ELSEVIER

Journal of Ethnopharmacology 60 (1998) 133–139

Journal of ETHNO-PHARMACOLOGY

Endothelium-dependent vasorelaxing activity of aqueous extracts of *Ilex paraguariensis* on mesenteric arterial bed of rats

A.L. Muccillo Baisch *, K.B. Johnston, F.L. Paganini Stein

Setor de Farmacologia, Depto. de Ciências Fisiológicas, Fundaçao Universidade do Rio Grande, Rio Grande-RS, Brazil

Received 10 July 1997; received in revised form 13 September 1997; accepted 14 November 1997

Abstract

The effects of aqueous extracts of *Ilex paraguariensis* leaves (AEIp) were studied. Mesenteric arterial bed (MAB), precontracted by methoxamine with or without intact endothelium, was mounted on a tissue bath and exposed to plant extracts (bolus). The bolus injections of AEIp (300-1050 μg) significantly inhibited, in a concentration-dependent manner, the maximal contractile response induced by methoxamine (30 μm) in MAB. The endothelium-dependent relaxations were reversed by N^G-nitro-L-arginine methyl ester (10 mM), whereas methylene blue (100 μM) was not capable of effectively inhibiting the AEIp-induced vasodilatation of MAB. The vasorelaxing effect of AEIp persisted in the presence of indomethacin (10 μM). These results suggest the involvement of NO of endothelial source (or others factors) in this vasodilatory effect.

Keywords: Endothelium; *Ilex paraguariensis*; Mesenteric arterial bed; Nitric oxide

1. Introduction

The genus *Ilex* (Aquifoliaceae) consists of over 400 species. They have alternate simple leaves, single or clustered small flowers and red black or yellow berries (Alikaridis, 1987), and grow as trees or shrubs.

The genus *Ilex* is composed of species of the so-called holly plant: *Ilex aquifolium* (European or English holly), *Ilex opaca* (American holly), *Ilex cornuta* (Chinese holly) and *Ilex cremata* (Japanese holly).

In Brazil, Argentine, Uruguay and Paraguay, one of the most important species of *Ilex*, *Ilex paraguariensis*, is cultivated and used in the preparation of a traditional beverage called maté.

The effects of this species on the circulatory system are appreciated by those who drink the maté to reduce hypertension as a diuretic agent (Mazzafera, 1994). It is also recommended for people with poor blood, weak circulation and varices (Cruz, 1982).

* Corresponding author.

0378-8741/98/$19.00
PII S0378-8741(97)00140-2

The depurative, stimulant and diuretic actions for which *I. paraguariensis* leaves have been traditionally used (Hegnauer, 1964) could be ascribed to their high purine content (Baltassat et al., 1984). Graham (1984) reported that maté constitutes the primary source of methylxanthines in the diet of differents populations in South America.

Phytochemical investigations of various species of *Ilex*, including *I. paraguariensis*, have found many classes of chemical constituents (Alikaridis, 1987), such as: flavonoides (quercetin glycosides and rutin) in the leaves (Roberts, 1956); terpenoids (ursolic acid) in the leaves (Nooyen, 1920; Hauschild, 1935; Mendive, 1940); purine alkaloids (caffeine) in the seeds and leaves (Lendner, 1918; Bohinc and Korbar-Smid, 1978); amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glycine, histidine, isoleucine, leucine. lysine, methionine, tryptophan, tyrosine and valine) in the leaves (Cascon, 1995); fatty acids (lauric, palmitoleic, oleic, linoleic) in the seeds (Cattaneo et al., 1952); carbohydrates (Chlamtac, 1955), vitamins and carotenoids (Villela, 1938).

Several studies have shown that many edible plants may have compounds that may be protective against cardiovascular diseases (Block and Langseth, 1994).

The lower incidence of coronary artery disease in the French population (the ''French paradox''), despite a diet rich in saturated fatty acids, has been attributed to the high rate of red wine consumption by this population (Renaud and de Lorgeril, 1992), as well as to a decrease in platelet aggregation and an increase in high-density lipoprotein (Seigneur et al., 1990)

Fitzpatrick et al. (1993) investigated the effects of various components of grapes on rat aortic rings. These authors found that grape skin extracts (which contain tannin and quercetin) had a relaxing effect on rings that were previously contracted with phenylephrine and that this action only occurred with an intact endothelium. Additionally, the level of cGMP in the vascular tissue increased significantly by this treatment and the effect was decreased by selective inhibitors of NO synthase such N^G-nitro-L-arginine and N^G-monomethyl-L-arginine.

