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PHARMACEUTICAL SCIENCE | RESEARCH ARTICLE

Fractionation and bioassay-guided isolation of antihypertensive components of *Senecio serratuloides*

Charlotte Mungho Tata[1,2], Derek Ndinteh[2], Benedicta Ngwenchi Nkeh-Chungag[3], Opeopluwa Oyehan Oyedeji[4] and Constance Rufaro Sewani-Rusike[1]*

Abstract: *Senecio serratuloides* commonly referred to as "two day cure" is used in folk medicine for treating hypertension and wounds in South Africa. This study was aimed at isolating and testing the antihypertensive effects of bioactive compounds from *S. serratuloides*. *Senecio serratuloides* was serially extracted using solvents of increasing polarity. Phytochemical analysis, antioxidant capacity and antihypertensive properties of fractions were investigated. Bioactive compounds were isolated from ethyl acetate and methanol fractions, their antihypertensive effects and effect on urine norepinephrine concentration were determined. Ethyl acetate and methanol fractions had all eight phytochemicals tested, better antioxidant capacity and significantly ($p < 0.001$) prevented the increase in blood pressure induced by N^{ω}-Nitro-L-arginine methyl ester hydrochloride. The isolated bioactive compounds were phytosteroids and Estran-3-one, 17-(acetyloxy)-2-methyl-, (2à,5à,17á) – which was isolated from methanol fraction had significantly ($p < 0.001$) better antihypertensive effects through the 4-h period of the study. *Senecio serratuloides* may be a potential source of antihypertensive lead compounds.

Subjects: Pharmacology Systems; Medicinal & Pharmaceutical Chemistry; Hypertension

Keywords: Senecio serratuloides; serial extraction; norepinephrine; oxidative stress; hypertension; antioxidants

ABOUT THE AUTHOR

Our group is interested in probing for novel treatments for non-communicable diseases like hypertension and Diabetes from indigenous medicinal plants. In a bid to determine the mechanism of action of these plant extracts and isolates, we investigate their *in vitro* and *ex vivo* antioxidant capacities and equally carry out several Biochemical, immunological and histopathological assays on samples obtained from experimental animals after treatment with the extracts or isolates.

PUBLIC INTEREST STATEMENT

Senecio serratuloides is prescribed by traditional healers in Eastern Cape, South Africa for treating hypertension. Since these traditional healers claim they have been having positive results over the years, it is likely that this plant has phytoconstituents that can be exploited by pharmaceutical industries. In this study, *Senecio serratuloides* was extracted by sequential fractionation using four solvents (hexane, dichloromethane, ethyl acetate and methanol). The ethyl acetate and methanol fractions had better antioxidant capacity and antihypertensive properties and thus were subjected to thin layer and column chromatography for isolation of bioactive compounds. Three phytosteroids were isolated; two from ethyl acetate fraction and one from the methanol fraction. The phytosteroid isolated from the methanol fraction had better antihypertensive properties.

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1. Introduction

Hypertension (HTN) is the central pathophysiologic contributor to cardiovascular morbidity and mortality (Dharmashankar & Widlansky, 2010). Increased sympathetic nervous system (SNS) activity and reactive oxygen species (ROS) are implicated in the pathogenesis of HTN (Campese, 2010). The role of the SNS in HTN is confirmed by increase in circulating plasma levels of catecholamines like norepinephrine in normotensive individuals with a family history of HTN or people with borderline HTN (Mancia & Grassi, 2014). Several factors are potentially capable of activating the SNS, some of which include baroreflex dysfunction, chemoreceptor activation, renin-angiotensin system and other humoral systems (Mancia & Grassi, 2014; Saino et al., 2000).

Activation of the SNS and other systems like the renin angiotensin system results in increased formation of reactive oxygen species (ROS) which in turn activate the SNS even further (Campese, 2010). Increased production of ROS decreases nitric oxide (NO) bioavailability by direct inactivation through formation of peroxynitrite (Gonzalez, Valls, & Brito, 2014) and also by inhibition of eNOS activity through oxidation of 4-tetrahydrobiopterin leading eNOS uncoupling (Dinh, Drummond, Sobey, & Chrissobolis, 2014). NO is known to mediate vasodilation, inhibit platelet aggregation, and prevent leukocyte adhesion to endothelial cells (Adaramoye, Nwosu, & Farombi, 2012). Therefore, inhibition of NO has deleterious effects on the cardiovascular system. For instances inhibition of NOS using N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) results in NADPH activation and subsequent production of ROS (Berkban et al., 2015; Bunbupha, Pakdeechote, Kukongviriyapan, Prachaney, & Kukongviriyapan, 2014). In experimental models and human subjects, administration of antioxidant compounds such as vitamin C, Vitamin E, Polyphenols, Allopurinol and Selenium have been shown to have antihypertensive effects through decreasing ROS formation or increasing levels of NO (Baradaran, Nasri, & Rafieian-Kopaei, 2014; Kadkhodaee & Sedaghat, 2014; Kizhakekuttu & Widlansky, 2010).

There are major advances in the development of therapeutic treatments of HTN (Cushman et al., 2016). However, despite these advances, the global prevalence of HTN is on the increase due to multiple factors one of which is directly associated with antihypertensive therapy, mainly involving compliance problems (Armario & Waeber, 2013; Jarari et al., 2016). Non-compliance is a major problem attributed to associated side effects of current antihypertensive drugs. Most of these drugs are not accessible and/or affordable and in many cases, none of them can control HTN singly (Marshall, Wolfe, & McKevitt, 2012; Mugabo & Raji, 2013). In addition to lack of compliance, it is estimated that up to 30% of patients with HTN are unresponsive to available drug regimens (Calhoun et al., 2014). Therefore, there is need for novel agents with better efficacy and little or no side effects.

