Studies on Anti-Allergic Constituents in the Leaves and Stems of *Anchietia salutaris* var. *martiana* (Violaceae)

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The anti-allergic active fractionation of hexane extracts of the leaves and stems of *Anchietia salutaris* var. *martiana* (family Violaceae) was performed by monitoring their activities with an *in vitro* bioassay system measuring the inhibitory effects on induced histamine release from guinea pig lung cells. Three known pentacyclic triterpenes (friedelin, α-amyrin, β-amyrin) were isolated, but these compounds were inactive. Aliphatic hydrocarbons and methyl esters of fatty acids (palmitic, oleic, linoleic, linolenic acids) were detected in active fractions. All compounds isolated were detected for the first time in this medicinal plant.

Key words   *Anchietia salutaris*; anti-allergy; histamine release; fatty acids

Anchietia salutaris St. Hil. var. *martiana* (Violaceae) is a woody liana abundant in South America. The leaves and stems of the woody liana have been traditionally used in Brazil to treat skin and allergic diseases.[2] In a preliminary screening study, the stem and leaf aqueous extracts and their partitioned extracts (hexane, dichloromethane, methanol/water) of this species were studied in a bioassay of the inhibition of histamine release induced by compound 48/80, ionophore A23187 or antigen from rat peritoneal cells and rat and guinea pig mast cells.[3] The leaf and stem hexane extracts of *Anchietia salutaris* were found to be highly active in the inhibition of histamine release, while dichloromethane and methanol/water extracts were inactive.[3b]

According to a biomonitored fractionation of the hexane extract of the leaf and stem with vacuum liquid chromatography (VLC) and silica gel column chromatography (CC), we studied the effect of all fractions of the stems and leaves of *Anchietia salutaris* var. *martiana* on the histamine release induced in guinea pig lung cells. We also analyzed the chemical composition of the active fractions by high resolution gas chromatography/mass spectrometry (HR-GC/MS).

We first reinvestigated the biological activities of the stem and leaf hexane extracts. At a concentration of $100 \mu g/ml$, the leaf and stem extracts inhibited histamine release 21.0 and 40.5%, respectively. These extracts were inactive at a concentration of $30 \mu g/ml$. Repeated fractionation of both hexane extracts by VLC afforded several active fractions that inhibited histamine release at a concentration of $100 \mu g/ml$ (Charts 1, 2).

As shown in Chart 1, at a concentration of $100 \mu g/ml$, fractions S1, S2, S3 and S4 from stem hexane extract inhibited histamine release 63.4, 58.9, 56.0 and 28.3%, respectively, while fraction S5 was inactive. At a concentration of $30 \mu g/ml$, only fractions S1 and S2 inhibited histamine release, while other fractions were inactive.

Chart 2 shows the results obtained with 30 and $100 \mu g/ml$ of the hexane extract and their VLC fractions (L1 to L5) from the leaves of the plant. Only fractions L1, L2 and L5 inhibited histamine release 25.2, 81.6 and 29.5%, respectively. Fractions L3 and L4 were inactive. At a concentration of $30 \mu g/ml$, only fraction L2 inhibited histamine release, by 26.1%.

HR-GC/MS analyses of the hexane extracts of leaves re-

vealed the presence of the three known pentacyclic triterpenes (friedelin, α-amyrin, β-amyrin), as well as phytol and aliphatic hydrocarbons (not fully identified), while HR-GC/MS of the hexane extract of the stem afforded only a series of known methyl ester of palmitic, oleic, linoleic and linolenic acids. All these compounds were detected for the first time in this medicinal plant.

Next, we investigated fractions L2 and S1, which inhibited histamine release 81.6 and 63.4%, respectively. These fractions were selected for sequential fractionation in silica gel CC. All CC fractions of hexane extracts of both the leaf and stem were monitored by mast cell bioassay (Charts 1, 2).

