Spergularia marina: a potential source of a novel calcium channel blocker with antihypertensive and diuretic activities

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Abstract. – **OBJECTIVE:** *Spergularia marina* (L.) Griseb. (*S. marina*) is a sub-cosmopolitan species used as traditional phytotherapy based on diverse biological activities. It is native and widespread in the northern hemisphere, though introduced also into the southern hemisphere. The extract of another species '*Spergularia purpurea*' has been traditionally used in Morocco against various diseases and *S. marina*, itself, is a local popular food in South Korea. In this context, we evaluated the potential antihypertensive and diuretic effects of *S. marina* water and n-butanol extracts in L-NAME-induced hypertensive rats *vs.* the well-known diuretic, furosemide.

MATERIALS AND METHODS: After toxicity studies, selected doses were administered orally daily for one week. Mean arterial blood pressure (MABP), water/electrolyte clearance, renal functions, and serum electrolytes were assessed. Vascular reactivity of isolated aortic rings was evaluated under different incubating settings against various antagonists to unravel the mechanism of action.

RESULTS: Both extracts significantly reduced the MABP. Only, the *n*-butanol fraction exerted a significant aquaresis, increasing electrolyte free-water clearance with a significantly decreased urinary Na$^+$, K$^+$, and C$^-$ excretion. The water extract significantly augmented the ACh-induced relaxation and attenuated the NE-induced aortic rings' contractile response. It also exhibited a direct relaxant effect on the NE-precontracted rings with intact or denuded endothelium. Blocking the vascular calcium channels by preincubation with nifedipine prevented the *S. marina*-induced relaxation, denoting a calcium channel blocking activity.

CONCLUSIONS: The vasorelaxant and the differential diuretic effects of both extracts introduce *S. marina* as a potential novel antihypertensive agent with calcium channel blocking activity. To enrich cardiovascular therapeutics, human studies to confirm the efficacy and safety of *S. marina* in hypertension are warranted.

Key Words:
Aortic rings, Blood pressure, Furosemide, Endothelium, L-NAME.
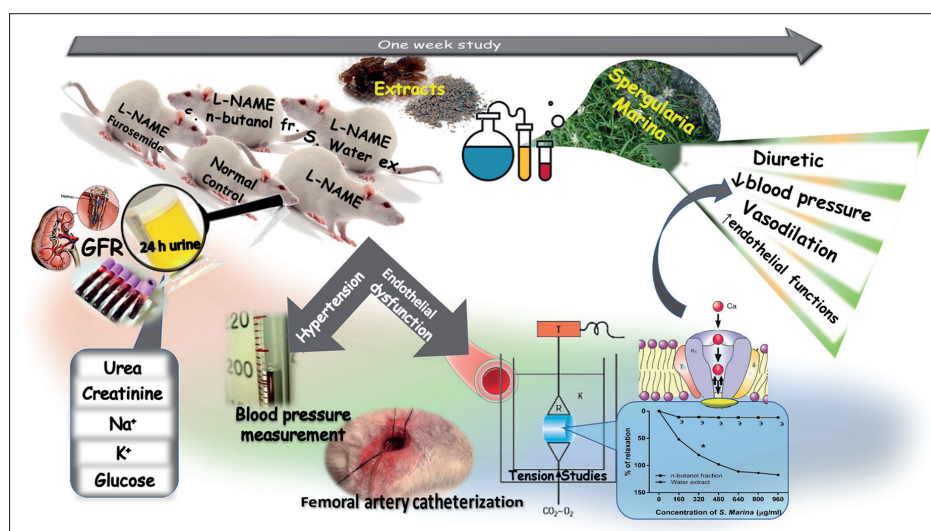
Graphical Abstract. The ethnopharmacological utility of the extracts of *Spergularia marina* with antihypertensive, diuretic and vasculoprotective properties was investigated in reference to furosemide.

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Abbreviations

S. marina: Spergularia marina; MABP: mean arterial blood pressure; L-NAME: N^G-nitro-L-arginine methyl ester; E-Cosm: electrolyte clearance; E-CH$_2$O: electrolyte free water clearance; GFR: Glomerular filtration rate; HPLC-UV/Vis: ultraviolet visible HPLC detector.

Introduction

Hypertension is one of the major risk factors for cardiovascular illnesses, as well as for end organ damage. Consequently, it bears a tremendous socioeconomic burden worldwide. Vasodilators play a major role in cardiovascular therapeutics. However, they did not expand over the last decades, and concern has been raised on their tolerability[1]. Investigations of the traditionally used herbal medicine could be a valuable tool to enrich novel vasodilators, more preciously if they combine a diuretic action and a more tolerable profile. It has been demonstrated that flavonoids extracted from some medicinal plants could exhibit antihypertensive and possible diuretic activity. Of interest is the Genus *Spergularia (S)* that belongs to the family *Caryophyllaceae* and includes more than 50 species distributed worldwide mostly in the Mediterranean region[2]. Some of the *S.* species were found to have several biological activities. For many years, the water extract of the whole plant of *S. purpurea* has been traditionally used in Morocco against various diseases, like hypertension, diabetes, renal, and cardiac diseases[3-6].

Of these *S.* species, *S. marina* (L.) Griseb. is a sub-cosmopolitan species documented in www.theplantlist.org (Record 6301824). It is native and widespread in northern hemisphere but also introduced in southern hemisphere. It is widely distributed in most of the countries in the Mediterranean region and north Africa[2]. Moreover, it has been used as a local vegetable in South Korea to prevent several chronic diseases[7-9].

