

Regular Article

Acute and Chronic Antihypertensive Effect of Fractions, Tiliroside and Scopoletin from *Malva parviflora*

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Received May 11, 2018; accepted October 5, 2018

Hypertension is a disease of high prevalence and morbidity where vascular inflammation and associated oxidative stress (endothelial dysfunction) is the underlying cause of this pathology. We are reporting the antihypertensive activity of extracts and fractions of *Malva parviflora* in mice with chronic and acute hypertension. Also, the treatments of this plant were able to counteract the kidney inflammation and associated oxidative stress. The chronic hypertension model consisted of administration of angiotensin II (AGII) during 12 weeks, causing a sustained increase in systolic (SBP) or diastolic (DBP) pressure, with values of pharmacological constants of: $ED_{50} = 0.038$ mg/kg y $E_{max} = 135$ mmHg for SBP and $ED_{50} = 0.046$ mg/kg y $E_{max} = 98$ mmHg for DBP. The chronic hypertension caused the inflammation and lipid peroxidation in kidneys, measured by of tissue level of cytokines such as interleukin-1 β (IL-1 β), IL-6, Tumor Necrosis Factor- α (TNF- α), IL-10 and malondialdehyde, and treatments for *M. parviflora* were able to modulate these parameters. The chemical fractionation allowed to identify three compounds: oleanolic acid, tiliroside and scopoletin, which were tested in a model of acute hypertension. The pharmacodynamic parameters for SBP were $ED_{50} = 0.01$ and 0.12 mg/kg while $E_{max} = 33.22$ and 37.74 mmHg for scopoletin and tiliroside, respectively; whereas that for DBP data were $ED_{50} = 0.01$ and 0.02 mg/kg; with an $E_{max} = 7.00$ and 6.24 mmHg, in the same order. *M. parviflora*, is able to counteract the effect of chronic and acute administration of AGII, on hypertension, but also the inflammatory and oxidative damage in the kidney. The oleanolic acid, scopoletin and tiliroside are the compounds responsible for such activities.

Key words *Malva parviflora*; antihypertensive; angiotensin II; coumarin; flavonoid

INTRODUCTION

Systemic arterial hypertension (SAH) is a serious global public health problem¹⁾; in Mexico a third of the population suffers from this disease.²⁾ SAH is one of the main risk factors for the development of other diseases with high mortality rates, such as renal failure, cardiac and cerebral infarction.³⁾ As a consequence of hypertension, several damage components are present in the patients, such as the endothelial dysfunction (ED) that underlying to brain and heart strokes.⁴⁾ ED is a chronic degenerative disease, characterized by the loss of the vasorelaxant capacity of the arterial tree, due to the decrease in the availability of nitric oxide (NO), because of the increment of oxidative stress,⁵⁾ and the proinflammatory state,⁶⁾ derived from the overstimulation of the renin angiotensin aldosterone system that is tightly related to SAH.⁷⁾

Malva parviflora (Malvaceae) is an original plant from Europe, which grows from 1000 to 3900 m.a.s.l. This plant has been used in European and African traditional medicine to treat bruises, sores and swelling.^{8,9)} In Mexico this plant is distributed all over the country and the ethnobotanical uses are diverse, it is used for treating wounds, bruises, abscesses, fever, headaches, cold sores, infections and for renal problems.¹⁰⁾ This plant has not been widely studied, some pharma-

cological effects are attributed to it such as, anti-inflammatory activity on cyclooxygenase-1 and -2 (COX-1 and COX-2) and other studies demonstrated that the extracts inhibited ear oedema induced with croton oil,⁸⁾ as well as, inhibiting the vascular permeability induced with acetic acid, also presenting in a dose dependent form a strong scavenging activity against free radical 2,2-diphenyl-1-picrylhydrazil (DPPH).⁹⁾ Methanol extract of *M. parviflora* also reduces mucosal damage in an experimental colitis assay.¹¹⁾ Another study evaluated the diuretic activity of this plant.¹²⁾ Even though *M. parviflora* is widely used in traditional medicine and pharmacological studies have been made, the chemical composition has not been completely identified; only some fatty acids: malvalic, sterculic and vernolic acids, as well as, some anthocyanins and phenolic compounds have been reported.^{10,13)}

In this work were evaluated the anti-hypertensive activity of extracts and fractions from *M. parviflora* by using a chronic model of hypertension induced by angiotensin II (AGII), and in this same assay was measured the capacity of the plant for to modified the concentration of cytokines in kidneys, as well as the lipid peroxidation. And the other hand, was made a chemical separation of the most active fraction and two compounds were isolated by chromatographic column, these substances were tested in an acute hypertension-induced with

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AGII in mice.

MATERIALS AND METHODS

Plant Material *Malva parviflora* was acquired by purchase in the municipal market of Cuernavaca, Morelos Mexico. The plant was dried in darkness and at room temperature. One specimen was deposited in the herbarium of Ethnobotanical Garden of Museum of Traditional Medicine and Herbal medicine of INAH Morelos. The species was identified by Margarita Aviles and Macrina Fuentes with the registration number 2048.

Extract Preparation The plant material was pulverized in an electric mill (Pulvex), obtaining particles of <4mm. The extraction was performed thorough the exhaustive maceration in 60% ethanol, conducting three successive extractions of 24h each. The ethanol extract (MpEtOH) was concentrated by evaporation at reduced pressure and controlled temperature and then lyophilized. Fifty grams of the MpEtOH was separated and subjected to a bipartition where it was suspended in a mixture of ethyl acetate/water for three times, thereby, obtaining the organic fraction (MpAcOEt) and the aqueous fraction (MpAq) which were concentrated by evaporation at reduced pressure and controlled temperature following freeze-drying.