Fractionation of L2 by CC yielded 9 fractions (L2A to L2I), in which only fraction L2A inhibited histamine release, 60.4%, at a concentration of $100 \mu g/ml$. In the same concentration, fractions L2B, L2C, L2D, L2E, L2F and L2H were inactive. Surprisingly, fractions L2G and L2I at a concentration of $100 \mu g/ml$ increased the histamine release, 94.99 and 95.21%, respectively, but this result was due to the fluorescence of the fractions and their consequently detection in the fluorometric assay used. The HR-GC/MS analyses of fraction L2A revealed phytol and aliphatic hydrocarbons, which were also first detected in the hexane extract of the leaf. On the other hand, the known pentacyclic triterpenes (friedelin, α-amyrin, β-amyrin) were not detected in this active fraction. Therefore, these pentacyclic triterpenes are not the active compounds of this medicinal plant.

Fractionation of S1 by CC yielded 12 fractions (S1A to S1L), in which S1A, S1D, S1F, S1G, S1H, S1I and S1L were active at a concentration of $100 \mu g/ml$, while the other fractions were inactive. At a concentration of $30 \mu g/ml$, only one fraction, S1D, inhibited histamine release. Thus, this fraction was selected as a representative for HR-GC/MS analyses, which revealed the presence of the methyl esters of palmitic, oleic, linoleic and linolenic acids. These compounds are also present in the active hexane extract of the stem. Preliminary data in our laboratory show that the methyl ester of linoleic acid (10, 30, $100 \mu g/ml$) inhibited histamine release, respectively, 35.2, 53.5 and 57.4%, and that other fatty acids and methyl esters also inhibit histamine release. These data suggest that the methyl esters of fatty acids may be the chemical constituents responsible for the inhibition of histamine release.

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VLC   CC

AS_SHex c/f - 2.16g[a]/24.1%[b]   S1 – 955.8mg[a]/45.2%[b]   S1A – 46.7mg[a]/4.9%[b]
ns[c]/40.5[d]   26.9[c]/63.4[d]   ns[c]/13.93[d]

                                       S2 – 692.8mg[a]/32.1%[b]   S1B – 70.8mg[a]/7.4%[b]
                                           23.4[c]/58.9[d]   ns[c]/ns[d]

                                       S3 – 218.9mg[a]/10%[b]   S1C – 61.9mg[a]/9.6%[b]
                                           ns[c]/56.0[d]   ns[c]/ns[d]

                                       S4 – 59.7mg[a]/2.8%[b]   S1D – 65.0mg[a]/6.8%[b]
                                           ns[c]/28.3[d]   15.0[c]/23.34[d]

                                       S5 – 101.2mg[a]/4.7%[b]   S1E – 20.7mg[a]/2.2%[b]
                                           ns[c]/ns[d]   ns[c]/ns[d]

                                                                            S1F – 14.6mg[a]/1.5%[b]
                                                                               ns[c]/19.16[d]

                                                                            S1G – 13.6mg[a]/1.4%[b]
                                                                               ns[c]/12.89[d]

                                                                            S1H – 18.9mg[a]/2.0%[b]
                                                                               ns[c]/18.12[d]

                                                                            S1I – 17.6mg[a]/1.9%[b]
                                                                               ns[c]/18.46[d]

                                                                            S1J – 69.4mg[a]/7.3%[b]
                                                                               ns[c]/ns[d]

                                                                            S1K – 43.3mg[a]/4.5%[b]
                                                                               ns[c]/ns[d]