The plant kingdom may be an alternative for novel agents because it includes a large number of species which produce diverse bioactive compounds with different biological activities (Atanasov et al., 2015). These bioactive compounds include flavonoids, polyphenols, saponins, alkaloids, tanins, triterpenoids, phytosteroids, and glycosides. Flavonoids are scavengers of free radicals (Kooshki & Hoseini, 2014) and they prevent oxidation of low density lipoproteins (de Paula et al., 2012). They are associated with improvement of sympatho-vagal balance, decrease systolic blood pressure (SBP) and heart rates thus reducing cardiovascular risk and mortality (Duarte, Mostarda, Irigoyen, & Rigatto, 2016). Polyphenols have vasorelaxant effects, decreasing BP by increasing endothelial nitric oxide bioavailability via their antioxidant action and their capacity to activate vascular endothelial nitric oxide synthase (Zhao, Wang, Ballevre, Luo, & Zhang, 2012). Saponins block the renin-angiotensin-aldosterone system resulting in decrease total peripheral resistance and consequently decrease systemic HTN (Chen et al., 2013). Some alkaloids bind strongly to protein receptors on the membrane of secretary vesicles found in the intracellular cytosol of presynaptic neurons and prevent neurotransmitters from being incorporated into the presynaptic vesicle. This prevents and dampens the promulgation of nervous signals in the primary sympathetic neurons of the brain and peripheral nervous system (Lobay, 2015). Triterpenoids and phytosteroids lower serum lipid levels thus reducing the risk of atherosclerosis and hence HTN (Machaba et al., 2014).

An example of a plant which is used in folk medicine for treating HTN in Eastern Cape, South Africa and thus maybe a source of antihypertensive agents is *Senecio serratuloides* that is used singly or in combination with other herbs (Personal communication, Mahlakata). *Senecio serratuloides* is also used singly or in combination with other plants to treat wounds such as cuts, internal and external sores (including those resulting from sexually transmitted infections), burns, swollen gums and chest pain (De Wet, Nciki, & van Vuuren, 2013; De Wet, Nzama, & Van Vuuren, 2012; Fawole et al., 2010; Gould, Penny, Patel, & Candy, 2015). A study in our laboratory reported the antihypertensive effect of the hydoethanolic extract of *S. serratuloides* (Tata et al., 2019). The plant has also been reported to have phenols, tannins, flavonoids, and gallotannins and to possess anti-inflammatory, anticholinesterase, antioxidant, and wound healing properties (Fawole et al., 2010; Gould et al., 2015). This study was aimed at serially fractionating *S. serratuloides* using solvents of increasing polarities in order to simplify fractions and enhance isolation of bioactive compounds from the fractions since each solvent extracts different phytochemical groups. The antihypertensive properties of the fractions and bioactive compounds were investigated.

2. Materials and methods

2.1. Chemicals and drugs
Nω-Nitro-L-arginine methyl ester, 2,2′- azinobis (3-ethylbenzothiazoline-6-sulfonic acid), 1,1-diphenyl-2-picryl-hydrazil, gallic acid, ascorbic acid, 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (trolox) and quercetin were purchased from Sigma-Aldrich Chemical Co. (St Lois, Mo, USA), Captopril was purchased from Pharmacare Ltd. (South Africa) and Norepinephrine ELISA kit from Cloud-Clone Corp. (Texas, USA). All solvents (hexane, dichloromethane, ethyl acetate, methanol) were of analytical grade.

2.2. Plant material
Senecio serratuloides whole plant (stems, leaves, and roots) was supplied by Mr Fikile Mahlakata of Lusikisiki, Eastern Cape, South Africa. It was authenticated by Dr Immelman of the Kei Herbarium, Walter Sisulu University where a voucher specimen (Tata 1/13,967) was deposited. Whole plant material was air-dried in the laboratory and crushed using a mortar and pestle.

2.3. Serial exhaustive extraction
Serial extraction of 1221 g of the crushed plant was done using non polar and polar solvents in the order n-hexane, dichloromethane, ethyl acetate, and methanol. The dry material was extracted (three times) with 5 L of n-hexane for 7 days at room temperature. The filtrate was collected by passing the mixture through Whatman No.1 filter paper using a Büchner funnel. The filtrate was concentrated under reduced pressure using a rotatory evaporator (Heidolph Laboroto 4000, Germany) at temperatures not exceeding 40°C (Ruttoh et al., 2009). The marc was further extracted three times with dichloromethane. The procedure was repeated with ethyl acetate and methanol. Once concentrated to small volumes, the fractions were placed in pre-weighed labelled beakers and allowed to dry completely; hexane, dichloromethane and ethyl acetate fractions were dried at room temperature while the methanol fraction was dried at 35°C. The total mass of fraction extracted by each solvent was calculated as percentage yield using the formula:

%Yield = mass of fraction/mass of plant material*100

2.4. Phytochemical characterisation
Phytochemical screening of fractions for the presence of phytoconstituents was done following the procedures as described by Mir, Sawhney, and Jassal (2013). Phenolic compounds were quantified employing Folin's reagent using gallic acid as standard (Yadav, Yadav, & Yadav, 2014). Flavonoid content was quantified following procedures as described by Irshad, Zafaryab, Singh, and Rizvi (2012) using quercetin as standard.

3. Antioxidant capacity of extract fractions

3.1. Radical scavenging activity
Radical scavenging activity was evaluated by 2 methods; DPPH (1,1-diphenyl-2-picryl-hydrazil) and ABTS (2,2′- azinobis (3-ethylbenzothiazoline-6-sulfonic acid)). The DPPH assay was done following the method described by Yadav et al. (2014) using ascorbic acid as standard and ABTS was done following method described by Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, and Hawkins Byrne (2006) using trolox as standard.

