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Phytochemical Profiling of *Clusia valerioi* (Standl.) Floral Resin and Its Correlation with the Chemical Composition of Propolis from Southern Costa Rica

Quimioprospección de la resina de la flor de *Clusia valerioi* (Standl.) y su relación con la composición química de propóleos de una región de la zona sur de Costa Rica

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Abstract
Propolis are resinous byproducts produced by bees, known for their wide range of bioactive properties. The chemical composition of propolis is closely linked to the botanical environment surrounding the hive. *Clusia valerioi* (Standl.), a plant species endemic to Costa Rica, is commonly found in the country's southern region and produces floral resins that are collected by bees for propolis production. This ecological relationship suggests that chemical traceability between *C. valerioi* floral resins and propolis produced in nearby apiaries is feasible.
In this study, ethanolic extracts from propolis samples collected at eight apiaries, resin loads transported by forager bees, and floral resin samples from *C. valerioi* at four sites near two of these apiaries were analyzed using high-performance thin-layer chromatography (HPTLC). The analysis revealed three chemical markers—referred to as compounds A, B, and C—that support the traceability of chemical constituents from floral resin to propolis. Compound A was identified as a terpene derivative, while compound C was determined to be a flavonoid derivative (specifically a flavonol or flavanone). Compound B was isolated and identified as a polyprenylated polycyclic benzophenone, and was conclusively characterized as nemorosone based on proton (^{1}H-NMR) and carbon-13 (^{13}C-NMR) nuclear magnetic resonance spectra.

Keywords: Propolis; *Apis mellifera*; *Clusia valerioi* (Standl.); HPTLC; Nemorosone.

Resumen
Los propóleos son subproductos apícolas elaborados por abejas del género *Apis mellifera* y de otras especies, que presentan muchos tipos de bioactividad. Su origen y composición puede relacionarse con el entorno botánico del sitio de anidación de las abejas, tal es el caso de la planta *Clusia valerioi* (Standl.), especie endémica de Costa Rica, cuya flor produce una resina utilizada por las abejas que la visitan para la producción de propóleo. Lo anterior implica la posibilidad de establecer una trazabilidad de la composición química de la resina de la flor de *C. valerioi* y de los propóleos recolectados de apiarios ubicados en la Zona Sur de Costa Rica, en términos de relacionar la presencia de compuestos tipo flavonoides, ácidos fenólicos y del tipo benzofenonas policíclicas polipreniladas (nemorosoma) en ambos tipos de matrices, para la identificación de posibles biomarcadores activos para futuros estudios. Extractos etanólicos de muestras de propóleo de ocho apiarios visitados y extractos etanólicos de resina floral de *C. valerioi* de cuatro sitios cercanos a dos de estos apiarios, muestreados en la zona de Coto Brus (Costa Rica), fueron analizados y derivatizados con técnicas de HPTLC. Se estableció la presencia de 3 marcadores químicos importantes en el análisis (denominados como compuestos A, B y C), a partir de espectros de resonancia magnética nuclear de protones (^{1}H-NMR) y carbono-13 (^{13}C-RMN), espectros de absorción y cromatografías para 16 extractos de propóleos, resinas y flores *C. valerioi*. Se identificó el compuesto A como un compuesto derivado de los terpenos, el compuesto B como nemorosona y el compuesto C como un derivado de los flavonoides del tipo flavonol o flavonona.

Palabras clave: Propóleos; *Apis mellifera*; *Clusia valerioi* (Standl.); HPTLC; Nemorosona.

Introduction

Propolis are resinous substances produced by honeybees (*Apis mellifera*) from plant exudates collected in the vicinity of the apiaries (defined as two or more hives). Bees manipulate these exudates using their mandibles, enriching them with enzymes from their mandibular glands, and then deposit the material inside the hive, where it is mixed with small amounts of sugars and beeswax secreted by the bees themselves (Chaillou et al., 2004; Fernández, 2008; Salas, 2005). This resulting product, known as propolis, serves not only as a protective material for the hive's internal walls and entrance but also functions as a chemical defense mechanism, forming barriers that inhibit the growth of fungi and bacteria (Vargas et al., 2018; Rossana et al., 2010). Chemically, propolis is a complex mixture composed of approximately 55% aromatic balsams and resins, 30% waxes, 10% essential oils, 5% pollen grains, and other minor constituents.

Among the principal bioactive compounds found in propolis are flavonoids, including flavones such as apigenin, and flavonols such as quercetin and kaempferol. These are commonly present in European propolis derived from Populus nigra species collected by *Apis mellifera* bees (Umaña, 2013). Overall, approximately 38 types of flavones, 12 benzoic acid derivatives, 14 derivatives of cinnamyl alcohol and cinnamic acid (including 3,5-diprenyl-4-hydroxycinnamic acid, also known as artepillin C, found in Brazilian green propolis), 12 various alcohols, ketones, and phenols, 7 terpenes, 11 steroids, 7 sugars, and 2 amino acids have been identified. Within this extensive set of metabolites, flavonoids, phenolic acids, and their esters stand out for their well-documented pharmacological activity (Tolosa & Cañizares, 2002; Umaña, 2013).

Propolis has been shown to possess antimicrobial, antifungal, antitumor, anti-inflammatory, hepatoprotective, antidiabetic, cardioprotective, and immunomodulatory properties. From a therapeutic standpoint, these effects are primarily attributed to the presence of polyphenolic compounds, including flavonoids and phenolic acids, along with their esterified forms (Fernández-León et al., 2022). Such characteristics have generated significant scientific interest in studying both the composition of propolis and the botanical environments from which it originates. Given its broad therapeutic potential and natural origin, propolis is considered a promising candidate for the treatment of various diseases, including certain types of cancer and cardiovascular conditions (Fernández, 2008).

Ethnobotanical studies have demonstrated that certain members of the Clusiaceae family possess notable anti-inflammatory activity. Many of these species are characterized by the production of secondary metabolites such as terpenoids and polyphenols. Within this family, the genus Clusia is of particular interest, as its flowers produce a resin that is actively collected by various bee species for nest construction, where it functions as an adhesive and water-resistant protective agent within the hive (Herbert, 2021; Hochwallner & Weber, 2006; Meranti, 2015). This relationship between Clusia floral resin and its incorporation into hive architecture suggests that the resin's chemical profile may directly influence the chemical constituents of the resulting propolis. This hypothesis is supported by studies conducted in Mexico and by Cuban researchers who have observed an enhancement of the therapeutic properties of propolis derived from Clusia resins (Herrera, 2019; Monzote et al., 2011).

Clusia valerioi (Standl.) is a facultatively epiphytic tree with dark brown to black branches, native to humid, very humid, and pluvial forests along Costa Rica's Caribbean slope. It is found in the Tilarán, Central, and Talamanca mountain ranges, as well as in the plains of San Carlos, Tortuguero, the Coto Brus Valley, and the Golfo Dulce region. This species is readily distinguishable as the only member of its genus that features leaf blades (the flat, typically green portion of the leaf attached to the stem) that turn almost black upon drying. Its flowers are pink or red and exhibit anthers marked with a distinctive ring-like structure (Ley, 2013; Zwicky, 1937).

Species in the genus Clusia are notable for their production of resins rich in polyprenylated polycyclic benzophenones (notably nemorosone), which bees actively use in the synthesis of propolis (Herbert, 2021; Herrera, 2019). A total of 55 different benzophenones

have been identified in this genus, occurring in flowers, fruits, stems, branches, and leaves. However, most of these compounds are concentrated in floral resins and fruits (Novais, 2016). Structurally, benzophenones can be classified into two categories: simple benzophenones, which consist of a 13-carbon skeleton featuring two aromatic rings joined by a carbonyl group, and polyprenylated benzophenones, which possess complex polycyclic frameworks with oxo bridges and isoprenyl groups (see Figure 1) (Novais, 2016; Hernández et al., 2014).

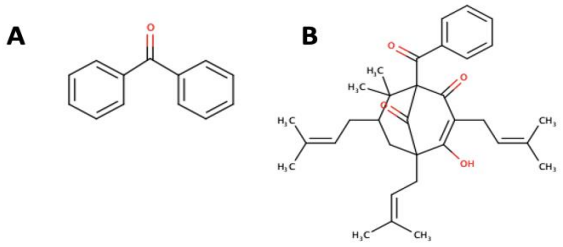

Figure 1. A) Chemical structure of a simple benzophenone. B) Chemical structure of nemorosone (a polyprenylated benzophenone). (Adapted from Novais, 2016).

In Costa Rica, three chemical types of propolis can be distinguished based on their overall composition—measured by Proton Nuclear Magnetic Resonance (^{1}H-NMR)—and their antioxidant capacity, assessed as the radical scavenging effect against 2,2-diphenyl-1-picrylhydrazyl (DPPH). Type I propolis primarily contains phenolic compounds such as n-coniferyl benzoate; Type II includes polyprenylated benzophenones like nemorosone; and Type III comprises terpenoid substances such as agatadiol. Propolis from Types I and II exhibit higher activity against the DPPH radical compared to Type III. Types I and III predominated in the North Pacific, Central Pacific, and Central Valley regions, whereas in the Southern Zone only Type II propolis was found. This Type II propolis was also present in the other studied regions but only incidentally. This geographical distribution pattern of the different propolis types was statistically significant. Based on these results, it is hypothesized that a species of Clusia sp. present in the southern region of the country plays a relevant role as a resin source for bees in propolis production (Umaña, 2013; Umaña et al., 2023).