                                                                            S1L – 27.8mg[a]/2.9%[b]
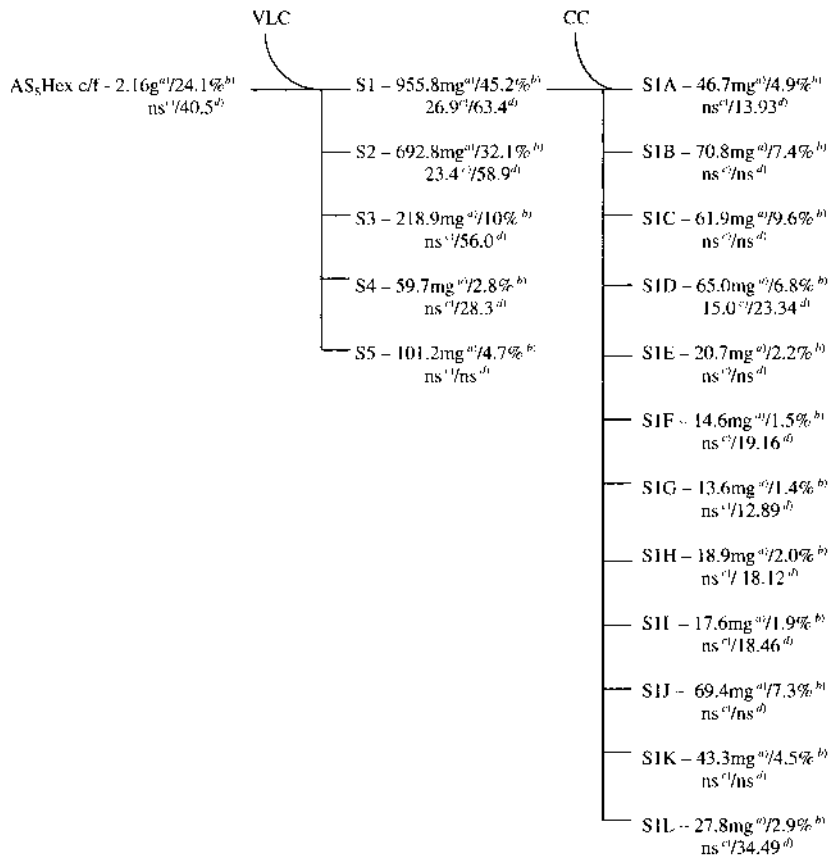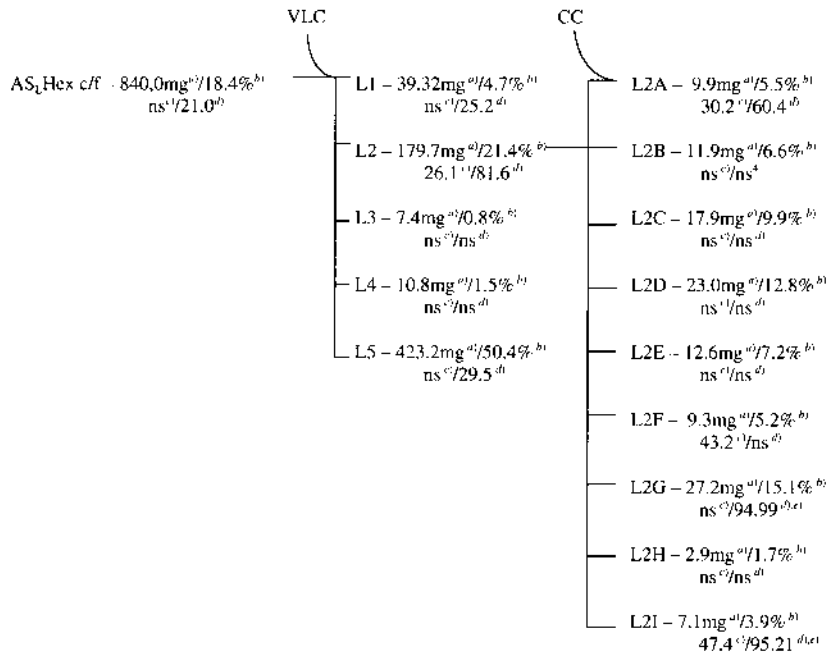   ns[c]/34.49[d]

Chart 1. Fractionation of the Stem Hexane Extract (AS_SHex) of *Anchietia salutaris*

a) Absolute yield, b) relative yield, c) inhibition of histamine release (%) with 30 μg/ml, d) inhibition of histamine release (%) with 100 μg/ml, c/f: chlorophyll-free extract, ns: no significant inhibition of histamine release.

