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ORIGINAL ARTICLE

Vasorelaxant and Antihypertensive Activities of Citroflavonoids (Hesperidin/Naringenin Mixture): Potential Prophylactic of Cardiovascular Endothelial Dysfunction

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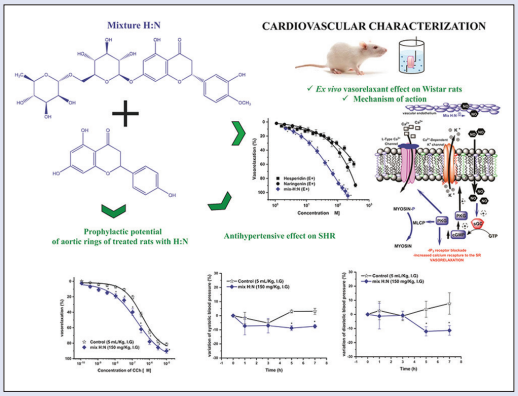
ABSTRACT

Background: Flavonoids in the *Citrus* genus have a positive influence in cardiometabolic parameters, preventing cardiovascular diseases (CVDs). The main flavonoids in sweet orange are hesperidin and naringenin. **Objective:** The aim of this study is to evaluate the cardiovascular effects of mixture of Hesperidin: Naringenin (mix-H:N). **Materials and Methods:** The relaxant effect and the mechanism of action of mix-H:N were studied on isolated aorta of Wistar rats. Aortic reactivity was determined through concentration-response curves of norepinephrine (NE) and carbamylcholine or carbachol (CCh) after intragastric administration of mix-H:N (150 mg/kg) for 30 days. The antihypertensive effect of a single dose of mix-H:N was studied on spontaneously hypertensive rats (SHR). **Results:** Mix-H:N produced concentration-dependent relaxation response in Wistar rat's aorta pre-contracted by NE. Inhibitors of NO production and inhibition of extracellular Ca^{2+} influx caused a significant blockade on the relaxation response to mix-H:N; besides, mix-H:N elicited a vasorelaxant effect on KCl (80 mM)-induced contraction. In addition, oral administration of 150 mg/kg of mix-H:N of SHR rats evoked a significant decrease in systolic and diastolic blood pressure at 5 h and 7 h after administration. Finally, sub-chronic oral administration of mix H:N for 30 days caused *ex vivo* vascular reactivity modification on NE-induced contraction and CCh-induced relaxation, improving endothelial function. **Conclusion:** The mix-H:N has the vasorelaxant and antihypertensive effect that may be attributed to an increase of NO production and a blockade of the Ca^{2+} channels on VSMCs. Furthermore, mix-H:N improves endothelial function of Wistar rats acting as a potential prophylactic against CVDs.

Key words: Antihypertensive, endothelial damage, hesperidin, naringenin, nitric oxide, vasorelaxant effect

SUMMARY

- The mixture of Hesperidin: Naringenin (mix-H:N) show vasorelaxant effect involves of nitric oxide production and a blockade of the Ca^{2+} channels
- The mix-H: N exhibited an antihypertensive effect in spontaneously hypertensive rats
- Subchronic administration of mix-H: N improves endothelial function of Wistar rats.



Abbreviations used: °C: Degree Celsius, 20-HETE: 20-hydroxyeicosatetranoic acid, µM: Micromolar, ANOVA: Analysis of variance, BK_{Ca}: Large conductance Ca^{2+} activated K^+ channel, Ca^{2+}: Calcium, $CaCl_2$: Calcium chloride, CCh: Carbamylcholine or carbachol, CO_2: Carbon dioxide, CRC: Concentration-response curves, CVDs: Cardiovascular diseases, DACS-UJAT: División Académica de Ciencias de la Salud of Universidad Juárez Autónoma de Tabasco, DBP: Diastolic blood pressure, DMSO: Dimethyl sulphoxide, E–: Aortic rings without-endothelium, E+: Aortic rings with intact-endothelium, EC_{50}: Median effective concentration, EDRFs: Endothelium-derivative relaxant factors, EDTA: Ethylenediaminetetraacetic acid, E_{max}: Maximum effect, FES: Facultad de Estudios Superiores, GC: Guanylate Cyclase, H: Hesperidin, H: Hours, HR: Heart rate, I.G: Intragastric via, i.p: Intraperitoneal via, K^+: Potassium, KCl: Potassium chloride, KH_2PO_4: Potassium phosphate monobasic, L-NAME: N_ω-Nitro-L-arginine methyl ester, LPH: Lactase phlorizin hydrolase, LTCC: Long-lasting (L-type) Ca^{2+} channels, $MgSO_4$: Magnesium sulfate, min: Minutes, mix-H: N: Mixture of Hesperidin: Naringenin, mL: Milliliter, mM: millimolar, N: Naringenin, NaCl: Sodium chloride, $NaHCO_3$: Sodium bicarbonate, NE: Norepinephrine, NO: Nitric oxide, NOM: Norma Oficial Mexicana, NOS: Nitric Oxide Synthase, O_2: Oxygen, ODQ: 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one, PKG: Protein kinase G, S.E.M: Standard error of the mean, SAGARPA: Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación, SBP: Systolic blood pressure, SERCA: Sarcoplasmic reticulum Ca^{2+}-ATPase, sGC: Soluble Guanylyl cyclase enzyme, SGLT1: Sodium-dependent glucose transporter, SHR: Spontaneously hypertensive rats, SR: Sarcoplasmic reticulum, TEA: Tetraethylammonium chloride, VDCC: Voltage-dependent Ca^{2+} channels, VSMC: Vascular smooth muscle cells.