The secondary metabolites present in the flowers of *Clusia valerioi* (Standl.) represent a source of natural products with pharmaceutical potential, both within the plant matrix and in derivative products. However, given the limited studies on this flower, the objective of the present research was to establish whether there is a correlation between polycyclic polyprenylated benzophenone compounds (such as nemorosone) and other compound classes found in the resin of *C. valerioi* flowers from the Southern Region of Costa Rica and those present in propolis from honeybee hives collected in the same area. To achieve this, the chemical nature of the main compound families in the flower resin and in the extracted propolis was determined using High-Performance Thin-Layer Chromatography (HPTLC). The results were further confirmed through melissopalynological analysis of pollen found in the most frequent resin loads transported by bees to the hives.

MATERIALS Y METHODS

Sampling Sites

Samples of flower receptacles and propolis from surrounding apiaries were collected in the Southern Region of Costa Rica, specifically during the flowering period of *Clusia valerioi* (Standl.) in this region (June–July 2022). A random sampling scheme was followed. This strategy involved the random selection of eight apiaries out of the total in the southern region, representing approximately 9% of the registered apiaries in the area (Ministry of Agriculture and Livestock, 2021). At each apiary, two propolis samples were randomly selected from different hives, yielding a total of 16 samples. Figure 2 details the coding used for the extracts prepared from the collected samples and the geographical locations of their origin.

Código de Muestra	Descripción	Provincia, Cantón, Distrito	Número de Ubicación en Mapa
CDRF 1	Propóleos de apiario	Puntarenas, Coto Brus, Pittier	5
CDRF 2	Extracto a partir de resina en corbículas de una abeja	Puntarenas, Coto Brus, Pittier	5
CDRF 3	Extracto de flores de *C. valerioi*	Puntarenas, Coto Brus, Pittier	5
CDRF 4	Extracto de flores de *C. valerioi*	Puntarenas, Coto Brus, Pittier	5
CD 02A	Extracto de flores de *C. valerioi*	Puntarenas, Coto Brus, Limoncito	10
CD 03B	Extracto de flores de *C. valerioi*	Puntarenas, Coto Brus, Limoncito	10
CD 05	Cúmulo de resinas en colmena (sin procesar)	Puntarenas, Coto Brus, Limoncito	10
CD 06A	Propóleos de apiario	Puntarenas, Coto Brus, Limoncito	10
CD 2A	Propóleos de apiario	Puntarenas, Buenos Aires, Buenos Aires	2
CD 3A	Propóleos de apiario	San José, Pérez Zeledón, Platanares	3
CD 4A	Propóleos de apiario	Puntarenas, Buenos Aires, Potrero Grande	4
CD 5A	Propóleos de apiario	Puntarenas, Coto Brus, Pittier	5
CD 7B	Propóleos de apiario	Puntarenas, Golfito, Puerto Jiménez	7
CD 8A	Propóleos de apiario	Puntarenas, Osa, Palmar	8
CD 9A	Propóleos de apiario	Puntarenas, Coto Brus, Gutiérrez Braun	9
CD 10B	Propóleos de apiario	Puntarenas, Coto Brus, Limoncito	10

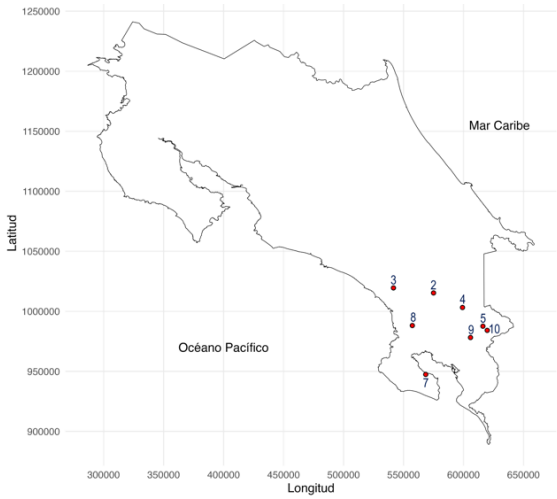

Figure 2. Coding of the analyzed extracts from flowers, resins, and propolis, along with their geographical locations based on GPS coordinates of the sampled apiaries (Global Positioning System).

Collection and Identification of Botanical Material

To determine and confirm the botanical origin of the samples, leaves, flowers, and flower buds of *Clusia valerioi* (Standl.) were collected from four sampling sites located in the localities of Santa Elena and Santa Fe de Pittier, as well as Limoncito de Coto Brus, in the province of Puntarenas. The plants sampled were located within the foraging areas of the visited apiaries. Additionally, samples of the same plant species were collected from another site outside the bees' foraging area, in the same region, but specifically in Santa Fe de Pittier.

Taxonomic verification of the material was performed by comparing the collected and pressed specimens with reference samples from the National Herbarium collection of the Department of Natural History at the National Museum of Costa Rica. This was supported by consultation of specialized literature (Hochwallner and Weber, 2006; Hochwallner et al., 2012; Alencar et al., 2020) and a final confirmation at the Juvenal Valerio Rodríguez Herbarium of the Faculty of Earth and Marine Sciences at the National University.

Collection of Resins from Bees and Apiaries

Two methods were employed to collect resin loads from bees. The first method involved collecting resin loads carried by foraging bees on their corbiculae. In this case, with the aid of forceps, the resin load was separated from ten individual bees from each of ten randomly selected hives at every apiary. The second method involved collecting recently deposited resin loads (unprocessed resin) from the upper frames of ten randomly selected hives in each apiary.

Determination of the Botanical Origin of the Resins

Resin samples or corbicular loads were sorted by color, and the frequency of each load type was established according to the dominance of a specific color relative to the total loads present. The most dominant loads were then analyzed microscopically to identify pollen grains via palynological analysis. For this, samples were stained with safranin and centrifuged in a 50% glycerol solution (v/v). The pollen grains, once precipitated and dried in an oven, were mounted on fixed slides for microscopic observation at 10X and 100X magnification. Microphotographs were then taken of the pollen grains present in the resins collected from corbiculae and hive frames. Subsequently, pollen from the anthers of identified flowers was prepared to compare and contrast the images with those of pollen from the resin samples (modified method of Erdtman, 1986).

Sample Processing and Extract Preparation

Once collected, all samples were transported at room temperature to the Tropical Apicultural Research Center (CINAT), where they were stored under refrigeration (4°C) prior to transfer

and processing at the Natural Products and Biological Assays Laboratory (LAPRONEB) of the School of Chemistry at the National University (UNA).

Propolis samples were dehydrated for 24 hours in a lyophilizer. From this point, they were processed in duplicate following the methodology described by Umaña (2013). The samples were then pulverized using a mortar, and 400 mg of each were weighed on an analytical balance and placed into separate test tubes. Each sample was extracted with 10.0 mL of methanol (99.9%, Sigma-Aldrich) and subjected to ultrasonic bath treatment for 15 minutes at room temperature. Samples were subsequently centrifuged at 5000 rpm for 5 minutes at room temperature. The supernatants were concentrated, dispensed into capped vials, and stored refrigerated until use.

Samples from the flower receptacles of *C. valerioi* (Standl.) and resin samples were macerated in methanol (100%, Sigma Aldrich) in triplicate. These extracts were then concentrated under vacuum at 40°C using a rotary evaporator and subsequently refrigerated. Solutions were prepared at a concentration of 2 mg/mL from the concentrated extracts of the flowers, resin from a bee's corbicula, unprocessed resin obtained from hives, and the sampled propolis.

High-Performance Thin-Layer Chromatography (HPTLC)

High-Performance Thin-Layer Chromatography analyses were conducted using a CAMAG HPTLC system located at CINAT, National University (UNA). The system consisted of an ATS 4 Automatic TLC Sampler, ADC 2 Automatic Developing Chamber, Visualizer imaging system, Derivatizer, TLC Plate Heater, TLC Scanner 4 densitometer, and a computer running CAMAG VisionCATS 2.5 software for data management.

The stationary phase used was HPTLC silica gel 60 F254 plates, sized 20 cm × 10 cm on glass support (Merck). Aliquots of each sample, ranging from 1 to 20 µL, were applied to each plate using the ATS 4 sampler (15 samples per plate). Samples were applied 8 mm from the bottom edge of the plate, with an 8 mm application band length. Nitrogen gas was used by the ATS 4 for aerosolized sample application.

Chromatography was performed in the ADC 2 chamber. The mobile phase consisted of a dichloromethane:methanol solution (9:1). The chamber was saturated with 25 mL of mobile phase, and 10 mL were used for development. The procedure included 20 minutes of chamber saturation, 10 minutes of activation with $MgCl_2$ (to achieve 33% relative humidity), chromatographic development to 70 mm, and plate drying for 5 minutes.