3.2. Total antioxidant capacity
FRAP (Ferric Reducing Antioxidant Power) was done following the method described by Irshad et al. (2012) using ascorbic acid as standard.

3.3. Chromatography of ethyl acetate and methanol fractions
Thin layer and column chromatography were done following protocols described by Bajpai, Majumder, and Park (2016). Aluminium-backed TLC plates (Merck Silica F254 plates) were used. Plates were developed under ultraviolet (UV) light at 254 nm and 356 nm (CAMAG universal UV lamp). For visualization of non-fluorescing spots plates were dipped in concentrated sulphuric acid, incubated at 60°C for 5 min. The column for column chromatography was packed by slurry packing and solvents of different polarities were passed through the column at uniform rate under gravity to further fractionate the fractions. Each fraction was collected separately in a beaker (250 ml) and numbered consecutively for further analysis on TLC. The fractions were concentrated to approximately 1/100 of original volume using a rotatory evaporator (BUCHI, Germany) at 80°C. TLC was done on concentrated fractions and those that had the same bands on chromatoplates were mixed and all the fractions were allowed to dry in vials. Crystals were formed in some of the vials and were referred to as bioactive compounds while the fractions that dried up into pastes were referred to as sub-fractions. Two bioactive compounds (CSSA and CSSB) and two sub-fractions (CSSX1 and CSSX2) were isolated from ethyl acetate fraction (SSEA) and one bioactive compound (CSSD) and two sub-fractions (CSSY1 and CSSY2) from methanol fraction (SSMOH).

3.4. Identification of bioactive compounds and sub-fractions
Characterization of bioactive compounds and GC/MS of sub-fractions was done in Department of Applied Chemistry, University of Johannesburg, Doornfontein Campus, South Africa.

3.5. Animals
Swiss albino mice weighing 20–25 g were used for acute toxicity and female Wistar rats weighing 200–240 g were used for HTN prevention study. Animals were housed six per cage in animal holding facilities of Walter Sisulu University which were maintained at 23–24°C. The rooms were lit by day light and dark at night. The animals had free access to rat chow (Epol, grade-BR 1, SA) and water. All animal procedures were in accordance with South African National Standards (NSPCA) and EU committee guidelines and were approved by the Research and Ethics Committee of the Faculty of Health Sciences, Walter Sisulu University (Protocol # 051/15).

3.6. Hypertension study design for fractions, sub-fractions and bioactive compounds
Animals in group 1 were treated with normal saline, those in group 2 were treated with L-NAME and normal saline while those in groups 3 to 7 were co-treated with L-NAME (20 mg/kg) and fraction (150 mg/kg) or sub-fraction (5 mg/kg) or bioactive compound (5 mg/kg) orally once daily for 2 days (Table 1) (Biancardi, Bergamaschi, Lopes, & Campos, 2007).

3.7. Measurement of blood pressure
Blood pressure was measured in conscious rats, using non-invasive tail-cuff plethysmography (CODA[TM] 8 Non-Invasive Blood Pressure System, Kent Scientific Corporation, USA) as per manufacturer's instructions. Baseline BP was measured for all groups. 9–16 h after the last treatment with fractions, BP was measured. Meanwhile on day 2, BP was measured at 1, 2 and 4 h after treatment (Tavares, Sevilla, Montero, Carron, & Malcata, 2012) for the groups treated with sub-fractions or bioactive compounds.

Table 1. Animal treatment groups			
Group (n = 6)	**Fractions**	**Sub-fractions**	**Bioactive compounds**
1 (NT)	Normal saline	Normal saline	Normal saline
2 (LN)	Normal saline+L-NAME	Normal saline+L-NAME	Normal saline+L-NAME
3	Captopril+L-NAME	Captopril+L-NAME	Captopril+L-NAME
4	SSHex+L-NAME	CSSX1 + L-NAME	CSSA+L-NAME
5	SSDCM+L-NAME	CSSX2 + L-NAME	CSSB+L-NAME
6	SSEA+L-NAME	CSSY1 + L-NAME	CSSD+L-NAME
7	SSMOH+L-NAME	CSSY2 + L-NAME	

NT—normotensive group, LN—L-NAME group, SSHex—hexane fraction, SSDCM—dichloromethane fraction, SSEA—ethyl acetate fraction, SSMOH—methanol fraction CSSX and CSSY—fraction codes; CSSA, CSSB and CSSD—compound codes.

3.8. Urine collection

Twenty-four hours urine was collected in acidified (300 μl of 3 M HCl) graduated cylinders by placing rats individually in metabolic cages. Collected urine was stored at −20°C for later analysis. The quantity of water consumed was also monitored.

3.9. Determination of norepinephrine concentration in urine

Norepinephrine (NE) concentration in urine was determined using an ELISA kit (CEA907Ge; Cloud-Clone Corp., USA) which employed a competitive inhibition enzyme immunoassay technique, as per manufacturer's instructions. All samples were run in one assay. Intra-assay coefficient of variability was < 10%. There was no significant cross reactivity or interference between NE and analogues. Detection range of assay was between 61.7 and 5000 pg/ml.

3.10. Statistical analysis

Results were expressed as mean ± standard error (SEM). Statistical analyses were carried out using Graphpad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's *posthoc* test for multiple comparisons were performed to determine differences between treatment groups. A p-value less than 0.05 were considered statistically significant.

4. Results

4.1. Percentage yield of fractions

Serial extraction using hexane, dichloromethane, ethyl acetate, and methanol yielded four fractions SSHex, SSDCM, SSEA, and SSMOH, respectively. The highest yield was obtained with methanol with a percentage of 12.74%, followed by dichloromethane (1.15%), hexane (0.92%) and ethyl acetate (0.81%).

