

Chemical Composition of *Tipuana tipu*, a Source for Tropical Honey Bee Products

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Tipuana tipu (Benth.) Kuntze is a tree from the leguminosae family (Papilionoideae) indigenous in Argentina and extensively used in urbanism, mainly in Southern Brazil. The epicuticular waxes of leaves and branch, and flower surface were studied by high temperature high resolution gas chromatography. Several compounds were characterized, among which the aliphatic alcohols were predominant in branch, leaves and receptacle. Alkanes were predominant only in the petals and the aliphatic acids were predominant in stamen. In branches and leaf epicuticular surfaces, six long chain wax esters series were characterized, as well as lupeol and b-amyrin hexadecanoates.

Key words: Epicuticular Wax, *Tipuana tipu*, High Temperature Gas Chromatography

Introduction

Tipuana tipu (Benth.) Kuntze is a tree from the leguminosae family (Papilionoideae) indigenous in Argentina and extensively used in urbanism, mainly in Southern Brazil. It can be used as a supplementary food to small ruminants (*e.g.* goats), mainly the dried leaves proved to have high nutritive value (Norton and Waterfall, 2000). This plant is visited by numerous insects, mainly due to its pollen, including Brazilian bees (*e.g.* *Tetragonisca angustula*, *Trigona spinipes*, *Paratrigona subnuda*, *Plebeia droryana*, and *Plebeia remota*) and the European bee (*Apis mellifera*) (Pirani and Cortopassi-Laurino, 1994).

Several tropical bees produce honeys more appreciated than the one of *Apis mellifera*. Some of them are used for therapeutic purposes in popular medicine. A Brazilian honey that is very much appreciated for therapeutic use is the commonly known Jataí honey (produced by *Tetragonisca angustula*). Propolis (CAS No. 9009-62-5) is another bee product, often used in popular medicine in several parts of the world and the variations in its chemical composition and biological activity has been attributed to the floral origin and bee species (Bankova *et al.*, 2000).

Therefore, the composition of the epicuticular surface from the flower, branch and leaf of *Tipuana tipu*, by high temperature high resolution gas chromatography coupled to mass spectrometry

(HT-HRGC-MS), was evaluated with the aim to improve our understanding of the interspecific relationship between plants and bees. The choice for the use of HT-HRGC-MS is because this methodology permits a fast and thorough evaluation of molecular composition, since it is possible to characterize low as well as high molecular weight compounds in the same analysis (Pereira and Aquino Neto, 1999).

Experimental

Material

The *Tipuana tipu* (Benth.) Kuntze parts were collected in October 2001 in Jundiaí (São Paulo State, Brazil) during the pollination period.

Plant extracts

The flowers were carefully divided in petal, receptacle, anther, and filament. The surfaces of these parts as well as epicuticular waxes of the branches and leaves were separately extracted. The samples (3 g with the exception of anther which was only 0.9 g) were extracted sequentially three times each with 20 ml of dichloromethane. The extractions were performed using ultrasonic agitation for 30 min at room temperature. The combined extracts for each solvent were concentrated under vacuum, and the resulting crude extracts were analyzed by HT-HRGC.

Crude extracts were weighed after solvent removal under vacuum and drying in vacuum desiccators with P_2O_5 , and gave values of 210 mg (7.0%; petal); 170 mg (5.7%; receptacle); 40 mg (4.4%; anther); 90 mg (3.0%; filament); 95 mg (3.2%; branch) and 120 mg (4.0%; leaf).

Gas chromatography

An on-column injector (Carlo Erba, Rodano, Italy) was mounted on a Hewlett-Packard (Palo Alto, USA) model 5890-II gas chromatograph. Fused silica capillary columns (15 m $\times$ 0.25 mm *i. d.*; J. & W., Folsom, CA, USA) coated with 0.2 μ m of DB-5HT (5%-phenyl-95%-methylpolysiloxane).

The column temperature was maintained at 40 °C during injection, then programmed at 10 °C/min to 390 °C and held for 10 min. The flame ionization detector (FID) and the on-column injector were operated at 400 °C and room temperature, respectively. Hydrogen was used as carrier gas at a linear velocity of 50 cm/s and the sample volume injected was 0.5 μ l. GC data were acquired and processed with a HP 3396-II integrator.

Mass spectrometry conditions

HTHRGC-MS analyses were carried out on a HP 5973 MSD spectrometer (Hewlett Packard,

Palo Alto, USA), under electron impact ionization (70 eV). The GC operating conditions were as described above. The on-column injector and the transfer line temperatures were set to 40 °C and 350 °C, respectively, and the ion source temperature to 300 °C (MS scan range was 40 to 800 Da). Helium was used as carrier gas at a linear velocity of 38 cm/s.