VLC   CC

AS_LHex c/f - 840.0mg[a]/18.4%[b]   L1 – 39.32mg[a]/4.7%[b]   L2A – 9.9mg[a]/5.5%[b]
ns[c]/21.0[d]   ns[c]/25.2[d]   30.2[c]/60.4[d]

                                       L2 – 179.7mg[a]/21.4%[b]   L2B – 11.9mg[a]/6.6%[b]
                                           26.1[c]/81.6[d]   ns[c]/ns[d]

                                       L3 – 7.4mg[a]/0.8%[b]   L2C – 17.9mg[a]/9.9%[b]
                                           ns[c]/ns[d]   ns[c]/ns[d]

                                       L4 – 10.8mg[a]/1.5%[b]   L2D – 23.0mg[a]/12.8%[b]
                                           ns[c]/ns[d]   ns[c]/ns[d]

                                       L5 – 423.2mg[a]/50.4%[b]   L2E – 12.6mg[a]/7.2%[b]
                                           ns[c]/29.5[d]   ns[c]/ns[d]

                                                                            L2F – 9.3mg[a]/5.2%[b]
                                                                               43.2[c]/ns[d]

                                                                            L2G – 27.2mg[a]/15.1%[b]
                                                                               ns[c]/94.99[d,e]

                                                                            L2H – 2.9mg[a]/1.7%[b]
                                                                               ns[c]/ns[d]

                                                                            L2I – 7.1mg[a]/3.9%[b]
                                                                               47.4[c]/95.21[d,e]

Chart 2. Fractionation of the Leaf Hexane Extract (AS_LHex) of *Anchietia salutaris*

a) Absolute yield, b) relative yield, c) inhibition of histamine release (%) with 30 μg/ml, d) inhibition of histamine release (%) with 100 μg/ml, e) sample that increases histamine release, c/f: chlorophyll-free extract, ns: no significant inhibition of histamine release.

Methyl esters of fatty acids, aliphatic hydrocarbons and phytol are trivial constituents with wide occurrence in plant species. Fatty acids and aliphatic hydrocarbons are usual chemical constituents of surface waxes and are generally detected in close association.[4] There is also an intricate biogenetic relationship between fatty acids and aliphatic hydrocarbon.[5] On the other hand, the phytol with the ester of fatty acids has been abundantly detected in plants.[6] All these compounds show several chemical similarities and a wide relationship in biogenesis or natural occurrence. This fact can explain the similar pharmacological activity produced by the leaves and stems of *Anchietia salutaris* in the inhibition of histamine release.

Several reports show that fatty acids and their methyl esters, present as plant constituents, have several related pharmacological activities: the linoleic acid isolated from *Asarum forbesii* posses an inhibitory effect in passive cutaneous anaphylaxis,[7] whereas the mixture of palmitic, miristic, stearic, oleic, linoleic, araquidic, linolenic and eicosadienoic acids obtained from *Nigella sativa* oil show inhibitory eicosanoid formation *via* cyclooxygenase and lipooxygenase inhibition.[8] The interference of fatty acids and their methyl esters on eicosanoid metabolism has been intensively studied, since recent data show that hydroperoxidoeicosatetraenoic acids produce histamine release and increase the release of anaphylactic mediators.[9] It has been shown that modifications involving fatty acid in the diet produce alterations in the fatty acid composition of mast cell membranes, and these alterations led to a modified level of prostagladins and fatty acid levels in mast cells stimulated by ionophore A23187.[10]

The present data clearly show that *Anchietia salutaris* produces several chemical constituents which inhibit histamine release and also suggest that the methyl ester of fatty acids and phytol are among the active principles that could be useful in the treatment of allergic diseases. These data are very important because the anti-allergic activity is attributed to known and usual compounds, which means easy acquisition and a lower price. Therefore, new studies will be performed in order to determine the role of these compounds on endogenous mediators related to the allergic process. On the other hand, it is important to note that all the isolated compounds were detected here for the first time in *Anchietia salutaris*, and that a polar extract of the stem and leaves of this medicinal plant were inactive in the same bioassay.[3] The activity found in the fractions of both hexane extracts was not completely explained by the chemical compounds determined, as is clear from the distribution of activities by several fractions and yields. Many other unisolated compounds in this study may contribute to the activities of the hexane extracts of this medicinal plant. Finally, these results were also in good accord with the traditional use of *Anchietia salutaris* for anti-allergy purposes.