Few phytochemical studies of *S. marina* were done and reported the presence of amino acids, vitamins, minerals, flavonoids, glycoside, phenolics, saponins, alkaloids, and tannins[10-13]. Several biological effects of this plant had already been reported. These include antioxidant, antibacterial, antifungal, anti-inflammatory, anti-adipogenic, pro-osteoblastogenic, and hypoglycemic activities, as well as reducing insulin resistance[7-10,12,14]. However, its antihypertensive and diuretic effects have not been evaluated. Thus, the aim of the present study was to investigate the antihypertensive and diuretic potential of *S. marina*. The activity of the water extract and the *n*-butanol fraction of this species was evaluated on N^G-nitro-L-arginine methyl ester (L-NAME)-induced hypertensive rats versus the loop diuretic, furosemide. Effects on vascular reactivity and renal functions were also investigated. Lastly, we explored the mechanism(s) behind *S. marina* vascular effects to provide a scientific background to its medicinal use.

Materials and Methods

Plant Materials

Specimens of *Spergularia marina (L.) Griseb.* were collected in April 2017 during flowering stage from Rosetta 40-km East of Alexandria-Egypt. The plant origin was identified by prof. Rokia M. Abdallah and voucher specimens of *S. marina* were deposited in the Herbarium of Department of Botany, Faculty of Science, Alexandria University-Egypt [SPMA.2017]. Phytochemical investigations of *S. marina* crude extract were carried out qualitatively for the presence of alkaloids, saponins, tannins, and anthraquinones according to a standard method[5].

Extracts Preparation

Water extract was prepared according to the traditional method used in Morocco for preparation of *S. purpurea* water extract[4]. For *n-butanol fraction,* fresh aerial part (500 g) of *S. marina* was chopped and exhaustively extracted with 70% ethanol (2 L) at room temperature. The extract was concentrated under reduced pressure to 200 ml, then fractionated successively with petroleum ether (3 x 200 ml), chloroform (2 x 200 ml), ethyl acetate (2 x 200 ml), and finally *n*-butanol (2 x 200 ml). The *n*-butanol fraction was evaporated under reduced pressure to 1.0 g[15]. Both extracts were reconstituted in 2% gum acacia daily just before administration.

HPLC Profiling for Preliminary Phytochemical Constituents' Comparison of the Two Extracts

HPLC analysis was performed with Dionex UltiMate 3000 system coupled to a UV-VIS photodiode array detector. Routine detection was at 235, 254, 280, and 340 nm. The separation column (125×4 mm, Knauer, Berlin, Germany)

was prefilled with Eurosphere 100-5 C18. The autosampler injected 30 μL of each solution in a separate 60 min. run. A step gradient elution of MeOH and H_2O with a flow rate of 1 mL/min was used as follows: 5 min. (10% MeOH); 30 min. (from 10% to 100% MeOH); 10 min. (100% MeOH); 5 min. (from 100% to 10% MeOH); and 10 min. (from 10% to 0% MeOH). UV spectra were measured using a Perkin-Elmer Lambda 25 UV/vis spectrometer. Maximum absorbance was detected at 235 nm channel. The UV spectra of individual peaks from the 235 nm channel were scanned with wavelength ranging from 200-595 nm. Common compounds were identified by their similar retention times and UV spectra. Flavonoids were identified by their characteristic UV spectra and comparative quantification were done by comparing individual and total peak area (mAU*min) between the two extracts.

Experimental Procedures

Animals

The study was carried out on 71 male Wistar rats, weighing 200-230 g, and purchased from the Experimental Animal Centre in the Faculty of Medicine, Alexandria University. Animals were housed in a temperature and light-controlled conditions with food and water ad libitum. Procedures involving animals comply with the ARRIVE guidelines and the experiments were conducted in conformity with the NIH guide for care and use of laboratory animals. Study protocol was approved by the Ethics Committee of the Faculty of Medicine, Alexandria University (Protocol approval number: 0303690).

Toxicity Studies[16]

Toxicity studies were conducted on 27 rats for dose establishment of the *S. marina* extracts. Animals were divided into 3-equal groups: normal control, water extract-treated and *n*-butanol-treated rats. The later groups were divided into subgroups of 3-rat each, that were subjected to 3 different oral doses within a logarithmic range of (10-1000 mg/kg) for the water extract or (2-200 mg/kg) for the *n*-butanol fraction, respectively. Close daily observation of the rats for 3 weeks was performed for mortality or pre-set humane endpoints. At the end, rats were sacrificed for gross necropsy, and hematological and biochemical studies to identify possible target organ(s) toxicity[16].

Experimental Design

After acclimatization, animals were randomly divided into normal control (8-rat, receiving oral vehicle) and hypertensive group (36-rat). Hypertension was induced by oral administration of the nitric oxide synthase (NOS) inhibitor L-NAME (Sigma-Aldrich) in a dose of 40 mg/kg/day for 7 days[17]. After one week, the hypertensive group was further subdivided into 4-equal subgroups, according to the treating agents; water extract (400 mg/kg/day), *n*-butanol fraction (50 mg/kg/day), furosemide (16 mg/kg/day, Lasix-Sanofi-Aventis), or oral gum acacia as a vehicle for one week[18]. Only animals with MABP between 110-130 mmHg were included in the study. Doses of both extracts were extrapolated from both toxicity and pilot (data not shown) studies.

