to denote statistical significance of differences. Statistical comparisons were assessed by Student's t-test, or one-way analysis of variance (ANOVA), followed by Tukey's test. pEC50 values (-logEC50, concentration producing half-maximal effect) were obtained by non-linear regression of the individual concentration-response curves.

3. Results

3.1. In vivo effect of AI treatment on urine output and systolic blood pressure

Baseline means of urine output values in treated and untreated groups were 27.2 ± 5.4 ml/day and 25.7 ± 3.0 ml/day, respectively. Our results showed that the oral treatment of SHR-SP rats with AI extract did not significantly change urine output in comparison to baseline as well as to the control group (Fig. 3A).

At the baseline, SBP of untreated and AI-treated group was 234 ± 12.1 mmHg and 237 ± 9.3 mmHg, respectively. The plant extract induced a progressive decrease of SBP starting at day 3. At day 7, SBP in AI-treated rats was decreased significantly ($p < 0.01$) compared to the pre-treatment value. After the end of treatment, SBP returned progressively to its pre-treatment value (Fig. 3B).

3.2. Ex vivo study

3.2.1. Effect of AI treatment on the contractile responses to noradrenaline

In order to investigate whether oral treatment with AI has an effect on reactivity of SHR-SP rats aorta, noradrenaline (Nad; 10 - 9 - 3 10^{-6} M) induced contractile response curves were performed without (Fig. 4A) and with (Fig. 4B) the NO-synthase inhibitor N-nitro-L-arginine (L-NNA, 100 μM). In the absence of L-NNA, the contractile responses of aortic rings from AI-treated rats were significantly reduced compared to those of untreated-rats arteries: Nad Emax was reduced by 44.3% in AI-treated rats compared to control rats [from 13.10 ± 0.55 mN/mg (control) to 7.34 ± 0.12 mN/mg (treated-rats), $P < 0.001$, $n = 8$] (Table 1). Treatment with AI produced a slight but not significant displacement of the concentration-response curve to Nad (Table 1).

As expected, in the presence of L-NNA, Nad-induced contraction was significantly enhanced compared to responses measured in the absence of L-NNA and pEC50 values were significantly increased, in both control rats and AI-treated rats aortic rings (Table 1). In the presence of L-NNA, contraction induced by Nad was reduced in AI-treated rats compared to control rats. However, the effect of AI treatment on the Nad Emax was slightly less pronounced in the presence (reduction of 34%) than in the absence of L-NNA (reduction of 43.3%). The potency of Nad (given by the pEC50) in the presence of L-NNA was not significantly affected by AI-treatment compared to control rats (Table 1).

3.2.2. Ex vivo effect of AI treatment on acetylcholine-induced relaxation

To ascertain whether oral daily administration of AI extract (500 mg/kg BW) can influence the endothelium-dependent relaxation induced by acetylcholine, experiments were performed in aortic rings isolated from AI-treated SHR-SP. As shown in Fig. 5, Nad-precontracted aortas from treated as well as from untreated rats exhibited similar relaxation in response to acetylcholine, and the concentration-response curves were not shifted. The maximum relaxation evoked by acetylcholine 3 μM in both untreated and treated rats was nearly equal. Similarly, the pEC50 value of acetylcholine was not significantly changed (Table 1).

3.3. In vitro experiments

To identify active fractions and potential bioactive compounds of AI aqueous extract, fractionation was conducted with immiscible solvents (see Fig. 1). Among all resulting fractions tested *in vitro*, only A1 fraction

