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Antihypertensive and vasorelaxant effects of tilianin isolated from *Agastache mexicana* are mediated by NO/cGMP pathway and potassium channel opening

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ABSTRACT

Current investigation was undertaken to elucidate the mode of action of tilianin, isolated from *Agastache mexicana*, as a vasorelaxant agent on *in vitro* functional rat thoracic aorta test and to investigate the *in vivo* antihypertensive effect on spontaneously hypertensive rats (SHR). Tilianin (0.002–933 μM) induced significant relaxation in a concentration- and endothelium-dependent and -independent manners in aortic rings pre-contracted with noradrenaline (NA, 0.1 μM), and serotonin (5-HT, 100 μM). Effect was more significant ($p < 0.05$) in endothelium-intact (+E) aorta rings than when endothelium was removed (−E). Pre-treatment with N-nitro-L-arginine methyl ester (L-NAME; 10 μM) or 1-*H*-[1,2,4]-oxadiazolo-[4,3a]-quinoxalin-1-one (ODQ, 1 μM) produced a significant change of the relaxant response and activity was markedly inhibited, but not by indomethacin (10 μM) or atropine (1 μM). Furthermore, tilianin (130 μM) provoked a significant displacement to the left in the relaxation curve induced by sodium nitroprusside (SNP; 0.32 nM to 0.1 μM). Moreover, tilianin induced significant *in vitro* NO overproduction (1.49 ± 0.86 μM of nitrites/g of tissue) in rat aorta compared with vehicle ($p < 0.05$). In addition, pre-treatment with tetraethylammonium (TEA, 5 mM) and 2-aminopyridine (2-AP, 0.1 μM) shifted to the right the relaxant curve induced by tilianin ($p < 0.05$). Finally, a single oral administration of tilianin (50 mg/kg) exhibited a significant decrease in systolic and diastolic blood pressures ($p < 0.05$) in SHR model.

Results indicate that tilianin mediates relaxation mainly by an endothelium-dependent manner, probably due to NO release, and also through an endothelium-independent pathway by opening K^+ channels, both causing the antihypertensive effect.

1. Introduction

Since the studies of the late 1970s to early 1980s which underlined the obligatory role of the endothelium in mediating acetylcholine-induced vasodilatation, nitric oxide (NO) has been recognized as an endogenous nitrovasodilator that mediates the local regulation of basal arterial tone [1]. NO, a short-lived gaseous molecule is produced by the endothelial enzyme nitric oxide synthase (eNOS) [2]. During the last 20 years, substantial evidence has been accrued that all major cardiovascular risk factors, individually and/or jointly may profoundly alter endothelial function by impairing NO synthesis or, in more general terms, by decreasing the bioavailability of this molecule at vascular level [3]. Many of the physiological functions of NO in the cardiovascular, neuronal, gastrointestinal and other systems are mediated through its primary receptor, soluble guanylyl cyclase (sGC). sGC, a key protein in the NO/guanylyl monophosphate cyclic (GMPc) signaling pathway, is an obligatory heterodimeric protein composed of one α and one β subunits. The α1/β1 sGC heterodimer is the predominant form expressed in various tissues, and is regarded as the major isoform mediating NO-dependent effects such as vasodilation [1]. The heme-containing sGC heterodimer converts guanosine triphosphate into the second messenger guanosine 3′:5′-cyclic monophosphate (cGMP). The sGC activity increases more than 200-fold in response to NO [1].

There exist a lot of compounds with NO-release stimulation properties that produce a strong vasodilation in vessels, such as

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ursolic and oleanolic acids [4,5], galangin [6] epigallocatechin gallate [7], *inter alia*. In this context, it is well known that the study of medicinal plant species had allowed the isolation of several agents, used as leads for the development of new therapeutic drugs [8]. Although there is availability of low-cost therapy, the Mexican folk medicine policies promote the use of medicinal plants for the treatment of different diseases, and some herbal medicines are real choices for treatment of hypertension [9,10]. *Agastache mexicana* is one of these plants used as antihypertensive agent [10].

In this framework, the present investigation was undertaken to elucidate the mode of action of tilianin, a flavonoid isolated from *A. mexicana*, as a vasorelaxant agent on *in vitro* rat thoracic aorta test, and to investigate the *in vivo* antihypertensive effect on spontaneously hypertensive rats (SHR).

2. Materials and methods

2.1. Chemicals and drugs

Noradrenaline bitartrate (NA), serotonin chloride (5-HT), atropine, N-nitro-L-arginine methyl ester (L-NAME), indomethacin, sodium nitroprusside (SNP), carbamylcholine chloride (carbachol), 1-*H*-[1,2,4]-oxadiazolo-[4,3a]-quinoxalin-1-one (ODQ), nifedipine, tetraethylammonium chloride (TEA), 2-aminopyridine (2-AP), sulfanilamide and napthylethylenediamide were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All other reagents were analytical grade from local sources.

2.2. Plant material and extraction

A. mexicana plant material was collected in September 2006 in Morelos, México, and identified by Dr. Patricia Castillo-España of the CEIB-UAEM. A voucher specimen (26336) was deposited at the HUMO-Herbarium of the Centro de Estudios Ambientales e Investigación "Sierra de Huautla" (CEAMISH) of the Morelos State University. Briefly, the dried plant material was pulverized (225.6 g) and crude extracts were prepared by successive maceration with C_6H_{14}, CH_2Cl_2 and MeOH (3 times for 72 h at room temperature). After filtration, extracts were concentrated *in vacuo* at 40 °C.

2.3. Isolation of tilianin

During the maceration process of MEAm a white-yellowish amorphous solid (Compound 1) was obtained by precipitation.

2.4. Animals

Male Wistar and SHR rats were used and maintained under standard laboratory conditions with free access to food and water. All animal procedures were conducted in accordance with our *Federal Regulations for Animal Experimentation and Care* (SAGARPA, NOM-062-ZOO-1999, México), and approved by the Institutional Animal Care and Use Committee Recommendation.