Experimental

Plant Material Leaves and stems of *Anchietia salutaris* St. Hil. var. *martiana* (Violaceae) were collected (February 1992) in the Botanical Garden of the Instituto de Biociências, Botucatu, Unesp, São Paulo, Brazil. The plant was identified by Dr. L. H. Bicudo of the Herbarium BOTU, Departmento of Botany, Instituto de Biociências, Botucatu, Unesp, São Paulo, Brazil (Voucher number 05675).

Preparation of the Crude Hexane Extract Leaves (70.0 g) and stems (240.0 g) were separated, dried (37 °C), powdered and extracted with methanol (2.0 l for leaves and 3.5 l for stems) for 48 h. The methanol ex-

tracts were separately concentrated under a vacuum, up to 20% of the volume, and submitted to a chlorophyll elimination.[11] For each 100 ml of the methanol chlorophyll-free extract of the leaves and stems, 30 ml of hexane was used for extraction (separator funnel) yielding the hexane extracts that were submitted to bioassays.

Fractionation of the Crude Hexane Extracts The hexane extracts of the leaves (840.0 mg) and stems (2.16 g) were initially fractionated by VLC using 7.5 g of Silica gel 60 for thin-layer chromatography and 100 ml of the following eluents: hexane, hexane/ethyl acetate 9 : 1, 7 : 3 and 1 : 1, ethyl acetate and methanol. The collected fractions were monitored by thin layer chromatography (Silica gel F60, 0.25 mm) and grouped in five fractions, respectively designated as L1 to L5 for leaves and S1 to S5 for stems. The active fractions L2 (179.72 mg) and S1 (955.78 mg) were selected for new fractionation by liquid CC using Silica gel 60 (70—230 mesh) in the following dimensions: 1.60 cm diameter and 15.0 cm high. The chromatography of fraction L2 yielded 52 fractions of 8.0 ml grouped in 9 fractions designated as L2A to L2I, and the CC of fraction S1 yielded 63 fractions of 8.0 ml grouped in 12 fractions designated as S1A to S1L.

HR-GC/MS HR-GC/MS analyses were performed on a Hewlett-Packard 5988 chromatograph equipped with a silica column WCOT 15 m×0.25 nm, DB-1 from J&W Scientific, CA, coupled to a mass spectrometer Hewlett-Packard 5970. Conditions: injector temperature: 250 °C; temperature range: 70 to 300 °C, 4 °C/min; carrier gas He 0.5 bar, 1 ml/min.; sample volume: 1—2 μl, and ionization energy of 70 eV. The compounds were identified by comparison of their mass spectrometric data with those of authentic standards and a computer search in the NBS library (*ca.* 70000 compounds) as well as by comparison of their fragmentograms with literature data.[12—17]

Friedelin: $C_{30}H_{50}O$, M.W. 426, *m/z* (%): 55 (88), 69 (96), 191 (40), 205 (48), 218 (55), 232 (36), 246 (43), 273 (82), 302 (55), 341 (37), 426 (M^+, 72); retention time: 55.7 min.

α-Amyrin: $C_{30}H_{50}O$, M.W. 426, *m/z* (%): 44 (17), 147 (11), 203 (18), 218 (100), 408 (2), 411 (5), 426 (M^+, 18); retention time: 53.9 min.

β-Amyrin: $C_{30}H_{50}O$, M.W. 426, *m/z* (%): 95 (13), 175 (5), 203 (44), 218 (100), 408 (2), 411 (5), 426 (M^+, 9); retention time: 53.3 min.

Methyl Ester of Palmitic Acid: $C_{17}H_{34}O_2$, M.W. 270, *m/z* (%): 43 (31), 59 (7), 74 (100), 87 (60), 143 (14), 199 (4), 227 (8), 239 (6), 241 (2), 270 (M^+, 13); retention time: 10.5 min.

Methyl Ester of Linolenic Acid: $C_{19}H_{32}O_2$; $\Delta^{9,12,15}$, M.W. 292, *m/z* (%): 55 (57), 67 (70), 74 (12), 79 (100), 87 (16), 93 (50), 95 (56), 107 (24), 121 (16), 236 (4), 263 (3), 292 (M^+, 5); retention time: 14.1 min.