The present study was designed to investigate the effect of aqueous extract of *Ilex paraguariensis* (AEIp) on isolated mesenteric arterial bed (MAB) precontracted with methoxamine for possible vascular relaxing activity in intact and de-endothelialized rat MAB.

2. Materials and methods

2.1. Plant materials

Ilex paraguariensis St. Hil. was collected in Lajeado, state of Rio Grande do Sul-Brazil, in July 1995. A voucher specimen (001154) has been deposited at the Herbarium of the Morphobiological Department of the Fundaçao Universidade do Rio Grande, Brazil.

2.2. Preparation of extract

The fresh plant material (leaves) was washed, and dried at 37°C. Coarsely powdered dried plant material (30 g) was extracted with 130 ml of bi-distilled water using a Soxhlet extractor, yielding after lyophilization 3.1 g of powder (31%). The extracts were dissolved in Krebs Ringer bicarbonate immediately before all tests were performed. All results are shown as micrograms of lyophilized powder.

2.3. Animals

Male Wistar rats, weighing 426.5 ± 8.0 g ($n = 62$), were obtained from the Animal House of the Fundaçao Universidade do Rio Grande. The rats were housed in temperature-controlled rooms (20-22C), with a 12:12 h light/dark photoperiod and $55 \pm 1\%$ relative humidity. Standard laboratory chow (Nuvital, Nuvital Nutrientes, Colombo, Paraná, Brazil) and drinking water were provided ad libitum.

2.4. Drugs

The following drugs were used: acetylcholine chloride, indomethacin, L-arginine hydrochloride, N^G-nitro-L-arginine methyl ester hydrochloride,

methylene blue, methoxamine from Sigma (St. Louis, MO, USA). The stock solution of indomethacin was prepared in 5% $NaHCO_3$; all other compounds were dissolved freely in distilled water.

2.5. Statistical analysis

Data are reported as mean ± standard error with the number of observation in parentheses. Statistical differences between means were measured using Student's *t*-test for unpaired and paired observations. The level of significance was $P < 0.05$.

2.6. Preparation of mesenteric arterial vascular bed (MAB)

The MAB was removed from animals under brief ether anaesthesia and perfused as described by McGregor (1965). The superior mesenteric artery with its nerve plexus was carefully separated from the surrounding tissue at a point 2 cm distal to the aorta. A stainless steel cannula (1.5 cm long) was inserted into the artery. The associated vascular bed was covered with gauze moistened with Krebs Ringer bicarbonate and perfused by means of a peristaltic pump (Milan, Colombo, Brazil) at 5 ml/min with Krebs Ringer bicarbonate. The composition of the perfusion solution was (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 3.3; KH_2PO_4, 1.2; $MgSO_4$, 2.4; $NaHCO_3$, 25; glucose, 10; EDTA, 0.03; ascorbic acid, 0.1. The Krebs Ringer bicarbonate solution was gassed with 95% O_2 and 5% CO_2 to obtain a pH of 7.2-7.4 and was maintained at 37°C.

Perfusion pressure was measured with a transducer (Hewlett-Packard, USA) on a side arm just before the perfusing cannula and continuously recorded on a polygraph inscriber (Hewlett-Packard, USA). Since the flow rate was constant throughout the whole experimental period, any pressure alteration reflected changes in vascular resistance. All preparations were allowed to equilibrate for at least 30 min before the start of experiments.

Vasoconstriction is expressed as the absolute increase in perfusion pressure (mmHg) above the baseline.

Drug- and extract-induced relaxation was expressed as a percentage of the increase in perfusion pressure induced by methoxamine (30 μM) (Fig. 1).

After contraction was induced with methoxamine, all drugs were administered as a bolus (50 μl).