2.5. Functional studies

Rats (250–350 g body weight) were sacrificed by cervical dislocation. The thoracic aorta was cleaned of adhering connective tissue and was cut into 3–5 mm length rings. In some rings, the endothelium was removed. Then, tissue segments were mounted in stainless steel hooks, under an optimal tension of 3 g, in 10 mL organ baths containing warmed (37 °C) and oxygenated (O_2/CO_2, 19:1) Krebs solution (composition, mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; EDTA, 0.026 and glucose, 11.1, pH 7.4). Changes in tension were recorded by Grass-FT03 force transducers (Astromed®, West Warwick, RI, USA) connected to a MP100 analyzer (Biopac® Instruments, Santa Barbara, CA, USA), as previously described [4]. After equilibration, arterial rings were contracted by NA (0.1 μM) and washed every 30 min for 2 h. The absence of endothelium was confirmed by the lack of a relaxing response to carbachol (1 μM). After pre-contraction with NA (0.1 μM) and 5-HT (100 μM), the test samples (HEAm, DEAm, MEAm, tilianin, vehicle and positive control) were added to the bath in a volume of 100 μL; then cumulative concentration–response curves were obtained for each ring (0.01–1000 μg/mL for organic extracts and 0.002–933 μM for tilianin). In order to avoid fatigue of the arterial preparation, a 30 min recovery period was allowed between curves. The relaxant effect of extracts and positive controls were determined by comparing the muscular tone of the contraction before and after addition of the test materials. Muscular tone was calculated from the tracings, using Aqcknowledge software (Biopac®).

2.6. Determination of mode of action

For this, four sets of experiments were conducted [4,11]:

1. Aortic rings (+E) were incubated with indomethacin (10 μM) L-NAME (10 μM), atropine (1 μM) or ODQ (1 μM) added to the organ bath, and incubated during 15 min before contracting the arterial rings with NA (0.1 μM). Then, tilianin was added at different concentrations and cumulative concentration–response curves were obtained.
2. Aortic rings (+E) were incubated with tilianin (130 μM) during 15 min before contracting them with NA (0.1 μM). Then, carbachol (0.1 nM to 10 μM) and SNP (0.32 nM to 0.1 μM) were added at different concentrations and cumulative concentration–response curves were obtained.
3. Aortic rings were incubated with tilianin (40 and 130 μM) during 15 min, and then NA and 5-HT (+E) or $CaCl_2$ (−E) were added at different concentrations (1.15 nM to 3.36 μM, 10 nM to 200 μM and 0.06–20.4 mM, respectively), and cumulative concentration–response curves were obtained.
4. In order to know the role of K^+ channels on tilianin-induced relaxation, (+E) and (−E) arterial rings were pre-incubated with the K^+ channel blockers, TEA (5 mM) and 2-AP (100 μM), 15 min before NA-induced contraction (0.1 μM), then tilianin was added at different concentrations and cumulative concentration–response curves were obtained.

2.7. Determination of NO synthesis

NO production was measured by monitoring levels of released nitrite (NO_x) from aorta tissue using the Griess reagent [12,13]. Briefly, tissue segments were incubated during 15 min with NA (10 μL, 0.1 μM) in Krebs solution (950 μL). Then, 40 μL of tilianin (10 mM) was added to the medium, SNP (1 mM) or vehicle (DMSO 100%), allowing them to interact with aorta segments. After 30 min, 100 μL of medium were obtained, centrifugated (2300 × *g*) and combined with an equal volume of the Griess reagent (1% sulfanilamide and 0.1% napthylethylenediamide in 5% phosphoric acid), and the optical density was measured at 550 nm using a Microplate Reader (Ultramar, BIORAD®). Each experiment was conducted in triplicate.

2.8. Determination of antihypertensive effect

Antihypertensive activity study of tilianin was conducted in SHR rats (250–300 g; 408.5 ± 4.1 bpm for hearth rate (HR); 152.6 ± 2.4 and 122.1 ± 1.9 mmHg for systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively). Animals were allotted into two groups (six animals each): Control rats [SS

(group 1)] and treated rats [tilianin (50 mg/kg), group 2]. Measurements (blood pressure and heart rate) were recorded before and after the treatment (by intragastric route) of test samples at 0, 1, 2, 4 and 6 h by a tail cuff method using a LE 5007 automatic blood pressure recorder (Letica®, PanLab, Barcelona, Spain). Percent decrease in heart rate (HR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were calculated.

2.9. Data analysis

Results are expressed as the mean of six experiments ± S.E.M. concentration–response curves (CRC) were plotted and the experimental data from the CRC were adjusted by the nonlinear, curve fitting program (ORIGIN® 8.0). For NO_x release, results are expressed as the normalized mean of three assay's ± S.E.M. The statistical significance ($p < 0.05$) of differences between means was assessed by Student's *t*-test and ANOVA followed by Tukey's test.

3. Results

3.1. Yield and relaxant effect of organic extracts

3.8, 4.85 and 14.4 g of hexanic (HEAm), dichloromethanic (DEAm) and methanolic (MEAm) extracts were obtained, respectively. Then, extracts were evaluated in order to establish their relaxant effect. DEAm (E_{max} = 76.27 ± 3.26% and IC_{50} = 189.06 μg/mL) induced a significant vasorelaxant effect in a concentration-dependent and endothelium-independent manners (Fig. 1A). On the other hand, MEAm (E_{max} = 62.01 ± 4.63% and IC_{50} = 232.09 μg/mL) also showed a significant relaxant effect after NA (0.1 μM) action on endothelium-intact aortic rings (Fig. 1B). However, DEAm and MEAm were less potent than carbachol (a muscarinic acetylcholine receptor agonist) and nifedipine (a calcium channel blocker) used as positive controls in aortic rings +E and −E, respectively (Fig. 1A and B).