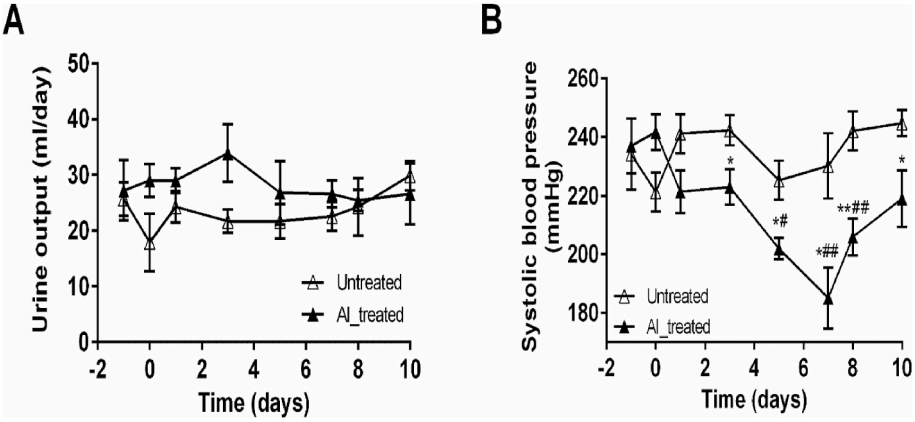

Fig. 3. Effect of treatment with AI extract on urinary output (A) and systolic blood pressure (B). SHR-SP rats were divided into two groups: treated rats received AI extract (500 mg/kg BW, n = 6), control rats received a same volume of water (n = 6). All rats were treated from day 1 to day 7. Mean values ± SEM are shown. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs pre-treatment value of the same group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control after the same period of treatment.

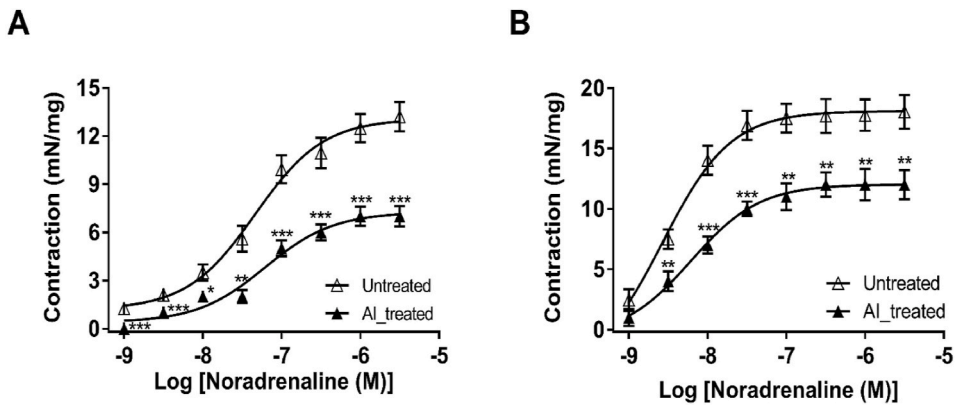

Fig. 4. *Ex vivo* effect of AI on contractions evoked by noradrenaline in aortic rings incubated without (A), and with (B) N-nitro-l-arginine (L-NNA). *Ex vivo* effect was determined in aorta isolated from SHR-SP rats which had received either AI extract (500 mg/kg) or water for 8 days orally. Each point is the mean of 8 determinations. Significant difference between control and AI-treated rats was indicated as: *p < 0.05, **p < 0.01, ***p < 0.001.

Table 1
Responses to the endothelium-dependent vasodilator acetylcholine (ACh) and the α1-adrenoceptor agonist noradrenaline (Nad) in aortic rings from untreated and AI-treated SHR-SP rats.

	Nad		Ach	
	E_{max} (mN/mg)	pEC_{50}	E_{max} (%)	pEC_{50}
Without L-NNA				
Untreated	13.1 ± 0.55	7.34 ± 0.12	57.1 ± 4.2	8.07 ± 0.67
Treated	7.2 ± 0.31[c]	7.21 ± 0.11	55.7 ± 4.0	7.74 ± 0.43
With L-NNA				
Untreated	18.1 ± 0.6[b]	8.5 ± 0.17[a]		
Treated	12.0 ± 0.5[c b]	8.2 ± 0.17[a]		

[a] Significantly different from the value obtained in the absence of L-NNA ($p < 0.05$).
[b] Significantly different from the value obtained in the absence of L-NNA ($p < 0.01$).
[c] Significantly different from untreated rats ($p < 0.001$).

