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Full Length Research Paper

Intraspecific variability of dihydrochalcone, chromenes and benzoic acid derivatives in leaves of *Piper aduncum* L. (Piperaceae)

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Chemical analysis carried out in leaves of 18 specimens of *Piper aduncum* L. (Piperaceae) occurring at Ripasa Reserve, Araraquara, SP, Brazil indicated two distinct populations when investigated over a period of 14 months (January 2000 to February 2001) and then submitted to cluster analysis. The two groups were characterized by accumulation of prenylated benzoic acids, chromenes and dihydrochalcone, respectively. A total of seven compounds were identified by HPLC analysis and compared with standards including two prenylated benzoic acid [aduncumene (1) and 3-(3'-7'-dimethyl-2'-6'-octadienyl)-4-methoxy-benzoic acid (5)], four chromenes [methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (4), methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (2b), methyl 8-hydroxy-2,2-dimethyl-2*H*-1-chromene-6-carboxylate (3) and 2,2-dimethyl-2*H*-1-chromene-6-carboxylic acid (2a)] and one dihydrochalcone [2',6'-dihydroxy-4'-methoxy-dihydrochalcone (6)].

Key words: Instraspecific variability, *Piper aduncum*, chemotypes and HPLC analysis.

INTRODUCTION

Piper aduncum is the most widespread Piperaceae species in the world being encountered from Mexico to Argentina. The species, a shrub or slender tree, can be commonly found in secondary vegetation and it has been considered an aggressive alien in Southeast Asia and the Pacific. *P. aduncum* grows in humid areas and it is moderately intolerant to shade. It can survive and grow slowly under a moderate under story but requires at least partial exposure to light in order to develop and to produce flowers. Phenological pattern is of the continuous type producing flowers and fruit throughout the year. The pollination involves wind and small insects, mainly Diptera and their seeds are reported to be dispersed by bats, birds and possibly by arboreal rodents (Figueiredo et al., 2000; Orjala et al., 1993).

Chemical studies carried out so far on *P. aduncum* described one amide, eight phenylpropanoids, twelve terpenes, eight chalcones and dihydrochalcones, two flavones and further compounds including benzoic acid derivatives and chromenes. Major biological activities include insecticidal, antibacterial, molluscicidal, antitumoral, antifungal, growth inhibition of *Leishmania amazonensis* and anti-trypanocidal activity (Batista et al., 2008; Lago et al., 2004; Baldoqui et al., 1999; Torres-Santos et al., 1999; Moreira et al., 1998; Parmar et al., 1998; Parmar et al., 1997; Okunade et al., 1997; Orjala et al., 1993; Nair et al., 1986; Smith et al., 1979).

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In previous work dealing with the chemical composition of *P. aduncum*, we have described the occurrence of chromenes with antitumoral, antifungal and anti-trypanocidal activities [2,2-dimethyl-2*H*-1-chromene-6-carboxylic acid (2a), methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (2b), methyl 8-hydroxy-2,2-dimethyl-2*H*-1-chromene-6-carboxylate (3), methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (4)] and prenylated benzoic acid derivatives [aduncumene (1), 3-(3'-7'-dimethyl-2'-6'-octadienyl)-4-methoxy-benzoic acid (5)] with antifungal and antitumoral activities, respectively. The antifungal activity was investigated against *Cladosporium cladosporioides* and *Cladosporium sphaerospermum* and the antitumoral activity was investigated against mutant strains of *Saccharomyces cerevisiae* which indicated moderate antitumoral potential (Batista et al., 2008; Lago et al., 2004; Baldoqui et al., 1999).

The quantitative variation of six kavalactones and eight isoenzymes in 121 cultivars of *Piper methysticum* from 51 different islands of the Pacific revealed the occurrence of six chemotypes, which are not affected by environmental factors but genetically controlled (Telascrea et al., 2007; Barazani et al., 2002; Lebot et al., 1996).

In case of *P. aduncum*, previous study was addressed the determine circadian changes in which maxim of both chromene content and prenyltransferase were detected at dawn of the day indicating the biosynthetic process to be light activated (Morandim et al., 2005). In spite of the widespread occurrence of *P. aduncum* in the tropics and to the occurrence of biological activities ascribed to chromenes there is no data regarding characterization of populations or possible seasonal changes in secondary metabolites content. Herein, we describe the chemical variability within a natural population of 18 specimens of *P. aduncum* by monitoring the quantitative variation of seven compounds including, chromenes, prenylated benzoic acid derivatives and one dihydrochalcone.