Diuretic Activity

Three days before the end of the study, animals were individually placed into metabolic cages and 24-hour urine was collected in graduated cylinders. The mean daily collected urine output was calculated in relation to the body weight and expressed as ml/100 g.bw/day. For diuretic effect, three parameters; urinary water excretion, diuretic action, and diuretic activity were calculated using the following formulae[19]:

$$\text{Urinary excretion} = \frac{\text{Total urinary output}}{\text{Total liquid administered}} \times 100\%$$

$$\text{Diuretic action} = \frac{\text{Urinary excretion of treated group}}{\text{Urinary excretion of negative control group}}$$

$$\text{Diuretic activity} = \frac{\text{Diuretic action of test drug}}{\text{Diuretic action of standard drug}}$$

Where, total urinary output: urine volume in ml/100 g.bw/24 hours, total liquid administered: range of 35-40 ml/24-h, normal control diuretic action equals 1, and diuretic activity of furosemide equals 1

After measurement of urine volume, a portion of filtered urine was centrifuged at 1870 g for 10 min. and the supernatant was used for determination of the urinary parameters (urea, creatinine, electrolytes, glucose, and osmolality). The elec-

trolyte clearance (E-Cosm) and electrolyte free water clearance (E-C_{H2O}) that provide further details about natriuretic and aquaretic effects of S. marina extracts, respectively were calculated via the following formulae[20]: E-Cosm = [(U_{Na} + U_K) UV]/S_{Na}

$$E-C_{H2O} = UV - E-Cosm$$

Where UV: urine volume, U_{Na}: urinary sodium concentration, U_K: urinary potassium concentration, and S_{Na}: serum sodium concentration (Serum K^+ is negligible).

Measurement of MABP

At the end of the 1st week, animals were anaesthetized with sodium thiopental (50 mg/kg) for blood pressure measurement via femoral artery catheterization[21] using polyethylene tubing, PE-50 and -10 (Intradmedic Clay Adams®, Becton Dickinson Primary Care Diagnostics). The tubes were connected to a physiological pressure transducer (MLT844, ADInstruments) and Powerlab system (PowerLab 8/35 with Lab-Chart-Pro Module Software ADInstrument). Systolic and diastolic pressures were daily recorded, and mean values were calculated for the last three days.

Serum Biochemical Analysis

At the end of the study, rats were anesthetized, and blood samples were collected for analysis of the serum electrolytes (Na^+ and K^+), glucose, urea, creatinine, and osmolarity. The glomerular filtration rate (GFR) was estimated using the following formula[22]: GFR (ml/min/100 g.bw) = Ucr × UV/ (Scr × 1440)

Where, Ucr: urine creatinine concentration (mg/dl), UV: 24-h urine volume (ml/100 g b.wt), Scr: serum creatinine concentration (mg/dl), and 1440: number of minutes/day.

In Vitro Isometric Tension Studies on Isolated Aortic Rings

Isometric tension studies[23-29] followed two protocols. The first one aimed to compare the vascular reactivity of aortic rings isolated from hypertensive rats treated with either *S. marina* extracts versus those from non-treated hypertensive or normal rats. The second protocol included series of experiments aimed to test the direct effect of both extracts on isolated normal rings, least present, and to unravel its underlying mechanism(s)[23-29].

Protocol I

From each rat, aortic rings were prepared from isolated segments of endothelium-intact thoracic aortae. Rings were then suspended in organ baths, where aortic ring tension was recorded with sensitive force isometric transducers (MLT0202, ADinstruments) connected to Powelab 8/35 data acquisition system (PL3508/P, ADinstruments). Cumulative contractile dose-response curves to increasing doses of Norepinephrine (NE: 1 nM-0.1 mM, Sigma-Aldrich) were initially obtained. Maximally contracted rings were then exposed to serial increasing concentrations of acetylcholine (ACh: 1 nM-0.1 mM, Sigma-Aldrich). Finally, relaxant responses were expressed as a percent reduction of the tension relative to the NE peak response[23].

Protocol II

The first series of experiments: After confirming the endothelial integrity of isolated rings, the direct effect of *S. marina* serial doses on NE-precontracted rings was examined (5-960 µg/ml final concentration in organ bath for both extracts) and dose-response curves were obtained. The next set of experiments was continued with water extract only, since it was the one that gave a significant direct relaxant effect.

The second series of experiments aimed to discover whether the observed direct relaxant effect of water extract was endothelium-dependent. Therefore, previous experiments were repeated on aortic rings with denuded endothelium as confirmed by lack of ACh-relaxant effect on NE-precontracted rings.

The third series of experiments aimed to find out the possible mechanism(s) behind the direct relaxant effect of *S. marina*. Water extract dose-response curves were obtained after 20-min incubation of endothelium-intact normal aortic rings with L-NAME (3×10^{-4} M), nifedipine (3×10^{-5} M) glibenclamide (1×10^{-5} M), indomethacin (3×10^{-5} M), atropine sulphate (1×10^{-6} M), or propranolol (1 uM) (all purchased from Sigma-Aldrich). Our goal was to detect possible links to NOS, calcium channel, potassium channel, prostaglandin, cholinergic, or beta-adrenergic receptors, respectively[24-27].

In the fourth series of experiments, S. marina effect on intra- and extracellular Ca^{+2} influx was examined. This was done by pre-incubation of endothelium-denuded aortic rings with a submaximal dose of water extract (480 µg/ml final concentration in organ bath) for 20-min. Then, their cumulative contractile responses to NE

were compared to that of non-incubated normal aortic rings. The same experiments were repeated after washing and incubating the aortic rings with Ca^{+2} free solution rather than the standard Kreb's solution. Finally, the role of extracellular Ca^{+2} influx was inspected by recording the contractile response to NE maximal dose (10 µM) triggered by cumulative concentrations of Ca^{+2} (0.1-3.0 mM) in the presence or absence of *S. marina* in the organ bath[26, 29].