Chemical Separation of the Most Active Extract The fraction with the best antihypertensive and antiinflammatory activity (MpAcOEt, 7g) was fractionated in a chromatographic column (4×50cm) previously packed with 100g of silica gel (60–230 mesh, from Merck, Whitehouse Station, NJ, U.S.A.). To effect the separation, a mobile phase was used which consisted of a gradient of acetone-methanol. At the beginning the column was eluted with 100% acetone and gradually the system was changed to finish with 100% methanol. The entire chromatographic separation procedure was monitored by TLC. The fractions in which the major compounds were identified were separated for testing in the antihypertensive activity assay. The major compounds obtained from the chromatographic process were identified by HPLC, comparing the retention time and the spectrum of UV absorption against pure compounds.

Animals All procedures were conducted in accordance with Official Mexican Norm (NOM-062-ZOO-1999) regarding technical specifications for the production, care, and use of laboratory animals, and the *Guide for the Care and Use of Laboratory Animals* and the international ethical guidelines for the care and use of laboratory animals. ICR male albino mice weighing between 25–30g, (Harlan, México City, México) were used for this assay. All animals were housed eight per cage and maintained under laboratory conditions at 25°C, with a normal 12h:12h light/dark schedule (lights on at 7:00a.m.) and free access to water and food (pellets, Envigo rodent lab diet). The mice were allowed 3 weeks to adapt to the laboratory environment prior to the trial period. Experiments were carried out between 8:00a.m. and 12:00p.m. This research study was approved by the ethical committee of the Mexican Social Security Institute (R-2010-1701-63). Minimum number of animals and minimum duration of observation required to obtain consistent data were employed.

Drugs and Treatments AGII, Telmisartan (Sigma-Aldrich Corporate, St. Louis, MO, U.S.A.). Pentobarbital (PISA Agropecuaria, México City, México).

Chronic Hypertension-Induced by AGII A group of 40 mice was randomly assigned to five groups. Each of these groups received a dose of AGII (0.0064, 0.016, 0.04, 0.10 and 0.25 µg/kg) intraperitoneally (i.p.) daily for six weeks. A sixth group called baseline received i.p. a daily administration of a 100 µL of isotonic saline (IS) for the same period. Determinations of systolic and diastolic blood pressure (SBP and DBP) were performed at the beginning of the sixth week trial and then every two weeks following. Blood pressure (BP) measurements were performed under the following procedure: under surgical anaesthesia (pentobarbital 60 µg/kg, i.p.) 8 BP lectures were taken, the BP was monitored by a non-invasive BP detector (LE 5002 Storage Pressure Meter, Biopac Systems mp 150°C).

Antihypertensive Activity of Extracts and Fractions of *Malva parviflora* Once it was established that after the fourth week of daily i.p. administration of AGII at a dose of 0.1 µg/kg, caused hypertension in mice, the following assay was carried out: a lot of 56 mice were randomly assigned to seven groups, each group received one of the following treatments: Group 1 (baseline) received daily 100 µL of isotonic saline solution (ISS) orally and 100 µL of ISS i.p., Group 2 (negative control) received a daily dose of AGII 0.1 µg/kg i.p. for 10 weeks, after the fourth week of the administration of the AGII treatment the animals were administered a 100 µL of ISS orally. Group 3 (positive control) received the same schedule of administration than the previous group, except that instead of oral ISS, it received a daily oral dose of Telmisartan 40 mg/kg. Groups 4 to 6 (experimental groups) received the same dose of AGII for ten weeks and likewise, from the fourth week each group was administered the following oral treatments (20 mg/kg): MpEtOH, MpAcOEt, and MpAq, respectively. BP measurements were taken at the end of the treatments using the already described method.

Blood Samples and Kidneys Dissection After 10 weeks of treatment and BP acquisition, mice were deeply anaesthetized with sodium pentobarbital at a dose of 80 mg/kg i.p., blood samples were quickly obtained throughout the infraorbital sinus of each mouse. After this procedure, each mouse was perfused throughout intra-cardiac pathway with phosphate buffered saline (PBS) and the femoral vein was sectioned for the elimination of the washing solution; perfusion was carried out until the remaining blood was completely clear. The plasma was obtained by centrifugation (2500rpm for 15min) at room temperature, and then the samples were stored individually at –20°C until use. On the other hand, animal organs were obtained (both kidneys), these were identified and transferred in to 15mL tubes placed on dry ice and containing 0.1mM of phenyl methyl sulfonyl fluoride (PMSF; Sigma-Aldrich Mexico) and in PBS (1mL/200mg of tissue). The samples were homogenized (Potter-Elvehjem homogenizer) and then centrifuged for 10min at 14000rpm at 4°C, the supernatants were transferred in to 1.5mL Eppendorf tubes and were ready for the cytokine and malondialdehyde (MDA) determination. Cytokine determination was carried out following the manufacturer instructions (BD OptEIA™ ELISA kit, U.S.A.) by enzyme-linked immunosorbent assay (ELISA) method. For tissue MDA quantification, 50 µL samples of tissue homogenates supernatants were added in equal volume of trichloroacetic acid (TCA) and Tris–HCl buffer (50mM, pH 7.4), keep at room temperature for 10min. Then

50 μL of 0.75% thiobarbituric acid (TBA) in a 2M sodium sulphate solution were added. The mixture was heated to boiling point for 45 min, then, they were left to cool down and centrifuged at 15500 rpm for 7 min. The supernatant was read spectrophotometrically at 532 nm and the concentration of MDA was determined using the molar absorptivity coefficient ($\epsilon = 1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) (Stat Fax[®] 2100).