MATERIALS AND METHODS

Reagents and standards

Acetonitrile (HPLC grade), methylene chloride and methanol from Mallinckrodt (Baker, Xalostoc, Mexico) were used. Nanopure water (>18 MOhm) was produced using a Millipore (Bedford, MA, USA) purifier. The following list of standard compounds were isolated from *P. aduncum* using previously described methods: aduncumene (1) and 3-(3'-7'-dimethyl-2'-6'-octadienyl)-4-methoxy-benzoic acid (5), four chromenes [2,2-dimethyl-2*H*-1-chromene-6-carboxylic acid (2a), methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (2b), methyl 8-hydroxy-2,2-dimethyl-2*H*-1-chromene-6-carboxylate (3) and methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (4)] and one dihydrochalcone [2',6'-dihydroxy-4'-methoxy-dihydrochalcone (6)] (Lago et al., 2004; Baldoqui et al., 1999).

Plant material

Leaves from 18 specimens of *P. aduncun* L. (Piperaceae) were collected at Fazenda Fortaleza of Ripasa, Araraquara – SP, Brazil. The specimens were identified by Dr Inês Cordeiro (Instituto de Botânica, SP, Brazil). Vouchers PA0, PA2, PA3, PA4, PA5, PA6, PA7, PA9, PA11, PA12, PA13, AP16, PA17, PA18, PA19, PA20, PA21 and PA22 were deposited at Herbário do Estado "Maria Eneyda P. Kaufmann Fidalgo" (HBSP). The samples were collected from plants growing in two habitats: nine specimens were located at a stational semidecidual forest – denominated "Hill" – and another set with nine specimens were located in a gallery forest along the Anhumas creek – herein denominated "River". These two areas were 500 m apart.

HPLC analyses and identification of compounds from the leaves of *P. aduncum*

A Varian (Palo Alto, CA, USA) ProStar liquid chromatographic system, consisting of a model 410 auto-sampler, a model 240 quaternary pump coupled to a Varian Microsorb column (C-18, 250 x 4.6 mm i.d.; 5 μm) and pre-column (20 x 4 mm i.d.; 5 μm), and a model 230 UV - visible photodiode array detector (PAD) were employed. Eluent consisted of water containing 0.1% acetic acid (solvent A) and acetonitrile (solvent B) in a linear gradient from 65:35 (A:B) to 20:80 in 20 min followed by isocratic elution with 20:80 for a further 15 min. The flow rate was 0.7 $mlmin^{-1}$, and the total analytical run time was 35 min. All solvents and samples were filtered through a 0.2 μm nylon membrane before analysis. Solutions of extracts (1 $mg.ml^{-1}$, 30 μl aliquots) were applied to the column by automatic injector, and the column effluent was monitored at 270 nm. The samples were analyzed in duplicate. Fourteen peaks were monitored: four chromenes (2a, 2b, 3 and 4), one dihydrochalcone (6), two prenylated benzoic acid derivatives (1 and 5) and seven unidentified compounds. The identification of the compounds was based on their retention time, UV spectra and by comparing with authentic standards previously obtained from the leaves of *P. aduncum* (Lago et al., 2004; Baldoqui et al., 1999).

Statistical analysis

In order to compare the overall similarity between the seven major compounds in *P. aduncum*, all extracts were analyzed by HPLC and the retention time of peaks were compared to the standards and the corresponding UV spectra. The average between area data of the peaks of the each sample were evaluated by analyzing groups in terms of their similarity according to Ward's clustering method using Pearson's correlation to measure linkage distances (Kube et al., 2007; Laitiner et al., 2000)

The box-chart graph depicts the maximum/minimum concentration of the seven major compounds over the 14 months of this research (Kube et al., 2007).

Sample preparation and extraction

Leaves (two or three) from each of the 18 specimens of *P. aduncum* were collected from 8.00 to 10.00 am on a monthly basis from January 2000 to February 2001. The leaf surfaces were cleaned mechanically with a cloth and frozen in liquid N_2 and kept in dry ice for transportation. Then the material was freeze-dried for 24 h.