Photographic records under visible light, UV light at 254 nm and 366 nm, derivatizations, and Rf calculations were performed with the Visualizer and VisionCATS 2.5 software.

Derivatizations

Derivatizing agents were applied using the Derivatizer. The following reagents were employed: natural products reagent (2-aminoethyl diphenylborinate), anisaldehyde, vanillin, and Dragendorff reagent. These reagents were prepared and applied according to the manufacturer's instructions for the Derivatizer. When necessary, chromatograms were heated using the TLC Plate Heater to complete derivatization.

Densitometry

Absorption spectra (ranging from 200 nm to 900 nm), densitometric assays at 206 nm and 307 nm, correlation tests, and spectral purity evaluations were conducted using the TLC Scanner 4 in combination with VisionCATS 2.5 software.

Creation of Chemical Fingerprints

From the chromatograms and densitometric data, image comparison views were generated using VisionCATS 2.5 software. These composite images, known as chemical fingerprints, were used in this study to identify the chemical families present in each sample. Interpretation of derivatizations was based on comparisons with specialized literature, particularly the works of Burnstein (1953), Conde et al. (1992), Foti et al. (2004), Matteini et al. (2011), Spangenberg (2008), Wagner and

Bladt (1996), and Waksmundzka-Hajnos et al. (2008).

Isolation and Purification of Compounds of Interest

Preparative Thin-Layer Chromatography (PTLC)

Compounds of interest were initially isolated using preparative thin-layer chromatography (PTLC) with Silica Gel 60 F254+366 plates (2 mm thickness, Merck) as the stationary phase and a dichloromethane:methanol (9:1) solution as the mobile phase, following the method described by Umaña (2013). After chromatographic development, the bands of interest were scraped off. The scraped silica gel was mixed with 100 mL of methanol and filtered through a funnel fitted with a Whatman #40 filter. The collected fraction was then concentrated by rotary evaporation at 40°C under reduced pressure, and the isolated fraction was dissolved in 4 mL of methanol. Finally, this solution was passed through a 0.45 µm syringe filter (Fisher Scientific) and dispensed into a vial, which was stored refrigerated (2–5°C) until use.

Isolation and Purification by Medium-Pressure Liquid Chromatography (MPLC)

The compounds of interest were isolated and purified by medium-pressure liquid chromatography. For this, a flash chromatograph (SepaCore®, Büchi) equipped with a reversed-phase column (Büchi RPC18ec) (12 mm × 150 mm), a detector with a monochromator, and an automatic fraction collector was employed. A 2 mL volume of a 20 mg/mL solution of the fractions to be purified was injected into the MPLC. A flow rate of 5 mL/min was applied using a mobile phase composed of methanol and water (75:25) for the purification of a compound referred to as B, and methanol and water (95:5) for the purification of a substance designated C. Chromatographies were performed under isocratic conditions. Based on the presumed structure, the absorbance of compound B was monitored at 307 nm and compound C at 206 nm. Fraction volumes of 5 mL were collected. Fractions were selected post-chromatography primarily based on purity criteria. The mobile phase was removed from the collected fractions by rotary evaporation under reduced pressure at 40°C followed by lyophilization. This procedure successfully isolated and purified two compounds of interest. The purity of the isolates was verified by HPTLC and their derivatization with a 1 mg/mL potassium permanganate solution. Purity was defined by the presence of only the band corresponding to the compound of interest on the chromatogram.

Structural Elucidation of the Isolated Substances

Of the two isolated substances, the first showed a spectral purity greater than 99.97% and was analyzed by infrared (IR) spectroscopy and nuclear magnetic resonance (NMR). The molecular structure of compound B was determined by comparing and correlating the NMR and IR data obtained with those reported in the literature (Cuesta-Rubio et al., 2001; Umaña, 2013; Umaña et al., 2023; Uwamori et al., 2012).

Infrared Spectrum

The infrared spectrum was recorded using an attenuated total reflectance (ATR) instrument, Thermo Scientific Nicolet iS50 FT-IR model.

Nuclear Magnetic Resonance (NMR)

Fifteen milligrams of compound B were dissolved in 750 µL of deuterated methanol (CD_3OD). The final solution (20 mg/mL) was transferred into NMR tubes. Spectra were recorded on a Bruker Avance III spectrometer equipped with a 50 mm probe, at 25°C, using a magnetic field of 400.13 MHz for proton NMR (^{1}H-NMR) and 100.62 MHz for carbon-13 NMR (^{13}C-NMR). In both cases, a 30° pulse was applied. Relaxation times were 1 s for ^{1}H-NMR and 2 s for ^{13}C-NMR. The free induction decay (FID) comprised 64K points in both cases.

RESULTS

Taxonomic Identification of the Species *Clusia valerioi* (Standl.)

The botanical material collected during the different field trips was used for the identification and classification of the sampled species.

Analysis of the resin samples carried by individual bees and those collected from the upper parts of the hive frames showed that the dominant loads (93%) were whitish in color and, after some time, turned pale orange or brown once deposited into vials. The other loads, considered incidental or accessory (7%), were pale yellow, red, and greenish, and retained their coloration over time.

Microscopic mounts of the dominant loads revealed that they corresponded almost entirely to the pollen of *C. valerioi* (see Figures 3 and 4). The pollen grains of *C. valerioi* are tricolporate, with circular to elliptical apertures, a tectate exine, and granular sexine. The grains are small, spheroidal in shape, measuring 22–24 µm (CINAT Pollen Image Database, 2024; Roubik and Moreno, 1991).

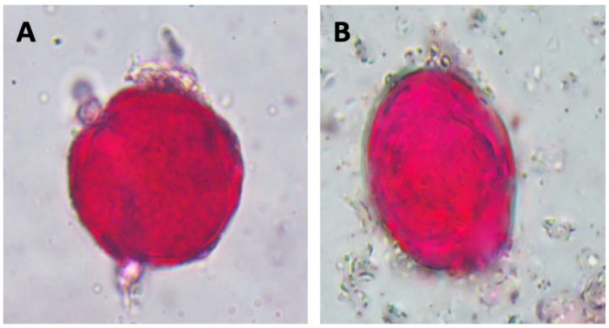

Figure 3. Microphotograph of *C. valerioi* pollen grains obtained from floral anthers (100X magnification). A) Polar view; B) Equatorial view.

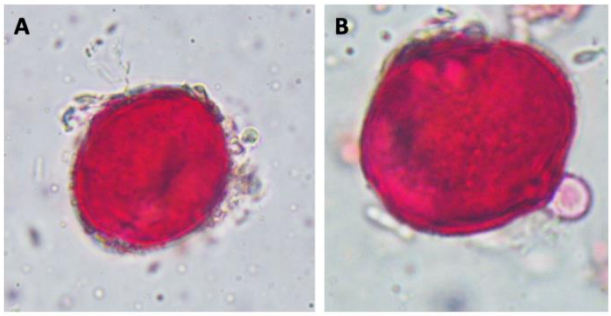

Figure 4. Microphotograph of polar views of *C. valerioi* pollen grains (100X magnification). A) Grain from resin collected by bees; B) Grain from resin collected on the upper parts of hive frames (unprocessed resin).

High-Performance Thin-Layer Chromatography (HPTLC) Assays

The results of the high-performance thin-layer chromatography assays and derivatizations are presented in Figures 5 and 1A of the Appendix. The chemical families of the compounds present in the extracts were identified based on the colors developed after exposure to the derivatizing reagent, and by comparison with information available in the literature (Burnstein, 1953; Conde et al., 1992; Foti et al., 2004; Matteini et al., 2011; Spangenberg, 2008; Wagner and Bladt, 1996; Waksmundzka-Hajnos et al., 2008).