Nitric Oxide Assay

The *S. marina* NO-releasing potential was evaluated. The NO production from lysates of rings incubated with *S. marina* was measured, using the *Griess* reaction, according to the manufacturer's instructions[30].

Statistical Analysis

Statistical comparisons between the different groups were made with One-way analysis of variance (ANOVA), while Data obtained from dose-response isometric tension studies were analyzed by the two-way repeated-measure ANOVA, followed by least significant difference (LSD) criterion for comparison. All data were expressed as mean ± SEM. Statistics were done using Graph-Pad Prism software (version 7.0). *p*-values < 0.05 were considered significant.

Results

Comparison of the Phytochemical Constituents of S. marina Extracts

Phytochemical analysis revealed a difference in the number of compounds between the water and *n*-butanol extracts. However, the main finding is the significant higher *n*-butanol flavonoids content and number of detected compounds, as well (Table I, **Supplementary Table I** and **Supplementary Figure 1, 2** and **3**). The two extracts share five compounds, which constitute 62 and 65% of the total detected areas for water and *n*-butanol extract, respectively (Table I). Four out of them are preliminarily identified as flavonoids from their characteristic UV spectra (**Supplementary Figure 2** and **Supplementary Figure 3**) and represent 36 and 64% of the total content in water and *n*-butanol extracts, respectively.

Effect of S. marina Extracts on Rats' General Condition

Tested doses did not reveal any signs of toxicity or organ damage. No mortality was observed during the study period. Therefore, a dose of 400 mg/kg for water extract was chosen like that used in *S. purpurea* study, while a dose of 50 mg/kg was chosen for *n*-butanol based on a pilot study.

Effect of S. marina Extracts on MABP

Inhibition of NO synthesis by L-NAME was associated with a significant rise in MABP. The administration of either extracts was associated with a nearly comparable significant decrease in MABP versus non-treated hypertensive rats, although significantly less than furosemide (Figure 1*a*).

Effect of S. marina Extracts on Urinary and Serum Electrolytes, and Renal Functions

In contrast to serum parameters, urinary electrolytes showed statistically significant differ-

Table I. Preliminary comparison of phytochemical constituents of *S. marina* extracts.

Parameters	Water extract	*n*-butanol
Total area of all detected compounds (mAU*min)	32.008	952.030
Total number of detected compounds (peaks)	9	19
Number of suggested flavonoids compounds	5	12
Sum of flavonoids percentage	42%	82%
Number of common non-flavonoids compounds	1	
Percentage of the common non-flavonoids compounds	26%	1%
Number of common flavonoids	4	
Sum of the percentage of common flavonoids	36%	64%

The water and *n*-butanol extracts contained 9 and 19 compounds, respectively and total peak areas were 32.008 and 952.030 mAU*min, respectively. The majority of n-butanol extract content (82%) is suggested to be flavonoids (12 out of 19) *vs*. 42% for water extract (5 out of 9). Across the two extracts, only 5 compounds (4 of which are suggested to be flavonoids) are common and the 4 flavonoids represent 36 and 64% of total content in water and n-butanol extracts, respectively. mAU*min: Milli-Absorbance Units*minute.
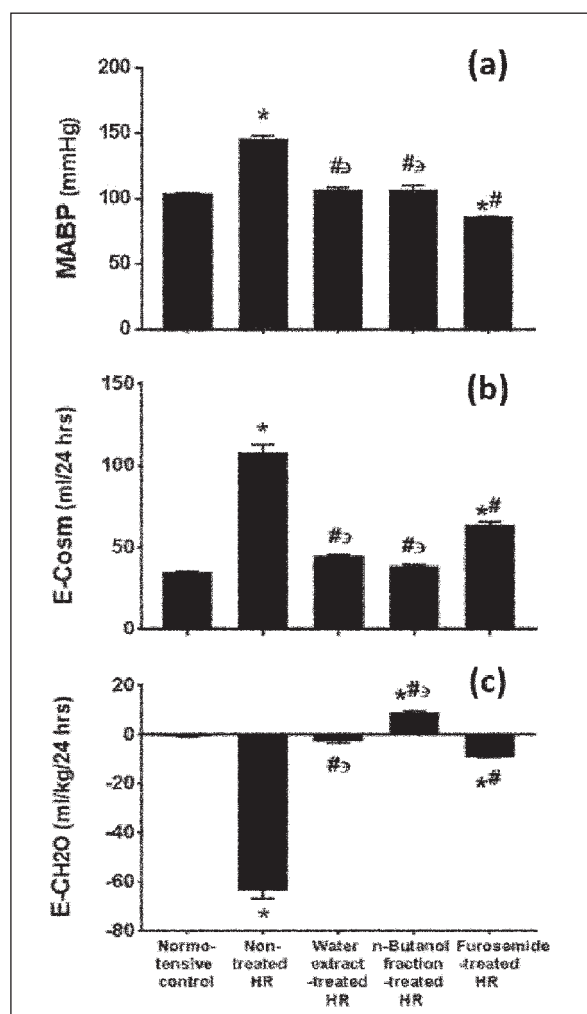
Figure 1. Effect of *S. marina* extracts on mean arterial blood pressure in (**a**), electrolyte clearance (E-Cosm) in (**b**), and on electrolyte free water clearance (E-CH2O) in (**c**). MABP: mean arterial blood pressure, HR: hypertensive rats. *: Significant difference *vs.* normotensive control rats, #: Significant difference *vs.* non-treated hypertensive rats, ᵓ: Significant difference *vs.* furosemide-treated hypertensive rats; n=8. Data are expressed as mean ± SEM. *p*< 0.05.

ence between groups as shown in Table II. A non-significant change of urinary urea and creatinine was observed in both extracts versus normotensive control in the contrary to other hypertensive rats. Regarding urinary electrolytes particularly Na⁺, all treated groups showed a significant increase versus normotensive control. However, the *n*-butanol fraction yielded the least increase versus all groups. Both extracts did significantly decrease GFR versus other hypertensive groups.