4.2. Phytochemical constituents

Qualitative phytochemical screening showed that SSEA and SSMOH had the highest number of phytochemicals followed by SSHex and SSDCM (Table 2). Results from quantitative analysis of phenols and flavonoids showed that SSMOH and SSEA had the highest phenol contents while SSDCM had higher flavonoid content (Table 2).

4.3. Antioxidant capacity

Results from ABTS and DPPH assays showed that SSEA and SSMOH had lower IC_{50} values and hence better scavenging properties. Results from FRAP assay showed that SSMOH and SSEA equally had better reducing power than SSDCM and SSHex (Table 3).

Table 2. Phytochemical constituents of fractions from S. serratuloides

Phytochemical	SSHex	SSDCM	SSDCM	SSMOH
Alkaloids	–	+	+	+
Phenols	–	–	+	+
Steroids	+	+	+	+
Tannins	–	–	+	+
Saponins	+	+	+	+
Flavonoids	–	–	+	+
Terpenes	+	–	+	+
Glycosides	+	–	+	+
Polyphenols (µgGAE/mg extract)	64.3 ± 1	47.3 ± 3	114.5 ± 2	185.9 ± 1
Flavonoid (µgQE/mg extract))	20.5 ± 0.1	61 ± 0	26.8 ± 0.3	34.8 ± 0.8

\+ Phytochemical present; -Phytochemical absent; GAE—gallic acid equivalent; QE—quecertin equivalent

Table 3. Radical Scavenging (IC$_{50}$) and Total Antioxidant Capacity of Fractions

	SSHex	SSDCM	SSEA	SSMOH
ABTS (IC50 mg/ml)	11.79	2.38	1.09	0.41
DPPH (IC50 mg/ml)	#	#	0.61	0.18
FRAP (µgAAE/mg extract)	37.8 ± 2	52.4 ± 0.4	61.1 ± 1	157.6 ± 1

AAE—ascorbic acid equivalent, #—very weak scavenging properties as percentage inhibition at the concentrations examined were far lower than 50. SSHex—hexane fraction, SSDCM—dichloromethane fraction, SSEA—ethyl acetate fraction, SSMOH—methanol fraction.

4.4. Sub-fractions and compounds isolated from ethyl acetate and methanol fractions

Two bioactive compounds (CSSA and CSSB) were isolated from SSEA and one (CSSD) from SSMOH. Sub-fractions CSSX1 and CSSX2 were isolated from SSEA while CSSY1 and CSSY2 were isolated from SSMOH. GC-MS analysis revealed that compounds with relative abundance of 1% and above were 17 in CSSX1, 6 in CSSY1 and 3 in CSSY2. The most abundant compounds are shown in Table 4. The three bioactive compounds identified by NMR are shown in Table 5.
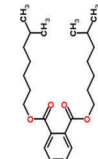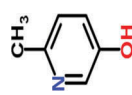
4.5. Effects of fractions on systolic and diastolic blood pressure

Administration of L-NAME (20 mg/kg) to female Wistar rats for 2 days significantly increased SBP and DBP in LN group by 16% and 27% respectively compared to NT group that only observed 0.4% and 3% increase in SBP and DBP, respectively. SSEA and SSMOH significantly prevented this increase in BP compared to SSDCM and SSHex. It was observed that SBP increased by 7% and 8% in rats treated with L-NAME and SSEA or SSMOH, respectively, compared to 16 % increase in LN group. DBP increased by 4% in SSEA rats and decreased by 5% in SSMOH rats compared to 27 % increase in LN group (Figure 1).

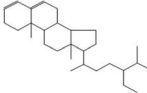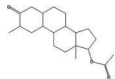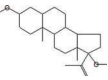## 4.6. Effects of sub-fractions on blood pressure, heart rate, and norepinephrine concentration

Sub-fractions CSSX1 and CSSX2 isolated from SSEA and CSSY1 and CSSY2 from SSMOH were investigated for acute antihypertensive activity over a period of 2 days. Results from co-treatment of rats with L-NAME and sub-fractions showed that L-NAME significantly increased SBP and DBP by 23% and 37% respectively compared to NT group that observed 2 and −1% change in SBP and DBP, respectively. CSSX1 and CSSX2 from SSEA significantly (p < 0.001) prevented this increase in BP in the first hour after treatment while CSSY1 and CSSY2 from SSMOH had no significant effect on BP (Table 6).

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Table 4. Prominent compounds in sub-fractions from ethyl acetate and methanol fractions