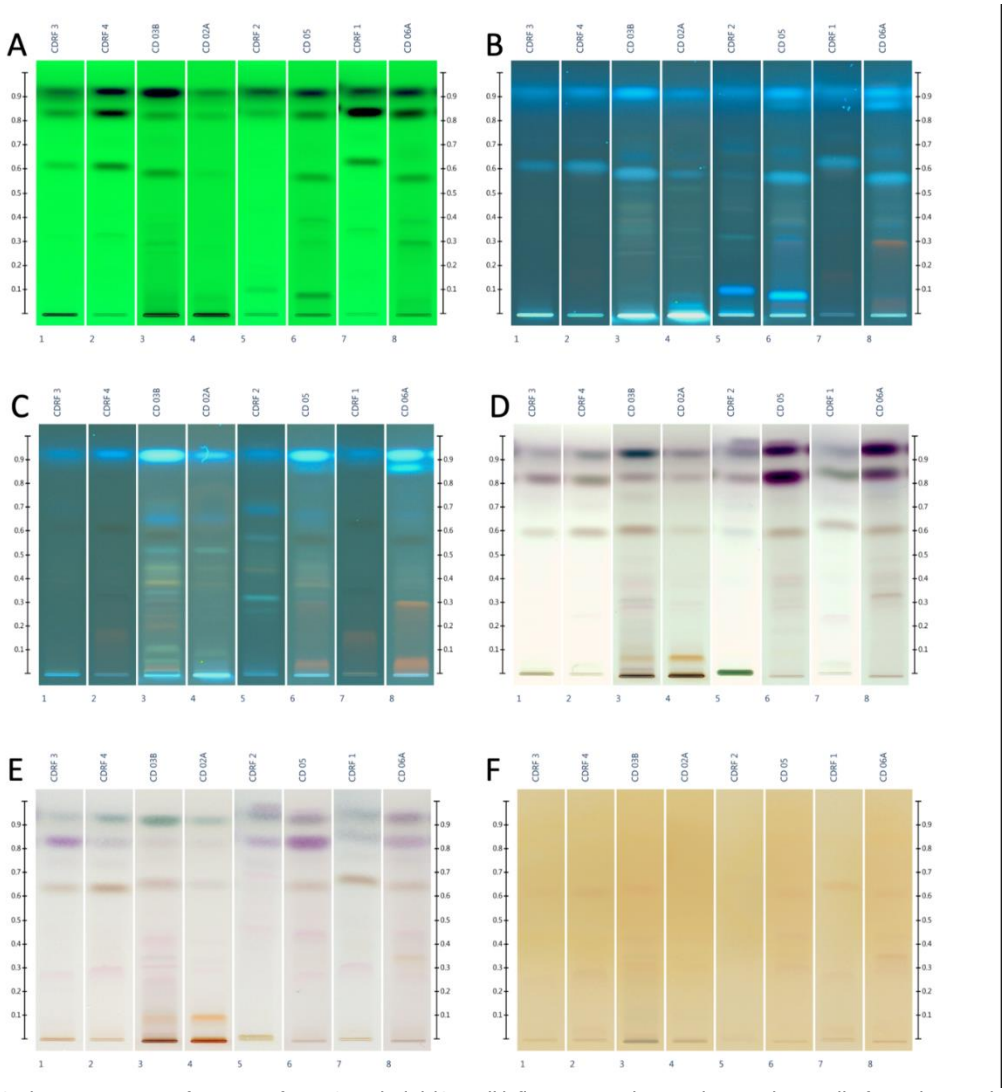

Figure 5. HPTLC chromatograms of extracts from *C. valerioi* (Standl.) flower samples, resins, and propolis from the southern region of Costa Rica, showing the results under various conditions: exposure to ultraviolet light at 254 nm (A), exposure to ultraviolet light at 366 nm (B), derivatization with the Natural Products Reagent under 366 nm light (C), derivatization with the anisaldehyde reagent under visible light (D), derivatization with the vanillin reagent under visible light (E), and derivatization with the Dragendorff reagent under visible light (F).

All of the extracts analyzed (100%) tested negative with the Dragendorff reagent, which is used to detect the presence of alkaloids. Therefore, the absence of alkaloids is reported in the analyzed samples.

Derivatization with the Natural Products Reagent revealed bands in yellow, orange, red, blue, and green hues within the Rf range of 0.01 to 0.60 in 77% of the samples. These colorations indicate the presence of phenolic compounds in the majority of the extracts, particularly flavanones and flavonols (see Figures 5 and 1A in the Annexes).

In 91% of the samples, three bands with Rf values between 0.61 and 1.00 were observed (Figure 6). The upper band (Rf = 0.93), assigned to a compound referred to as A, fluoresces under UV light at 366 nm and shows a blue hue when derivatized with the Natural Products Reagent. Upon reaction with the anisaldehyde reagent, it turns a very dark purple, and, based on its lack of response to the Dragendorff reagent, it is not an alkaloid. This chemical fingerprint suggests that compound A may be a terpene derivative. However, in some chromatograms, two closely adjacent intense blue bands were observed

under UV light at 366 nm. Derivatization with vanillin yielded green tones in some bands and purple hues in others (see Figures 5 and 6). These results suggest the presence of two distinct compounds with very similar Rf values under the chromatographic conditions used. Therefore, a more in-depth analysis of compound A would first require an appropriate separation of its constituents.

The middle band (Rf = 0.83), assigned to a compound referred to as B, absorbs at 254 nm but is not visible under UV light at 366 nm and does not react with the Natural Products Reagent. However, upon derivatization with anisaldehyde and vanillin reagents, it develops purple hues. Like compound A, this behavior suggests that compound B may also be a terpene derivative.

The lower band (Rf = 0.63), attributed to a compound referred to as C, shows absorption under UV light at 254 nm, is not visible under UV light at 366 nm, and reacts with the Natural Products Reagent to produce an intense blue color. It also develops orange hues upon derivatization with anisaldehyde and vanillin reagents. Based on its chemical fingerprint, compound C is likely a phenolic compound, particularly a flavonol or flavanone. The chemical fingerprints of compounds A, B, and C are shown in Figure 6.

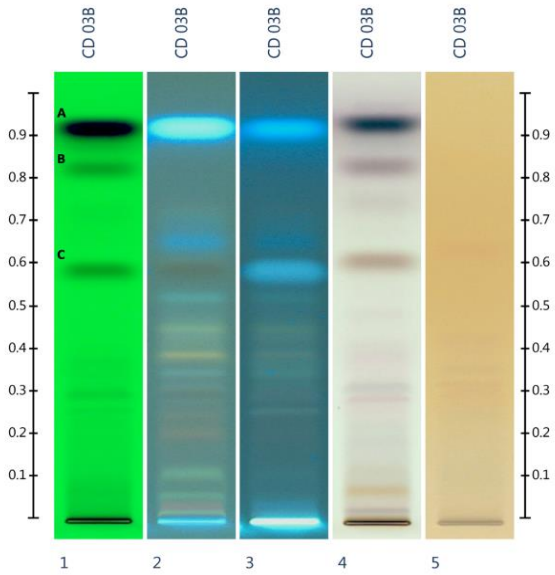

Figure 6. Chemical fingerprint of the flower resin extract from *C. valerioi* (sample CD 03B). The figure shows the results of various exposures to ultraviolet light and derivatizations for compounds A (Rf 0.93), B (Rf 0.83), and C (Rf 0.63). 1) exposure to UV light at 254 nm, 2) exposure to UV light at 366 nm, 3) derivatization with the Natural Products Reagent (viewed under 366 nm UV light), 4) derivatization with the anisaldehyde reagent (visible light exposure), 5) derivatization with the Dragendorff reagent (visible light exposure).

Subsequently, based on sample CD03B—derived from the resin extracts obtained from the flowers of *C. valerioi*—the absorption spectra of compounds B (Rf 0.83) and C (Rf 0.63) were determined through densitometric assays. Compound B exhibited a maximum absorption peak at 307 nm, while compound C showed a maximum absorption at 206 nm (see Figures 2A and 3A in Annexes).

Densitometric analyses were then performed at 206 nm and 307 nm. The results were subjected to peak integration analyses corresponding to the absorbance signals of compounds A, B, and C. This approach optimized the visualization of the constituents and enabled a more accurate assessment of the presence or absence of the target compounds. The results are presented in Figure 7 and Figure 4A of the Annexes.

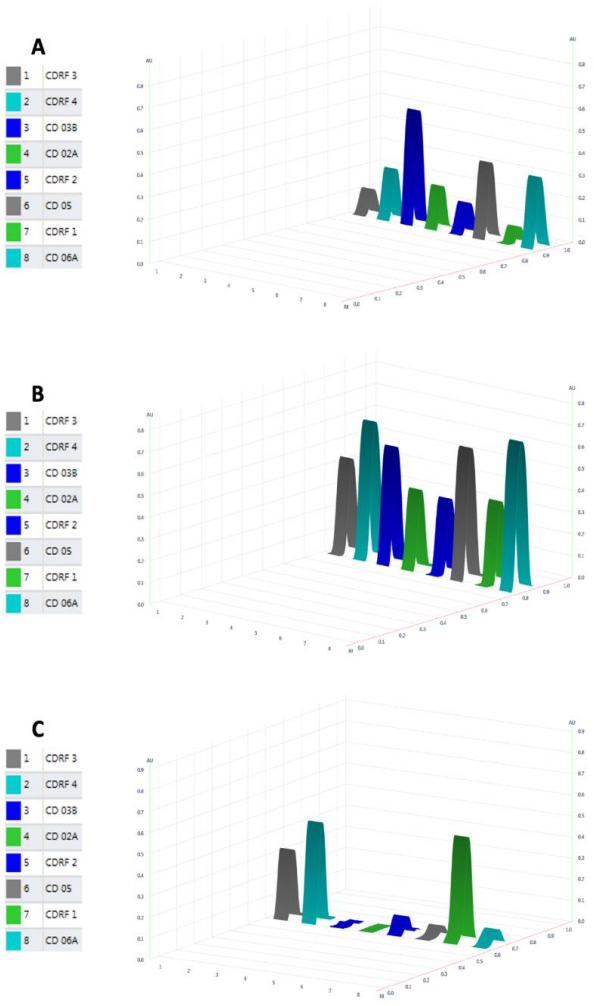

Figure 7. Densitometric analyses based on HPTLC chromatograms of extracts from *C. valerioi* flower samples, resins, and propolis collected from the southern region of Costa Rica. Absorption peaks corresponding to compounds A, B, and C were integrated to assess their presence in the samples. A) Densitometry at 307 nm for the evaluation of compound A. B) Densitometry at 307 nm for the evaluation of compound B. C) Densitometry at 206 nm for the evaluation of compound C.