Acute Hypertension-Induced by AGII ICR mice of 25 g of weight were randomly divided in to 5 groups ($n=6$) for the scopoletin trial and many others for the trial of tiliroside. In addition, three additional control groups were used. Each group received, orally, the corresponding treatment since 24, 18 and 1 h before the hypertensive challenge.

After the oral administration of the corresponding treatments, all animals received sodium pentobarbital (60 mg/kg) and once anesthetized were applied for the challenge, 100 μL of ISS intravenously (i.v.) for the group 1; and 100 μL of AGII (dose 2.0 $\mu\text{g/kg}$ i.v.) for all other groups:

Group 1 received 250 μL of ISS

Group 2 (negative control) received 250 μL of ISS

Group 3 (positive control) Telmisartan (dose 10 mg/kg)

Groups 4 to 8 (experimental groups) were administered orally with scopoletin isolated from *M. parviflora* (to different dose 0.01, 0.1, 1, 2 and 5 mg/kg).

Groups 9 to 13 (experimental groups) were administered with tiliroside isolated from *M. parviflora* (to different dose 0.01, 0.1, 1, 2 and 5 mg/kg).

The blood pressure was measured as indicated before. The results are presented as the difference of BP (for systolic ΔSBP and for diastolic ΔDBP) in mmHg.

Statistical Analysis Data were analyzed with ANOVA of one way, and the Dunnett (post-test) with a significance level of $*p < 0.05$ when it was compared against the group of damage, to which only ISS (VEH) was administered as treatment. For this analysis was used an SPSS statistical software program (ver. 11.0).

RESULTS

Composition of Extract of *M. parviflora* Figure 1 shows the chromatographic profile of the compounds identified in the MpEtOH extract and the active ethyl acetate fraction of *Malva parviflora*. **1** Scopoletin, **2** tiliroside and **3** oleanolic acid were used as standards, with retention times of 9.44 min, 14.21 min and 25.86 min, respectively. In this chromatographic profile the standard compounds were identified in both the MpEtOH extract and the MpAcOEt fraction. This last fraction presents a more complex mixture of compounds than those present in the MpEtOH extract and the MpAq fraction. In the active extract MpAcOEt, the following compounds were quantified: scopoletin 23 μg , tiliroside 212 μg , and oleanolic acid 55 μg , all of them per gram of extract.

Hypertension-Induced by Chronic Administration of AGII Figure 2 shows the results of the dose response curves of chronic intraperitoneal administration of AGII. To establish the conditions that cause hypertension, increasing dosages of AGII were administered daily to different groups of mice for ten weeks. After this period, it was observed that the application of increasing doses of AGII, increased blood pressure in a dose-dependent relation, for both values

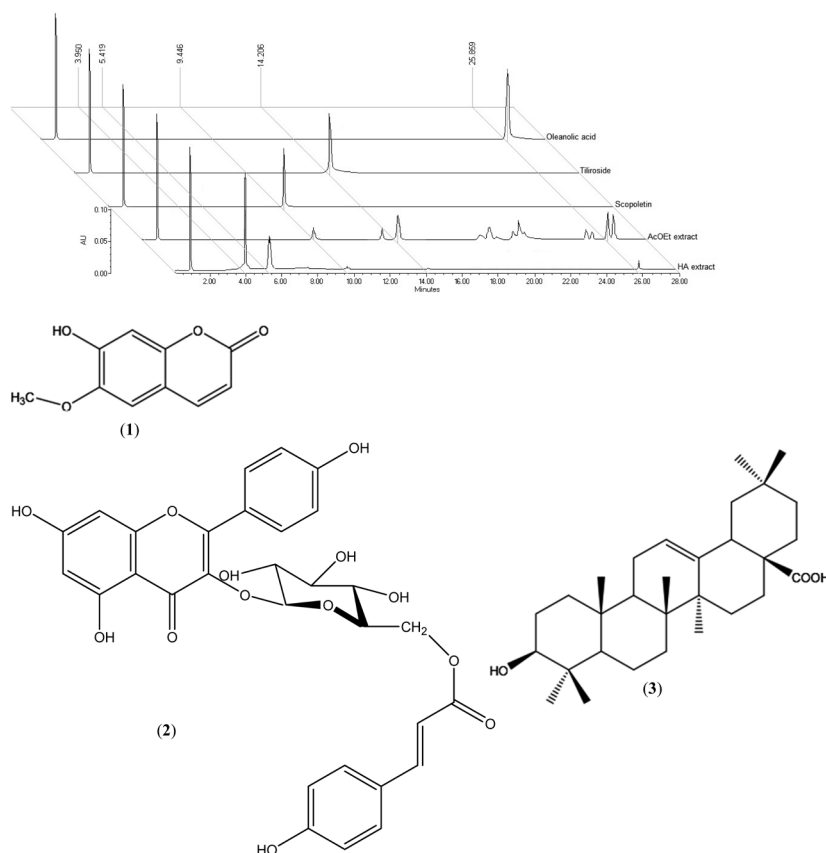


Fig. 1. Chromatographic Profile of the Composition of the Hydroalcoholic Extract and the Ethyl Acetate Fraction of *Malva parviflora*