3. Results

3.1. Vasodilatation in response to aqueous extract of Ilex paraguariensis and acetylcholine in MAB precontracted with methoxamine

The basal perfusion pressure of the rat MAB was 22.3 ± 0.6 mmHg ($n = 56$). In the study of the control vasodilator responses, the tone of the preparation was raised by constant perfusion with methoxamine (30 μM) added to the perfusate. This procedure increased the perfusion pressure to 72.1 ± 3.1 mmHg ($n = 56$). After the tonus of the MAB was increased with methoxamine, exposure to bolus (300, 450, 600, 750, 900 and 1050 μg/50 μl) of AEIp induced a dose-dependent relaxation (Fig. 2).

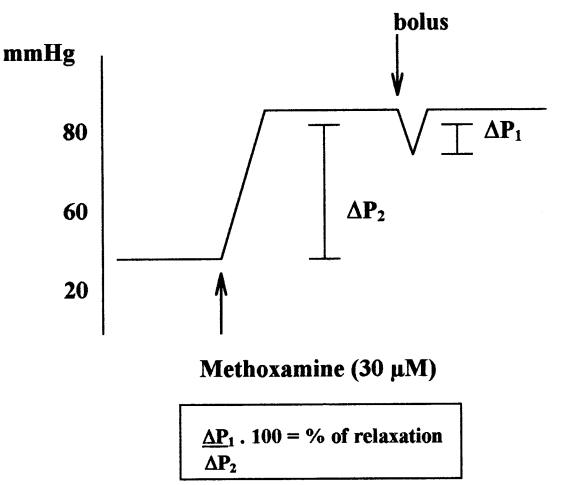

Fig. 1. Relaxation in the rat mesenteric arterial bed in response to acetylcholine or AEIp on methoxamine-induced contraction. ΔP_1, drop in perfusion pressure (mmHg) induced by acetylcholine or AEIp; ΔP_2, increase in perfusion pressure (mmHg) induced by methoxamine.

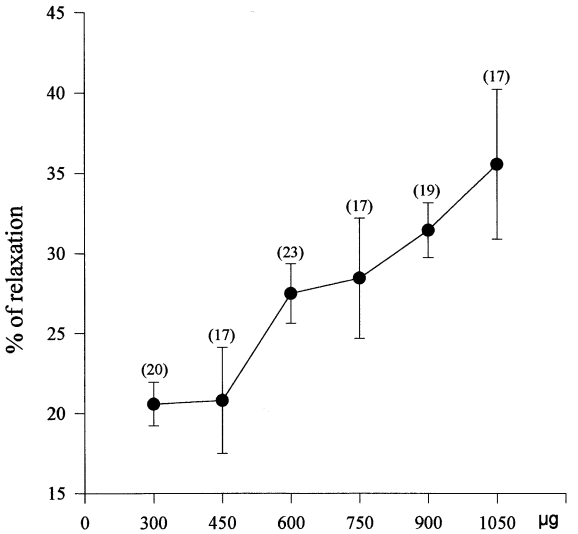

Fig. 2. Concentration–response curve for the relaxation induced by *Ilex paraguariensis* aqueous extract in the methoxamine-precontracted mesenteric arterial beds. Each data point is mean ± S.E.M. Numbers of experiments are shown in parentheses.

3.2. Effect of cyclo-oxygenase pathway inhibitor on AEIp-induced vasodilatation in MAB precontracted with methoxamine

The involvement of prostaglandins in the vasodilator responses induced by AEIp were recorded in the absence and in the presence of indomethacin (10 μM). The cyclo-oxygenase inhibitor did not alter significantly the responses to AEIp, which were $20.6 \pm 1.3\%$ ($n = 20$) to 300 μg, $27.5 \pm 1.9\%$ ($n = 23$) to 600 μg, $31.5 \pm 1.7\%$ ($n = 19$) to 900 μg, and $19.8 \pm 3.0\%$ ($n = 9$) to 300 μg, $23.5 \pm 3.4\%$ ($n = 6$) to 600 μg, $33.5 \pm 5.1\%$ ($n = 9$) to 900 μg, before and after the addition, respectively. Further experiments were similarly performed without indomethacin.

3.3. Effect of removal of endothelium on changes in perfusion pressure induced by AEIp and acetylcholine in MAB precontracted with methoxamine

To exclude the involvement of the endothelium on extract-induced vasodilatation, the mesenteric was perfused with distilled water for 10 min to removal endothelium (Criscione et al., 1984). In intact MAB contracted with methoxamine (30 μM), AEIp caused immediate relaxation. After the endothelium was removed, the vascular relaxing ability of *I. paraguariensis* bolus of 600 μg and 900 μg was significantly changed (Fig. 3).