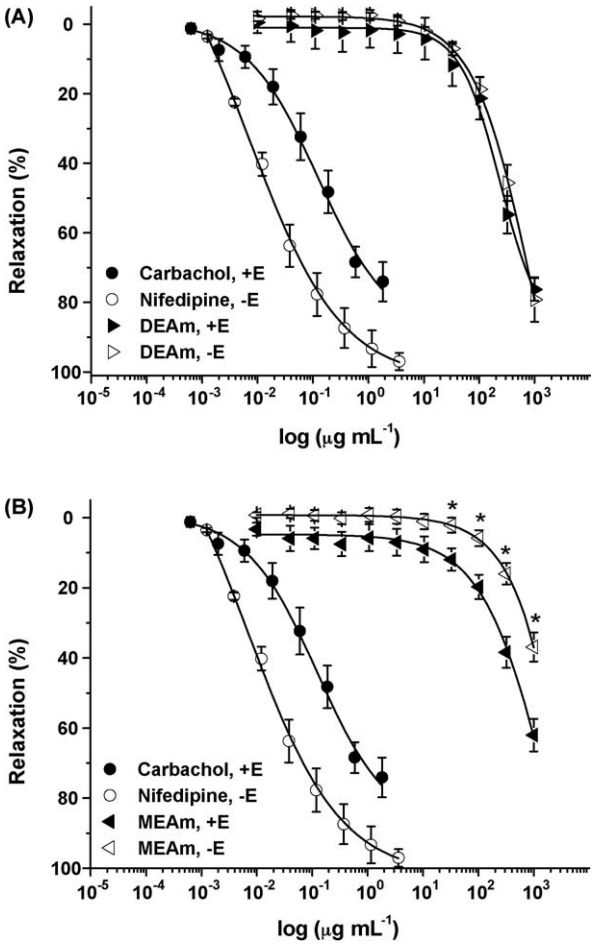

Fig. 1. (A) Relaxant effect of DEAm and (B) relaxant effect of MEAm on isolated rat aortic rings pre-contracted with NA (0.1 μM) in the presence and absence of endothelium. Results are expressed as the mean ± S.E.M. of six experiments ($^{*}p < 0.05$ vs. control).

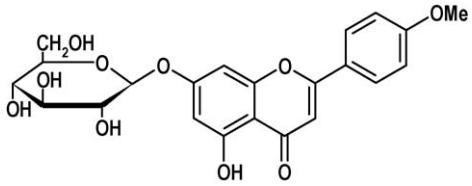

Fig. 2. Structure of tilianin.

3.2. Isolation and relaxant effect of tilianin

Tilianin (compound 1) was obtained as a white-yellowish amorphous solid. Spectroscopic and spectrometric data (IR, NMR ^{1}H, ^{13}C and GC-MS) of 1 were compared with values previously obtained for a flavonoid named tilianin [14], and both were similar (Fig. 2, Table 1). Tilianin (Fig. 3A and B) showed a significant relaxant effect on endothelium-intact aortic rings after contraction with NA (0.1 μM) and 5-HT (100 μM) (E_{max} = 84.78 ± 6.68%, IC_{50} = 240 μM and E_{max} = 75.22 ± 5.20%, IC_{50} = 275.5 μM, respectively). Relaxant effect of tilianin showed less potency and efficacy on aortic rings without endothelium (Fig. 3A and B).

3.3. Effects of tilianin on the contractile concentration–response curves for NA and 5-HT on aortic rings with endothelium

Forty and 130 μM of tilianin inhibited the concentration–response contraction of NA and 5-HT in a nonparallel manner, and depressed the maximal responses (Fig. 4A and B). On the other hand, $CaCl_2$ concentration–response contraction was not modified (Fig. 4C).

3.4. Role of relaxant factors derived from endothelium: participation of muscarinic receptor, eNOS and COX on tilianin induced relaxation

Pre-treatment with L-NAME (10 μM, a non selective NOS inhibitor) produced a significant change of the response and

Table 1
RMN-^{13}C chemical shifts of tilianin [14].

No.	δ previously described	δ obtained
2	162.83	162.8
3	103.61	103.66
4	181.79	181.74
5	156.77	156.75
6	99.4	99.47
7	163.63	163.6
8	94.29	94.79
9	162.25	162.22
10	105.22	105.31
1′	122.47	122.55
2′,6′	128.23	128.17
3′,5′	114.45	114.39
4′	160.92	160.96
1	99.8	99.94
2″	73	72.98
3″	77.03	77.09
4″	69.48	69.49
5″	76.28	76.35
6″	60.48	60.59
4′-OMe	55.41	55.43

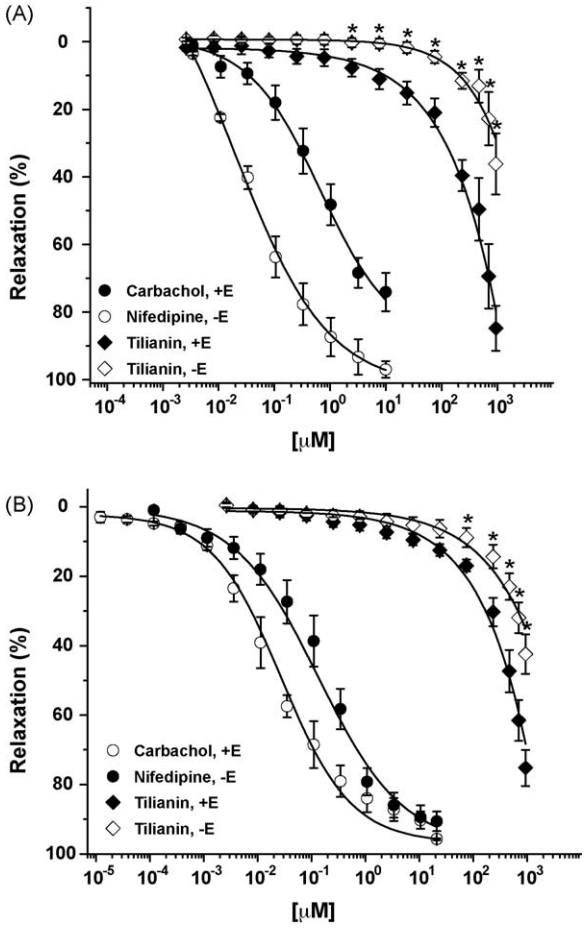

Fig. 3. Relaxant effect of tilianin on isolated rat aortic rings pre-contracted with (A) NA (0.1 μM) and (B) 5-HT (100 μM) in presence and absence of endothelium. Results are expressed as the mean ± S.E.M. of six experiments ($^{*}p < 0.05$ vs. control).