1 Scopoletin; **2** tiliroside and **3** oleanolic acid.

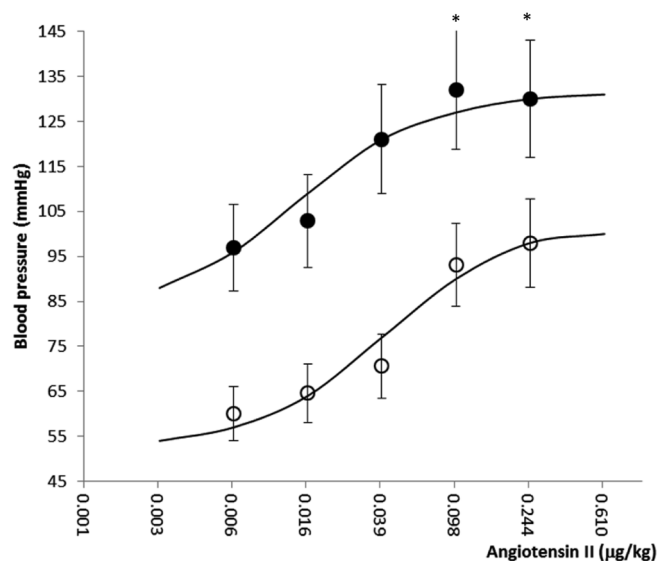


Fig. 2. Dose-Response Curve of the Chronic i.p. Administration of AGII (0.006, 0.016, 0.039, 0.098 and 0.244 $\mu\text{g/kg}$), through BP Measurements after Six Weeks of Treatment

Baseline values of SBP (●) $85 \pm 7.7 \text{ mmHg}$ and DBP (○) $53 \pm 5.6 \text{ mmHg}$. ANOVA, *post hoc* Tukey, * $p < 0.05$ compared with the basal group $n = 6$.

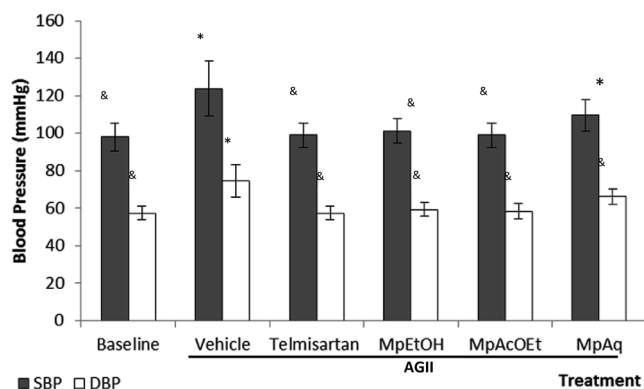


Fig. 3. Effect of the Extract and Fractions of *Malva parviflora* on SBP and DBP of Hypertensive Mice

ANOVA, *post hoc* Tukey (average \pm S.D., $n = 12$); * $p < 0.05$ (in comparison with the baseline group), & $p < 0.05$ (in comparison with the vehicle group).

of systolic blood pressure (SBP) and diastolic blood pressure (DBP). In both curves, it was possible to determine the pharmacological constant resulting from this behavior, where SBP: $\text{ED}_{50} = 0.038 \mu\text{g/kg}$ and $E_{\text{max}} = 135 \text{ mmHg}$, DBP: $\text{ED}_{50} = 0.046 \mu\text{g/kg}$ and $E_{\text{max}} = 98 \text{ mmHg}$. Despite the steady increase in the values of both pressures that were observed, only statistically significant differences were established from the baseline values from doses greater than $0.05 \mu\text{g/kg}$ for SBP and for DBP a higher dose was needed, having to use $0.1 \mu\text{g/kg}$ of AGII.

Effect of *M. parviflora* on Hypertension-Induced by Chronic Administration of AGII Figure 3 showed that chronic administration of AGII caused an increase of SBP of 20% from baseline, likewise for DBP that had a relative increase of 23%. In both cases the increment in blood pressure was statistically significant (* $p < 0.05$, Fig. 3). It is worth mentioning that prior to the administration of the treatments the animals were exposed to AGII for 28 d. Therefore, the administration of the different treatments, positive control

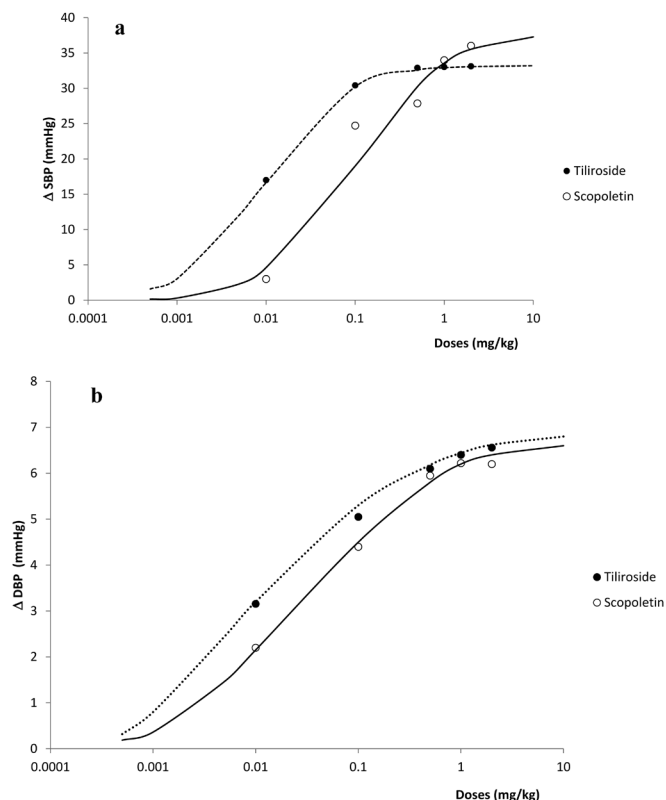


Fig. 4. Effect of Tiliroside (●) and Scopoletine (○) on the Transient Systolic Blood Pressure Differential (ΔSBP ; Panel a) and the Transient Systolic Blood Pressure Differential (ΔDBP ; Panel b)

Each point is the average of the difference in blood pressure reading with and without treatment. The dose-dependent behavior of tiliroside and scopoletin compounds was characterized by the following parameters: ΔSBP $\text{ED}_{50} = 0.01$ y 0.12 mg/kg ; $E_{\text{max}} = 33.22$ y 37.74 mmHg , respectively. For ΔDBP $\text{ED}_{50} = 0.01$ y 0.02 mg/kg ; $E_{\text{max}} = 7.00$ y 6.24 mmHg in the same order.