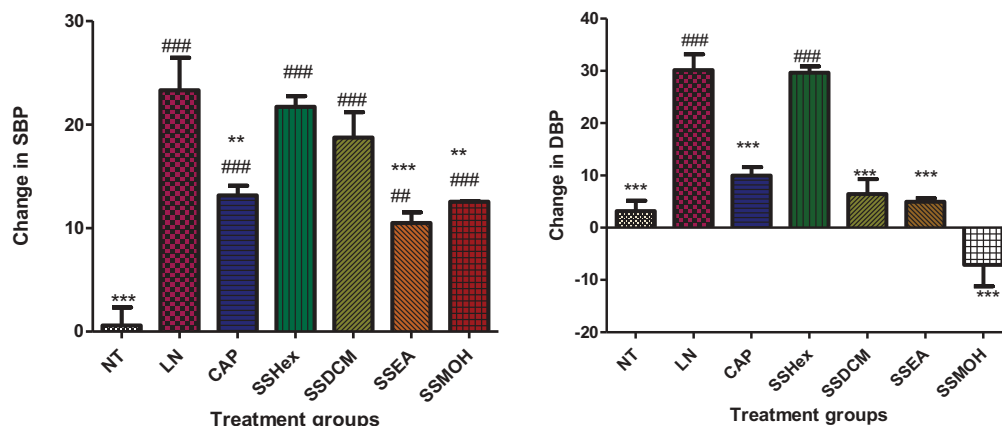| Code	Name	% and RT	Structure	Properties
CSSX1	1,2-benzenedicarboxylic acid, diisooctyl ester	11.33% 21.64 mins		H bond acceptor; MW-390 g/mol MF-$(C_8H_{17}COO)_2$ C_6H_4
	6-methyl-3-pyridinol	8.56% 11.75 mins		H bond donor and acceptor; MW-109 g/mol MF-C_6H_7NO
	Hexadecane	7.42 % 11.41 mins		MW-226 g/mol; MF-$C_{16}H_{34}$
	Pentadecane	6.71 % 10.14 mins		MW-212 g/mol; MF-$C_{15}H_{32}$
CSSY1	2-butoxy-ethanol	3.7 % 3.65 mins		H bond donor and acceptor; MW-118g/mol MF-$C_6H_{14}O_2$
CSSY2	Cis-9-oxabicyclo[6.1.0] non-2-yne	3.02 % 2.61 mins		H-bond acceptor; MW-122 g/mol MF-$C_8H_{10}O$

CSSX and CSSY—fraction— codes; MW—molecular weight; MF—molecular formula; RT—retention time; structures and properties from ChemSpider and PubChem database.

Table 5. Bioactive compounds: structures and Properties

Code	Name	structure	Properties
CSSA	Stigmastan- 3, 5-diene		MW—396 g/mol; MF- $C_{29}H_{48}$
CSSB	Pregnan-20-one, 3,17-bis[oxy]-, O-methyloxime, (3á,5à)-		MW—332.48 g/mol; MF—$C_{21}H_{32}O_3$
CSSD	Estran-3-one, 17-(acetyloxy)-2-methyl-, (2à,5à,17á)-		MW—332.52; MF $C22H_{36}O_2$

CSSA, CSSB and CSSD—compound codes; MW-molecular weight, MF-molecular formula

Figure 1. Changes in systolic and diastolic blood pressure in rats treated with fractions. Values are expressed as mean ±SEM. n = 6; SSHex-hexane fraction; SSDCM-dichloromethane fraction; SSEA-ethyl acetate fraction; SSMOH-methanol fraction.
* indicates comparison between the treatment groups and L-NAME (LN) control group; # indicates comparison between the treatment groups and nor-motensive control group (NT). ** p < 0.01, *** p < 0.001; ##p < 0.01, ### p < 0.001.

L-NAME caused progressive decrease in heart rates in all treatment groups. CSSX1, CSSY1, and CSSY2 significantly (p < 0.001) lowered HR even further from the 1, 2 h to 4 h compared to LN control (Figure 2). L-NAME also significantly (p < 0.001) decreased norepinephrine concentration in the LN group compared to the NT group and all the groups that were co-treated with L-NAME and sub-fractions or captopril equally had significantly (p < 0.001) lower norepinephrine levels compared to NT control group (Figure 2).

4.7. Effect of sub-fractions on water intake and urine output
LN treatment group consumed significantly (p < 0.05) lower volume of water compared to NT control group. Water intake in CSSX2 group was significantly (p < 0.05) higher compared to LN control group and this was reflected in significantly higher urine output in this group compared to NT and LN control groups (Table 7).

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Table 6. Effect of sub-fractions on systolic and diastolic blood pressure

	Time/h	NT	LN	CPT	CSSX1	CSSX2	CSSY1	CSSY2
SBP	0	146 ± 3	146 ± 1	147 ± 1	149 ± 2	146 ± 1	146 ± 0.4	149 ± 2
	1	149 ± 4	180 ± 3	168 ± 4a	141 ± 1c	161 ± 3c	169 ± 2	171 ± 1
	2	147 ± 3	175 ± 5	170 ± 3	166 ± 5	166 ± 3	174 ± 4	170 ± 2
	4	140 ± 1	171 ± 2	160 ± 3	151 ± 5b	160 ± 4	158 ± 2	165 ± 2
DBP	0	113 ± 5	110 ± 2	117 ± 3	114 ± 1	119 ± 4	120 ± 3	117 ± 5
	1	112 ± 2	152 ± 4	141 ± 5	108 ± 7c	130 ± 3a	132 ± 4a	142 ± 1
	2	117 ± 2	142 ± 6	140 ± 4	130 ± 8	136 ± 5	142 ± 7	138 ± 3
	4	112 ± 1	129 ± 2	128 ± 5	122 ± 3	127 ± 3	167 ± 3	135 ± 3

Values are expressed as mean±SEM. n = 6; NT = normotensive control; LN = L-NAME control; CPT = captopril; CSSX1 & CSSX2-sub-fractions from SSEA, CSSY1 & CSSY2—sub-fractions from SSMOH. a = * $p < 0.05$, b = ** $p < 0.01$, c = *** $p < 0.001$ compared to L-NAME (LN) control group.