vasorelaxation was markedly inhibited (Fig. 5); in contrast, the presence of indomethacin (10 μM, a non selective cyclooxygenase, COX, inhibitor) and atropine (1 μM, an antagonist of muscarinic receptors) did not show a significant influence in tilianin effect (Fig. 5).

3.5. Role of sGC on tilianin induced relaxation on aortic rings with endothelium

Pre-incubation with ODQ (1 μM, a soluble guanylyl cyclase inhibitor) provoked a significant displacement to the right of the relaxant curve of tilianin ($p < 0.05$, Fig. 6).

3.6. Effect of tilianin on the concentration-relaxant effect of SNP and carbachol

Pre-treatment with tilianin 130 μM significantly shifted to the left ($p < 0.05$) the relaxant curve of SNP (Fig. 7A). However, relaxant curve to carbachol was not modified (Fig. 7B).

3.7. Role of K^+ channel in tilianin-induced relaxation on aortic rings with endothelium and without endothelium

K^+ channel blockers TEA (5 μM) and 2-AP (100 μM) significantly shifted to the right the tilianin relaxant curves in endothelium-intact and -denuded rings pre-contracted by NA (0.1 μM) (Fig. 8A and B).

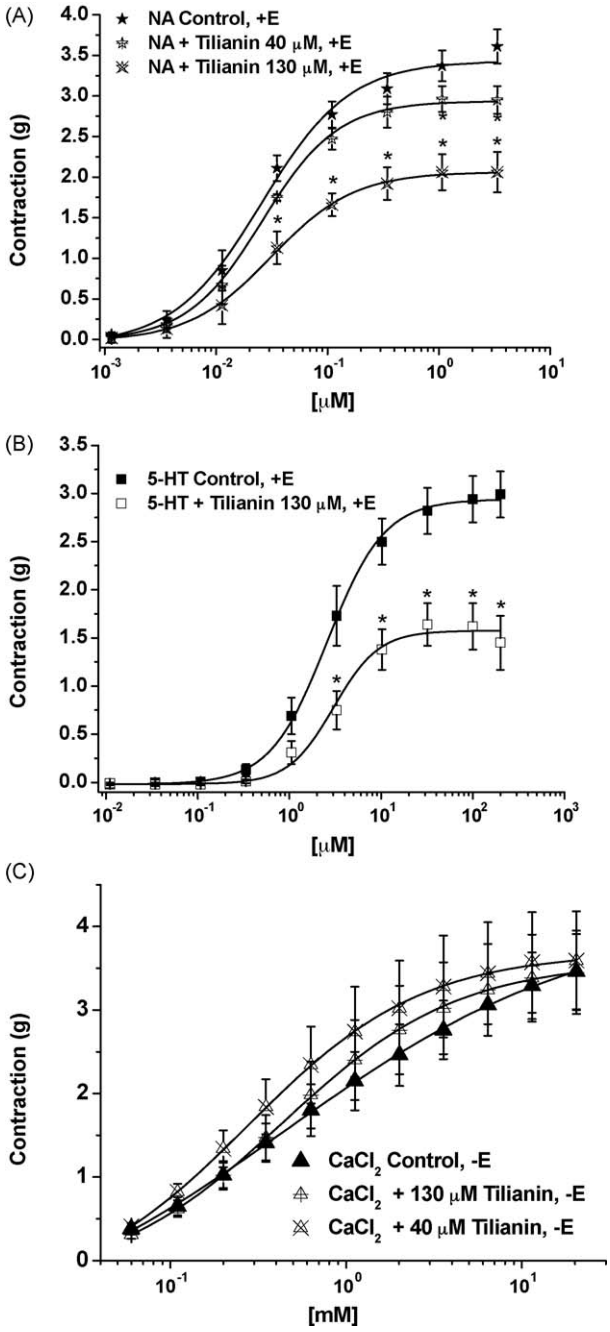

Fig. 4. (A) Effect of 40 and 130 μM of tilianin on NA accumulative concentration–response curves (1.15 nM to 3.36 μM) on rat aorta rings with endothelium. (B) Effect of 130 μM of tilianin on 5-HT accumulative concentration–response curve (10 nM to 200 μM). (C) Effect of 40 and 130 μM of tilianina on $CaCl_2$ accumulative concentration–response curve (0.06–0.40 mM). Each point represents the mean ± S.E.M. of six experiments ($^{*}p < 0.05$ vs. control).

3.8. Nitric oxide production

Incubation of rat aortic rings with tilianin (10 mM) induced a significant overproduction of NO (1.49 ± 0.86 μM of nitrites/g of tissue) compared to control group and was less effectiveness than SNP (Fig. 9).