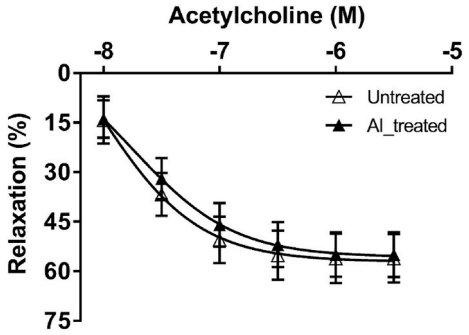

Fig. 5. *Ex vivo* effect of AI extract treatment on the relaxation curves to acetylcholine in aorta. Aortic rings isolated from control rats and from AI-treated rats (500 mg/kg) were pre-contracted by Nad (3 μM) and acetylcholine was added into the solution when the contraction had reached a plateau. Each point is the mean from 8 determinations.

reduced the Nad maximal contraction by 26%, ($p < 0.001$) at 100 μg/ml (Fig. 6A).

The relation concentration-effect of A1 fraction was examined on aorta segments incubated in presence and in absence of L-NNA (Fig. 6B). Without L-NNA, the fraction produced a dose-dependent relaxation of noradrenaline-induced contraction. The highest dose tested (600 μg/ml) caused 53% relaxation ($p < 0.001$) which was approximately two times more than with the lower dose (26% at 100 μg/ml). No effect was observed in the presence of L-NNA.

3.4. HPLC chromatography

AI and A1 samples were analyzed by HPLC using the method described by Boudjelal et al. (2015). As they indicated that naringenin and apigenin were present and showed that the major compound was naringin, but also found apigenin 7-O-glucoside, we used these compounds as standards. Our results showed that, besides many unidentified peaks, two peaks attributed to naringin and apigenin 7-O-glucoside were detected in the chromatograms, by comparison with their corresponding reference standards. Typical chromatogram of standards, AI and A1 samples are shown in Fig. 7. Naringin, apigenin 7-O-glucoside, naringenin and apigenin eluted at retention times of 25, 27, 40.5 and 42 min, respectively.

While chromatograms of AI and A1 extracts showed many unidentified peaks that often co-elute before 25min, they did not show significant peaks after 35 min. We can thus conclude to the absence of genines, as naringenin or apigenin and the presence of mainly glycosylated compounds as glycosylated flavonoids. The chromatograms of both AI extract (Fig. 7I) and A1 fraction (Fig.7II), spiked by 4 standards, confirmed the presence of naringin and apigenin-7-O-glucoside. We also observed that both extracts have qualitative comparable profiles for the compounds identified or detected with this LC-DAD method, particularly flavonoids and phenolic compounds and that profiles were more complex than those found by Boudjelal et al.

4. Discussion

AI is a medicinal plant which is widely used as a panacea to treat a plethora of ailments including hypertension (Tahraoui et al., 2007). Additionally, in northern and south-eastern Morocco, hypertensive and diabetic patients take AI alone (Tahraoui et al., 2007) or in association with *Artemisia herba-alba* or *Olea europea* leaves; a teaspoon of powder mixture is taken orally three times a day to treat diabetes (Boudjelal et al., 2015; El-Hilaly et al., 2003). This dosage and the optimum pharmacological dose used in this work remain much lower than the NOAEL for AI (El Hilaly et al., 2004b). Besides, the antihypertensive dose of 500 mg/kg applied in these *in vivo* tests seems realistic since the decoction of AI yielded nearly 22%.

This study investigated the diuretic, antihypertensive, and *ex vivo* vasorelaxant effects of oral treatment of SHR-SP with AI extract. We also studied the *in vitro* activity of several fractions of AI decoction and quantified naringin in the crude extract and the most active fraction.