Results and Discussion

Hydrocarbons

All plant parts studied showed n-alkanes from 23 up to 31 carbons, with odd carbon number predominance and a maximum at C_{29} . This is the usual distribution of the plant wax alkanes fraction (Kolattukudy, 1969), however a great variation of the relative concentration of the total n-alkanes was observed according to plant part (Fig. 1), as a significant variation of the alkane weight distribution (Fig. 2). For example in the dichloromethane extract of epicuticular waxes of the branches, the n-alkanes amount represent only 0.2%, however for the petals surface it reached 50% (Fig. 1), with nonacosane responsible for 26.4% of the total chromatogram area. Squalene was detected in epi-

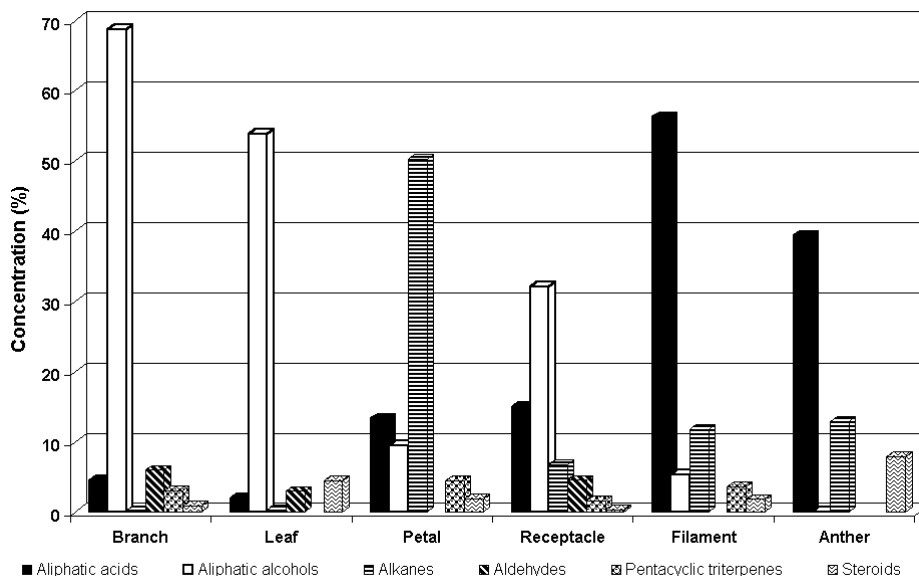


Fig. 1. Chemical distribution of main constituents from epicuticular waxes of branch and leaves, and surface of the petal, receptacle, anther and filament of the *Tipuana tipu* (Benth.) Kuntze obtained by dichloromethane ultrasonic extraction.

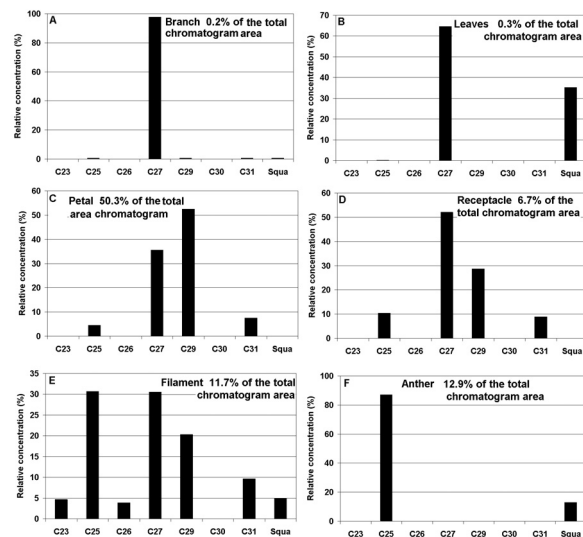


Fig. 2. Alkanes relative distribution from epicuticular waxes of branch (A) and leaves (B), and surface of the petal (C), receptacle (D), filament (E) and anther (F) of the *Tipuana tipu* (Benth.) Kuntze obtained by dichloromethane ultrasonic extraction. Squa = squalene.

cuticular wax of the leaves, anther and filament epicuticular surfaces.

The high concentration of the alkanes in the surface of petals (50%) could be due to a variant in alkane biosynthesis; normally it is accepted that the alkanes are formed by a classical mechanism, of head-to-head condensation type, however there is a second pathway by an elongation-decarboxylation mechanism, present, *e.g.*, in leaves of *Brassica oleracea* (Kolattukudy *et al.*, 1972). The biosynthesis variant can justify the highest relation alkane/acid ratio (3.8 based in the chromatogram areas) found in petals surface, while for other plant parts they were: 0.04 (branch), 0.16 (leaf), 0.45 (receptacle), 0.21 (filament) and 0.33 (anther), Fig. 1.