3.9. Antihypertensive effect of tilianin

In this series of experiments, baseline of HR, SBP and DBP before oral administration of a single dose of tilianin (50 mg/kg) were

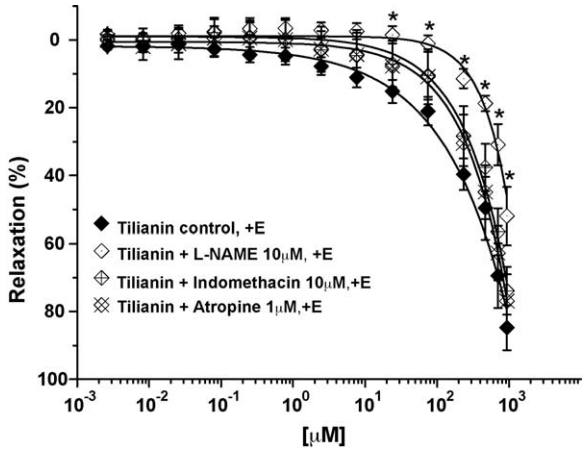

Fig. 5. Relaxant effect of tilianin on isolated rat aortic rings pre-contracted with NA (0.1 μM) in the presence of L-NAME (10 μM), indomethacin (10 μM) and atropine (1 μM), in presence of endothelium. Results are expressed as the mean ± S.E.M. of six experiments ($^{*}p < 0.05$ vs. control).

recorded at zero hour, and were considered as 100% of activity. Two hours after tilianin administration (Fig. 10A and B), we observed a significant decrease in SBP and DBP compared to control ($p < 0.05$); this effect lasted for at least 6 h. On the other hand, HR was increased about 10% after 2 h post-administration, but 4 h later HR recovered to control values ($p < 0.05$, Fig. 10C).

4. Discussion

A. mexicana is a medicinal plant that is used in Mexican folk medicine for the treatment of hypertension and related diseases [10]. This species is commonly named "toronjil" and the aerial part is prepared by decoction and is orally taken to control the disease. To the best of our knowledge, this is the first study that describes the pharmacological basis of the uses of *A. mexicana* as antihypertensive agent, and also is the first time that isolation of compound 1 from this species is described. In this context, extracts obtained from maceration of plant material, with exception of hexanic extract (data not shown), induced a significant vasorelaxant action. DEAm produced its effect in a concentration-dependent and endothelium-independent manners; suggesting that the dichloromethanic extract induced its relaxant effect through mechanisms contained in vascular smooth

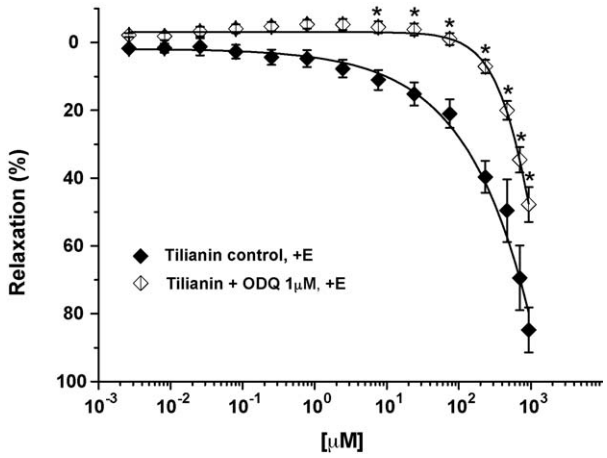

Fig. 6. Relaxant effect of tilianin on isolated rat aortic rings pre-contracted with NA (0.1 μM) in the presence of ODQ (1 μM), in presence of endothelium. Results are expressed as the mean ± S.E.M. of six experiments ($^{*}p < 0.05$ vs. control).

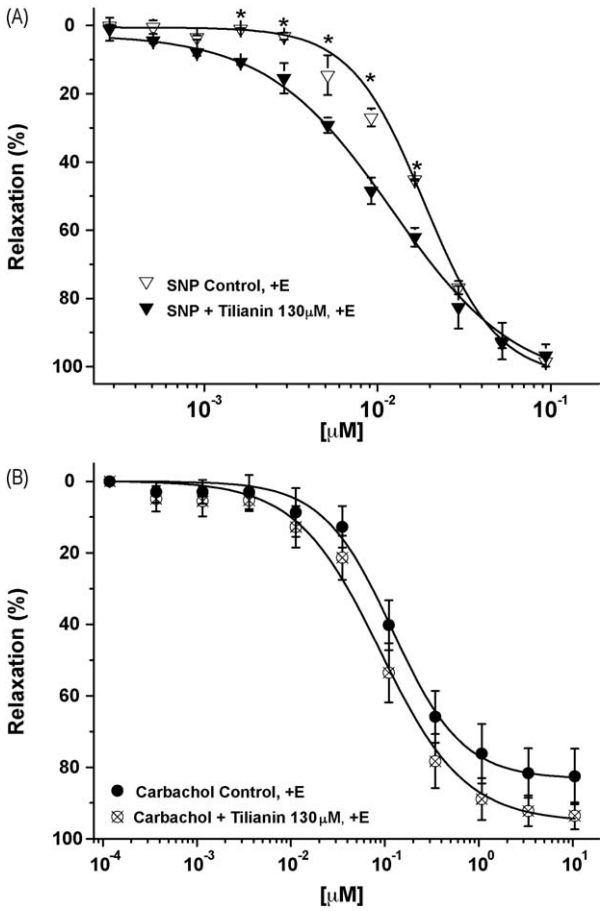

Fig. 7. (A) Relaxant effect of SNP on isolated rat aortic rings pre-contracted with NA (0.1 μM) in presence of tilianin (130 μM). B) Relaxant effect of carbachol on isolated rat aortic rings pre-contracted with NA (0.1 μM) in presence of tilianin (130 μM). Each point represents the mean ± S.E.M. of six experiments ($^{*}p < 0.05$ vs. control).

muscle cells, such as the interference in a common pathway which several receptor agonists stimulate, i.e., as the augment of free cytosolic Ca^{2+} levels [15,16]; or by the blockade of the intracellular Ca^{2+} release from sarcoplasmic reticulum Ca^{2+} channels activated by IP_3 [17,18]; or by inhibition/activation of enzymes that catalyze the synthesis of second messengers into the muscular cells [19]. Further experiments are in progress in order to determine its mechanism of action and to isolate the active principles.