All samples exhibited an absorption peak corresponding to compound A. Absorption peaks for compounds B and C were detected in all samples, with two exceptions: one propolis sample from Puerto Jiménez, Golfito (CD 7B) did not contain compound B, and one flower extract from Limoncito, Coto Brus (CD 02A) did not show evidence of compound C.

Absorption spectra (ranging from 200 nm to 900 nm) were recorded from chromatogram bands with Rf values corresponding to compounds B and C. Based on these results, correlation analyses of the absorption spectra were conducted. Pearson correlation coefficients of 0.995055 and 0.987449 were obtained for the bands assigned to compounds B and C, respectively. These values strongly suggest that both compounds are present in all samples, with the exception of those specifically mentioned above.

The constituents identified as compounds A, B, and C were found in *Clusia valerioi* (Standl.) flowers, in the resins carried by bees in their corbiculae, and within the hive, both in the unprocessed resin deposits and in the processed propolis.

Structural Identification of Compound B

The isolated substance with the highest purity, referred to as compound B, was identified as nemorosone. Its molecular structure was determined based on the consistency and comparison of data obtained from ^{1}H-RMN, ^{13}C-RMN, and IR spectroscopy with those previously reported in the literature by Cuesta-Rubio et al. (2001), Umaña (2013), Umaña et al. (2023), and Uwamori et al. (2012). The data supporting the structural elucidation of this compound are presented in Figures 5A to 8A and Tables 1A to 3A of the Annex. The optimization of the purification processes and subsequent structural elucidation of compounds A and C will be addressed in future studies.

DISCUSSION

The joint efforts in sampling and botanical identification of the plant collected during the various field trips, carried out by Lic. Luis Jorge Poveda and M.Sc. Luis Sánchez Chaves, confirm with full certainty that the specimen sampled near the apiaries corresponds to the species *Clusia valerioi* (Standl.). This finding aligns with the report by Hochwallner et al. (2012), who indicated that this species is common in the southern region of Costa Rica.

Furthermore, field observations by beekeepers in the southern region of Costa Rica (Bermúdez, 2019), corroborated by botanist Luis J. Poveda and confirmed by our own sampling visits, documented a high visitation rate by *Apis mellifera* bees to the flowers of *C. valerioi* (Standl.) for resin collection. These observations are supported by our analysis of the resins transported by bees and those

collected from the hive frames, where 93% of the dominant resin load corresponded to Clusiaceae (Figure 3). In addition, Hochwallner et al. (2012) reported frequent visits of *Apis mellifera* to both male and female flowers of *C. valerioi* (Standl.) for resin collection. This confirms that the pollen and resins of this plant are effectively transported to the apiaries, raising the possibility of their use in the production of bee-derived products—an aspect to be further evaluated.

The most direct way to determine whether the materials collected by the bees (pollen and resin) from *C. valerioi* (Standl.) flowers are incorporated into beehive products, specifically propolis, is by detecting the presence of shared chemical constituents—known as chemical markers—across these matrices. This is confirmed by the results shown in Figures 4 to 6.

The HPTLC chromatographic results of extracts from *C. valerioi* floral tissues, resins collected from the bees' corbiculae, unprocessed resin aggregates inside the hives, and the propolis samples from apiaries, generally show an absence of alkaloid-type compounds across all matrices. They also confirm the inconsistent presence of phenolic compounds, particularly flavanones and flavonols, with low to intermediate Rf values (below 0.60) in most samples (77%). However, at higher Rf values (above 0.60), a large majority of the samples (91%) exhibited three common bands that may serve as chemical tracers linking the chemical composition of *C. valerioi* floral resins, the resin collected by bees from these flowers, the resins transported to the hive, and the propolis ultimately produced within the apiary (Figures 4 to 6).

These three chemical tracers were designated as **Compound A**, **Compound B**, and **Compound C**. **Compound A** (Rf = 0.93), based on its derivatization behavior, appears to be a terpene derivative. Under different chromatographic conditions, it seems to consist of a mixture of two closely related substances—possibly isomers or tautomers—warranting further chromatographic separation and structural elucidation. **Compound B** (Rf = 0.83) was identified as the polyprenylated benzophenone nemorosone, based on its behavior during chemical derivatization and comparison with proton nuclear magnetic resonance (NMR) spectroscopy (Figures 5 and 6; Figures 5A and 6A; Table 1A of Annex), carbon-13 NMR (Figure 7A; Table 2A of Annex), and infrared (IR) spectroscopy (Figure 8A; Table 3A of Annex). Finally, **Compound C** (Rf = 0.63), based on its chemical reactivity with derivatizing agents, is likely a phenolic compound, specifically a flavonol or flavanone.

It is worth noting that previous studies have shown other *Clusia* species to serve as resin sources for *Apis mellifera* in the production of propolis, such as *C. major* (syn. *C. rosea*), *C. minor*, and *C. scrobiculata* in Venezuela, and *C. major* (syn. *C. rosea*) in Cuba—these species are known for their richness in polyprenylated benzophenones like nemorosone and scrobiculactones A and B (Trusheva et al., 2004; Cuesta-Rubio et al., 2000).

In our case, the present study constitutes the first report of *C. valerioi* (Standl.) as a primary source of resins for *Apis mellifera* propolis in Costa Rica. Three chemical markers were identified in 94% (15 extracts) of the propolis samples analyzed, collected from apiaries both with and without confirmed presence of *C. valerioi* (Standl.) in their surroundings.

CONCLUSIONS

Based on the results obtained for the beekeeping sector or ecosystem studied—geographically located in the southern region of Costa Rica, specifically in Pittier, Coto Brus, and surrounding areas—it is concluded that the confirmed botanical identification of *Clusia valerioi* (Standl.) positions this species as a key floral source. The resin produced by its flowers is collected, transported, deposited, and processed by honey bees, and its chemical composition directly influences the chemical profile of the propolis produced.

This conclusion is supported by the HPTLC results, which demonstrate the presence of at least three relevant chemical markers, identified in this study as Compound A, Compound B, and Compound C. These findings were further validated by the presence of *C. valerioi* (Standl.) pollen in the resin loads

predominantly transported by the bees to the hives.

Based on derivatization tests, Compound A was identified as a terpene derivative, potentially a mixture of isomers or tautomers, similar to Compound B, which also exhibited a terpenoid nature. Compound C, in turn, was identified as a phenolic derivative, likely a flavonol or flavanone.

Spectroscopic analysis of Compound B enabled the elucidation of its chemical structure, which was determined to be that of nemorosone. In the context of the broader research project, further spectroscopic studies are recommended for Compounds A and C—once the isomeric or tautomeric mixture of the former is resolved—to allow for more detailed structural identification of both chemical markers.

ACKNOWLEDGEMENTS

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Special thanks to Guillermo Ramírez Arias, technician at the Tropical Apicultural Research Center (CINAT), for his valuable assistance during the sampling trips, and to Professor Emeritus Lic. Luis Jorge Poveda Álvarez of the National University for his essential support during the botanical identification phase at the Juvenal Valerio Herbarium of the School of Environmental Sciences, National University.

Annexes

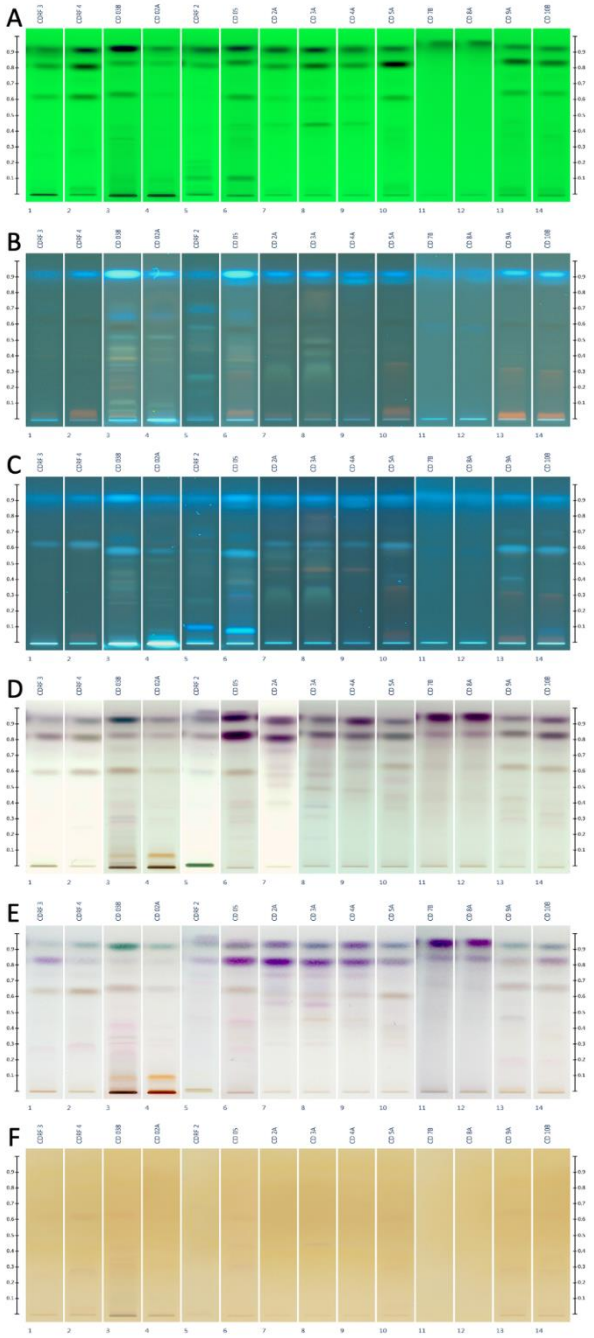

Figure 1A. HPTLC chromatograms of extracts from samples of *C. valerioi* (Standl.) flowers, resins, and propolis from the Southern Region of Costa Rica (part 2). A) Exposure to ultraviolet light at 254 nm, B) exposure to ultraviolet light at 366 nm, C) derivatization with the natural products reagent (exposure at 366 nm), D) derivatization with the anisaldehyde reagent (exposure under visible light), E) derivatization with the vanillin reagent (exposure under visible light), F) derivatization with the Dragendorff reagent (exposure under visible light).