In a centrifuge tube, 100 mg of dried and ground leaves were extracted with 5 ml of methylene chloride with sonication (20 min). The tube was centrifuged for 10 min (2800 rpm), an aliquot (3 ml) was taken, evaporated under reduced pressure, dissolved in 3 ml of MeOH:H_2O (95:5), submitted to solid phase extraction in a C18 cartridge, and finally eluted with 3 ml of MeOH:H_2O (95:5). The eluted

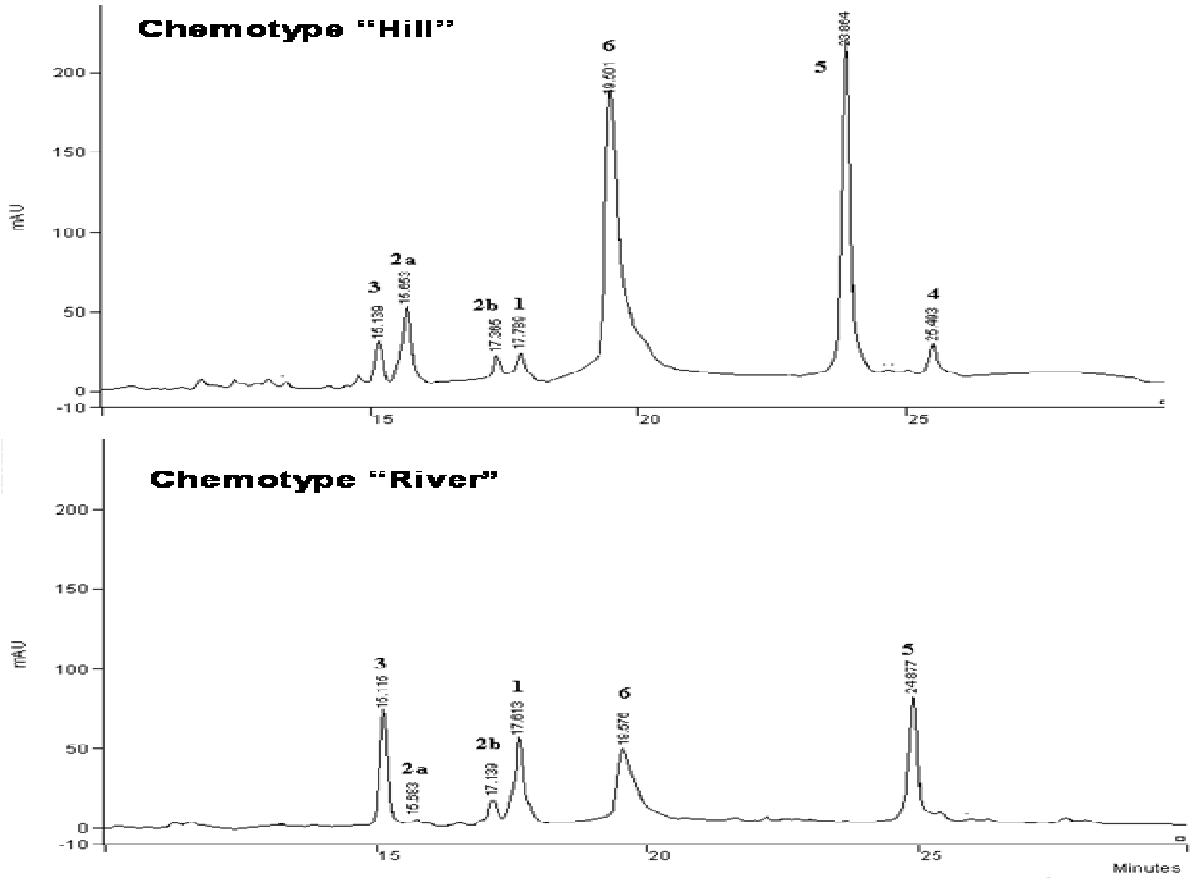

Figure 1. Typical HPLC chromatogram of *P. aduncum* leaf extracts for chemotypes "Hill" and "River".

volume was dried under reduced pressure and dissolved in 2 ml of MeOH and analyzed using a Varian ProStar HPLC unit with a photodiode-array detector. Every extract was prepared in duplicate and analyzed by HPLC using external standard.