Figure 2. Effect of sub-fractions on heart rates and norepinephrine concentration in urine. Values are expressed as mean±SEM. n = 6; NT = normotensive control; LN = L-NAME control; CPT = captopril; CSSX1 & CSSX2- sub-frations from SSEA; CSSY1 & CSSY2- sub-frations from SSMOH. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to L-NAME (LN) control group; #$p < 0.05$, ## $p < 0.01$, ###$p < 0.001$ compared to normotensive control group.
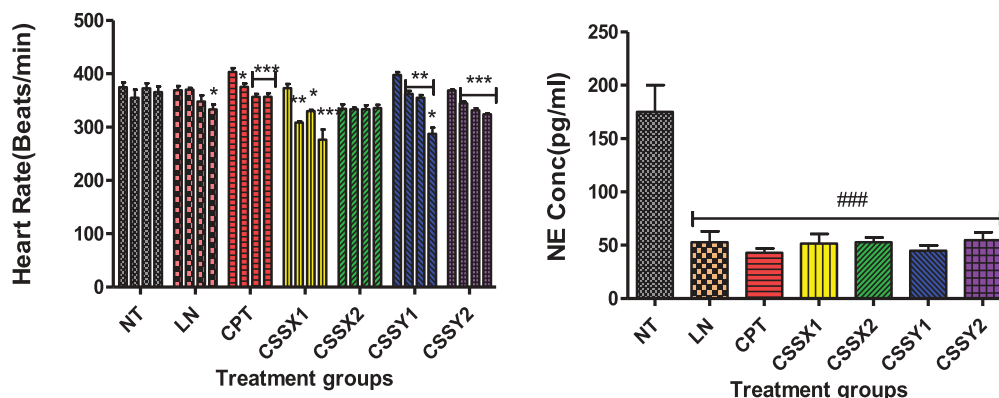
Table 7. Effect of sub-fractions on water intake and urine output

	NT	LN	CPT	CSSX1	CSSX2	CSSY1	CSSY2
Water intake/ml	30 ± 2	22 ± 2#	28 ± 2	27 ± 2	31 ± 2*	21 ± 0.2#	24
Urine output/ml	8 ± 1	8 ± 0.8	13 ± 0.7	13 ± 0.9	14 ± 2#*	12 ± 0.9	11 ± 1.6

Values are expressed as mean±SEM. n = 6; NT = normotensive control; LN = L-NAME control; CPT = captopril; CSSX1, CSSX2, CSSY1 and CSSY2 = sub-fractions consisting of several phytochemicals. * $p < 0.05$, compared to L-NAME (LN) control group; #$p < 0.05$ compared to normotensive control group.

4.8. Effects of bioactive compounds on blood pressure, heart rates, and norepinephrine concentration

Bioactive compounds CSSA and CSSB isolated from SSEA and CSSD from SSMOH were investigated for acute antihypertensive activity over a period of 2 days. Results showed that L-NAME significantly increased BP 1, 2 and 4 h after treatment by 23%, 20%, and 17% for SBP and 37%, 29%, and 17% for DBP compared to NT group with 2, 0.4 and −4% for SBP and −1, 3 and −1 for DBP, respectively. CSSD significantly prevented L-NAME-induced increase in SBP at 1, 2 and 4 h after treatment but its effect on DBP was not significant at the 4th hour after treatment. CSSB was significantly active in preventing increase in SBP from 2 h to 4 h ($p < 0.01$) after treatment and its effect on DBP was noticed at 1 and 2 h after treatment. CSSA only had significant effect on SBP and DBP 1 hr after treatment (Table 8).

Comparing HR after treatment with HR at baseline, L-NAME caused progressive decrease in heart rates in all treatment groups with a significantly ($p < 0.05$) lower HR observed 4 h after treatment. CSSA showed

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Table 8. Effect of bioactive compounds on systolic and diastolic blood pressure							
	Time/h	NT	LN	CPT	CSSA	CSSB	CSSD
SBP	0	146 ± 3	146 ± 1	147 ± 1	147 ± 2	147 ± 6	147 ± 2
	1	149 ± 4	180 ± 3	168 ± 4a	163 ± 2b	171 ± 2	153 ± 1c
	2	147 ± 3	175 ± 5	170 ± 3	170 ± 2	160 ± 4a	153 ± 2c
	4	140 ± 1	171 ± 2	160 ± 3	163 ± 1	151 ± 3c	157 ± 2b
DBP	0	113 ± 5	110 ± 2	117 ± 3	121 ± 3	117 ± 7	113 ± 4
	1	112 ± 2	152 ± 4	141 ± 5	130 ± 4b	135 ± 3a	108 ± 3c
	2	117 ± 2	142 ± 6	140 ± 4	140 ± 3	123 ± 4a	115 ± 3c
	4	112 ± 1	129 ± 2	128 ± 5	131 ± 2	123 ± 3	115 ± 6

Values are expressed as mean±SEM. n = 6; NT = normotensive group; LN = L-NAME group; CPT = captopril; CSSA = stigmastan-, CSSB = pregnan-, CSSD = estran-. a-* p < 0.05, b-** p < 0.01, c-*** p< 0.001 compared to L-NAME (LN) control group.

the same trend found in LN group whereas CSSD significantly (p < 0.001) decreased HR from the 1st to the 4th h. L-NAME significantly (p < 0.001) decreased norepinephrine concentration in the LN group (52.76 ± 10 pg/ml) compared to the NT group (175.04 ± 25 pg/ml). All the groups that were co-treated with L-NAME and bioactive compounds or captopril equally had significantly (p < 0.001) lower norepinephrine levels compared to NT control group (Figure 3).

4.9. Effect of bioactive compounds on water intake and urine output
Rats treated with CSSD had significantly (p < 0.05) higher water intake than rats treated with L-NAME. This was reflected in significantly higher urine output in this group compared to NT and LN control groups. There was however no significant difference in water intake and urine output in CSSA and CSSB compared to NT and LN controls (Table 9).