The same procedure decreased the acetylcholine-induced vasodilatation (5 nmol) from $61.45 \pm 2.61\%$ to 11.86 ± 1.84 (Fig. 3), which is consistent with results reported by Muccillo Baisch et al. (1994).

3.4. Effect of endothelium-derived NO-cGMP pathway inhibitor on AEIp- and acetylcholine-induced vasodilatation in MAB precontracted with methoxamine

The effects of n^{G}-nitro-L-arginine methyl ester (L-NAME, 10 mM) and methylene blue (MB, 100 μM) on the vasodilatation induced by acetylcholine and AEIp were examined.

As shown in Fig. 3, AEIp-induced vasodilatations of the MAB were significantly ($P < 0.05$) inhibited by treatment with L-NAME. Conversely, the AEIp-induced vasodilatation (300 and 600 μg) was not inhibited by subsequent perfusion of methylene blue (Fig. 4).

Acetylcholine-induced vasodilatation of MAB was significantly reduced ($P < 0.05$) after treatment with L-NAME (Fig. 3) and methylene blue (Fig. 4). In the endothelium-intact preparations precontracted by methoxamine (30 μM), the vasorelaxation by acetylcholine was $61.5 \pm 2.6\%$ ($n = 14$). In contrast, after L-NAME and MB, the acetylcholine-induced vasorelaxation was $26.9 \pm 3.2\%$ ($n = 8$) and $26.5 \pm 4.7\%$ ($n = 11$), respectively.

4. Discussion and conclusions

This study aimed to show the involvement of NO (or a related compound) in the vasodilator responses of AEIp. The results obtained indicated that AEIp (leaves) contain active substance(s) which produced a dose-dependent and reversible inhibition of methoxamine-induced increase of vascular tone.