On the other hand, MEAm also induced vasodilatation mainly in a concentration- and endothelium-dependent manners; this effect could be related to endothelium-derived factors such as COX-produced prostanoids, endothelium dependent hyperpolarization factor (EDHF) or nitric oxide synthase [20]. As described above, tilianin was obtained by spontaneous precipitation from MEAm and it was the major component of the extract. Functional pharmacological evaluation of tilianin showed that this flavonoid caused both concentration- and endothelium-dependent relaxation of rat thoracic aortic rings, pre-contracted with NA and the effect was less potent than carbachol (positive control). This effect suggested that COX and/or NOS pathways were involved in the response [20]. However, since vasorelaxation was blocked by L-NAME and ODQ, while indomethacin and atropine did not inhibit the effect, endothelium-derived NO seems to be involved in tilianin action [21], and allowed us to discard the possible role of COX or a direct action on cholinergic muscarinic receptor in the endothelium-dependent relaxation [22]. Relaxation due to SNP and carbachol was shifted to the left in the presence of tilianin, but

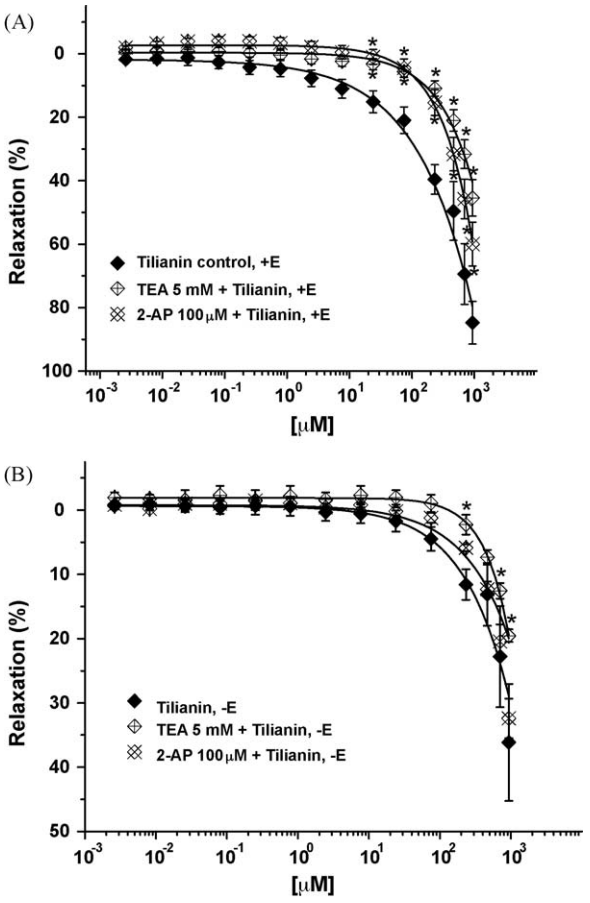

Fig. 8. Relaxant effect of tilianin on rat aorta rings with (A) and without (B) endothelium pre-contracted with NA (0.1 μM) in the presence of TEA (5 mM) and 2-AP (0.1 μM). Results are expressed as the mean ± S.E.M. of six experiments ($^{*}p < 0.05$ vs. control).

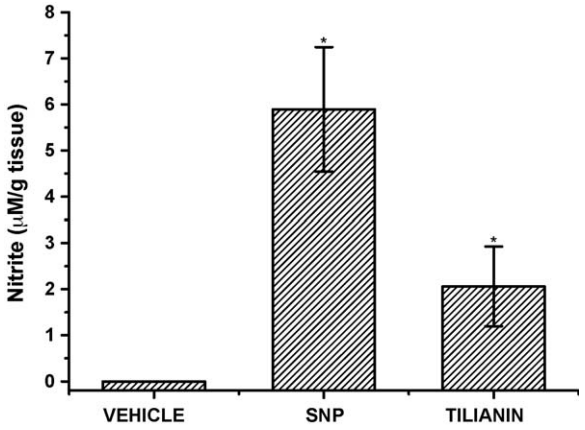

Fig. 9. Effect of tilianin (10 mM), SNP (1 mM) and DMSO (100%) on NO production in rat aortic segments. Each point represents the mean ± S.E.M. of three experiments ($^{*}p < 0.05$ vs. control).

only in the presence of SNP was significant, indicating a synergic effect on the vasorelaxant activity. Finally, tilianin caused a marked depression of the E_{max} in the contraction of aortic rings (+E) stimulated by NA and 5-HT, suggesting that receptor blockade is not the mode of action of this compound; but offering strong evidence that tilianin stimulated the eNOS and/or NO release [23]. In this context, we decided to determine NO production stimulating tissue with tilianin and SNP. Tilianin induced a significant production of nitrites, supporting the idea that tilianin relaxant effect is mainly mediated by NO/cGMP pathway. NO release from endothelium could be related with several mechanisms of regulation in eNOS activity such as caveolin-mediated, AMPK-activated, Ca^{2+}-CaM complex-activated, and/or receptors-mediated (estrogenic, insulinic or muscarinic) activation [12]. Last results are in according with previous reports were described that several flavonoids have vasorelaxant activity through NO release, e.g. naringenin [24], flavone [24], quercetin [24], galangin [6] and epigallocatechin gallate [7], although, there is not exists any report to describe how is produced.

However, the observation that the vasorelaxant effect of tilianin was still observed in aortic endothelium-denuded rings or in those treated with NOS inhibitor, suggests that compound 1 has a direct effect on vascular smooth muscle cells. The opening of K^+ channels or Ca^{2+} channel blockade in the vascular smooth muscle cells provide important mechanisms to dilate arteries [11,25]. The present results also suggest that K^+ channels opening are involved in the mechanism of tilianin's action. As we can see, relaxant effect was modified in the presence of unspecific K^+ channel blocker TEA more than in the presence of voltage-dependent K^+ channel blocker 2-AP in intact- and denuded-endothelium, which indicates that the effect could be unspecific for different potassium channel types. Thus, these results indicate that the vasorelaxant effect of tilianin is partially mediated by K^+ channels opening in vascular smooth muscle cells. Furthermore, $CaCl_2$ contraction curve was not modified by tilianin, which suggested that the relaxant effect of compound 1 on endothelium-denuded aortic preparations is only related with K^+ channels opening. In a previous investigation, Calderone et al., showed that acacetin, the aglycone of tilianin, induced a full vasorelaxing effect, but they did not describe its mode of action [26].