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INTRODUCTION

Cardiovascular diseases (CVDs) are responsible for a third of all deaths worldwide, and the number of deaths attributable to high blood pressure among adults have increased from 32% in 2017 to 46% in 2018.[1,2] Hypertension is the most important modifiable risk factor for CVDs such as cerebrovascular disease, coronary disease, heart failure, chronic kidney disease, and peripheral vascular disease[1-3] and is also associated with metabolic abnormalities such as diabetes and dyslipidemia.[4] Vascular endothelium is a monolayer of cells that covers the luminal wall of blood vessels. It protects the arterial wall against the development of lesions and contributes to vascular homeostasis through the production of vasodilators and vasoconstrictors, and other factors such as pro-coagulants and anticoagulants, inflammatory and anti-inflammatory, fibrinolytics and antifibrinolytics, oxidizing and antioxidizing, among others.[5,6]

Recent studies provide growing evidence that flavonoid-rich food may play a role in the regulation of diseases that compromise metabolic processes in cells. This represents an alternative in the treatment of many diseases, including the CVDs.[7,8] Thus, the foods that have the highest flavonoid content are citrus fruits, nuts, red fruits, herbs, spices, and grains. The daily consumption of flavonoids in the human diet varies, ranging between 23 mg and >500 mg/day.[9] The *Citrus* genus is characterized as one of the largest accumulators of phenolic compounds such as glycosylated flavanones and polymethoxylated flavones.[10,11]

Epidemiological evidence and clinical and pre-clinical studies suggest that *Citrus* genus' flavanones have a positive influence in cardio-metabolic parameters, preventing CVDs.[12-14] This positive influence on human health has increased the citrus consumption and results in the accumulation of high amounts of by-products such as peel, seed and membrane residues. *Citrus* by-products are a good source of phenolic compounds, especially flavanones. Previous studies indicate that the sweet orange's major flavanones are hesperidin and naringenin which are particularly concentrated in the peel (albedo and flavedo) rather than the pulp.[10,15,16] Cabañas 2013 reports presence of hesperidin and naringenin mixture in orange peel[15] and González-Rivero 2018, reports synergic vasorelaxant effect of seven mixtures of hesperidin and naringenin and its effect was endothelium-dependent through increase of nitric oxide,[17] In this context, the vascular endothelium plays an important role in controlling blood pressure. It has been reported that both flavanones exert vasorelaxant activity through the activation of the endothelium. Hesperidin enhances NO production by phosphorylation of the protein Akt and inhibits endothelin-1, a potent vasoconstrictor; and naringenin enhance the production of NO and prostacyclin.[18,19] Besides the vasorelaxant effect, both, hesperidin and naringenin, have demonstrated beneficial effects on blood pressure, inflammation, atherosclerosis, dyslipidemia, and diabetic neuropathy, among others.[20-26] Hesperidin and naringenin have been studied individually. However, there are no experimental data that evaluates them together, as they are found in nature. Therefore, the aim of the present study was to determinate the cardiovascular effect through vasorelaxant ability, mechanism of action, antihypertensive activity, and prophylactic potential against endothelial damage in the medium term of a mixture of Hesperidin: Naringenin with the proposal to contribute to the growing knowledge about citroflavonoids for the treatment and prevention of CVDs.

MATERIALS AND METHODS

Chemicals and drugs

The flavonoids hesperidin (H) and naringenin (N), and the reagents (+/-)-norepinephrine bitartrate hydrate (NE), carbamylcholine chloride (carbamylcholine or carbachol [CCh]), tetraethylammonium chloride (TEA) and dimethyl sulfoxide (DMSO), N_{ω}-Nitro-L-arginine methyl ester (L-NAME) and 1H-[1,2,4] oxadiazolo[4,3-a] quinoxalin-1-one (ODQ), were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents were analytical grade from local sources. Stock solutions of hesperidin (H) and naringenin (N), and mix-H:N were dissolved with DMSO (10%) and prepared the same day of experimentation.

Ex vivo and *in vivo* pharmacological evaluation

Animals

Male Wistar rats (250–300 g bodyweight) were obtained from the Animal House of "Unidad de Producción, Cuidado y Experimentación Animal" of the División Académica de Ciencias de la Salud of Universidad Juárez Autónoma de Tabasco in Mexico. Spontaneously hypertensive rats (SHR) (250–300 g bodyweight) were provided by Facultad de Estudios Superiores Iztacala animal facilities, from Universidad Nacional Autónoma de México. Animals were housed in polycarbonate cages and maintained under standard laboratory conditions (12-h light/dark cycle, at a temperature of 25°C ± 2°C, and with a humidity of 45%–65%), and were fed with standard rodent diet and water *ad libitum*. All animal procedures were conducted in accordance to our Federal Regulations for Animal Experimentation and Care (SAGARPA, NOM-062-ZOO-1999, México)[27] and approved by the Institutional Animal Care and Use Committee based on the US National Institute of Health publication (No. 85-23, revised 1985). All experiments were carried out using five or nine animals per group according to the experiment. They were euthanized by cervical dislocation after deep anesthesia with phenobarbital (65 mg/kg, i.p).