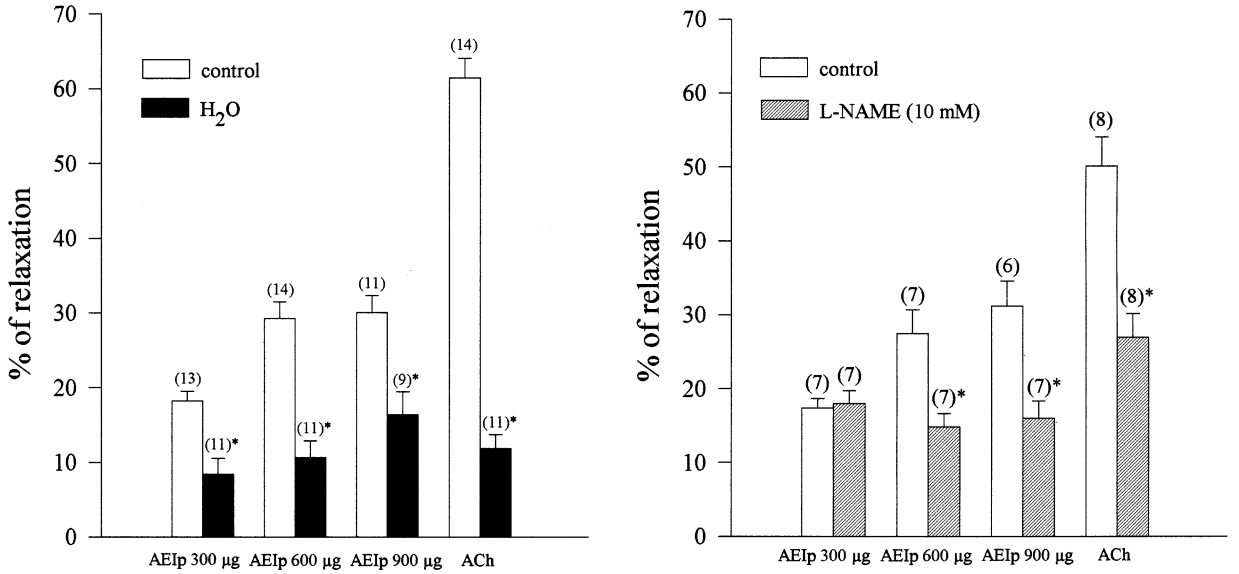

Fig. 3. Effects of a 10 min infusion period with distilled water (H_2O), to remove the endothelium, and of N^G-nitro-L-arginine methyl ester (L-NAME, 10 mM) on the aqueous extract from *Ilex paraguariensis* (AEIp, 300, 600 and 900 μg) or acetylcholine (ACh, 0.05 mmol) induced vasodilatation of mesenteric arterial bed precontracted by 30 μM methoxamine. Relaxation is expressed as a percentage of the contraction induced by methoxamine. Numbers of experiments are shown in parentheses. *Values significantly different from control ($P < 0.05$).

This relaxation was not affected by indomethacin, which indicates that it was not caused by prostaglandin production.

When the MAB was perfused with distilled water, both acetylcholine- and AEIp- (300, 600 and 900 μg) induced vasodilatations were significantly reduced. As exposure to distilled water destroys only the endothelial cells (Criscione et al., 1984), these results confirm that both acetylcholine- and extract-induced vasodilatations are mediated by release of substances derived from endothelium. Furthermore, the relaxation was reversed by L-NAME, which is an inhibitor of NO synthase.

Furchgott and Zawadzki (1980) discovered that the vasorelaxing action of acetylcholine required the presence of an intact endothelium. The binding of acetylcholine to muscarinic receptors on endothelial cells triggers the release of a potent vasodilator, endothelium-derived relaxing factor (EDRF).

Additional investigations have identified EDRF as NO or a related substance synthesized from guanidino groups of L-arginine (Palmer et al., 1987). Endothelium-derived NO (EDNO) has been reported to stimulate the production of cGMP (Winquist et al., 1984), which induces a vasorelaxation in smooth muscle cells.

The vasodilatatory effects of endothelium-dependent substances can be inhibited by several L-arginine analogues such as *N*-monomethyl-L-arginine (L-NMMA) and L-NAME (Palmer et al., 1988; Rees et al., 1989; Moore et al., 1990).

NO has been implicated in various physiological roles of the cardiovascular system, such as regulation of the vascular tone (Lowenstein et al., 1994) and maintaining endothelial integrity (Mellion et al., 1981). Therefore, dysregulation of NO production has widespread pathophysiological implications, including essential hypertension, atherosclerosis and the myocardial depression associated with septic shock (Lowenstein et al., 1994).

In the present study, the AEIp caused endothelium-derived relaxation which was reversed by L-NAME. The results strongly suggest that cer-

tain compounds present in the plant extract may cause relaxation by increasing NO or any other related compounds.

Adeagbo and Triggle (1993) have also reported that endothelium-dependent vasorelaxants such as acetylcholine released endothelium-derived hyperpolarizing factor (EDHF) from the endothelium, which induces vasorelaxation through membrane hyperpolarization. The possible existence of a novel endothelium-derived relaxing factor in the endothelium of rat MAB was examined in the study of Kamata et al. (1996). The authors suggested that one or more EDRFs must exist, other than EDNO or EDHF. The novel EDRF may relax the MAB through production of cAMP but not cGMP.

In our study these conclusions are supported by the finding that the vasodilatation was not inhibited by methylene blue, suggesting that plant extract may also produce one of more novel EDRFs.