Alcohols

Alcohols were characterized in all plant parts studied, however in the anther they were characterized in trace amounts (Fig. 1). They were predominant in the receptacle surface and epicuticular waxes of the leaves and mainly in the branch, with 68.7% of the total chromatogram area (Fig. 1) with a distribution from octadecanol up to

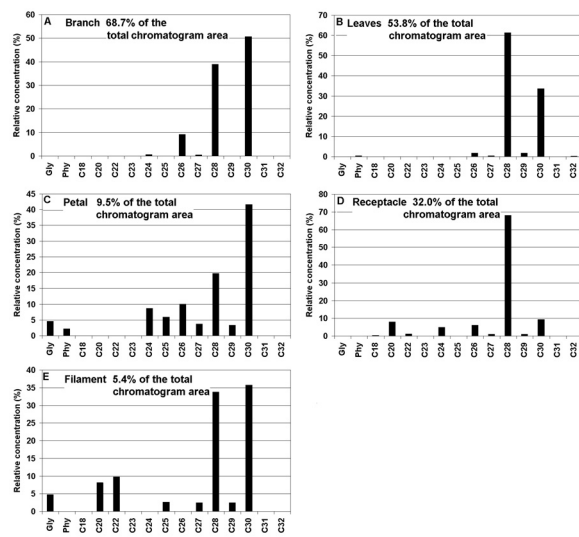


Fig. 3. Alcohols relative distribution from epicuticular waxes of branch (A) and leaves (B), and surface of the petal (C), receptacle (D) and filament (E) of the *Tipuana tipu* (Benth.) Kuntze obtained by dichloromethane ultrasonic extraction. Gly = glycerol and Phy = phytol.

dotriacontanol (Fig. 3), with even carbon number predominance and maxima at octacosanol or triacontanol (50.8% and 26.6%, respectively, in leaf and branch chromatogram areas). The fatty alcohols are derived from the reduction of acids, the acyl-reduction pathway produces aldehydes, primary alcohols, and wax esters derived from the esterification of fatty acids and primary alcohols (Bianchi *et al.*, 1985).

Phytol was characterized only in epicuticular wax of the leaf and petal surface and glycerol in petal and filament surfaces.

Aldehydes

These compounds were characterized only in the branch, leaf epicuticular waxes and mainly in receptacle surfaces (anther and filament), with a relative concentration of 4.5% (Fig. 1) with a distribution from tetracosanal up to dotriacontanal. Maxima were octacosanal (2.6%) for receptacle and triacontanal, 3.4% and 3.5%, respectively in branch and leaf chromatograms areas.

The slight accumulation of aldehydes, mainly in epicuticular waxes of leaves and branches (Fig. 1), could be due to the two steps reduction

of the fatty acids catalyzed by two separate enzymes. This mechanism was reported in several plants, as for example, in *Brassica oleracea* leaf (Kolattukudy, 1971; Bianchi *et al.*, 1985), when the two steps of the fatty acids reduction to primary alcohols are catalyzed by the same enzyme, the intermediate aldehydes do not accumulate (Pollard *et al.*, 1979).

Acids

All plant parts studied showed n-acids from 9 up to 30 carbons, with odd carbon number predominance and a maximum at hexadecanoic acid, with exception of the branches epicuticular waxes, with maximum at octaeicosanoic acid (Fig. 4). However a great variation of the relative concentration of the total alkyl acids was observed according to plant part (Fig. 1), these compounds were predominant in the stamen surface (in anther and filament). They represent as much as 56% of filament surface extract (Fig. 1) with a distribution from nonanoic up to triacontanoic acid, and maxima at hexadecanoic acid (27.6% and 26.5% for anther and filament chromatograms areas, respectively).

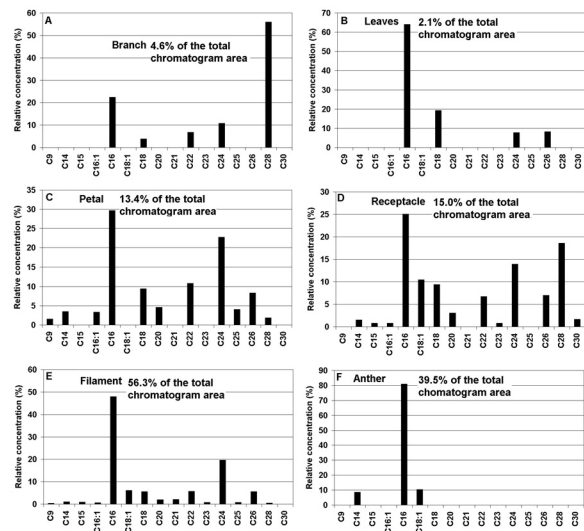


Fig. 4. Alkyl acids relative distribution from epicuticular waxes of branch (A) and leaves (B), and surface of the petal (C), receptacle (D), filament (E) and anther (F) of the *Tipuana tipu* (Benth.) Kuntze obtained by dichloromethane ultrasonic extraction.