Vasorelaxant activity of mix-H:N on aortic rings

For this purpose, a previous protocol of Estrada-Soto *et al.*[28] was used. After sacrifice, the thoracic aorta was removed, cleaned out of connective tissue and it was cut in rings of approximately 5 mm in length. In addition, for some aortic rings the endothelium layer was gently removed by the mechanic procedure. Each piece was suspended in organ baths containing Ringer–Krebs solution (10 mL), which chemical composition (mM) was: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; ethylenediaminetetraacetic acid, 0.026 and dextrose, 11.1. The solution was maintained at pH 7.4 and gassed with 95% O_2 and 5% CO_2 at 37°C. The rings were initially stretched to a basal tension of 3.0 g before allowing them to equilibrate in the bathing medium; tissues were allowed to stabilize for 20 min. The contractions were recorded with an isometric vertical force transducer (BIOPAC®) connected to a MP150 Data Analysis Software (Acknowledge®). After the stabilization period, the tissues were stimulated with NE (0.1 μM) during 10 min, and then they were washed with new Krebs solution. This sensitization process of the tissues was repeated three times, with intervals of 30 min, before starting the experiments. The absence of endothelium was confirmed by the lack of the relaxant response induced by the CCh (1 μM) during the last contraction. Finally, for the evaluation period, all tissues were contracted with NE (0.1 μM) and was added to the bath quarter-log cumulative concentrations (2.16–210 μM) of the mix-H:N or positive control (CCh [1.13×10^{-04} to 10 μM]), a cholinergic agonist, for the experiments with endothelium and nifedipine (1.13×10^{-04} to 10 μM), a calcium channel blocker agent, for experiments without endothelium).

Determination of mode of action of mix-H:N on aortic rings

To establish the mode of action of mix-H:N, after the stabilization and sensitization period, the following experiments were conducted.

- In order to know the role of Nitric Oxide (NO) or soluble Guanylyl Cyclase enzyme (sGC) in the vasorelaxant effect of the mix-H:N,

aortic rings with endothelium were incubated for 15 min with L-NAME (a Nitric Oxide Synthase [NOS] inhibitor, [100 μM]) or ODQ (an sGC inhibitor, [10 μM]), respectively. Then, mix-H:N was added at cumulative concentrations (2.16–210 μM), and the concentration-response curves (CRC) were obtained. The maximal relaxing effect of mix-H:N was compared in the absence and presence of L-NAME or ODQ.

- In order to know the role of potassium (K^+) channels in the vasorelaxant effect of the mix-H:N, aortic rings (E^+ and E-) were incubated for 15 min with an unspecific K^+ channel blocker (TEA) (100 μM), then mix-H:N was added at cumulative concentrations (2.16–210 μM), and CRC were obtained. The maximal relaxing effect of mix-H:N was compared in the absence and presence of TEA.
- To determine whether the vasorelaxant effect of mix-H:N is due to the inhibition of extracellular calcium (Ca^2+) influx, the experiments were carried out in Ca^{2+}-free Krebs solution. Aortic rings were washed with Ca^{2+}-free Krebs solution containing KCl (80 mM) and the cumulative CRC for $CaCl_2$ (0.1 μM to 10 mM) were obtained in the absence of mix-H:N (control group) or after 15 min incubation with (32 μM) of mix-H:N. Then, the contractile effect induced by $CaCl_2$ was compared in the absence and presence of mix-H:N.
- To establish a possible interaction of mix-H:N with L-type calcium channel blockade, the aortic rings without endothelium were pre-contracted with high KCl (80 mM), then mix-H:N was added at cumulative concentrations (2.16-210 μM) and was obtained the CRC of mix-H:N.

Determination of the antihypertensive effect of mix-H:N on spontaneously hypertensive rats

The antihypertensive effect of mix-H:N was conducted in SHR rats. Animals were allotted into two groups (five animals each): Control rats (Group 1) and treated rats (Group 2). Control group and treated group received a single intragastric dose of purified water (5 mL/kg) and a mix H:N (150 mg/kg), respectively. Measurements (blood pressure and heart rate) were recorded before (T0) and after the treatment of test compound at 1, 3, 5, and 7 h (T1–T7) by a tail-cuff method using a LE 5001 automatic blood pressure computer (PanLab®, Harvard Apparatus, Spain). The percentage decrease in heart rate (HR), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were calculated.

Determination of prophylactic potential of cardiovascular, endothelial damage of sub-chronic administration of mix-H:N on Wistar rats

Prophylactic effect of cardiovascular, endothelial damage of mix-H:N was conducted in normotensive Wistar rats. After acclimatization, the animals were randomly divided into two groups (nine animals each): A control group that was administrated with purified water (5 mL/kg b. w./day) and a treated group with mix H:N (150 mg/kg b. w./day). Both groups were administrated daily for 30 days, by intragastric via. At the end of the experimental period after overnight fasting, the animals were sacrificed by cervical dislocation, and their thoracic aorta was quickly dissected out. Aortic reactivity was determined through *ex vivo* curve CRC) of NE and CCh (3.60×10^{-11} to 1.00×10^{-05} μM). Maximum contraction or relaxation was determinate with E_{max} values.