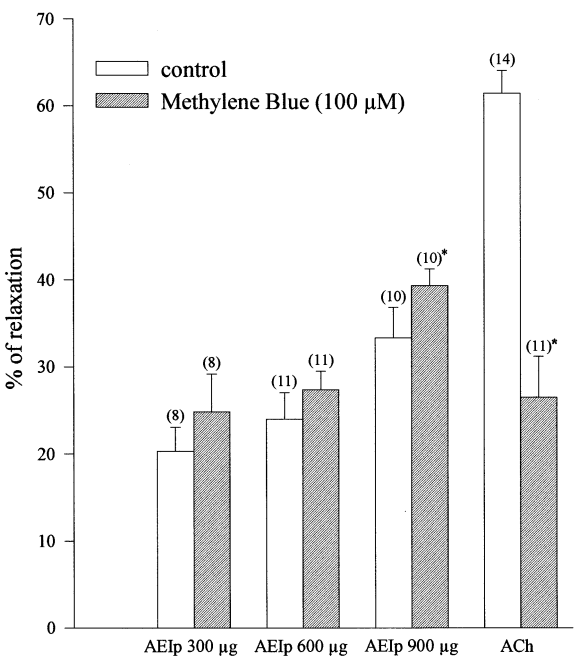

Fig. 4. Effect of methylene blue (MB, 100 mM) on the vasodilatation responses induced by a bolus of acetylcholine (0.05 mmol) and aqueous extract from *Ilex paraguariensis* (AEIp, 300, 600 and 900 μg) in the mesenteric arterial bed precontracted by 30 μM methoxamine. Relaxation is expressed as a percentage of the contraction induced by methoxamine. Numbers of experiments are shown in parentheses. *Values significantly different from control ($P < 0.05$).

The rate of elimination of endothelium-derived mediators may significantly regulate their response and this is critical for NO. As a short-lived radical mediator, its half-life is determined by ambient levels of superoxide anion. The latter reacts rapidly with NO, forming peroxynitrite (Ignarro, 1989).

Quercetin, a flavonoid found in *Ilex* species (Alikaridis, 1987) which possesses free radical scavenging and lipid antiperoxidation activity (Yuting et al., 1990), may relax intact aortic rings. Flavonoids are a group of antioxidants which occur naturally and are usually found in plants, fruits and vegetables. They are known to be excellent scavengers of oxygen free radicals and are used in the treatment of vascular endothelial damage and in diseases of the vascular wall involving inflammation (Van Acker et al., 1995).

In conclusion, this study shows that in *Ilex paraguariensis* there are easily extractable compounds that may cause endothelium-dependent NO-cGMP- or AMP-mediated (or related substances) vasorelaxation in vitro.

Studies are currently underway to isolate and identify the active compounds in the plant extracts.

Acknowledgements

The authors are grateful to Arcelino Fornari (Ervateira Fornari, Lajeado, RS, Brazil) for the donation of plant material and Fundaçao de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS) and Conselho Nacional de Pesquisa e Desenvolvimento (CNPq) for financial help. Thanks are also due to Dr. André C.S. Schmitt for the English revision of the manuscript.

References

Adeagbo, A.S.O., Triggle, C.R., 1993. Varying extracellular [K^+]: a functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. Journal of Cardiovascular Pharmacology 21, 423–429.

Alikaridis, F., 1987. Natural constituents of *Ilex* species. Journal of Ethnopharmacology 20, 121–144.

Baltassat, F., Darbour, N., Ferry, S., 1984. Study of purinr levels in caffeine drugs. Plantes Medicinales et Phytotherapie 18, 195–203.

Block, G., Langseth, L., 1994. Antioxidant vitamins and disease prevention. Food Technology July, 80-84.

Bohinc, P., Korbar-Smid, J., 1978. Xanthine alcaloids of holly plants. Acta Pharmaceutica Jugoslavica 28, 55–60.

Cascon, C.S., 1995. Amino acids in *Ilex paraguariensis*. Boletin del Instituto de Quimica Argentina 38, 7–15.

Cattaneo, P., De Sutton, K.G., Rodriguez, M.L., 1952. Chemical composition of the seed oil of *Ilex paraguariensis*. Anales de la Direccion Nacional de Quimica (Argentina) 5 (9), 9–12.

Chlamtac, E.B., 1955. Sugars in *Ilex paraguariensis*. Boletin del Instituto de Quimica Argentina 38, 17–24.

Criscione, L., Müller, K., Prescott, M.F., 1984. Endothelial cells loss enhances the pressor response in resistance vessels. Journal of Hypertension 2 (Suppl. 3), 441–444.

Cruz, G.L., 1982. Dicionário das Plantas Üteis do Brasil, 2nd ed. Bertrand Brasil, Rio de Janeiro, 599 pp.

Fitzpatrick, D.F., Hirschfield, S.L., Coffey, R.G., 1993. Endothelium-dependent vasorelaxing activity of wine and other grape products. American Journal of Physiology 265, H774–778.

Furchgott, R.F., Zawadzki, J.V., 1980. The obrigatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 299, 373–376.

Graham, H.N., 1984. Maté. Progress in Clinical and Biological Research 158, 179–183.