Telmisartan, MpEtOH extract and MpAcOEt fraction decreased SBP and DBP levels in comparison to the damage group (Vehicle) (& $p < 0.05$, Fig. 3). While, the MpAq fraction decreased both the SBP and DBP, compared to the damage group; this reduction was statistically significant only with respect to the DBP. Figure 4 shows the antagonism of scopoletin and tiliroside on transient hypertension caused by the administration of AGII $2 \mu\text{g/kg}$ i.v. In both cases, a dose-dependent effect of the oral administration of scopoletin and tiliroside was observed isolated from the MpAcOEt extract. The following pharmacological constants were calculated for tiliroside and scopoletin SBP $\text{ED}_{50} = 0.01$ and 0.12 mg/kg ; $E_{\text{max}} = 33.22$ and 37.74 mmHg , respectively. In DBP $\text{ED}_{50} = 0.01$ and 0.02 mg/kg ; $E_{\text{max}} = 7.00$ and 6.24 mmHg in the same order.

Effect of *M. parviflora* on Level of Cytokines in Kidney of Mice with Chronic Hypertension The chronic administration of AGII caused increased levels of pro-inflammatory cytokines in the kidneys of the negative control group (vehicle) compared with the kidneys of the baseline group. The increase in tissue levels of cytokines (Figs. 6, 7) were statistically significant (* $p < 0.05$) only for interleukin-6 (IL-6) and Tumor Necrosis Factor- α (TNF- α), meanwhile interleukin-1 β (IL-1 β) presented an increase in the tissue levels, however they were not statistically significant. The positive control (Telmisartan) group decreased IL-1 β levels below those of the positive control and the negative control although these decreases were not statistically significant (Fig. 5). Also, it

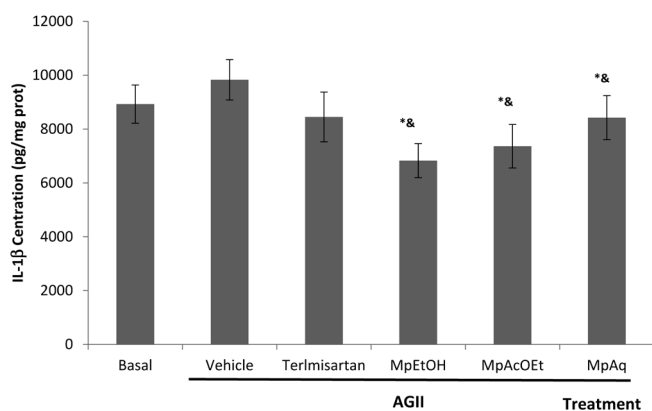


Fig. 5. Effect of the Extract and Fractions of *Malva parviflora* on the Inflammatory Response through the Quantification of Interleukin-1 β

ANOVA, *post hoc* Tukey (average \pm S.D., $n = 12$); * $p < 0.05$ (in comparison with the baseline group), & $p < 0.05$ (in comparison with the vehicle group).

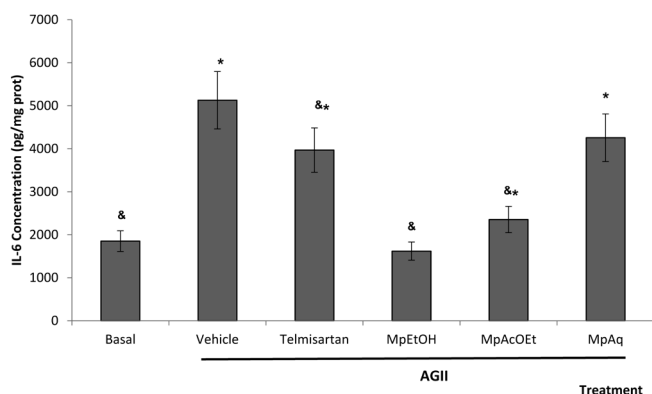


Fig. 6. Effect of the Extract and Fractions of *Malva parviflora* on the Inflammatory Response through the Quantification of Interleukin-6

ANOVA, *post hoc* Tukey (average \pm S.D., $n = 12$); * $p < 0.05$ (in comparison with the baseline group), & $p < 0.05$ (in comparison with the vehicle group).

decreased IL-6 in comparison to the negative control with a statistical significance of $p < 0.05$, even though IL-6 was decreased Telmisartan was not able to lower the levels to those of the baseline control group (Fig. 6). These last results contrasted with those of TNF- α levels, as Telmisartan did not only have no effect on TNF- α , it potentiated its production above the concentration of the negative control group as well as for the baseline control group (&* $p < 0.05$, Fig. 7).