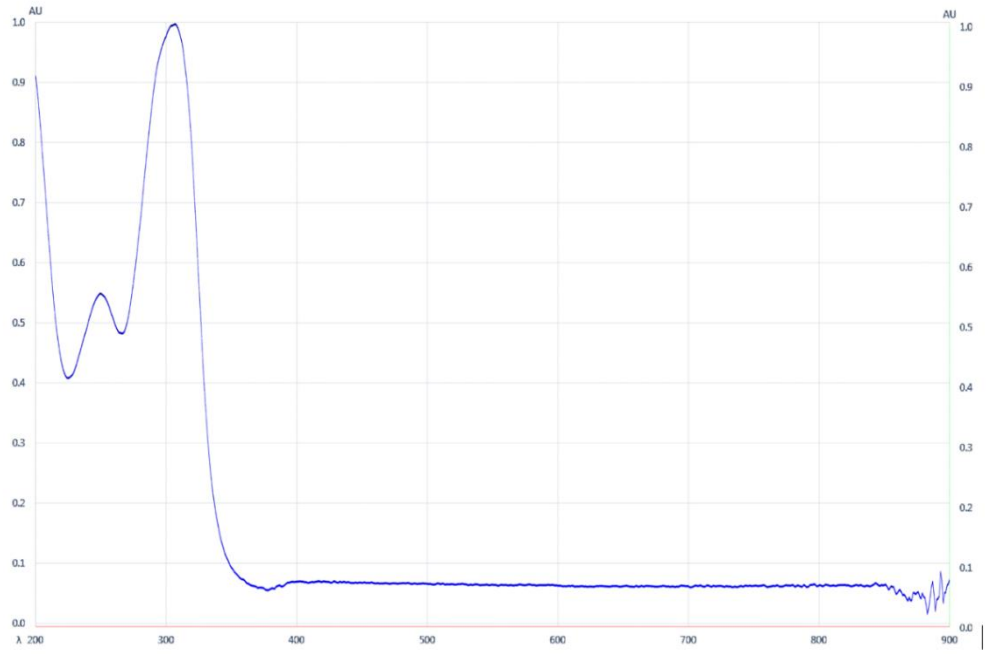

Figure 2A. Absorption spectrum from 200 nm to 900 nm for compound B.

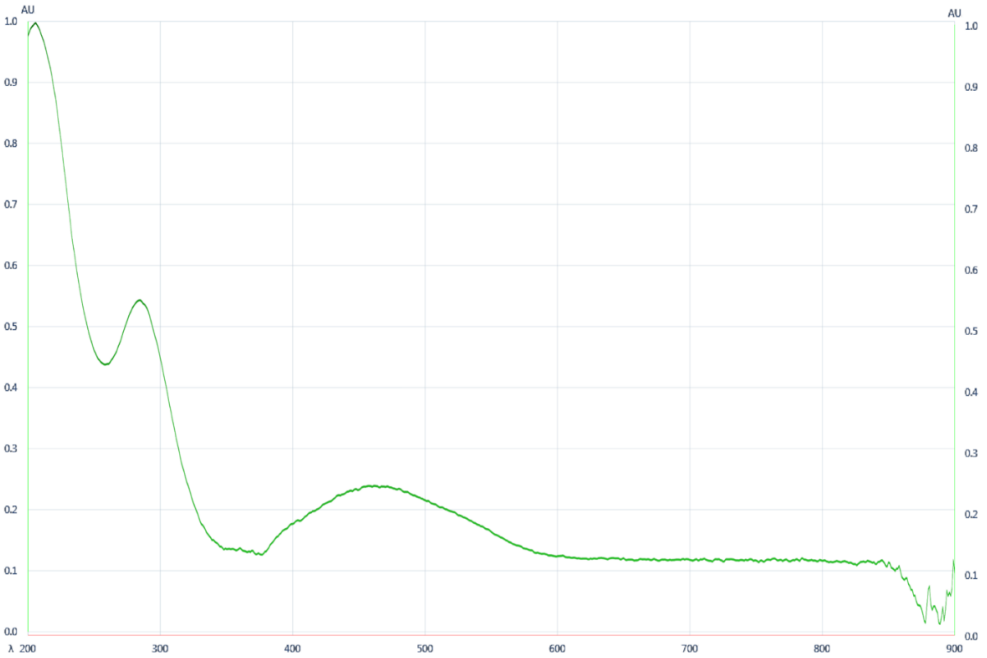

Figure 3A. Absorption spectrum from 200 nm to 900 nm for compound C.

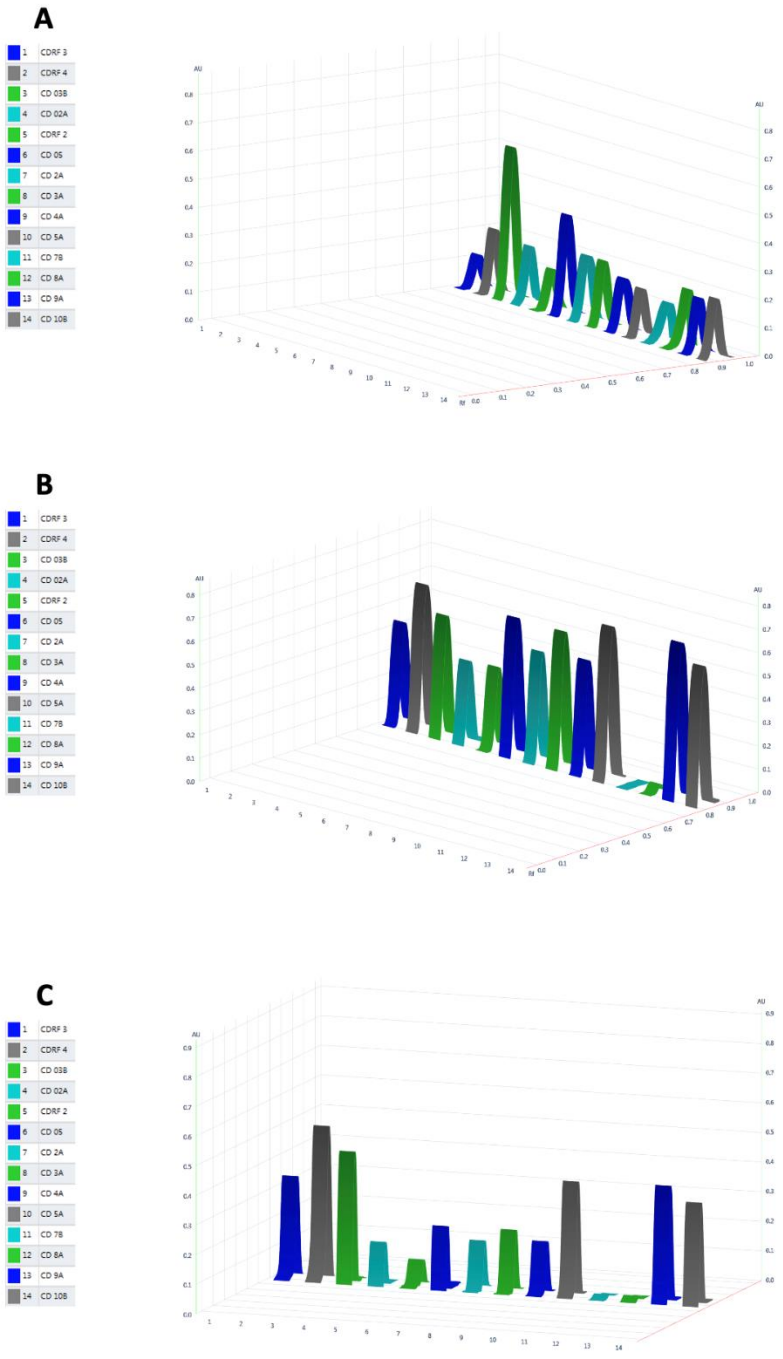

Figure 4A. Densitometric analyses from HPTLC chromatograms of extracts from samples of *C. valerioi* flowers, resins, and propolis from the southern region of Costa Rica (part 2). Absorption peaks corresponding to compounds A, B, and C were integrated to assess their presence in the samples. A) Densitometry at 307 nm for evaluating the presence of compound A. B) Densitometry at 307 nm for evaluating the presence of compound B. C) Densitometry at 206 nm for evaluating the presence of compound C.