Figure 3. Effect of bioactive compounds on heart rates and norepinephrine concentration in urine. Values are expressed as mean±SEM. n = 6; NT—normotensive control; LN—L-NAME control; CPT = captopril; CSSA—stigmastan-, CSSB—pregnan-, CSSD—estran-. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to L-NAME (LN) control group; #p < 0.05, ## p < 0.01, ###p < 0.001 compared to normotensive control group.
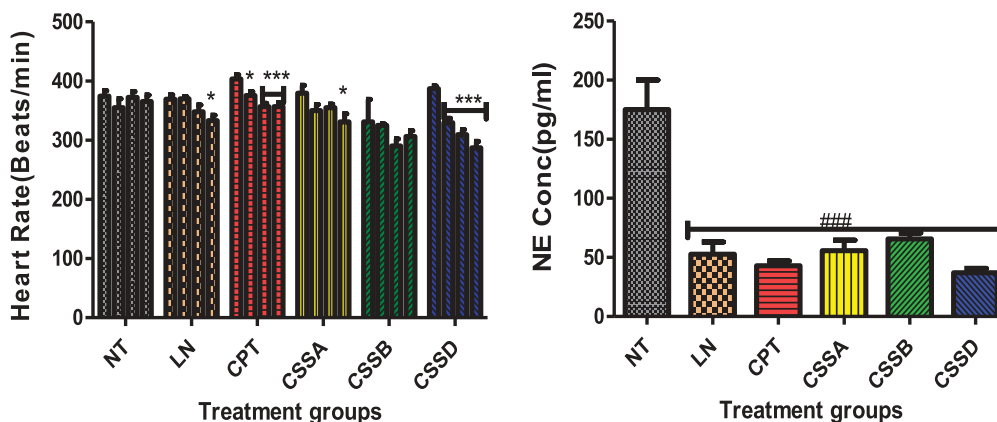

Table 9. Effect of bioactive compounds on water intake and urine output						
	NT	LN	CPT	CSSA	CSSB	CSSD
Water intake/ml	30 ± 2	22 ± 2	28 ± 2	25 ± 2	24 ± 4	31 ± 2*
Urine output/ml	8 ± 1	8 ± 0.8	13 ± 0.7#*	10 ± 1	10 ± 1	15 ± 1##***

Values are expressed as mean ± SEM. n = 6; NT = normotensive groug; LN = L-NAME group; CPT = captopril; CSSA = stigmastan-, CSSB = pregnan-, CSSD = estran-.* p < 0.05, *** p < 0.001 compared to L-NAME (LN) control group; #p < 0.05, ## p < 0.01, compared to normotensive control group.

4.10. Discussion

Results from this study revealed that the highest percentage yield was gotten with methanol and the least with ethyl acetate. Ethyl acetate and methanol fractions (SSEA and SSMOH) had more phytochemicals, better antioxidant, and antihypertensive properties than dichloromethane and hexane fractions (SSDCM and SSHex). L-NAME increased BP and decreased urinary norepinephrine concentration and heart rates. Among the three phytosteroid compounds isolated, estran-(CSSD) from SSMOH had better antihypertensive properties compared to the other compounds and CSSX1 and CSSX2 sub-fractions from SSEA had better antihypertensive properties compared to the other sub-fractions.

The polarity of solvents used in extraction determines the difference in type, composition, and bioactivity of phytochemicals extracted (Dehkharghanian, Adenier, & Vijayalakshmi, 2010). Ethyl acetate is a semipolar solvent that can dissolve sterols, alkaloids, glycosides, terpenoids, and flavonoids. Methanol is polar and can dissolve polar compounds such as sugar, amino acid, glycosides, phenolic compounds, flavonoids, terpenoid, saponin, tannin, flavone, phenone, and polyphenol (Sri Widyawati, Budianta, Kusuma, & Wijaya, 2014). Although the two solvents had great disparity in yield, they extracted similar phytochemicals some of which were not found in SSDCM and SSHex. Hexane is non-polar and can dissolve nonpolar compounds, such as lignin, wax, lipid, aglycon, sterol, and terpenoid (Sri Widyawati et al., 2014). This suggests that *S. serratuloides* had fewer phytochemicals with non-polar properties.

The high phytochemical content of SSEA and SSMOH was reflected in their antioxidant and antihypertensive capacities. Phytochemicals such as sterol, flavonoid, saponin, tannin, phenol, alkaloid and cardiac glycoside have been proven to have antioxidant activity (Bajpai et al., 2016; Biancardi et al., 2007). The mechanisms of action of these antioxidants include suppressing reactive oxygen species formation either by inhibition of enzymes or chelating trace elements involved in free radical production; scavenging reactive oxygen species; up-regulating or protecting antioxidant defences (Kumar & Pandey, 2013). The high antioxidant capacity of SSEA and SSMOH was reflected in their better antihypertensive properties. Their efficacy against acute L-NAME induced HTN suggested that they may have vasoactive properties. Previous studies have indicated the possibility of plant extracts in acting as vasorelaxants, for instance; extracts of saffron have been shown to decrease contractility and heart rate of guinea-pig isolated perfuse hearts by blocking Ca^{2+} channels, opening potassium channels and antagonizing β-adrenoreceptors (Boskabady, Shafei, Shakiba, & Sefidi, 2008). Extracts and constituents of celery have also been reported to lower arterial pressure in humans, possibly by lowering levels of circulating catecholamines and decreasing vascular resistance (Houston, 2005). The mechanism of action of extract components with vasoactive properties may be similar to that of neurotransmitters which modulate the activities of receptors directly by binding to the relevant receptor proteins or indirectly by diffusing into postsynaptic membranes and altering the membrane physicochemical properties (Kumar & Pandey, 2013; Tapas, Sakarkar, & Kakde, 2008). Besides interacting with functional proteins (enzymes, receptors, and ion channels) as the primary targets, bioactive phytochemicals like flavonoids, terpenoids, alkaloids have been presumed to act on lipid bilayers and modify membrane physicochemical properties (Tsuchiya, 2015).