The administration of the experimental treatments consisting of the MpEtOH extract and the fractions from the immiscible interface bipartition of ethyl acetate (MpAcOEt) and water (MpAq) presented the following effects: first, the MpEtOH and MpAcOEt caused a significant decrease in the concentration of IL-1 β (&* $p < 0.05$, Fig. 5), compared with the baseline and negative control groups; even though the effect was not as important for the MpAq fraction, it presented a decreased tendency as well. In the IL-6 quantification it was observed that MpEtOH and MpAcOEt decreased IL-6 in respect to the negative control, with a statistical significance of & $p < 0.05$ (Fig. 6). The effect of MpEtOH was strong enough to reduce the concentration to the levels of the baseline control group, whereas the MpAq fraction presented an opposite effect, it did not lower the levels of IL-6, this can be explained due to the absence in this fraction of the active compounds like scopoletin, tiliroside and oleanolic acid and.

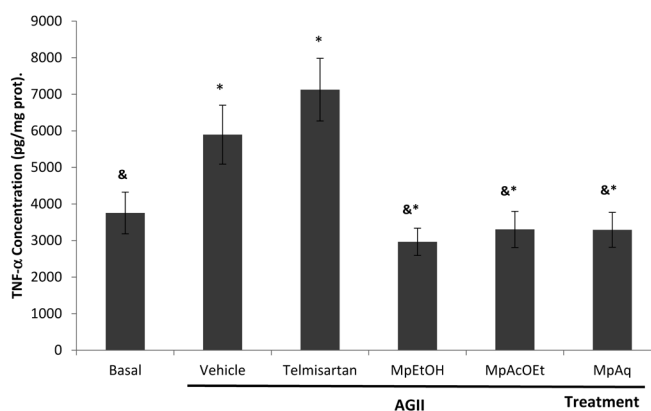


Fig. 7. Effect of the Extract and Fractions of *Malva parviflora* on the Inflammatory Response through the Quantification of Tumor Necrosis Factor- α

ANOVA, *post hoc* Tukey (average \pm S.D., $n = 12$); * $p < 0.05$ (in comparison with the baseline group), & $p < 0.05$ (in comparison with the vehicle group).

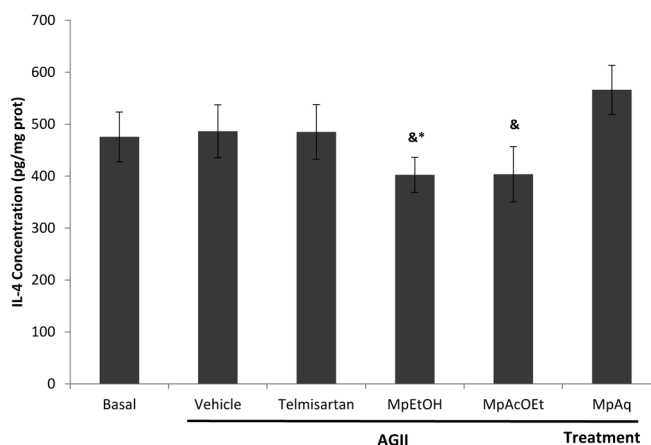


Fig. 8. Effect of the Extract and Fractions of *Malva parviflora* on the Inflammatory Response through the Quantification of Interleukin-4

ANOVA, *post hoc* Tukey (average \pm S.D., $n = 12$); * $p < 0.05$ (in comparison with the baseline group), & $p < 0.05$ (in comparison with the vehicle group).

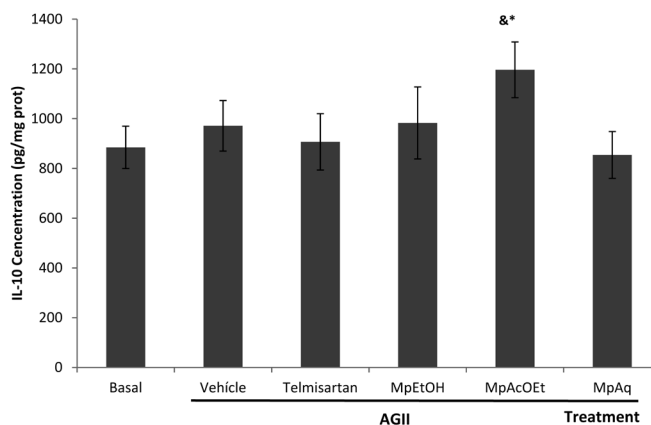


Fig. 9. Effect of the Extract and Fractions of *Malva parviflora* on the Inflammatory Response through the Quantification of Interleukin-10

ANOVA, *post hoc* Tukey (average \pm S.D., $n = 12$); * $p < 0.05$ (in comparison with the baseline group), & $p < 0.05$ (in comparison with the vehicle group).

Regarding TNF- α , the three experimental treatments could reduce the concentration of the pro-inflammatory cytokine with a statistical significance of &* $p < 0.05$ in comparison to

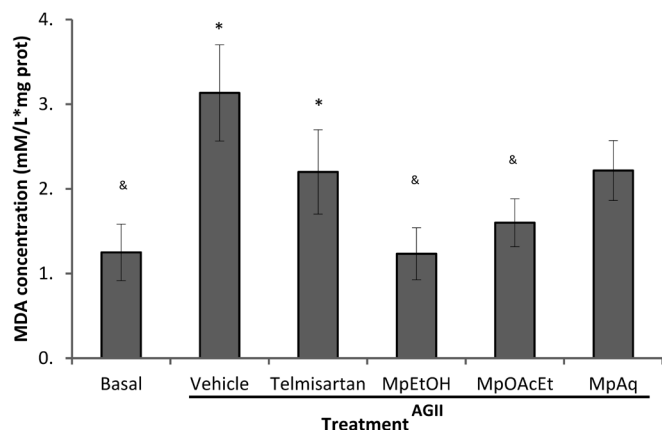


Fig. 10. Effect of the Extract and Fractions of *Malva parviflora* on the Concentration of MDA from the Homogenates of Right and Left Kidneys of Hypertensive Mice

ANOVA, *post hoc* Tukey (average \pm S.D., $n = 12$); * $p < 0.05$ (in comparison with the baseline group), & $p < 0.05$ (in comparison with the vehicle group).

the positive and negative controls. The MpEtOH was able to decrease the concentration of TNF- α more effectively (Fig. 7), when compared to the other two experimental treatments.