RESULTS AND DISCUSSION

The analysis of the secondary metabolite composition in a natural population of *P. aduncum* has shown the presence of dihydrochalcone, chromenes, and prenylated benzoic acids as major compounds in its leaves. A total of seven compounds were characterized as two prenylated benzoic acids [aduncumene (1) and 3-(3'-7'-dimethyl-2'-6'-octadienyl)-4-methoxy-benzoic acid (5)], four chromenes [2,2-dimethyl-2*H*-1-chromene-6-carboxylic acid (2a), methyl 2,2-dimethyl-2*H*-1-chromene-6-carboxylate (2b), methyl 8-hydroxy-2,2-dimethyl-2*H*-1-chromene-6-carboxylate (3), methyl 2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2*H*-1-chromene-6-carboxylate (4)] and one dihydrochalcone [2',6'-dihydroxy-4'-methoxy-dihydrochalcone (6)] (Figure 1). This profile was determined by the isolation of each compound via HPLC analysis and also by comparison with data in literature (Figure 1) (Lago et al., 2004; Baldoqui et al., 1999, Parmar et al., 1997).

The chemical compositions and concentration of all constituents from leaves of *P. aduncum* were monitored from early January 2006 through the end of February 2007 in order to determine the extension of seasonal changes on intraspecific variability (Figure 2). Since the composition observed during June 2006 was representative of such a profile, a cluster analysis using Pearson's correlation was carried out and then two major chemotypes (50% each) denominated "Hill" and "River" characterized specimens of *P. aduncum* (Figure 3). Flavanone 6 was ubiquitous in all specimens and the chemotype "River" was characterized by a relatively high content of prenylated benzoic acid 1 and 5, followed by chromene 3, while in case of chemotype "Hill" the geranylated benzoic acid 5 was the major compound along with flavanone 6. The prenylated chromene 4 in one chemotype (Hill) confirmed the occurrence of intraspecific variability in this species.

The production of all compounds occurring in specimens of *P. aduncum* was rationalized by a biosynthetic point of view (Figure 4). All compounds are derived from