Numeración de carbonos en Cuesta-Rubio et al (2001), Umaña et al (2023) y Uwamori et al (2012)	Numeración de carbonos en Umaña (2013)
1	P
2	D
3	O
4	C
5	Q
6	T
7	S
8	R
9	A
10	B
11	E
12, 16	J
13, 15	K
14	H
17	Z
18	L
19	I
20	B2
21	Y
22	U
23	N
24	F
25	D2
26	W
27	V
28	M
29	G
30	C2
31	X
32	A2
33	E2

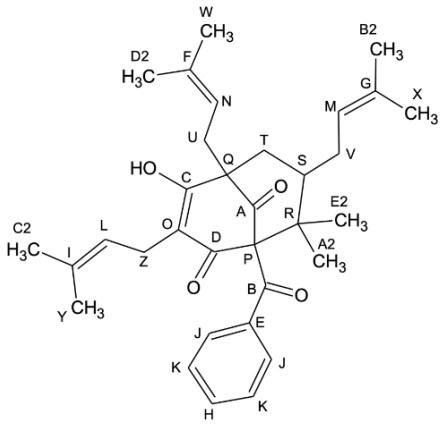

Figure 5A. Correspondence of alphanumeric assignments for carbon and hydrogen atoms used for nemorosone in the consulted literature.

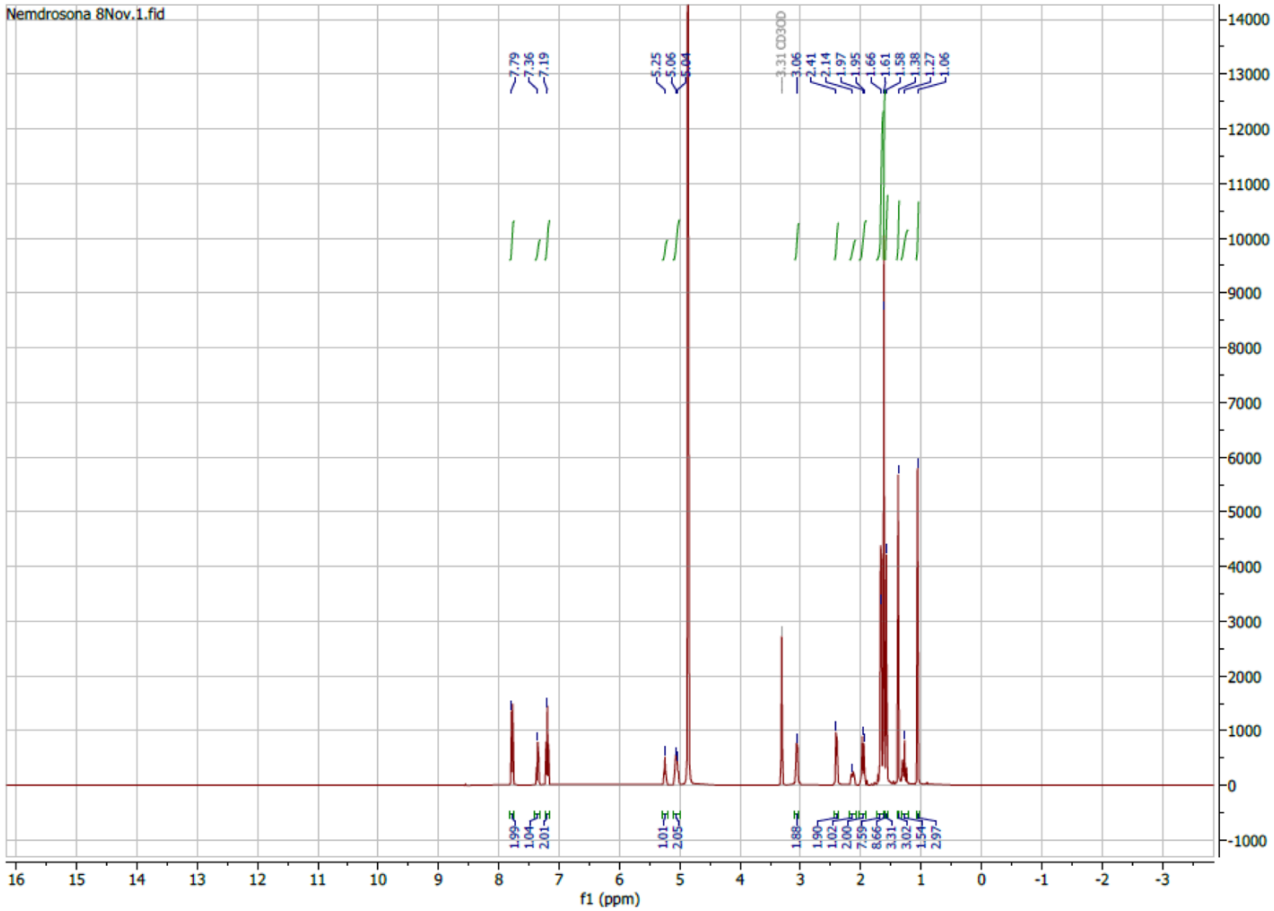

Figure 6A. Proton nuclear magnetic resonance (^{1}H-RMN) spectrum of nemorosone in CD_3OD (reference 3.31 ppm) measured at 400.13 MHz.

Table 1A. Comparison of the ^{1}H-RMN data of nemorosone isolated from the propolis extract CD 5A with data reported in the literature.

Hydrogen atom assignment	Nemorosone isolated from extract CD 5A / CD_3OD / ppm (integration), (multiplicity, coupling constant)	Umaña (2013), Umaña et al. (2023) / CD_3OD / ppm (integration), (multiplicity, coupling constant)	Cuesta-Rubio (2001) $CDCl_3$ / -55°C / Tautomer a / Tautomer b / ppm (integration), (multiplicity, coupling constant)	Synthetic nemorosone (Uwamori et al., 2012) / CD_3OD / ppm (integration), (multiplicity, coupling constant)
6 a (T1)	1.97 (1), (m)	1.96 (1), (m)	2.09 /1.97 (1), (dd, J = 13.5, 4 Hz)	2.02 (1), (dd, J =13.0, 3.5 Hz)
6 b (T2)	1.27 (1), (m)	1.27 (1), (m)	1.47/1.42 (1), (t, J = 13.3)	1.43 (1), (t, J = 13.0 Hz)
7 (S)	1.95 (1), (m)	1.96 (1), (m)	1.68/1.70 (1), traslapado	1.79-1.71, (m)
12,16 (J)	7.78 (2), (dd, J = 8.6, 1.2 Hz)	7.78 (2), (dbr, J=8.6 Hz)	7.53/7.67 (2), (dd, J = 7.5, 1 Hz)	7.56 (2), (d, J = 7.5 Hz)
13,15 (K)	7.20 (2), (tbr, J = 8.0 Hz)	7.20 (2), (dd, J = 8.4, 7.8 Hz)	7.31/7.35 (2), (t, J = 7.5 Hz)	7.26 (2), (t, J = 7.5 Hz)
14 (H)	7.36 (1), (tt, J = 7.4, 1.2 Hz)	7.36 (1), (tbr, J = 7.8 Hz)	7.47/7.51 (1), (t, J = 7.5 Hz)	7.43 (1), (t, J = 7.5 Hz)
17 (Z)	3.06 (2), (m)	3.06 (2), (dbr, J = 6.5 Hz)	3.14/3.20 (1), (dd, J = 16, 7) / (dd, J = 16.5, 7); 3.20/3.28 (1), (dd, J = 16, 7) / (dd, J = 16.5, 7)	3.13 (1), (dd, J = 15.0, 7.5 Hz); 3.08 (1), (dd, J = 15.0, 7.5 Hz)
18 (L)	5.25 (1), (tq, J = 7.2, 1.3 Hz)	5.24 (1), (tq, J = 7.0, 1.4 Hz)	4.89/5.23 (1), (m)	5.09 (1), (t, J = 7.5 Hz)
20 (B2)	1.62 (3), (s)	1.62 (3), (s)	1.56-1.76/1.56-1.76 (3), (s)	1.65 (3), (s)
21 (Y)	1.66 (3), (s)	1.66 (3), (s)	1.66-1.8/1.66-1.8 (3), (s)	1.66 (3), (s)
22 (U)	2.41 (2), (m)	2.40 (2), (dd, J = 6.6, 1.7 Hz)	2.47/2.47(1), (dd, J = 15, 7.5) / (dd, J =14, 7); 2.65/2.56 (1), (dd, J = 15, 7.5) / (dd, J = 14,7)	2.53 (1), (dd, J = 14.5, 6.5 Hz); 2.48 (1) (dd, J = 14.5, 6.5 Hz)
23 (N)	5.07 (1), (m)	5.06 (1), (m)	5.11/5.13 (1), (m)	5.01 (1), (sbr)
25 (D2)	1.58 (3), (s)	1.57 (3), (s)	1.56-1.76/1.56-1.76 (3), (s)	1.59 (3), (s)
26 (W)	1.62 (3), (s)	1.62 (3), (s)	1.66-1.8/1.66-1.8 (3), (s)	1.65 (3), (s)
27 a (V1)	2.14 (1), (m)	2.14 (1), (m)	2.16/2.11 (1), (dd, J = 13, 4) / (dd, J = 13.5, 4)	2.19-2.10, (m)
27 b (V2)	1.66-1.67 (1), (m)	1.66-1.67 (1), (m)	1.80/1.71 (1) (traslapados)	1.79-1.71, (m)
28 (M)	5.04 (1), (m)	5.04 (1), (m)	4.94/4.98 (1)	5.01 (1), (sbr)
30 (C2)	1.62 (3), (s)	1.62 (3), (s)	1.56-1.76/1.56-1.76 (3), (s)	1.65 (3), (s)
31 (X)	1.67 (3), (s)	1.67 (3), (s)	1.66-1.8/1.66-1.8 (3), (s)	1.69 (3), (s)
32 (A2)	1.38 (3), (s)	1.38 (3), (s)	1.35/1.40 (3), (s)	1.34 (3), (s)
33 (E2)	1.06 (3), (s)	1.06 (3), (s)	1.13/1.17 (3), (s)	1.11 (3), (s)