Also, interleukins 4 and 10 were determined and are shown in Figs. 8 and 9. These two cytokines are part of a self-regulation mechanism when pro-inflammatory cytokines like IL-1 β , IL-6 and TNF- α are produced and which participate by modulating the inflammatory system.

Figure 8 shows the quantification of IL-4, the chronic administration of the agonist AGII did not promote the production of this cytokine, since it does not present a statistical difference with the levels of the baseline control, as well as the result of the Telmisartan treatment. The MpEtOH and MpAcOEt treatments presented a decrease in the concentration of IL-4, with a statistical difference for MpEtOH of &* $p < 0.05$ in comparison to the baseline and AGII controls, meanwhile the MpAq fraction presents an increased tendency of the concentration of this interleukin in comparison to the baseline control without a statistical difference.

Meanwhile in response to the treatment with AGII, IL-10 quantification presents a slight increase in its concentration as a tendency, seen that this was not statistically significant (Fig. 9). Telmisartan treatment did not influence IL-10 concentration, as well as for the MpEtOH and MpAq treatments. On the other hand, MpAcOEt treatment promoted the production of this IL presenting a statistical difference of $p < 0.05$ in comparison with the baseline and vehicle controls.

Effect of *Malva parviflora* on Lipid Peroxidation of Mice with Chronic Hypertension In this study MDA was quantified in kidney homogenates (Fig. 10). The administration of Telmisartan decreased renal concentration of MDA in comparison to the negative control, although this decrease was not statistically significant. Interestingly the experimental treatments were able to decrease the concentration of MDA in respect to the negative control, especially the treatments corresponding to MpEtOH and MpOAcEt (&* $p < 0.05$, Fig. 10), MpAq treatment which also decreased MDA concentration, however this was not statistically significant.

DISCUSSION

Regarding the pharmacological model, there are reports in the literature using doses ten times higher of AGII to cause abnormalities in vascular tissue in less time (7d).¹⁴⁾ In our 10-week trial, using lower doses than what is reported caused vascular changes, which were evident by an increase in both SBP and DBP. This could be due to the administration of AGII at doses higher than 0.1 μ g/kg causing a hypertensive effect that is immediate and transitory which is secondary to the contraction of vascular smooth muscle cells (VSMC) that depend on the increase of the intracellular calcium concentration, which directly impacts on DBP and the increment of cardiac output that depends on the increase in sodium reabsorption,¹⁵⁾ simultaneously it increases the plasmatic volume, affecting the SBP, this increase in SBP is not immediate or permanent.¹⁶⁾ Therefore, the administration of lower dosages of AGII as the ones proposed in this work, do not have an immediate or evident hypertensive effect the activity of AGII is permanent. It depends on the vascular damage caused by the pro-inflammatory state and a pro-oxidant condition induced by AGII.¹⁷⁾

Telmisartan treatment had a very effective activity lowering SBP and DBP. The model proposed in this work allowed us to prove that angiotensin II type 1 receptor blockers (ARB) are effective to reduce the peripheral vascular resistance as well as the vascular damage and ED associated to the overstimulation of the renin angiotensin aldosterone system (RAAS) through the chronic i.p. administration of AGII.

With these results, it can be established that the sustained administration of AGII produces hypertension that is associated to endothelial dysfunction which has as its main component a vascular low intensity inflammatory process, where AGII stimulates the production and release of IL-6 initiating the inflammatory process.¹⁸⁾ The use of AT1 receptor antagonists can block or diminish the production and release of inflammatory cytokines like IL-6, TNF- α , and IL-1 β .¹⁸⁾ This insight can explain a possible action mode for the MpEtOH and MpAcOEt treatments, due to the presence of flavonoid compounds in the extract and fractions. Flavonoids have been described to block G-protein activity associated to membrane receptors,¹⁹⁾ like the ones of AT1 receptors. Even though IL-4 is considered as an anti-inflammatory cytokine the chronic administration of AGII develops vascular remodeling that causes secondary damage through oxidative stress and an inflammatory process, where IL-4 is an active participant in vascular remodeling interfering with the healing process of the vasculature, the decrease in the expression of IL-4 leads to attenuate the development of vascular damage and therefor,²⁰⁾ the activity of the treatments MpEtOH and MpAcOEt where IL-4 concentration is decreased can be considered not as an anti-inflammatory cytokine promoters, but as a provable vascular healing/regenerative activity.

IL-10 is considered an anti-inflammatory cytokine that is produced by different types of cells, such as, macrophages, mast cells, T cells (Tregs), among others. Its main function is to control the inflammatory response and manage the differentiation and proliferation of some immune cells. IL-10 receptors have been found in EC, this gives it, the ability to not only control pro-inflammatory cytokines as to potentially inhibit endothelial dysfunction.²¹⁾ Furthermore, the chronic

administration of AGII has been proven to decrease the number of regulatory Tregs and IL-10 levels,²²⁾ although, the negative modulation of the activity of NADPH oxidase (NOX) can increase the levels of IL-10 produced by Tregs.²³⁾ This mode of action can be part of the effects that *M. parviflora* presents, the MpAcOEt treatment increased the IL-10 levels and decreased the concentration of MDA that is evidently an oxidative stress marker, which is a sub-product of NOX, where AGII activates NOX unleashing the production of superoxide anion that oxidizes phospholipids producing MDA. Another mode of action of *M. parviflora* extract and fractions is to interrupt the interactions of AGII with its receptors and NOX, therefore controlling blood pressure and consequent inflammatory and oxidative stress.