Effect of S. marina Extracts on Diuretic Activity, Electrolyte Clearance (E-Cosm) and Electrolyte Free Water Clearance (E-C$_{H2O}$)

A significant diuresis and natriuresis were induced by L-NAME associated with an increase in GFR. The *n*-butanol significantly increased urinary excretion and diuretic action. Its diuretic activity was 0.87 that of furosemide (Table III). Meanwhile, the water extract diuretic action was non-significantly different versus the non-treated hypertensive rats with a diuretic activity 0.77 that of furosemide. Both extracts significantly decreased E-Cosm versus other hypertensive rats. However, only the *n*-butanol fraction expressed a significant aquaretic effect with a significant decrease in urinary Na⁺, K⁺, and Cl⁻ excretion versus natriuresis encountered in all other hypertensive rats. This was evidenced by the significant increase in E-C$_{H2O}$ to a positive value (Figure 1, Table III).

Effects of S. marina Extracts on Isolated Aortic Rings and Possible Underlying Mechanism

Primarily, NE cumulative concentrations induced vasoconstriction in a dose-dependent manner in normal control aortae, with maximum contraction achieved at 10^{-5} mol/L (Figure 2*a*). The maximally contracted rings showed a dose-dependent relaxant response to ACh cumulative doses (Figure 2*b*). The L-NAME induced endothelial dysfunction was evidenced by a significant enhancement of the NE-contractile response with left shift of EC$_{50}$. and by significant attenuation of ACh-induced relaxant responses. The mean E$_{max}$ value was significantly decreased to less than 20% of normal. Rings isolated from water extract-treated rats demonstrated a significant attenuation of the NE contractile responses even relative to normal control with a significant right shift of the dose-response curve. However, treatment with the *n*-butanol fraction almost showed non-significant change versus that of the L-NAME-treated rats. Alternatively, rings of the water extract-treated rats showed a significant augmentation of ACh-induced relaxation (Figure 2*b*). To confirm *S. marina* favorable effect on vascular reactivity, direct application of either extracts on normal aortic ring maximally precontracted with NE was done. We traced a dose-dependent relaxant response to the water extract (Figure 3*a*) with an E-$_{max}$ of

Table II. Effect of *S. marin*a extracts on urinary electrolytes and renal functions.

Rats variables	Urine						GFR (ml/min/ 100 g.bwt)
	Urea (mg/dl)	Creatinine (mg/dl)	Na+ (mmol/l)	K+ (mmol/l)	Cl- (mEq/l)	Osmolarity (mOsm/ Kg H_2O)	
Normotensive control	2780 + 104	15 + 0.62	6.5 + 0.44	131 + 2.38	175 + 8.29	1665 + 62.5	0.09 + 0.006
Non-treated HR	1928 + 165[a]	29 + 0.32[a]	67.5 + 1.72[a]	272 + 0.79[a]	140 + 1.42 [a]	1155 + 98.7[a]	0.22 + 0.013[a]
Water extract-treated HR	2565 + 165[bc]	19 + 0.32[bc]	51.5 + 1.29[ab]	94 + 5.68[abc]	125 + 0.69[ac]	1537 + 98.9[bc]	0.14 + 0.006[abc]
n-Butanol fraction-treated HR	2320 + 79.5[c]	13.8 + 1.15[bc]	15 + 1.83[abc]	97.5 + 3.14[abc]	104 + 1.93[abc]	1442 + 66.1[c]	0.12 + 0.004[abc]
Furosemide-treated HR	1579 + 99.4[ab]	27 + 1.96[a]	56.5 + 1.64[ab]	150 + 2.02[ab]	195 + 5.07[ab]	401 + 14.4[ab]	0.19 + 0.010[a]

GFR: glomerular filtration rate, HR: hypertensive rats [a]: Significant difference vs. normotensive control rats, [b]: Significant difference vs. non-treated hypertensive rats, [c]: Significant difference vs. furosemide-treated hypertensive rats. Data expressed as mean ± SEM. $p < 0.05$

640 µg/ml to achieve up to 117.3±6.6% relaxation (Figure 3*b*). Such relaxant effect was only minimally observed with the *n*-butanol fraction (11.91±0.66%) using the same dosing range (Figure 3*a*). Application of the water extract on endothelial denuded rings, showed no difference from rings with intact endothelium, denoting a non-endothelium dependent relaxation. To address the underlying mechanism, the *S. marina's* dose-response curves were retrieved after blockade of muscarinic, ß-adrenergic or prostaglandin receptors, or K+ channels. All revealed no significant difference. Only pre-incubation with the Ca^{+2} channel blocker, nifedipine, was associated with significant attenuation of ACh-induced relaxant activity of water extract. This pointed to an underlying role of Ca^{+2} in the *S. marina*-induced vasodilation (Figure 3*c*).

Role of Ca+ Flux in S. marina Relaxant Effect on NE-Precontracted Aortic Rings

Incubation of normal aortic rings with submaximal doses of the water extract significantly attenuated the contractile responses to NE in comparison to non-incubated rings, especially with the higher doses of NE (Figure 4*a*). This may denote a significant blockade of the slow-phase of NE-induced contraction, which is mainly dependent on extracellular Ca^{+2} influx. When each set of rings was washed with Ca^{+2} free solution, an immediate decrease in tension to near zero-level was observed (*data not shown*). Further addition of NE (10 µM) resulted in a minimal and non-sustained contraction in both sets of rings. However, rings incubated with the water extract showed a significant attenuation of NE-induced contractile responses versus non-incubated rings,

Table III. Diuretic effects of *S. marina* extracts vs. furosemide in L-NAME hypertensive rats.