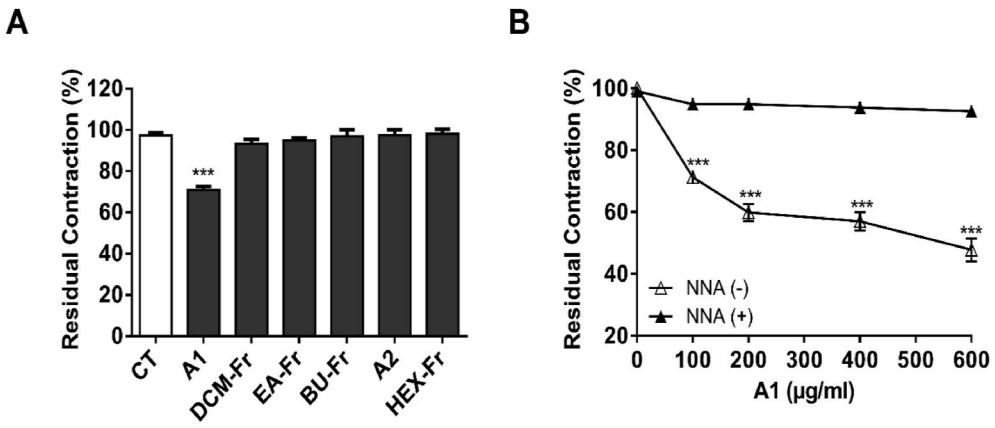

Fig. 6. (A) *In vitro* vasorelaxant activity of the six fractions (HEX-Fr = hexane fraction, DCM-Fr = dichloromethane fraction, EA-Fr = ethyl acetate fraction, Bu-Fr = butanol fraction, A2 = precipitate of the residual aqueous extract, A1 = lyophilized supernatant of the residual aqueous extract) resulting from liquid–liquid fractionation of the AI decoction on the contraction evoked by noradrenaline (3 µM) in aortic rings incubated with, or without L-NNA. Fractions extracts (100 µg/ml) were added into the bathing solution during the plateau phase of the contraction to noradrenaline. (B) Concentration effect relationship of the active fraction (A1) tested on noradrenaline-evoked contraction (3 µM) in the absence or in the presence of NNA. Each value is the mean ± S.E.M. from 8 determinations. ***p < 0.001 vs control group.