Data analysis

Experimental results were expressed as the mean ± standard error of the mean of five experiments for vasorelaxant and antihypertensive effects, and nine experiments for prophylactic effect. CRC were plotted, and experimental data in the CRC were adjusted using the fit-sigmoidal (Hill equation) in the program Microcal® Origin 8.6 (Microcal Software Inc., USA). The statistical analysis used to determine the significant difference between the control group and mix-H:N was a one-way analysis of variance, followed by a Tukey *post hoc* analysis. $P < 0.05$ were considered to be statistically significant.[29]

RESULTS

Vasorelaxant activity of mix-H:N on aortic rings pre-contracted with norepinephrine

The vasorelaxant effect of hesperidin and naringenin was determined in aortic rings with intact-endothelium (E+) and without-endothelium (E-) precontracted with NE (0.1 μM). Figure 1a shows Hesperidin (H) and Naringenin (N) vasorelaxant effect on E^+ rings (H: E_{max} = 59.7%; N: E_{max} = 89.5%,) and vasorelaxant effect on E-rings (H: E_{max} = 35.6%; N: E_{max} = 35.7%). Both flavonoids show partially endothelium-dependent effect. Naringenin was equiefficient with carbachol (positive control E+). Several reports have described vasorelaxant effect to naringenin,[30] but not to hesperidin, in addition, it has not been evaluated mixtures similar as they are found in nature. Vasorelaxant effect of mix-H:N was evaluated. Figure 1b and c show mix-H:N and individual flavonoids vasorelaxant effect on E^+ rings and E-rings, respectively; mix-H:N-CRC's were significantly shifted to left compared with H and N, this behavior suggest a potency synergism of mix-H:N. Table 1 describes maximum effect (E_{max}) and median effective concentration (EC_{50}), these values were calculated on the vasorelaxant effect-CRC of mix H:N. The effect of the mix-H:N was more efficient on E^+ rings (E_{max} = 107.6 ± 4.18%; EC_{50} = 32.0 ± 2.30 μM) than the effect on E-rings (E_{max} = 73.0 ± 4.67%; EC_{50} = 167.7 ± 1.26 μM), suggesting not only participation of endothelium-derivative relaxant factors (EDRFs) but also direct involvement of vascular smooth muscle cells (VSMC) which cause the vasorelaxation seen in Figure 1b and c.[31] According to the previous information, it was determined the mechanism of action of the mix-H:N.

Determination of mode of action of mix-H:N on aortic rings

Figure 2a shows mix-H:N vasorelaxant effect in the presence of L-NAME (100 μM) and ODQ (10 μM), two inhibitors of nitric oxide (NO) pathway. Mix-H:N-CRC (2.16-210 μM) was significantly shifted to the right compared with the control (mix-H:N vasorelaxant

Table 1: Pharmacological parameters calculated of vasorelaxant effect of mix- hesperidin: Naringenin, single flavonoids and positive control used

Test sample	With endothelium (E+)		Without endothelium (E−)	
	E_{max} (%)	EC_{50} (μM)	E_{max} (%)	EC_{50} (μM)
Carbachol (control E+)	86.7	0.3	ND	ND
Nifedipine (control E−)	ND	ND	99.8	0.03
H	59.7±4.05	248.0±4.08	35.6±1.98	>300
N	89.5±2.62	140.2±5.0	35.7±1.26	>300
Mix -H:N	107.6±4.18	32.0±2.30	73.0±4.67	167.7±1.26

Results are presented as the mean±SEM, *n*=5. Maximum effect (E_{max}) and median effective concentration (EC_{50}). ND: Nondeterminate; H: Hesperidin; N: Naringenin; SEM: Standard error of mean