Rats variables	Urine volume (ml/100 g.bw/24 hr)	Urinary excretion %	Diuretic action	Diuretic activity
Normotensive control	3.38 + 0.17	8.45 + 0.42	1	–
Non-treated hypertensive HR	4.01 + 0.09[a]	10.03 + 0.23a	1.19 + 0.03[a]	0.74 + 0.02[c]
Water extract-treated HR	4.19 + 0.16[ac]	10.48 + 0.40[ac]	1.24 + 0.05[ac]	0.77 + 0.03[c]
n-Butanol fraction-treated HR	4.72 + 0.14[abc]	11.80 + 0.34[abc]	1.39 + 0.04[abc]	0.87 + 0.03[bc]
Furosemide-treated HR	5.55 + 0.19[ab]	13.56 + 0.66[ab]	1.61 + 0.08[ab]	1
Sum of the percentage of common flavonoids	36%	64%		

HR: hypertensive rats [a]: Significant difference *vs*. normotensive control rats, [b]: Significant difference *vs*. non-treated hypertensive rats, [c]: Significant difference *vs*. furosemide-treated hypertensive rats. Data expressed as Mean ± SEM. $p < 0.05$.
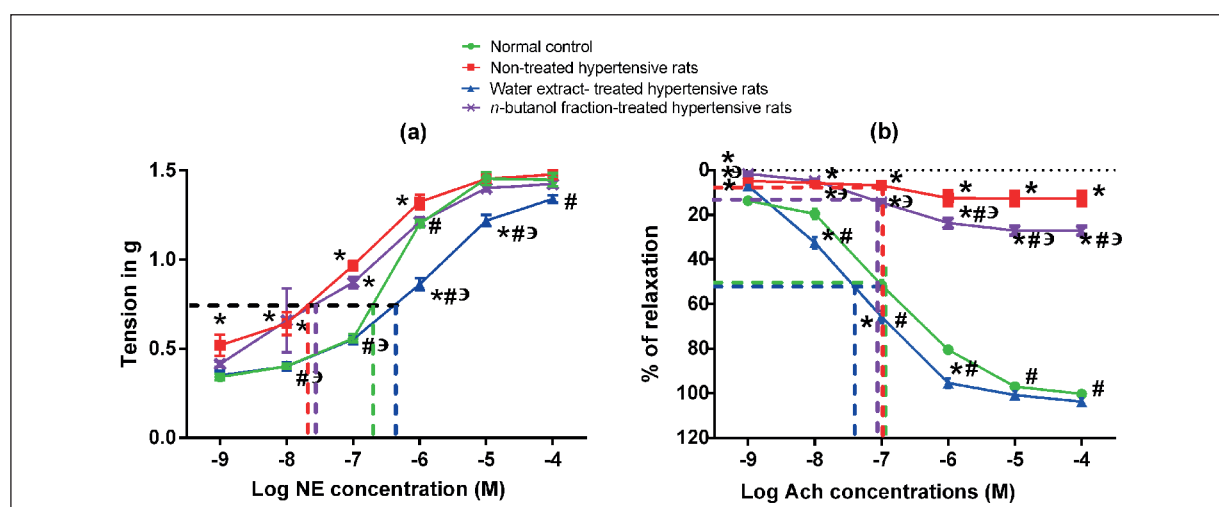
Figure 2. Effect of *S. marina* extracts on aortic ring vascular reactivity in L-NAME treated rats, showing contractile responses to cumulative doses of NE in (**a**)*,* percentage relaxation of maximally NE-precontracted rings in response to cumulative doses of ACh in (**b**). Data are expressed as means ± SEM for n=6. Colored dotted lines represent the EC50 relevant to NE in (**a**) or ACh in (**b**) in the corresponding-colored curve. *: Significant difference *vs.* normal control, #: Significant difference *vs.* non-treated hypertensive rats. ꝫ: Significant difference *vs. S. marina* water extract-treated hypertensive rats. ACh, Acetylcholine; NE, Norepinephrine. *p*< 0.05.

Discussion

The L-NAME-induced hypertension is a model of pressure overload mimicking human hypertension regarding the associated early cardiovascular alterations and endothelial dysfunction. In line with previous reports[18], inhibition of NO synthesis by L-NAME was associated with a significant rise in MABP, and a significant diuresis and natriuresis. This appears to involve a "pressure natriuresis" mechanism, as it was reported to be abolished when renal perfusion or interstitial hydrostatic pressure is prevented from being increased[18]. Indeed, GFR was increased herein by L-NAME administration. Another possible mechanism is the inhibition of renal NO, hindering its potential auto-regulatory roles on fluid and electrolyte transport in tubular segments[31]. Moreover, L-NAME treatment induced endothelial dysfunction in isolated aortae as evidenced by enhancement of NE-contractile response and attenuation of ACh-induced relaxant responses.

when such responses were triggered by a cumulative concentration of $CaCl_2$. This confirmed the assumed water extract blockade of extracellular Ca^{+2} influx induced by NE (Figure 4*b*). The role of the other known vasodilator mechanisms including NO pathway was not found to be involved based on the negative *in-vitro* NO assay.

The administration of either extracts of *S. marina* was associated with a comparable significant decrease in MABP, although significantly less than furosemide. Despite that the water extract dose is 8 times that of *n*-butanol fraction, their antihypertensive effect was similar; this could be explained by the higher *n*-butanol flavonoids content as detected by our phytochemical analysis. In support, it was previously demonstrated that flavonoids are responsible for the anti-hypertensive activity of *S. purpurea*[4].