Hauschild, W., 1935. The constituents of maté. Mitteilungen aus dem Gebiete der Lebensmitteluntersuchung und Hygiene 26, 329–351.

Hegnauer, R. (1964) Chemotaxonomie der Pflanzen, Band 3. Birkhäuser Verlag, Basel, p. 165.

Ignarro, L.J., 1989. Endothelium-derived nitric oxide: actions and properties. FASEB Journal 3, 31–36.

Kamata, K., Numazawa, T., Kasuya, Y., 1996. Characteristics of vasodilatation induced by acetylcholine and platelet-activating factors in the rat mesenteric arterial bed. European Journal of Pharmacology 298, 129–136.

Lendner, A., 1918. The seeds of *Ilex paraguariensis* St Hilaire. Schweizerische Apotheker-Zeitung 56, 565–569.

Lowenstein, C.J., Dinermann, J.L., Snyder, S.H., 1994. Nitric oxide: physiological messenger. Annals of Internal Medicine 120, 227–237.

Mazzafera, P., 1994. Caffeine, theobromine and theophyline distribution in *Ilex paraguariensis*. Revista Brasileira de Fisiologia Vegetal 6 (2), 149–151.

McGregor, D.D., 1965. The effect of sympathetic nerve stimulation on vasoconstritor responses in perfused mesenteric blood vessel of the rat. Journal of Physiology 177, 21–30.

Mellion, B.T., Ignarro, L.J., Ohlstein, E.H., et al., 1981. Evidence for the inhibitory role of guanosine 3′5′-monophosphate in ADP-induced human platelet aggegation in the presence of nitric oxide and related vasodilators. Blood 57, 946–955.

Mendive, J.R., 1940. Isolation of α-amyrin and ursolic acid in the leaves of *Ilex paraguariensis*. Journal of Organic Chemistry 5, 235–237.

Moore, P.K., Al-Swayeh, O.A., Chong, N.W.S., Evans, R.A., Gibson, A., 1990. L-N^G-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation in vitro. British Journal of Pharmacology 99, 408–412.

Muccillo Baisch, A.L., Larrue, J., Freslon, J.L., 1994. Involvement of endothelium-derived NO in the basal tone and in the vasodilator responses to muscarinic agonists in the rat isolated mesenteric arterial bed. Fundamental and Clinical Pharmacology 8, 54–63.

Nooyen, A.M., 1920. Urson and its distribution in the plant world. Pharmaceutisch Weekblad 57, 1128–1142.

Palmer, R.M.J., Ferridge, A.G., Mocada, S., 1987. Nitric oxide release accounts for the biological activity of rn-dothelium-derived relaxing factor. Nature 327, 524–526.

Palmer, R.M.J., Rees, D.D., Ashton, D.S., Moncada, S., 1988. L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochemical and Biophysical Research Communications 153 (3), 1251–1256.

Rees, D.D., Palmer, R.M.J., Moncada, S., 1989. Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proceedings of the National Academy of Sciences of the United States of America 86, 3375–3378.

Renaud, S., de Lorgeril, M., 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339, 1523–1526.

Roberts, E.A.H., 1956. Chlorogenic acids of tea and maté. Chemistry and Industry 37, 985.

Seigneur, M., Bonnet, J., Dorian, B., et al., 1990. Effect of the consumption of alcohol, white wine, and red wine on platelet function and serum lipids. Journal of Applied Cardiology 5, 215–222.

Van Acker, S.A.B.E., Tromp, M.N.J.L., Haenen, G.R.M.M.M., van der Vijgh, W.J.F., Bast, A., 1995. Flavonoids as scavengers of nitric oxide radical. Biochemical and Biophysical Research Communications 214 (3), 755–759.

Villela, G.G., 1938. Determination of vitamin B1 in yerba maté by the Schopfer-Jung method. Comptes Rendus des Seances de la Societé de Biologie et de ses Filiales 129, 987–989.

Winquist, R.J., Bunting, P.B., Baskin, E.P., Wallace, A.A., 1984. Decreased endothelium-dependent relaxation in New Zealand genetic hypertensive rats. Journal of Hypertension 2, 536–541.

Yuting, C., Rongliang, Z., Zhongjian, J., Yong, J., 1990. Flavonoids as superoxide scavengers and antioxidants. Free Radical Biology and Medicine 19, 19–21.