On the other hand, the present study shows that intragastric administration of tilianin in conscious SHR rats evoked a significant effect (1 h after administration) reducing systolic, diastolic and mean blood pressures ($p < 0.05$). Sustained antihypertensive effect induced by tilianin could be related with its possible long half-time life in plasma. So, recent study about the metabolism and pharmacokinetics of flavonoids concluded that flavonoid glucosides are better absorbed than the aglycone [27]. Moreover, latest report [28] indicates that flavonoids begin with the metabolic conjugation of a glucuronide moiety in intestinal cells. The flavonoid is then bound to albumin and transported to the liver. The liver can extend the conjugation of the flavonoid by adding a sulfate group, a methyl group, or both. The addition of these groups increases the circulatory elimination time and probably also decreases toxicity. These last reports is in according with our results, that showed a sustained antihypertensive effect of compound 1 on SHR, based on that tilianin has a glucoside substitution on 7 position (that could be better absorbed than other flavonoids), and also, posses a methyl group in OH-4′ position. As you can see, tilianin cannot be modified in both positions because it itself has those modifications. However, there does not exist any reports about tilianin's pharmacokinetic and neither any report about its induction on the production of NO in *in vivo* studies. So, we planned, as our next investigation, to make the pharmacokinetic studies of tilianin in rats and also to determine the production of NO as nitrites/nitrates formation on plasma. It is important to mention that this study, along with *in vitro* results, permitted us to draw conclusions regarding the site of action of tilianin, via oral administration.

With all of these data, we consider that the vasodilator effect showed by MEAm is related to the presence of tilianin, and experiments will be designed in order to provide new data to clarify the precise mechanism by which tilianin produce its vasorelaxant

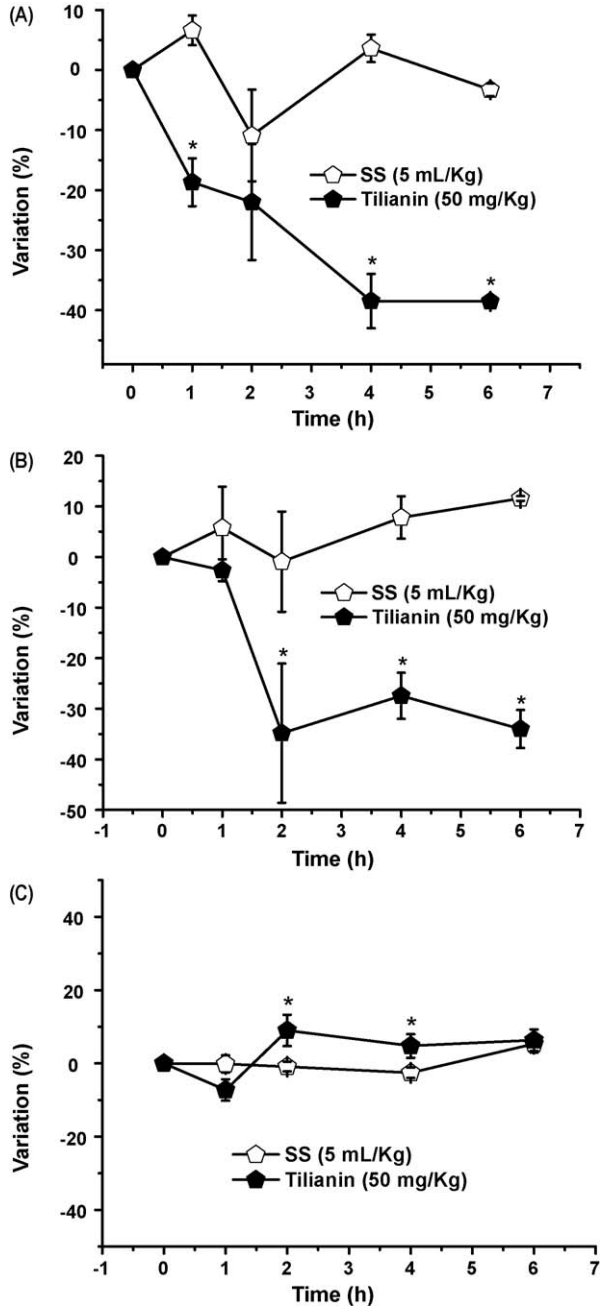

Fig. 10. Maximal decrease in (A) systolic blood pressure, (B) diastolic blood pressure, and (C) heart rate (%) elicited by oral administration of 50 mg/kg of tilianin in conscious rat. Each point represents the mean ± S.E.M. of six experiments ($^{*}p < 0.05$ vs. control).

effect. These experiments will determine the concentration of cGMP, NOS activity and NOS expression (PCR, Western Blot and/or microarrays) in the aorta to demonstrate the direct or indirect activation of eNOS in the endothelium-dependent vasorelaxation; and also, to evaluate tilianin's relaxant effect in the presence of selective potassium channel blockers.

In conclusion, tilianin induced relaxation in rat aortic rings through an endothelium-dependent pathway involving a NO release, and consequently an enhancement of cGMP concentration, and also through an endothelium-independent pathway by opening K^+ channels. These results confirm that vasorelaxation showed by MEAm is produced by the presence of tilianin and gives an additional support of the medicinal uses of *A. mexicana* for the treatment of hypertension. In addition, the results show that oral administration of tilianin in conscious SHR rats provoked an antihypertensive activity and a brief augment of heart rate in a time-dependent manner.