All the bioactive compounds isolated were phytosteroids. Since CSSD (estran-) was active from the first to fourth hour after treatment, it is possible that the compound and its metabolites had antihypertensive properties. On the other hand, it may have a long half-life, long clearance time and hence high bioavailability. Bioavailability is considered predictive of clinical outcomes (Chow, 2014). The activity of CSSB (pregnan-) only began 2 h after treatment suggesting that the activity may have been as a result of its metabolites. CSSA (stigmastan-) was only active in the first hour after treatment suggesting that its metabolites may not have antihypertensive properties or it may have a short half-life, fast rate of clearance and thus decreased bioavailability. The amphipathic nature of etran-(CSSD) and pregnan- (CSSB) may have been responsible for partitioning of the molecules into hydrophobic and hydrophilic media thus affecting their duration and

bioavailability. Pharmacologically, the parent drug and its metabolites may act by similar mechanisms, different mechanisms, or even by antagonism (Lin & Lu, 1997). CSSD (estran-) equally provoked excretion of higher amount of 24 h urine compared to the other compounds. This suggested that CSSD may have diuretic properties. Diuretics act by diminishing sodium reabsorption at different sites in the nephron, thereby increasing urinary sodium/water losses, decreasing blood volume and hence BP (Ribeiro et al., 2015). Studies have shown that phytosterols may act as adjuvants in the prevention and treatment of cardiovascular diseases by reducing blood cholesterol levels (Racette et al., 2010). This is achieved through competition between phytosterols and cholesterol in the intestinal lumen since they have similar chemical structures. The more hydrophobic plant sterols are retained, causing a decrease in cholesterol absorption and its consequent elimination in the faeces (Sanclemente, Marques-Lopes, Puzo, & Garcia-Otin, 2009). In addition to the hypocholesterolemic and antiatherosclerotic effects of phytosterols, some studies have shown that they exert other biological activities such as anti-inflammatory properties (Navarro, De Las Heras, & Villar, 2001) and antioxidant potential (Ferretti, Bacchetti, Masciangelo, & Bicchiega, 2010) all of which are important in preventing cardiovascular diseases. Considering Lipinki's rule of 5 on drug and drug candidates as stated by (Leeson, 2012): (molecular weight < 500 Da; lipophilicity, logP (the logarithm of the partition coefficient between water and 1-octanol) <5; H bond acceptors <10 and H bond donors < 5), these bioactive compounds may be considered as good drug candidates because their molecular weights are less than 500, they are not too polar and not too hyprophobic.

The presence of 1,2 benzenedicarboxylic acid-diisooctyl ester, 6-methyl-3-pyrinol, hexadecane, pentadecane and cis-9-[6.1.0]non-2yne in sub-fraction CSSX1 may be responsible for its better antihypertensive properties. Some of these compounds may be adrenergic antagonists while others like cis-9-oxabicyclo[6.1.0]non-2-yne, 2-butoxy-ethanol, 6-methyl-3-pyridinol and 1,2-benzenedicarboxylic acid-diisooctyl ester which are capable of donating and/or accepting hydrogen bonds may have antioxidant properties.

The decreased NE and heart rates observed in this study may suggest that the SNS had no role in the initiation of L-NAME induced HTN or maybe L-NAME augmented the release, reuptake and metabolism of NE. In line with the first suggestion, Fellet et al. (2003) showed that a bolus injection of L-NAME increased mean arterial pressure similarly in intact rats and in rats submitted to complete autonomic blockade. They proposed that the effect of L-NAME administration on BP may probably be due to a direct vasoconstrictor effect caused by the decreased vascular NO synthesis. From this proposal it is possible that decreased HR induced by L-NAME maybe as a result of activation of baroreceptor afferents by increased BP which resulted in activation of the parasympathetic vagal innervations of the heart. Considering the second suggestion, a study done by Kvetnansky et al. (1997) revealed significant elevation of NE metabolites in L-NAME treated animals in spite of unchanged levels of plasma NE thus suggesting that L-NAME increases release, turnover, reuptake and metabolism of NE in the sympathoneural system. In line with this suggestion, Saeed, Khan, & Shabbir (2012) found that the magnitude of interstitial NE can increase far greater than that in plasma and thus they suggested that NE movement into the circulation decreases with baroreceptor unloading. Studies have also shown that spillover of NE from the interstitium may be attenuated by decreased blood flow due to increased peripheral resistance leading to accumulation of interstitial NE (Ferretti et al., 2010; Leeson, 2012). Therefore, the decreased concentrations of NE in urine witnessed in this study may have resulted from interstitial accumulation and/or metabolism which resulted in increased BP. The increased vascular resistance may have triggered a compensatory reflex that overcame its direct stimulatory effects on the heart and resulted in decreased heart rates.

In line with the fact that L-NAME upregulates NE release and metabolism, the bioactive compounds and some sub-fractions that decreased BP may have acted as adrenergic antagonists or they had the ability to decrease interstitial NE concentration. Decreased heart rates induced by L-NAME were consistent with previous studies (Fellet et al., 2003; Kvetnansky et al., 1997).

Nevertheless, studies have also shown no change (Biancardi et al., 2007) or increased (Cunha, Cabral, & Vasquez, 1993) heart rates in this model. These discrepancies could be due to the dose of L-NAME or route of administration or duration of the study. Zatz and Baylis (1998) proposed that the relationship between NO and SNS is highly complex with direct interactions at various adrenergic receptor subtypes and indirect interactions through baroreceptor control of BP, providing numerous and sometimes opposing influences. This complexity may be responsible the controversies in literature.

5. Conclusion

Overall, our results provide evidence indicating that *Senecio serratuloides* may be a potential source of novel agents for treating HTN due to its wide range of phytochemicals and antioxidant properties. Its phytosteroids may serve as vital lead compounds for treating HTN and other cardiovascular diseases.

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