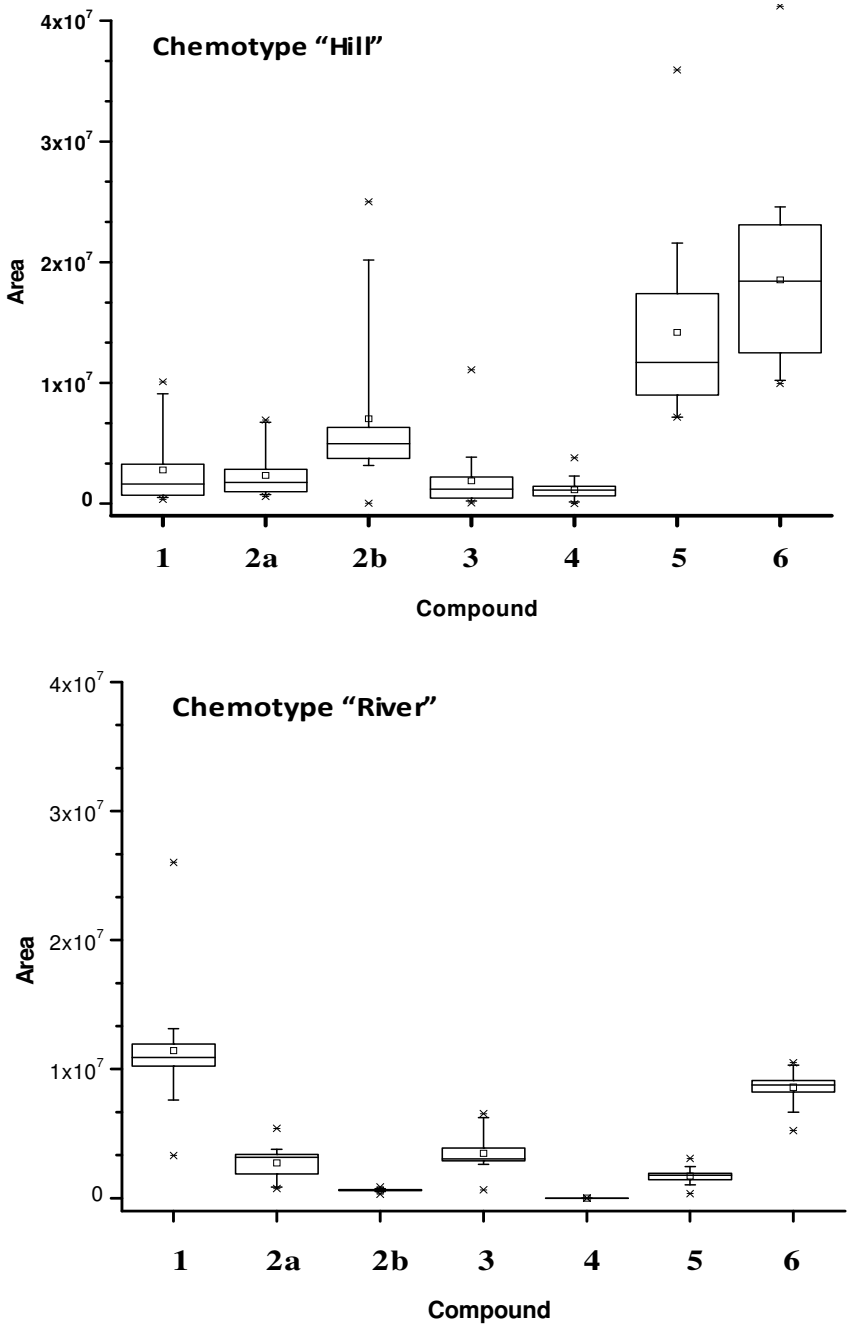

Figure 2. Box diagram indicating the variation in the concentration of the compounds identified for the chemotypes "Hill" and "River" of *P. aduncum*, over the period of January 2000 to February 2001.

prenylated benzoic acid at position C-3 or prenylated protocatechuic acid at position C-5. The *p*-hydroxybenzoic acid could be prenylated yielding the putative intermediate i, then stabilized by methylation of the C-4 hydroxyl and oxidized at the benzylic position yielding 1. The intermediate i can also be converted to 2a/2b. The *p*-hydroxybenzoic acid can be geranylated to 5 in the case of the specimens of chemotype "Hill"; and additionally, a second prenylation at position C-5 of chromene 2a can take place yielding 3. The specimens of chemotype "River" also contain chromene 3, which requires the protocatechuic acid as the precursor or the hydroxylation of 2a. The geranylated derivative of benzoic acid (5) reached its highest concentration in the

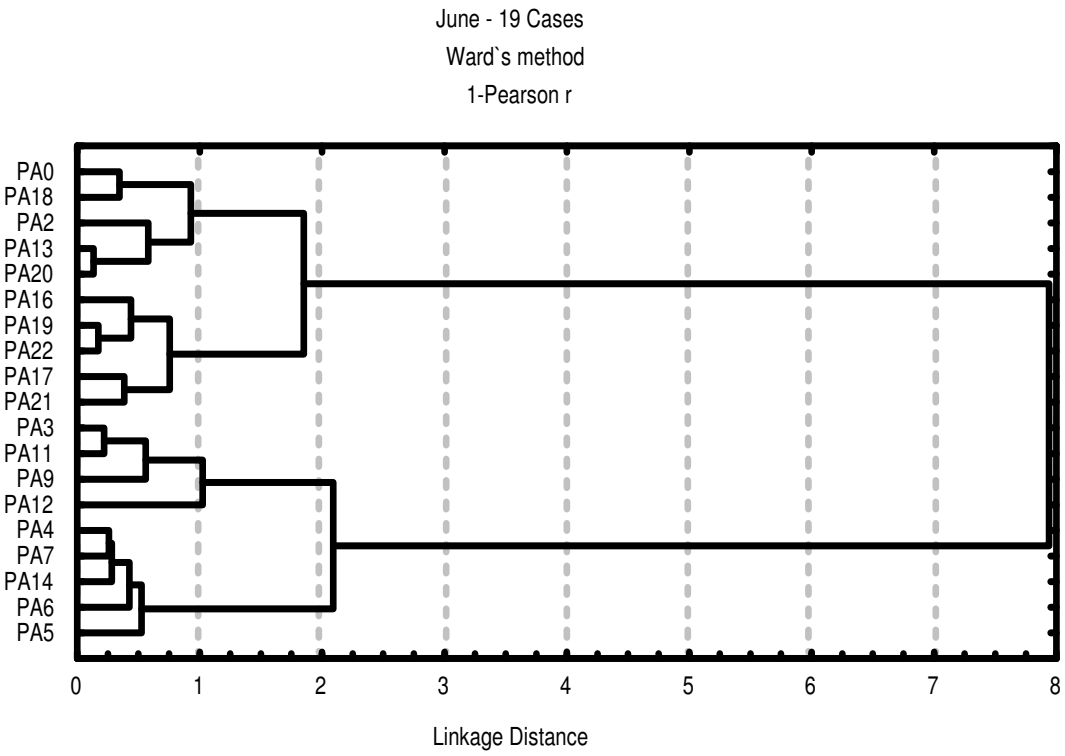

Figure 3. Cluster-analysis dendrogram of the specimens of *P. aduncum* collected in June of 2000.