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References

[1] Sharina IG, Jelen F, Bogatenkova EP, Thomas A, Martin E, Murad F. Alpha1 sGC splice forms as potential regulators of human sGC activity. J Biol Chem 2008;283:15104–13.
[2] Corbett SA, Hukkanen M, Batten J, McCarthy ID, Polak JM, Hughes SP. Nitric oxide in fracture repair. Differential localisation, expression and activity of nitric oxide synthases. J Bone Joint Surg Br 1999;81:531–7.
[3] Zoccali C. The endothelium as a target in renal diseases. J Nephrol 2007;20: S39–44.
[4] Aguirre-Crespo F, Vergara-Galicia J, Villalobos-Molina R, López-Guerrero J, Navarrete-Vázquez G, Estrada-Soto S. Ursolic acid mediates the vasorelaxant activity of *Lepechinia caulescens* via NO release in isolated rat thoracic aorta. Life Sci 2006;79:1062–8.
[5] Rodriguez-Rodriguez R, Herrera MD, Perona JS, Ruiz-Gutiérrez V. Potential vasorelaxant effects of oleanolic acid and erythrodiol, two triterpenoids container in 'orujo' olive oil, on rat aorta. Br J Nutr 2004;92:635–42.
[6] Morello S, Vellecco V, Alfieri A, Mascolo N, Cicala C. Vasorelaxant effect of the flavonoid galangin on isolated rat thoracic aorta. Life Sci 2006;78:825–30.
[7] Kim J, Formoso G, Li Y, Potenza MA, Marasciulo FL, Montagnani M, et al. Epigallocatechin gallate, a green tea polyphenol, mediates NO-dependent vasodilation using signaling pathways in vascular endothelium requiring reactive oxygen species and fyn. J Biol Chem 2007;282:13736–45.
[8] Gurib-Fakim A. Medicinal plants: traditions of yesterday and drugs of tomorrow. Mol Aspects Med 2006;27:1–93.
[9] Aguilar A, Camacho JR, Chino S, Jacquez P, Lopez ME. Herbario medicinal del instituto mexicano del seguro social. Mexico: Instituto Mexicano del Seguro Social; 1994. pp. 98–99.
[10] Monroy-Ortiz C, Castillo-España E. Plantas medicinales utilizadas en el estado de morelos, 2nd ed., Cuernavaca, Morelos, Mexico: Universidad Autónoma del Estado de Morelos; 2007. pp. 154–156.
[11] Vergara-Galicia J, Ortiz-Andrade R, Castillo-España P, Ibarra-Barajas M, Gallardo-Ortiz I, Villalobos-Molina R, et al. Antihypertensive and vasorelaxant activities of *Laelia autumnalis* are through calcium channel blockade and enhanced cGMP concentration. Vascul Pharmacol 2008;49:26–31.
[12] Papapetropoulos A, Andreopoulos S, Go CY, Hoque A, Fuchs LC, Catravas D. Regulation of the nitric oxide synthase-nitric oxide/cGMP pathway in rat mesenteric endothelial cells. J Appl Physiol 2001;91:2553–60.
[13] Nam KH, Choi JH, Seo YJ, Lee YM, Won YS, Lee MR, et al. Inhibitory effects of tilianin on the expression of inducible nitric oxide synthase in low density lipoprotein receptor deficiency mice. Exp Mol Med 2006;38:445–52.
[14] Itokawa H, Suto K, Takeya K. Structures of isoagastachoside and agastachin, new glicosylflavones isolated from *Agastache rugosa*. Chem Pharm Bull 1981; 29:1777–9.
[15] Huang Y, Ho IH. Separate activation of intracellular Ca^{2+} release, voltage-dependent and receptor-operated Ca^{2+} channels in the rat aorta. Chin J Physiol 1996;39:1–8.
[16] Zhang CY, Tan BKH. Vasorelaxation of rat thoracic aorta caused by 14-deoxyandrographolide. Clin Exp Pharmacol Physiol 1998;25:424–9.
[17] Zhu XM, Fang LH, Li YJ, Du GH. Endothelium-dependent and -independent relaxation induced by pinocembrin in rat aortic rings. Vascul Pharmacol 2007;46:160–5.
[18] Maciel SS, Dias KLG, Medeiros IA. Calcium mobilization as the endothelium-independent mechanism of action involved in the vasorelaxant response induced by the aqueous fraction of the ethanol extract of *Albizia inopinata* G.P. Lewis (AFL) in the rat aorta. Phytomedicine 2004; 11:130–4.
[19] Rho HE, Perkins JW, Lorenz RR, Warner OD, Jones AK. Differential effects of soluble and particulate guanylyl cyclase on Ca^{2+} sensitivity in airway smooth muscle. J Appl Physiol 2002;92:257–63.
[20] Vanhoutte PM. Endothelial adrenoceptors. J Cardiovasc Pharmacol 2001;38: 796–808.
[21] Moncada S, Higgs A, Furchgott R. XIV International union of pharmacology nomenclature in nitric oxide research. Pharmacol Rev 1997;49:137–42.
[22] Schlossmann J, Hofmann F. cGMP-dependent protein kinases in drug discovery. Drug Discov Today 2005;10:627–34.
[23] Aktan F. iNOS-mediated nitric oxide production and its regulation. Life Sci 2001;75:639–53.
[24] Ajay M, Gilani AH, Mustafa RM. Effect of flavonoids in vascular smooth muscle of the isolated rat thoracic aorta. Life Sci 2003;74:603–12.

[25] Nelson MT, Quayle JM. Physiological roles and properties of potassium channels in arterial smooth muscle. Am J Physiol 1995;268:C799–822.
[26] Calderone V, Chericoni S, Martinelli C, Testai L, Nardi A, Morelli I, et al. Vasorelaxing effects of flavonoids: investigation on the possible involvement of potassium channels. Naunyn-Schmiedeberg's Arch Pharmacol 2004;370: 290–8.
[27] Middleton E, Kandaswami C, Theoharides T. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and Cancer. Pharmacol Rev 2000;52:673–751.
[28] Nijveldt RJ, Van Nood E, Van Hoorn DEC, Boelens PG, Van Norren K, Van Leeuwen PAM. Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 2001;74:418–25.