Remarkably, both extracts expressed a different diuretic profile. The *n*-butanol significantly increased urinary excretion and diuretic action in contrast to the non-significant action of the water extract. Since the effect of both extracts on water and electrolytes clearance was assessed while administering L-NAME with its impact on glomerular functions, it seems herein that E-C$_{H2O}$ is a more reliable index than E-Cosm. In this view, E-C$_{H2O}$ will help to unravel the chief effect of the extracts on free water excretion by excluding the influence of ineffective osmoles. This is especially that both extracts raised urinary urea excretion in comparison to L-NAME effect. Despite the significant decrease of E-Cosm by both extracts, only the *n*-butanol fraction expressed a significant aquaretic effect. This finding could speculate a vasopressin receptors blockade by *S. marina*. This is as vasopressin is the hormone capable of selectively inducing water reabsorption
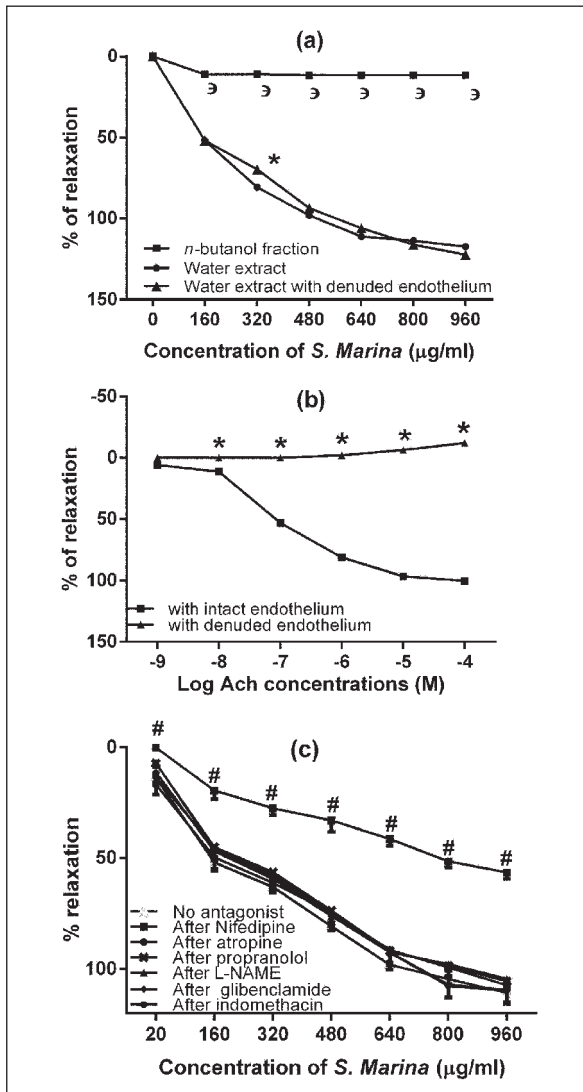
Figure 3. Relaxant effect of *S. marina* extracts on. NE-pre-contracted aortic rings in (**a**) *vs.* ACh-induced relaxation in (**b**), and with *vs.* without the presence of different antagonists in (**c**) on endothelial intact aortic rings. Data are expressed as means ± SEM for n=6. *****: Significant difference between rings with intact and denuded endothelium. ³: Significant difference between water extract and *n*-butanol fraction. **#**: Significant difference *vs. S. marina* alone. $p<0.05$ ACh, Acetylcholine; NE, Norepinephrine. $p<0.05$.

hypokalemia that could be worsen by natriuresis. Indeed, most phytodiuretics were reported to have an aquaretic effect[32].

In this study, the *S. marina effect* on diuresis was assessed after L-NAME administration. Therefore, it could be hypothesized that *S. marina* diuretic action is either through NO and higher L-NAME doses were mandatory to prevent its full action, or it may be acting through a different mechanism. However, the negativity of the *in-vitro* NO assay and the vascular reactivity responses after L-NAME incubation (*discussed below*) point to different mechanisms. In support, both extracts thrived to increase urinary urea nitrogen after NO blockade as discussed below.

Generally, since both extracts showed a different diuretic effect, it seems that their anti-hypertensive effect might depend on a non-diuretic mechanism, as well. Indeed, a promising
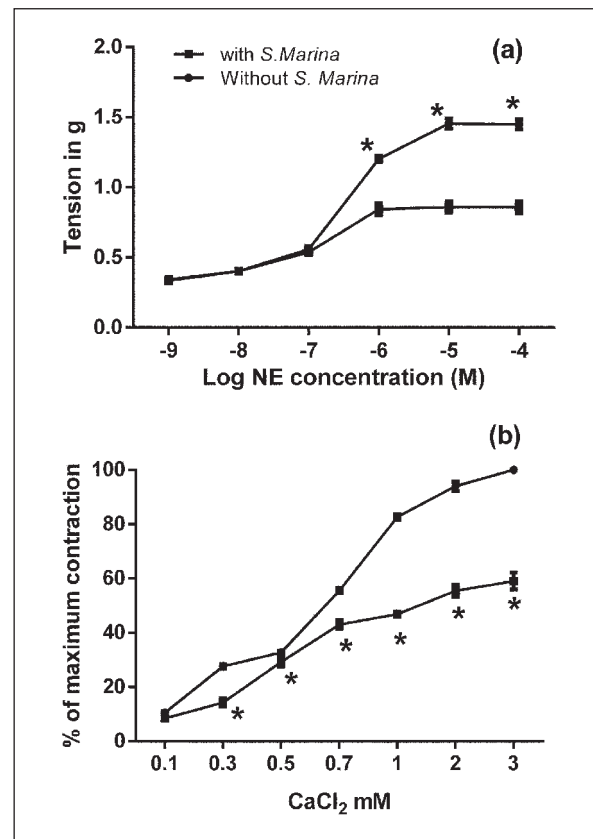