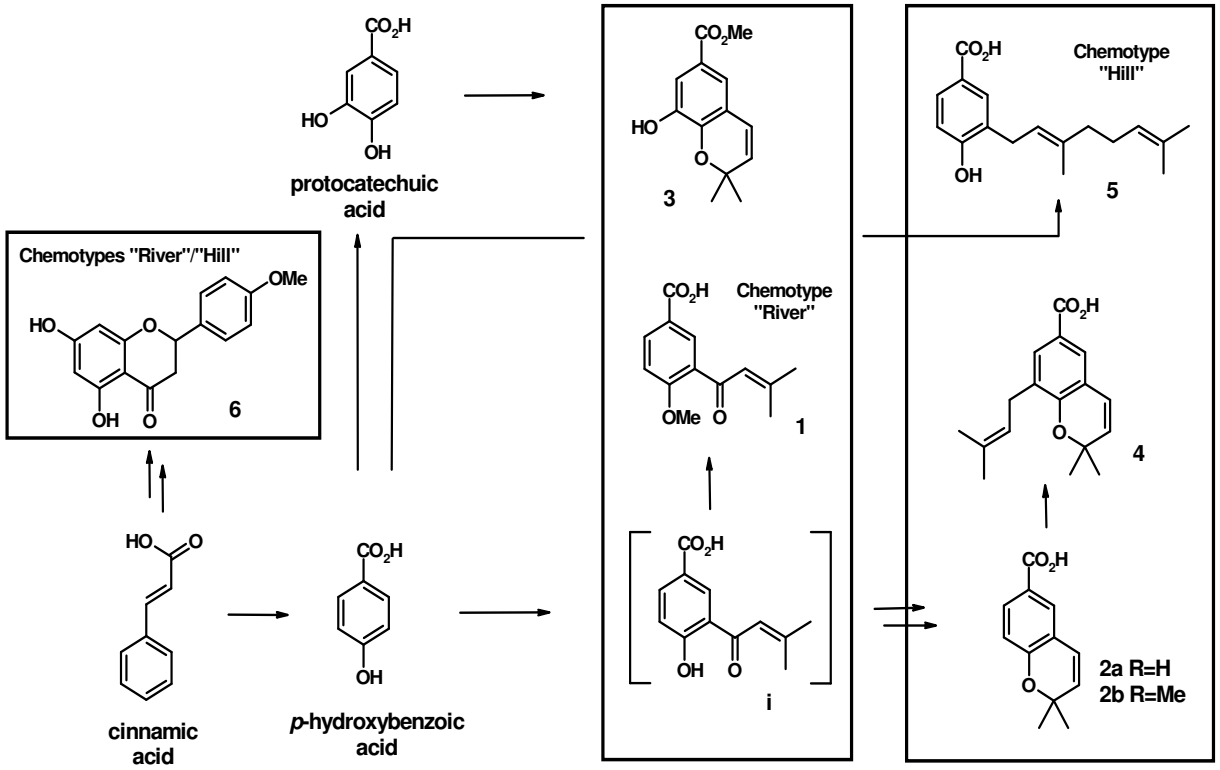

Figure 4. Biosynthetic relationship between major secondary metabolites in leaves of *P. aduncum*.

chemotype “Hill” possibly as a result of specific action of a geranyltransferase in the specimens of this group, while in the chemotype “River”, the major enzymatic activity is involved in the prenylation reaction (Morandim et al., 2005).

The two chemotypes, characterized by different secondary metabolites associated with distinct environmental conditions may have resulted from influences of the habitat on the composition and enzymatic activity within the leaves of *P. aduncum*. Further investigations regarding the regulation or partitioning of either geranyl or prenyltransferase activity should be carried out.

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REFERENCES

Baldoqui DC, Kato MJ, Cavalheiro AJ, Bolzani VS, Young MCM, Furlan M (1999) A chromene and prenylated benzoic acid from Piper aduncum. Phytochemistry, 51: 899-902.