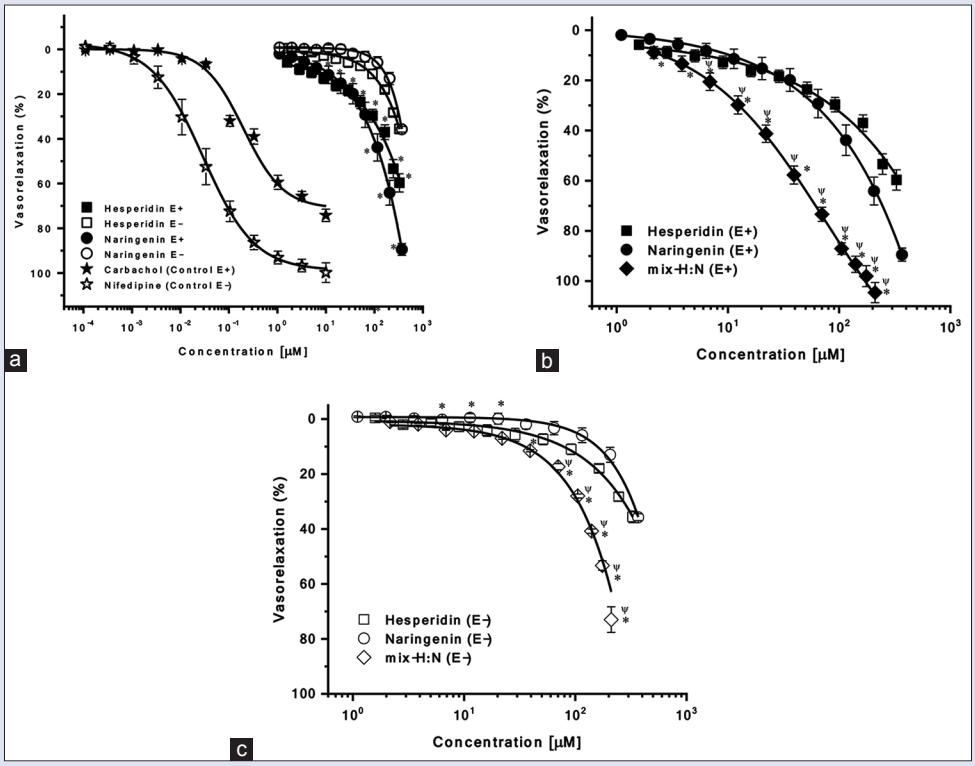

Figure 1: Concentration-response curves of vasorelaxant effect of (a) Hesperidin (h) and Naringenin (n) and positive controls used on intact-endothelium (E+) and denuded-endothelium (E-) (b) mix-H: N on aortic rings intact-endothelium (E+) and (c) mixture of Hesperidin: Naringenin on aortic rings denuded-endothelium (E-). Results are expressed as the means ± SEM of five experiments, $^{*}P < 0.05$ represents significant difference compared with endothelium-absence aortic rings and $^{\Psi}P < 0.05$ represents significant difference compared with single flavonoids

effect). Figure 2b shows mix-H:N's vasorelaxant effect in the presence of TEA (100 μM), an unspecific K^+ channel blocker. Mix-H:N-CRC was shifted to the right regarding the control on E^+ aortic rings, resulting in a reduction of 10% of the E_{max} and the increase of twice the EC_{50} (E_{max} = 90.3 ± 3.80%, EC_{50} = 59.2 ± 1.21 μM). On aortic rings without endothelium TEA did not modify the mix-H:N's vasorelaxant effect (data not shown).

Figure 2c shows CRC's contractile effect of $CaCl_2$ (0.1 μM to 10 mM) in the presence EC_{50} of the mix-H:N on E^+ aortic rings. The mix-H:N significantly shifted to the right the CRC to $CaCl_2$ (E_{max} = 1.3 ± 0.01 g; EC_{50} = 6.73 μM) regarding the control ($CaCl_2$ without mix-H:N: E_{max} = 2.4 ± 0.13 g, EC_{50} = 0.99 μM) and prevented it from reaching its maximum effect. On the other hand, Figure 2d shows the curve of Ca^{+2} contraction on E-aortic rings in the presence of EC_{50} of mix-H:N; the curve was slight shifted to the right without modifying the maximum effect (E_{max} = 2.0 ± 0.18 g; EC_{50} = 1.48 μM) with regard to the control (E_{max} = 1.9 ± 0.28 g; EC_{50} = 0.91 μM). Figure 2e shows the vasorelaxant effect exerted by the mix-H:N on the KCl (80 mM)-induced contraction in rings without endothelium, which reached 99% relaxation, as did nifedipine (E_{max} = 100%; positive control).

Determination of antihypertensive effect of mix-H:N on spontaneously hypertensive rats

Oral administration of 150 mg/kg of mix-H:N evoked a significant decrease of SBP in SHR at 5 h (8.78%) and 7 h (7.57%) after administration ($^{*}P < 0.05$) [Figure 3a]. Similarly, mix-H:N induced a significant decrease in DBP at 5 h (12.3%) and 7 h (11.37%) after administration [Figure 3b]. The heart rate was not modified compared with to the control (5 mL/kg; purified water) [Figure 3c].

Determination of prophylactic potential of cardiovascular, endothelial damage of sub-chronic administration of mix-H:N on Wistar rats

Sub-chronic oral administration (30 days) of 150 mg/kg of mix-H:N caused *ex vivo* vascular reactivity modification on NE-induced contraction and CCh-induced relaxation. Figure 4a shows NE-induced contractile effect on aortic rings of the treated group; here, the curve was slightly shifted to the right regarding the control group (aortic rings of rats administrated with purified water administrated), which indicates that the treated group was more resistant to the contraction of NE than the control group. On the other hand, Figure 4b shows CCh-induced relaxant effect on aortic rings of treated group, here the curve was shifted to left regarding the control group, this may indicate that the mix-H:N increases the relaxant effect of CCh.

DISCUSSION

Flavonoids are molecules widely studied. Numerous studies support their health benefits which include anti-viral/bacterial, anti-inflammatory, cardioprotective, anti-diabetic, anti-cancer and anti-aging properties.[32] The results of the present study showed the positive effect on cardiovascular health of flavonoids mixture (mix-H:N) previously found on the sweet orange peel using *ex vivo* and *in vivo* experimental models.