Methyl Ester of Linoleic Acid: $C_{19}H_{34}O_2$; $\Delta^{9,12}$, M.W. 294, *m/z* (%): 55 (69), 59 (15), 67 (100), 74 (10), 81 (85), 87 (10), 95 (56), 109 (34), 220 (2), 263 (10), 294 (M^+, 13); retention time: 13.9 min.

Methyl Ester of Oleic Acid: $C_{19}H_{36}O_2$; Δ^9, M.W. 296, *m/z* (%): 41 (88), 55 (100), 59 (20), 69 (58), 74 (54), 83 (50), 87 (47), 97 (48), 222 (12), 264 (16), 265 (12), 296 (M^+, 6); retention time: 14.1 min.

Phytol: $C_{20}H_{40}O$, M.W. 296, *m/z* (%): 43 (53), 71 (100), 81 (28), 123 (26), 278 (3), 296 (M^+, 2), retention time 29.7 min.

General Pharmacological Procedures The methodology used is carefully described elsewhere.[3b] Briefly, the crude hexane extract and fractions of the leaves and stem from *A. salutaris* were assayed on guinea pig cell suspensions containing mast cells obtained by enzymatic dispersion with collagenase type IA. The extracts were dissolved in dimethylsulfoxide (final concentration in the samples ≤0.2%) and then diluted in H_2O. The histamine release inducers (antigen and ionophore A23187) were added to the cell suspensions after preincubation with the extracts. The percentage of histamine release was calculated after the fluorometric assay through an automated apparatus (Technicon Autoanalyser II).

References

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2) Correa M.P., "Dicionário das plantas úteis do Brasil e das exóticas cultivadas," IBDF, Rio de Janeiro, 1984, pp. 303—304.
3) *a*) Gomes J. C., *Agents and Actions* **36** (suppl.): 87—95 (1992); *b*) Gomes J. C., Di Stasi L. C., Sgarbosa F., Barata L. E. S., *Int. Arch. Allergy Immunol.* **103**, 188—193 (1994).

4) Caldicott A. B., Eglinton G., "Phytochemistry," ed. by Miller L. P., V.N.R. Comp., New York, 1973, pp. 162—194.
5) Kolattukudy P. E., *Phytochemistry* **6**, 963—975 (1967).
6) Peisker C., Duggelin T., Rentsch D., Matile P., *J. Plant. Physiol.*, **135**, 428—432 (1989).
7) Kobayashi M., Asai M., Ishiguro T., Kishi T., Bian R., Xie Q., *Shoyakugaku Zasshi*, **43**, 230—234 (1989).
8) Houghton P. J., Zarka R., Heras B., Hout J. R. S., *Planta Med.*, **61**, 33—36 (1995).
9) Yamamoto S., *Free Rad. Biol. Med.*, **10**, 149—159 (1991).
10) Van-Haasten C. M. C. J., Engels W., Lemmens P. J. M. R., Hornstra G., Van-der-Vusse G. J., *Am. J. Clin. Nutr.*, **57**, 828S (1993).
11) Ferri P. H. "Química de Produtos Naturais: Métodos básicos," ed. by

Di Stasi L. C., Fundação Editora Unesp, São Paulo, 1996, pp. 129—156.
12) Ryhage R., Stenhagen E. J., *J. Lipid Res.*, **1**, 361—390 (1960).
13) Budzikiewicz H., Busker E., *Tetrahedron*, **36**, 255—266 (1980).
14) Halgreen B., Ryhage R., Stenhagen E., *Acta Chem. Scand.*, **13**, 845—847 (1959).
15) Budzikiewicz H., Wilson J. M., Djerassi C., *J. Am. Chem. Soc.*, **85**, 3688—3699 (1963).
16) Shannon J. S., McDonald C. G., Courtney J. L., *Tetrahedron Lett.*, **1963**, 173—179.
17) Djerassi C., Budzikiewicz H., Wilson J. M., *Tetrahedron Lett.*, **1962**, 263—270.