Esters

In filament only it was characterized a series of ethyl esters between hexadecanoic ethyl ester to heptacosanoic ethyl ester with maxima at the hexadecanoic ethyl ester; only this compound represents 0.6% of the total chromatogram area.

The ethyl alkyl esters characterized in filament has a similar distribution to the alkyl acids found also in filament (Fig. 1), however in anther methyl and/or ethyl esters were not characterized, which shows the specificity of these compounds in relation to the different parts of *Tipuana tipu*. Similar results were observed for the monoacylglycerols of hexadecanoic and octadecanoic acids, which were characterized in trace amounts in epicuticular waxes of the branch and leaves. In petals surface they are much more abundant: 2.3% and 2.8% of monoglycerols of hexadecanoic and octadecanoic acids, respectively. The triacylglycerols were not characterized in any extract, however the glyceril-1,2-dioleate-3-palmitate is a known brood pheromone for *Apis mellifera* (Koeniger and Veith, 1983).

In epicuticular waxes of the branch and leaves, six long chain wax esters series were characterized in trace amounts, from hexadecanoic acid (base peak m/z 257) to hexacosanoic acid (base peak m/z 397), the more abundant series being the eicosanoic acid one.

Fragmentation of long chain wax esters, gives as base peak a double rearrangement fragmentation of the ester group. The fragments ($C_nH_{2n-1}O_2H_2^+$) are formed by transfer of two hydrogen atoms to the acyl moiety, giving rise to protonated ionic species with a m/z equal to the acid + 1; this is a variation of the McLafferty rearrangement (Gülz *et al.*, 1994 and Reiter *et al.*, 1999).

More than 30 long chain wax esters were characterized forming a complex mixture of homologous series ranging from C_{40} to C_{56} . This composition is consistent with the distribution of the long chain wax esters (C_{36} to C_{54}) found in epicuticular waxes of leaves and fruits of many angiosperm species. There are good evidence supporting the hypothesis that the long chain wax esters of epicuticular waxes play an essential role in the epicuticular transport barrier that hinders the diffusion of water and solutes across the plant cuticle (Gülz *et al.*, 1994). On the other hand, these compounds

show nematocidal activity (especially against the root-knot nematode *Meloidogyne incognita*), causing paralysis and death of the juveniles (Nogueira *et al.*, 1996).

Triterpenes and triterpenyl alkanoates

Three pentacyclic triterpenes were characterized in the epicuticular wax of the branch and surface of the petal, receptacle and filament: β -amyrin, lupeol and lupenone.

In branch epicuticular wax and petal surface the proportion of β -amyrin and lupeol was 1:1 and between 1 to 2% of the total chromatogram area, however in filament surface the lupeol was found in 4.4% and β -amyrin in trace amounts, and lupeol was found in trace amount and β -amyrin in 1.5%. Lupenone only was found in branch epicuticular wax.

In the branch, receptacle and filament, lupeol and β -amyrin hexadecanoate were also characterized in trace amounts. β -amyrin alkanoates were previously characterized in leaves of *Simaruba amara* and *Bertholletia excelsa* (Siqueira *et al.*, 2000) while *Croton* species (Carbonell *et al.*, 2000) containing lupeol and its alkanoates were recently characterized in a Brazilian propolis (Pereira *et al.*, 2002). Despite their relatively complex structures, the mass spectra of triterpenyl fatty acid esters are quite simple. Basically, they are composed of molecular ion ($M^{\bullet+}$), $(M-CH_3)^+$, $(M-fatty\ acid)^{\bullet+}$, and the triterpenoid related fragments. The detailed interpretation of the mass spectra of β -amyrin fatty acid esters was reported

previously (Elias *et al.*, 1997). Recently it was reported that lupeol and lupeol linoleate have a marked anti-inflammatory activity (Geetha and Varalakshmi, 2001). The amyrin alkanoates in epicuticular waxes of the red raspberry (*Rubus idaeus* L.) have been associated with resistance to aphid infestation (Shepherd *et al.*, 1999).

Sterols

β -Sitosterol was characterized in all extracts, however in the anther it was the only sterol present in 7.9% of the total chromatogram area. Stigmasterol was present in all samples except the extract of the anther surface. Since insects are unable to biosynthesize the steroid nucleus, they require dietary sterols for structural and hormonal purposes. Cholesterol will satisfy this dietary need in most cases, but since phytophagous insects ingest little or no cholesterol from dietary materials, they must convert dietary C_{28} and C_{29} phytosterols to cholesterol or other sterols (Svoboda *et al.*, 1994).

The utilization and metabolism of sterols in the *Apis mellifera*, has been examined with radiolabeled sterols and it was demonstrated that this important beneficial insect is unable to remove C-24 alkyl groups from the sterol side chain (Svoboda *et al.*, 1994).

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