As it has been proven the chronic administration of AGII on experimental animals causes alterations in blood pressure and increases the production of pro-inflammatory agents and oxidative stress, lipid peroxidation is one of the biomarkers that can be observed under these conditions. Lipid peroxidation is the damage of lipids produced by their oxidation; polyunsaturated fatty acids are the most susceptible ones to ROS resulting in the production of cytotoxic products principally aldehydes like MDA.²⁴⁾ The production of MDA is generated through degradation of lipid peroxides after membrane lipid peroxidation induced by free radicals. Arachidonic acid present in cell membranes is very vulnerable to ROS attacks because of the presence of unsaturated bonds. MDA and other aldehyde products can act as toxic secondary messengers increasing the redox signals leading to cellular and tissue injury.²⁵⁾ The presences of tiliroside, scopoletin, oleanolic acid and other terpenoid compounds can explain the antihypertensive activity of *M. parviflora*, as these types of compounds have been reported to reduce oxidative stress and vascular inflammation in different models for hypertension, inflammatory and oxidative stress.^{26–28)} In the active extract MpAcOEt, the following compounds were quantified: scopoletin 23 μg , tiliroside 212 μg , and oleanolic acid 55 μg , all of them per gram of extract.

Based on this, the tiliroside and scopoletin isolated from MpAcOEt were evaluated on a model of transient hypertension induced by the acute administration of AGII i.v. In this trial, AGII induced an instantaneous and temporal increase on both SBP and DBP of 33 and 24 mmHg on average, respectively. The increment of blood pressure with AGII presented a statistically significant difference when compared with the base line control. Telmisartan did not present a significant difference when compared to the baseline treatment when i.v. administration of AGII, therefore, i.v. AGII did not have a vasoconstrictive effect on the positive control group. Regarding the antihypertensive effect of tiliroside administered orally in the *in vivo* model of transient hypertension, it should be considered that when tiliroside was applied to cultured VSMC, it produced an important vasorelaxant effect. This was mainly due to the abrupt decrease in the intracellular concentration of Ca^{2+} , since the flavonoid inhibited the activity of the L-type subunit of the voltage-dependent Ca^{2+} channel. However, the antihypertensive effect of tiliroside after intravenous administration of angiotensin II, only allowed to infer that there is an antagonism on rAT1 , without this being a direct evidence of receptor blockade. Although the indirect vasorelaxant effect, dependent on the release of mediators of the endothelium,

such as: nitric oxide, prostacyclin or endothelium-dependent hyperpolarizing factor cannot be ruled out.²⁹⁾

A pharmacokinetic study showed that at 10 min after the oral administration of 10 and 20 mg/kg of scopoletin, this substance is present in plasma at a concentration of 62.61 and 110.45 ng/mL, respectively.³⁰⁾ And in other set of experiments was presented an antihypertensive effect of this coumarin, when it is administered by i.v. and was obtained an ED_{50} of 229 $\mu\text{g}/\text{kg}$ and $E_{\text{max}} = 29 \text{ mmHg}$.³¹⁾ For the other hand, in the present work, the oral administration of scopoletin to different doses allows to obtain an $\text{ED}_{50} = 0.12 \text{ mg}/\text{kg}$ and $E_{\text{max}} = 37.74 \text{ mmHg}$; for DBP $\text{ED}_{50} = 0.02 \text{ mg}/\text{kg}$ and $E_{\text{max}} = 6.24 \text{ mmHg}$. For all this data, it can be assumed that scopoletin has antihypertensive activity by i.v. and oral (present work), that the potency of that effect is higher by this last administration pathway and that the concentration necessary in plasma for this effect should be low. The antihypertensive chronic effect of MpAcOEt, could also be due to the presence of scopoletin, due to it could owe its activity to that induce vasorelaxation dependent on the endothelial nitric oxide synthase (eNOS)/sGC/cGMP signaling pathways that are activated in the long-term treatment with this compound.³²⁾ In addition to tiliroside and scopoletin, the MpAcOEt to contain oleanolic acid, which could contribute with the effect of this fraction because this terpene induces endothelium-dependent vasorelaxation, by the activation of eNOS derived from the phosphorylation effected by protein kinase B/Akt.³³⁾

However, the chronic administration of the MpEtOH, MpAcOEt, and MpAq extracts for ten weeks could counteract the induced hypertension with the sub-effective administration of AGII, since oxidative stress and tissue damage by inflammation are the real cause of hypertension.³⁴⁾ Therefore, the antioxidant and anti-inflammatory capacity of scopoletin and eventually oleanolic acid could be the ones controlling hypertension that is secondary to endothelial dysfunction.^{35,36)}

Acknowledgments This work was supported by Coordination of Health Research-IMSS (FIS/IMSS/PROT/G11/988) grants. This paper is taken in part from the Ph.D. of Hipólita Lagunas Herrera (Programa de Doctorado en Ciencias Biológicas y de la Salud) belonging to Universidad Autónoma Metropolitana (UAM), with fellowship form CONACyT, Mexico (REG. 212744).

Conflict of Interest The authors declare no conflict of interest.

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