The effect of the mix H:N was more efficient than individual flavonoids, suggesting a possible potency synergism.[33] Besides, the vasorelaxant effect of the mix-H:N was partially endothelium-dependent, suggesting the participation of EDRFs and mechanisms in the VSMC.[31] The effects in the presence of L-NAME (nitric oxide synthase [NOS] inhibitor) and ODQ (soluble guanylate cyclase [GC] inhibitor) were significantly

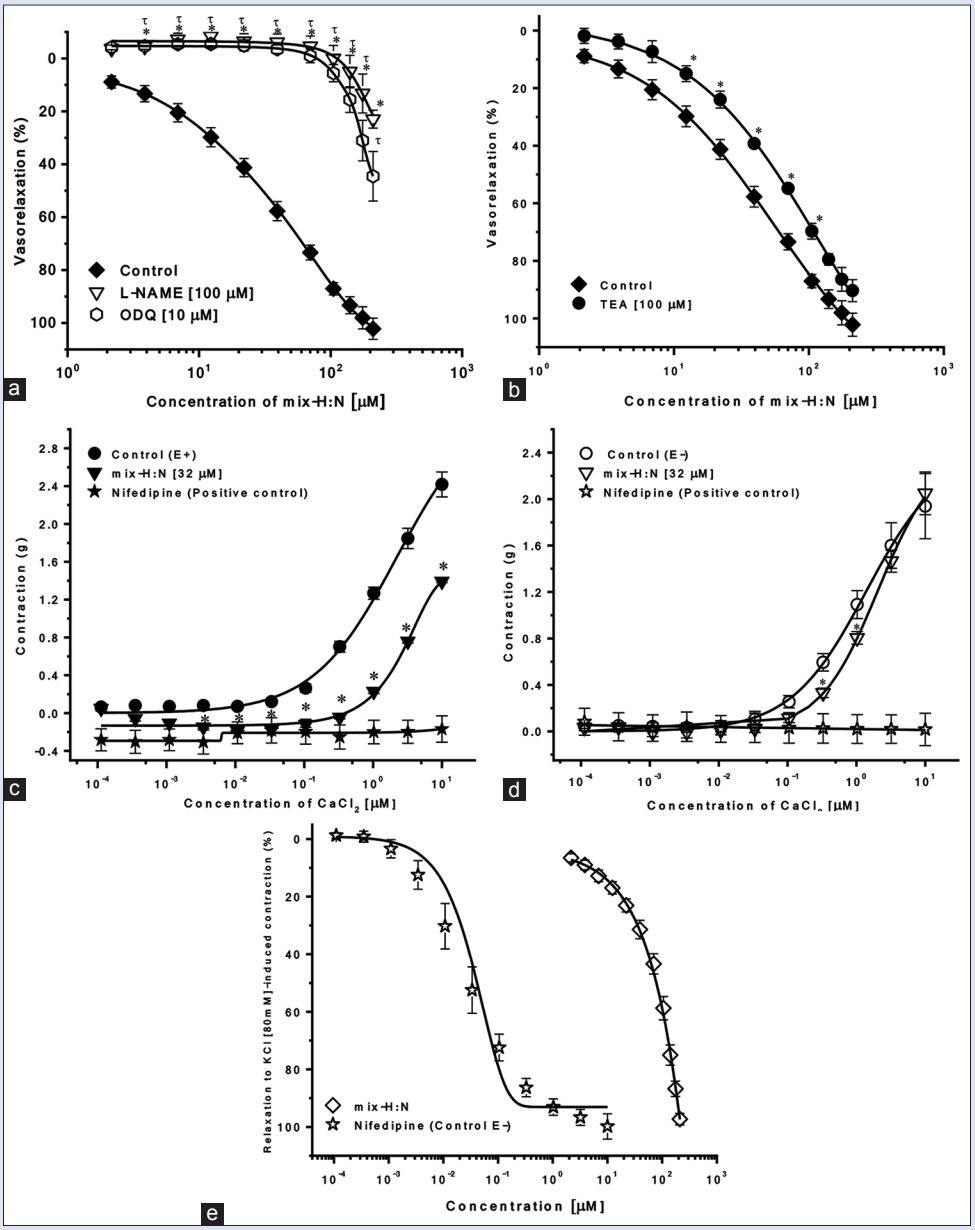

Figure 2: Vasorelaxant effect of mixture of Hesperidin: Naringenin (2.16 to 210 µM) on intact-endothelium aortic rings pre-contracted with NE (0.1 µM) (a) in presence of ODQ (10 µM) and L-NAME (100 µM) and (b) in presence of TEA (100 µM). Inhibitory effect of mixture of Hesperidin: Naringenin (32 µM) on the $CaCl_2$-induced cumulative-contraction curve on (c) aortic rings intact-endothelium and (d) on aortic rings denuded-endothelium. (e) Inhibitory effect of mixture of Hesperidin: Naringenin (2.16 to 210 µM) on the contraction induced by KCl (80 mM) in rat aortic rings denuded-endothelium. All results are expressed as the mean ± standard error of the mean of five experiments, (*) and (τ*) represent significant difference compared with mixture of Hesperidin: Naringenin curve without drugs (control) ($P < 0.05$)

modified, suggesting NO participation and cGMP increase as mechanism of action of mix H:N. Previous reports indicate that in both, hesperidin and naringenin, the vasorelaxant effect is attributed to NO production synthetized in the vascular endothelium.[18,19,34] NO nitrosylates the GC after it enters to the VSMCs. Once GC is activated it degrades the GTP to cGMP; the latter, activates the PKG, enzyme responsible for stimulating muscle relaxation.[34] In addition, Ajay *et al.*[18] reported that the vasorelaxant effect of naringenin depends as well on the release of prostaglandins, produced in the vascular endothelium.[19] These two mechanisms of endothelium-dependent vasorelaxation of naringenin could explain the fact that increasing the naringenin portion increases the vasorelaxant effect, and the synergic effect is exhibit for the mix-H:N.