Barazani O, Cohen Y, Fait A, Diminshtein S, Dudai N, Ravid U, Putievsky E, Friedman J (2002). Chemotypic differentiation in indigenous populations of Foeniculum vulgare var. vulgare in Israel. Biochem. Syst. Ecol., 30: 721-731.

Batista Jr JMB, Lopes AA, Ambrósio DL, Regasini LO, Kato MJ, Bolzani VS, Cicarelli RM, Furlan M (2008). Natural Chromenes and Chromene Derivatives as Potential Anti-trypanosomal Agents. Biol. Pharm. Bull. 31: 538-540.

Figueiredo RA, Sazima M (2000). Pollination biology of Piperaceae species in southeastern Brazil. Ann. Bot., 85: 455-460.

Kube S, Sokolowski A, Jansen JM, Schiedek D (2007). Seasonal variability of free amino acids in two marine bivalves, Macoma balthica and Mytilus spp., in relation to environmental and physiological factors. Comparative Biochemistry and Physiology - Part A: Mol. Integr. Physiol., 147: 1015-1027.

Lago JHG, Ramos CS, Casanova DCC, Morandim AA, Bergamo DCB, Cavalheiro AJ, Bolzani VS, Furlan M, Guilharães EF, Young MCM, Kato MJ (2004). Benzoic acid derivatives from Piper species and their fungitoxic activity against Cladosporium cladosporioides and C. sphaerospermum. J. Nat. Prod. 67: 1783-1788.

Laitiner ML, Julkunen-Tiitoo R, Rousi M (2000). Variation in Phenolic Compounds within a Birch (Betula pendula) Population. J. Chem. Ecol., 26: 1609-1622.

Lebot V, Levesque J (1996). Genetic control of kavalactone chemotypes in Piper methysticum cultivars. Phytochemistry, 43: 397-403.

Morandim AA, Bergamo DCB, Kato MJ, Cavalheiro AJ, Bolzani VS, Furlan M (2005). Circadian rhythm of anti-fungal prenylated chromene in leaves of Piper aduncum. Phytochem. Anal. 16: 282-286.

Moreira DL, Guimarães EF, Kaplan MAC (1998). A chromene from *Piper aduncum*. Phytochemistry, 48: 1075-1977.

Nair MG, Mansingh AP, Burke BA (1986). Insecticidal properties of some metabolites of Jamaican Piper spp., and the amides synthesized from 5,6-Z and E-butenolides of Piper fadyenii. Agric. Biol. Chem., 50: 3053-3058.

Okunade AL, Hufford CD, Clark AM, Lentz D (1997). Antimicrobial Properties of the Constituents of Piper aduncum. Phytotherapy Res. 11: 142-144.

Orjala J, Erdelmeier CAJ, Wright AD, Rali T, Sticher O (1993). Five new prenylated p-hydroxybenzoic acid derivatives with antimicrobial and molluscicidal activity from Piper aduncum leaves. Plant Med. 59: 546-551.

Parmar VS, Jain SC, Bisht KS, Jain R, Gupta S, Talwar S, Rajwanshi VK, Kumar R, Azim A, Malhotra S, Kumar N, Jain R, Sharma NK, Jha A, Tyagi OD, Lawrie SJ, Errington W, Howarth OW, Olsen CE, Singh SK, Wengel J (1998). Polyphenols and alkaloids from Piper species. Phytochemistry 49: 1069-1078.

Parmar VS, Jain SC, Bisht KS, Jain R, Taneja P, Jha A, Tyagi OD, Prasad AK, Wengel J, Olsen CE, Boll PM (1997). Phytochemistry of the genus Piper. Phytochemistry, 46: 597-673.

Smith RM, Kassim HNZJ (1979). The essential oil of Piper aduncum from Fiji. Sci. 22: 127-128.

Telascrea M, de Araújo CC, Marques MOM, Facanali R, Moraes PLR, Cavalheiro AJ (2007). Essential oil from leaves of Cryptocarya mandioccana Meisner (Lauraceae): Composition and intraspecific chemical variability. Biochem. Syst. Ecol. 35: 222-232.

Torres-Santos EC, Moreira DL, Kaplan MAC (1999). Selective effect of 2',6'-dihydroxy-4'-methoxychalcone isolated from Piper aduncum on Leishmania amazonensis. Antimicrob. Agents Chemother., 43: 1234-1241.