Figure 4. Role of modulation of ionic Ca^{+2} flux by *S. marina* water extract on its effect on NE-induced contraction. Endothelium-denuded aortic rings immersed in normal Kreb's solution in (**a**) and in Ca^{+2} free Kreb's solution and NE (10 μM) in (**b**). Data are expressed as means ± SEM for n=6. *****: Significant difference *vs.* non-*S. marina* incubated aortic rings. $p<0.05$.

from tubular fluid and thus decreasing renal free water clearance by generating the water channel, aquaporin-2[20]. However, this assumption needs further to be elucidated. Again, this unique *n*-butanol aquaretic effect could be due to its higher flavonoids content. This aquaretic action could be advantageous over conventional diuretics in patients with heart failure and liver cirrhosis, who already have dilutional hyponatremia and

improvement of endothelial function by water extract was observed in contrast to the *n*-butanol fraction. This was evidenced by attenuation of the NE contractile responses and augmentation of ACh-induced relaxation. The beneficial effect on endothelium could be, at least in part, due to the previously documented *S. marina* antioxidants and anti-inflammatory actions. Moreover, tricin, a potent non-selective cyclooxygenase inhibitor, was previously reported to be abundant in *S. marina,* and to possess superior biological actions over flavonoids and polyphenols[5, 33, 34].

The favorable effect of *S. marina* on vascular reactivity was further confirmed by the observed dose-dependent relaxant response of the water extract when directly applied on normal aortic rings maximally precontracted with NE. This direct relaxant response was proved to be non-endothelium dependent and was only minimally observed with the *n*-butanol fraction. On addressing the mechanism of the observed vasodilation, only pre-incubation of normal aortic rings with nifedipine, was associated with significant attenuation the relaxant effect of the water extract. This raised the interest of Ca^{+2} ionic movement as a potential mechanism of *S. marina*-induced vasodilation. The results of the last series of experiment confirmed that *S. marina* blocked the extracellular Ca^{+2} influx We demonstrated that incubation of aortic rings with the water extract attenuates the NE-induced contractile responses triggered by a cumulative concentration of $CaCl_2$ in Ca^{+2} free medium.

Taking these findings together, it seems that the blood pressure lowering effect of *S. marina* is a species effect. This is as the present findings are consistent with those obtained by Joaud et al.[4], who reported an antihypertensive effect of flavonoid extracted from *S. purpurea Pers*. They related the antihypertensive effect of their flavonoid extract to different mechanisms. Among these, is vasodilatation of both renal afferent and efferent arterioles, and inhibition of calcium influx and thus blocking of vascular excitation-contraction coupling. This could support our finding that *S. marina* vasodilation is through blockade of calcium influx. In fact, previous studies[10,14] reported that *S. marina* extracts have relatively high amounts of phenols and flavonoids, besides tannins and saponins that were known to have potent diverse biological benefits. The phenolic and flavonoids contents of both extracts were verified using the preliminary phytochemical constituents' comparison.

Noticeably, both extracts significant increased urinary urea and creatinine up to normal levels. It was reported that increase in urinary excretion of urea can offset its accumulation in serum. The primary mechanism for urea delivery into the inner medullary interstitium and hence to the blood is its reabsorption from the terminal inner medullary collecting duct (IMCD), which is regulated by hypertonicity. However, vasopressin has an additive stimulatory effect on urea permeability as it increases the IMCD plasma membrane accumulation of urea transporters[35]. This could support our speculation that *n*-butanol has a vasopressin blockade action since it significantly increased urinary urea.

Regarding renal functions, both extracts did significantly decrease GFR versus other hypertensive groups. Apart from serum creatinine, GFR is affected by blood pressure volume and glomerular capillary hydrostatic pressure. The only variable factor between hypertensive groups is the glomerular capillary hydrostatic pressure that is directly affected by the afferent and efferent arteriolar resistance and the renal artery pressure[36]. Thus, this reduction in GFR together with the observed decrease in MABP could support a potential vasodilatory action of *S. marina* that leads to pressure changes within the afferent and efferent arterioles, a common feature with furosemide, as well[20]. Finally, we note similar biological effects of both *S. marina* extracts, except for the significant direct vasodilation of water extract and the aquaretic effect of *n*-butanol fraction. This could highlight the different concentration of the active components of both extracts that are currently subjected for further investigations. The present preliminary phytochemical comparison indicated that the number of flavonoids and other detected compounds of *n*-butanol is much higher. This denotes that the observed vasodilatory activity is due to other compounds in the water extract that could not be detected by HPLC-UV/Vis. In line, Bhowmik et al[37] declared that the water extract expresses cumulative effect of several substances and/or yields secondary active metabolites. However, serial doses of both extracts should have been investigated before an accurate comparison is done.

Conclusions

Both *S. marina* extracts expressed an effective antihypertensive effect, which is due to direct va-

sodilatory action through a calcium entry blockade. Also, the differential diuretic activity of both extracts adds to their antihypertensive effect. *S. marina* thus represents a potential source for a novel antihypertensive agent with a favorable effect on vascular reactivity. Nevertheless, the precise molecular action of *S. marina*, as well as its long-term tolerability remain to be elucidated in human studies.

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