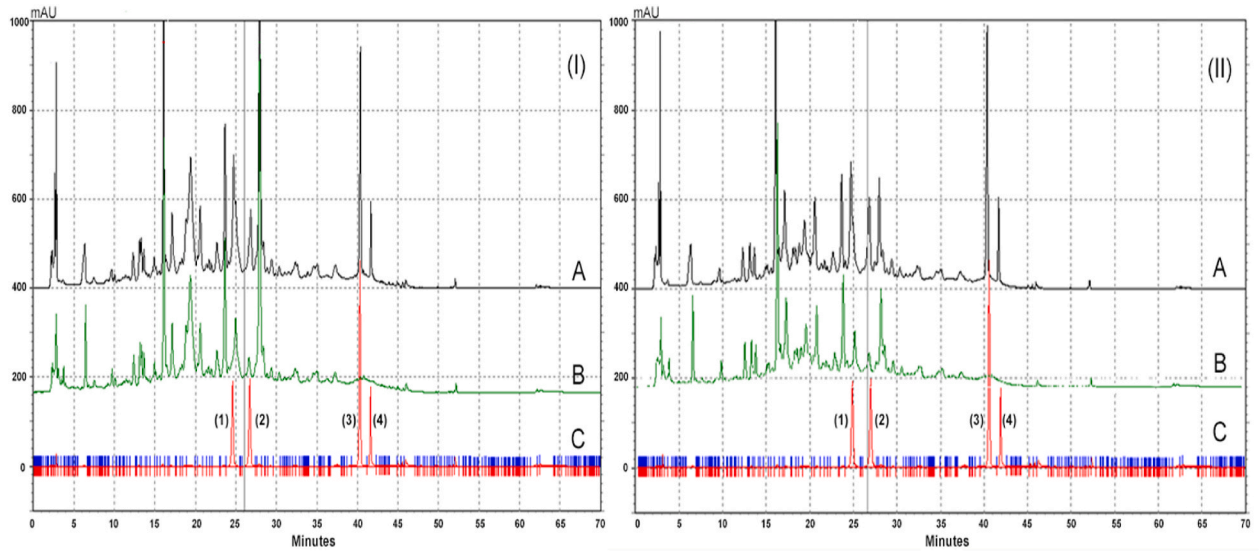

Fig. 7. (I) HPLC chromatograms of (A) spiked AI total aqueous extract, (B) AI total aqueous extract and (C) mixed standards solution of flavonoids (1 = naringin, 2 = apigenin 7-O-glucoside, 3 = naringenin, 4 = apigenin). (II) HPLC chromatograms of (A) spiked A1 residual aqueous extract total aqueous extract, (B) A1 residual aqueous extract and (C) mixed standards solution (1 = naringin, 2 = apigenin 7-O-glucoside, 3 = naringenin, 4 = apigenin).

The short-term treatment with AI extract to SHR-SP resulted in a significant lowering of systolic blood pressure, but did not change water diuresis. In contrast to our findings, previous studies carried out on other species of *Ajuga* have reported significant diuresis in mice (Hailu and Engidawork, 2014). Thus, the monitored antihypertensive effect appears unlikely to be related to a diuretic mechanism. In agreement with our previous study performed in normotensive Wistar rats (El-Hilaly et al., 2004a), short term AI treatment induced a decrease in vascular contraction to noradrenaline, while AI treatment did not modulate acetylcholine-evoked relaxation compared to untreated rats, neither the maximum relaxation nor pEC50 values of acetylcholine concentration-response curves. This observation suggests that AI extract does not interact either with the cholinergic receptor nor the endothelium-dependent NO-production and relaxing effect.

However, it is worth mentioning that Emax of acetylcholine for both treated and untreated groups of SHR-SP were only slightly smaller than what was observed in normotensive rats (El-Hilaly et al., 2004a). This contradicts some reports indicating that the acetylcholine induced relaxation of pre-contracted thoracic aortas from SHR-SP rats is markedly smaller than in normotensive rats (Sekiguchi et al., 2001). It can be postulated that the rats used in the present experiments had not yet fully developed aortic endothelium impairment. Indeed, SHR-SP exhibit progressive decrease in endothelium-dependent acetylcholine-induced relaxation with advancing age, so that significant alteration is not observed before 16 weeks (Lee et al., 2017; Sunano et al., 1996). In this line, it has been reported that SHR-SP rats develop oxidative stress, which increases free radicals, inactivates NO and therefore impairs the NO dependent vasodilatation (Suzuki et al., 1995). Some studies have indicated that AI aqueous extract reduces oxidative stress and exhibits antioxidant activity (Bouderbala et al., 2010; Hirooka and Sunagawa, 2017; Taleb-Senouci et al., 2009). It should be interesting to determine whether prolong treatment with AI could be able to prevent development of endothelium alteration in hypertensive models.

It is also interesting to note that short term AI treatment (8 days) markedly reduced SBP of stroke-prone spontaneously hypertensive rats, while it does not affect that of normotensive Wistar rats (El-Hilaly et al., 2004a). These results are congruent with other findings reporting that oral daily dose of quercetin reduced blood pressure, endothelial dysfunction, and oxidant status in spontaneously hypertensive (SHR) rats, but had no effect on normotensive (WKY) rats (Del Pino-García et al., 2017; Duarte et al., 2001). This observation suggests that AI should selectively affect a process involved in the development of

hypertension. It is presumably not relied on cell surface voltage-gated Ca^{2+} channels (VGCCs) because AI extract does not significantly inhibit KCl-induced contraction (El-Hilaly et al., 2004a). AI extract may show its hypotensive effect by acting through an α1-adrenoceptor blockage (Yin et al., 2014; Zhang et al., 2019), but other targets as ion channels cannot be rejected.

As all experiments were performed in the presence of indomethacin, a non-selective cyclooxygenase inhibitor, contribution of prostanoids to the vasodilatory effect of AI might be neglected.

It is well known that the relationship between blood pressure, cardiac output and systemic vascular resistance is modulated by vasorelaxation (Clark et al., 2015). Moreover, AI aqueous extract showed significant *ex vivo* NO independent vasorelaxant effect on hypertensive and normotensive aorta rats.