Besides the EDRFs, another ways to produce vasorelaxation are the hyperpolarization of the VSMCs and the reduction of $[Ca^{2}+]_i$.[35,36] The effect of mix-H:N was modified in the presence of TEA, an unspecific K^+ channel blocker. This suggests that the mechanism of action is through the opening of endothelium-dependent K^+ channels. Saponara *et al.*[35] reported that naringenin might activate the large conductance Ca^{2+} activated K^+ channels (BK_{Ca}) in VSMCs producing hyperpolarization of the membrane potential.[36] The main effect of the activation of these channels is a decrease in the influx of Ca^{2+} by reducing the opening of voltage-dependent Ca^{2+} channels (VDCC) and activating the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) that transfers Ca^{2+} from the cytosol of the cell to the lumen of the

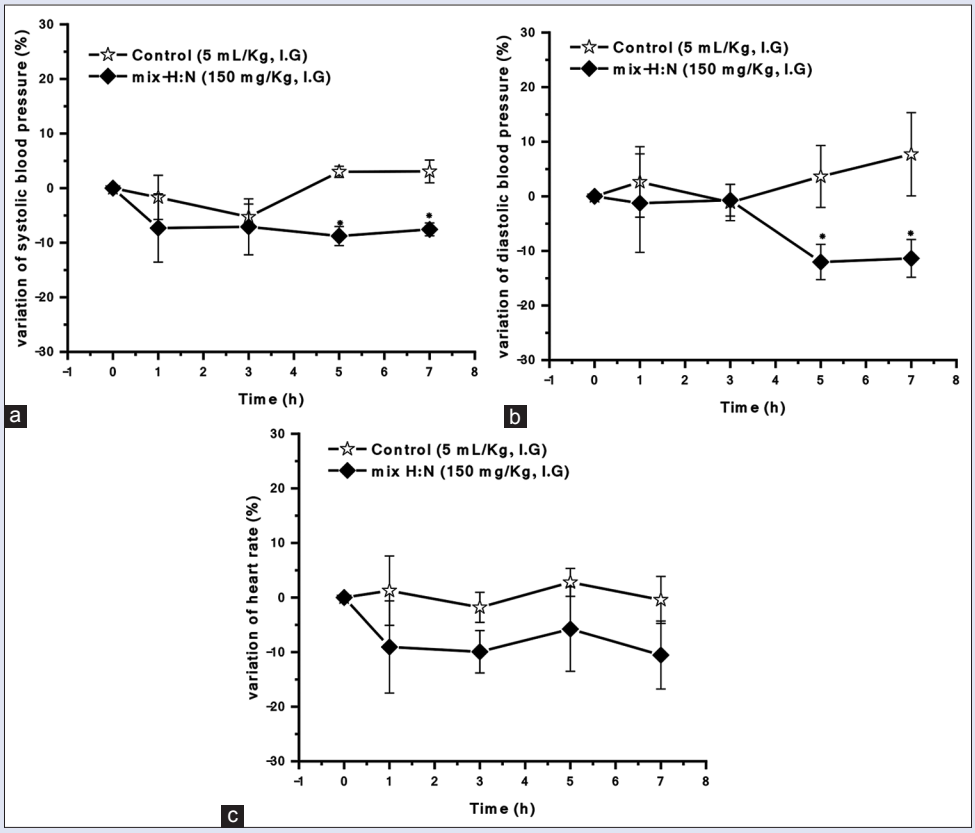

Figure 3: Maximal decrease in (a) systolic blood pressure, (b) diastolic blood pressure and (c) heart rate elicited by oral administration of 150 mg/kg of mixture of Hesperidin: Naringenin in conscious SHR rats. Results are expressed as the means ± SEM of five rats per group. (*) Indicates significance respect to purified water (control) ($P < 0.05$)

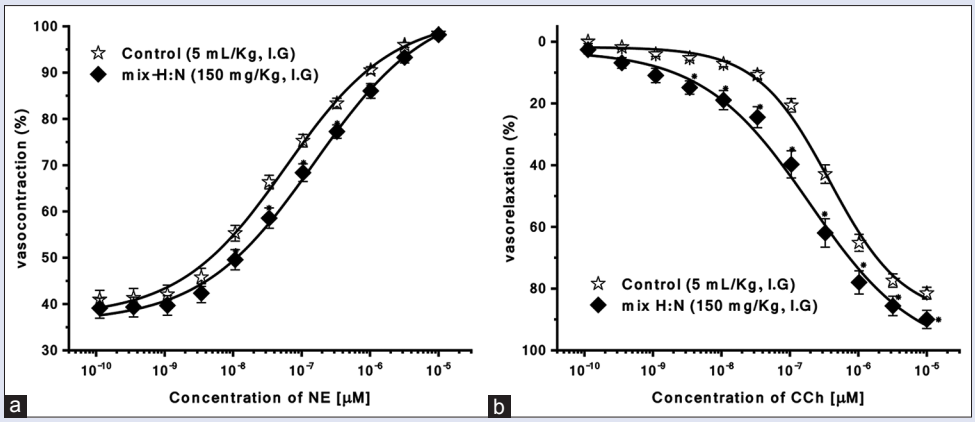

Figure 4: (a) Concentration-response curves of contractile effect of mixture of Hesperidin: Naringenin treated rats aortic rings. (b) Concentration-response curves of vasorelaxant effect of mixture of Hesperidin: Naringenin treated rats aortic rings. Results are expressed as the means ± SEM of nine rats per group. (*) Indicates significance respect to purified water (control) ($P < 0.05$)