Liquid-liquid fractionation showed that only the most polar fraction inhibited *in vitro* Nad contraction without NNA, but the activity disappeared when NNA was added. A1 fraction induced isolated Wistar aorta relaxation in an endothelium- and concentration-dependent manner with eNOS pathways mechanisms. Importantly, AI total extract showed previously an *in vitro* biphasic effect on the NO pathway in endothelial cells: a transient NO-dependent relaxation followed by a toxic effect resulting in the irreversible inhibition of the activity of the NO synthase, which may be related to the presence of different compounds (El-Hilaly et al., 2004a). Afterwards, bioguided fractionation of AI crude extract could have led to the elimination of the toxic effect and preservation of endothelial NOS-dependent vasorelaxation, which was found only in A1 fraction.

According to HPLC analysis, this fraction seems to contain mainly polar phenolic compounds, among which we identified flavonoid glycosides as naringin and apigenin 7-O-glucoside. The absence of active fractions with NO independent vasorelaxant effect could perhaps be attributed to the synergetic effect of active compounds that were separated upon their polarity. This could also be explained by metabolization as the extract was given by oral route. It has for example recently been shown than flavonoid metabolites can have good vasorelaxant properties with different modes of action (endothelium dependent and/or independent) than their parent compounds (Pourovaa et al., 2018). These suggestions seem plausible since the parent total extract exhibited dose-dependent, NO-mediated and NO- independent vasodilatory activities at relatively high doses (El-Hilaly et al., 2004a).

Taken together, these results suggest that the blood pressure may be lowered at least in part by flavonoids via modulating vascular reactivity. This is in accordance with previous data that considered flavonoids, largely present in AI (Boudjelal et al., 2015; Manguro et al., 2007), as potential compounds responsible for most plant induced vasorelaxation (Woodman and Chan, 2004; Xu et al., 2015). Some reports also showed that phenolic compounds and pure flavonoids reduce blood pressure in spontaneous hypertensive rats (Alam et al., 2013; Plotnikov et al., 2017; Waltenberger et al., 2016) and that the antihypertensive effect of flavonoids is due to their ability to improve endothelial function (Egert et al., 2009).

On other hand, other chemical compounds with reported vasodilatory properties have been identified in the genus *Ajuga* such as triterpenes, diterpenes (Cantrell et al., 1999), glycosides (Konoshima et al., 2000), flavonoids (Boudjelal et al., 2015), and tannins (Terahara et al., 1996). Therefore, it is speculative to extrapolate the effects observed *in vitro* to *in vivo* system, as most of these compounds are subjected to biotransformation and bioactivation enzymes (Walle, 2004). In this sense, *in vivo* antihypertensive studies of these subfractions and isolated compounds as well as an understanding of their metabolism and bioavailability are required to ascertain these hypotheses.

Comparing the composition of the crude extract and the most active fraction by HPLC-UV, we showed the presence of narigenin and apigenin 7-O-glucoside and many other unidentified peaks.

Although many studies on flavonoids of *Ajuga* genus are available, there is still a lack of data corresponding to AI species, and it would be interesting to determine those more related to the vasorelaxant activity.

5. Conclusion

In conclusion, AI aqueous extract exhibited antihypertensive effect in SHR-SP and decreased vascular contraction to noradrenaline. These effects may be due, at least in part, to polar phenolic compounds as flavonoid glycosides and/or their metabolites. These findings support the traditional use of AI to treat hypertension, and provide useful information for better use of AI in traditional medicine. Nevertheless, further studies are mandatory to determine the structure of these flavonoid glycosides and to ascertain their *in vivo* effect on blood pressure.

Author contribution

The design of the study was carried out by J Q-L, B L and N M. The experiments were done by J E-H, M-Y A and N M. The manuscript was written by J E-H and revised by N M, B L, M-Y A and J Q-L.

Declaration of competing interest

There is no competing financial interest for any author of this article, whether actual or potential.

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