SR.[37] The opening of K^+ channels in the VSMCs results in K^+ efflux and induces hyperpolarization of membrane potential, which provides an important mechanism to dilate arteries.[38] In this context, the vasorelaxant effect of the mix-H:N on endothelium-denuded rat aorta was not modified by TEA. This last result suggests that direct opening of K^+ channels in the VSMC is not involved in the mechanism of action of mix-H:N. Nevertheless, it has been reported that NO, an EDRF, activates BK_{Ca} through phosphorylation-dependent on PKG or directly by the binding of NO to cystine residues located at the α subunit of the channel. Similarly, NO inhibits 20-hydroxyeicosatetranoic acid, a potent inhibitor of BK_{Ca} activity.[39,40] This could explain the curve shift on E^+ aortic rings and not on E-aortic rings in the presence of TEA, suggesting that the mix-H:N induce vasorelaxation due to NO formation. Furthermore, BK_{Ca} are expressed in the endothelial cells and they contribute to Ca^{2+} entry, amplifying NO production.[35] Therefore, if these channels were blocked by TEA, the production of NO would be reduced or delayed; this could also explain the behavior observed in Figure 2b.

The intracellular flow of Ca^{2+} occurs, in the vast majority, through the long-lasting (L-type) Ca^{2+} channels (LTCC). The behavior of the $CaCl_2$ curve in the presence of the mix-H:N on aortic rings with intact endothelium is characteristic of a non-competitive antagonism.[33] It is known that the EDRFs can indirectly inhibit the LTCC channels in the VSMC. For example, NO inhibits these channels by the signaling mechanism that involves the NO-sGC-cGMP-PKG pathway. Another mechanism for blocking these channels is through the activation of PKA, using the COX-PGI_2-AC-cAMP-PKA relaxation pathway, or by cross-activation of PKG.[41,42] This is the reason by why removing the vascular endothelium, the effect is modified; nevertheless, in aortic rings without endothelium, there's a slight shift to the right, characteristic behavior of a competitive antagonism.[33] On the other hand, the mix-H:N was capable of inhibiting contractility induced by KCl (80 mM), suggesting a possible direct blockade of LTCC that decrease the entry of intracellular Ca^{2+}.

NO production and intracellular Ca^{2+} reduction on aortic rings may contribute to the decrease of arterial pressure. On the *in vivo* experiments, mix-H-N induced reduction of SBP and DBP after five and seven h of administration. The literature reports that these flavonoids have positive effects in the reduction of arterial pressure. Sánchez-Salgado reported it delayed antihypertensive effect of naringenin[42] and Yamamoto *et al*. reported SBP reduction of rats administrated with hesperidin for 8 weeks.[43]

Flavonoids are compounds with poor bioavailability and susceptible to biotransformation. Hesperidin is a glycoside flavonoid that, to enter into systemic circulation, suffers active uptake by sodium-dependent glucose transporter (SGLT1) with consequent deglycosylation by cytosolic β-glycosidase. Furthermore, this flavonoid can undergo luminal hydrolysis by lactase-phlorizin hydrolase (LPH) with subsequent passive absorption of released aglycones.[32] This biotransformation could explain that the antihypertensive effect of the mix-H:N took from 5–7 h after the administration was applied.

With the purpose to identify endothelium-dependent beneficial cardiovascular properties of mix-H:N sub-chronic oral administration, it was evaluated aortic rings reactivity to CCh and NE of rats treated with mix-H:N, showing an improvement of endothelial function. Previously, mix-H:N caused a vasorelaxant effect by increasing NO production. NO has a wide range of biological properties that maintain vascular homeostasis, such as modulation of vascular dilator tone, regulation of local cell growth and vessel's protection from injurious consequences of platelets and cells circulating in the blood, playing a crucial role in the normal function of the endothelium.[5] When endothelial cells lose their ability to maintain its delicate balance, fatty streaks appear in the intima of arteries. Fatty streaks consist of aggregates of lipoprotein-loaded macrophages, cholesterol and smooth muscle cells, which are the precursor lesion of atheroma that may become atheromatous plaque. If the situation persists, the progression of fatty streaks could break the plaque and set the condition for thrombogenesis or vascular occlusion.[5] Mix-H:N enhances CCh response and delay NE response on aortic rings due to augmented NO production. This suggests improvement of endothelial function and may act as prophylactic against the development of atherosclerosis, systemic hypertension, congestive heart failure, pulmonary hypertension, and the aging process.[44]

CONCLUSION

The mixture of Hesperidin: Naringenin had significant vasorelaxant *ex vivo* effect and antihypertensive activity. This activity seems to be a consequence of an increase of NO production on the endothelial cells and Ca^{2+} channel blockade on VSMC. In addition, sub-chronic intragastric administration of mix-H:N improves endothelial function by enhancing CCh response and delaying NE response on aortic rings acting as a potential prophylactic against CVDs. The citroflavonoids mixture H:N represents an important candidate with therapeutic potential in the development of new antihypertensive drugs with prophylactic properties against endothelial damage.

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Conflicts of interest

There are no conflicts of interest.

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