Notation: s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, tt = triplet of triplets, m = multiplet, br = broad

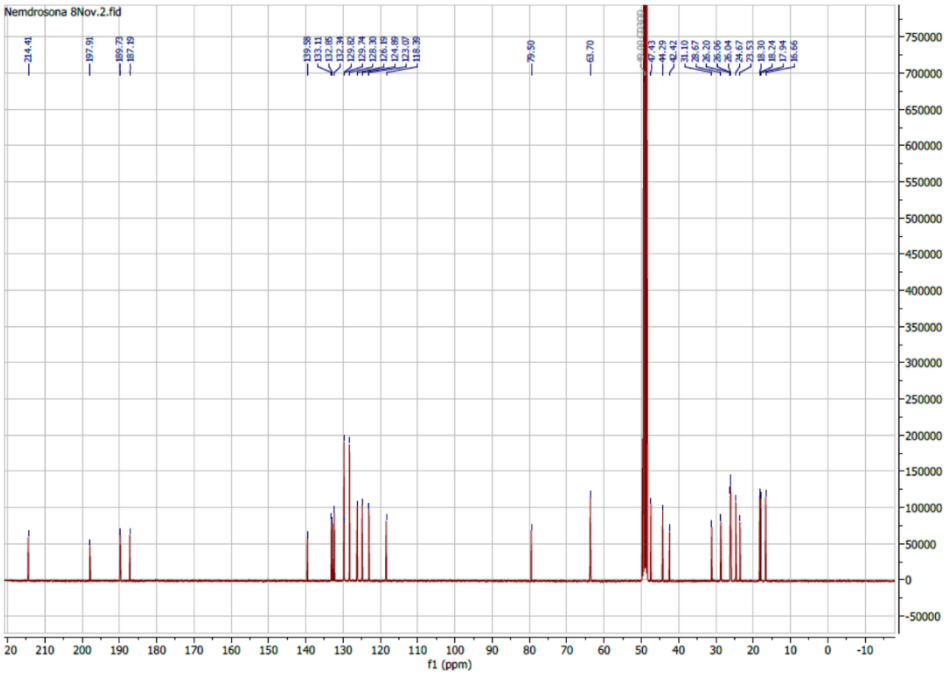

Figure 7A. The carbon-13 nuclear magnetic resonance (^{13}C-RMN) spectrum of nemorosone in CD_3OD (reference at 49.00 ppm) was measured at 100.62 MHz.

Table 2A. Comparison of the ^{13}C-RMN data of nemorosone isolated from the propolis extract CD 5A with data reported in the literature.

Assigned Carbon Atom	Nemorosone Isolated from CD 5A (CD_3OD, ppm)	Umaña (2013), Umaña et al. (2023) (CD_3OD, ppm)	Cuesta-Rubio et al. (2001) (CDCl3, -55°C, Tautomer a/Tautomer b, ppm)	Nemorosone Synthetic (Uwamori et al., 2012) (CD_3OD, ppm)
1 (P)	79.5	79.5	78.0/71.4	78.3
2 (D)	187.2	187.2	192.2/168.2	Not reported due to tautomerism.
3 (O)	118.4	118.4	117.6/117.2	121.07
4 (C)	189.7	189.7	170.9/195.0	Not reported due to tautomerism.
5 (Q)	63.7	63.7	57.1/64.8	62.1
6 (T)	42.4	42.4	39.4/42.1	42.4
7 (S)	44.3	44.3	42.9/42.1	44.7
8 (R)	47.4	47.4	48.2/47.3	49.1
9 (A)	214.4	214.4	207.3/207.6	209.7
10 (B)	197.9	197.9	193.2/193.1	195.2
11 (E)	139.6	139.5	136.6/135.9	138.4
12,16 (J)	129.7	129.7	128.1/128.0	129.7
13,15 (K)	128.3	128.3	127.9/127.9	128.9
14 (H)	132.3	132.4	132.4/132.6	133.5
17 (Z)	24.7	23.5	21.6/21.7	24.5
18 (L)	126.2	126.2	119.6/119.1	124.2
19 (I)	129.8	129.8	138.2/139.5	133.2
20 (B2)	18.3	18.3	17.9/17.9	18.4
21 (Y)	26.0	26.0	25.9/25.9	26.15
22 (U)	31.1	31.1	28.9/29.2	30.6
23 (N)	123.1	123.0	118.4/119.3	121.1

24 (F)	133.1 o 132.8	133.1 o 132.9	135.2/134.7	135.2
25 (D2)	18.2	18.2	18.0/18.0	18.3
26 (W)	26.2	26.2	26.0/26.0	26.4
27 (V)	28.7	28.7	26.4/27.3	28.3
28 (M)	124.9	124.9	122.1/122.1	122.5
29 (G)	133.1 o 132.8	133.1 o 132.9	133.6/133.6	134.3
30 (C2)	17.9	18.3	18.2/18.2	18.1
31 (X)	26.1	26.2	26.3/26.3	26.17
32 (A2)	23.5	23.5	23.2/24.1	23.9
33 (E2)	16.7	16.7	15.6/15.6	16.4

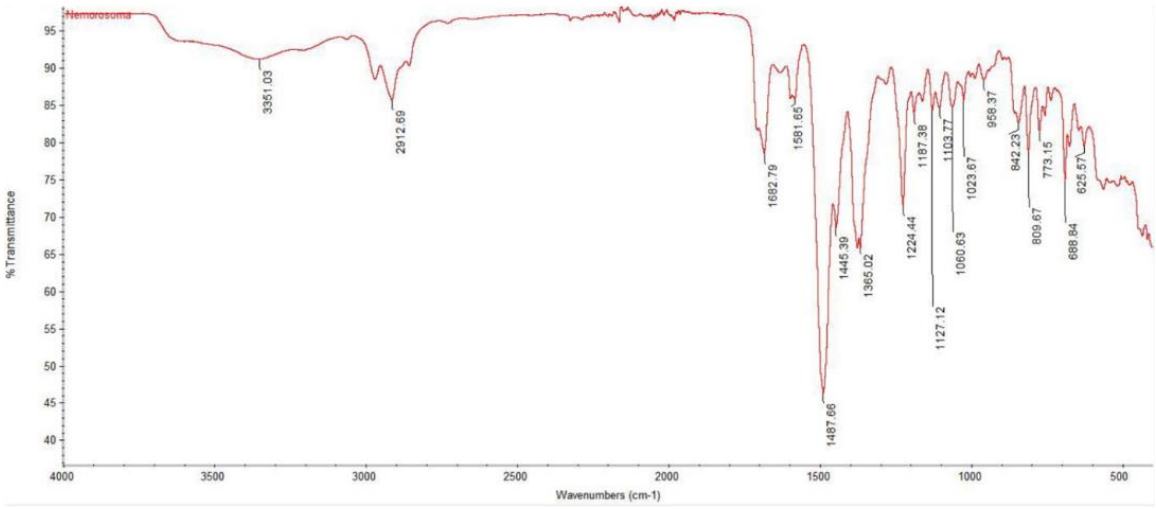

Figure 8A. Infrared spectrum (ATR) of nemorosone.

Table 3A. Comparison of the infrared spectra of nemorosone isolated from extract CD5A with literature data.

Signal (assignment)	Nemorosone isolated from extract CD 5A / cm^{-1}	Cuesta-Rubio et al. 2001 / cm^{-1}	Uwamori et al. (2001) / cm^{-1}
1 (O-H)	3351	3431	3297
2 (C-H)	2913	2969	2923
3 (C=O	1713	1708	1723
4 (C=O)	1683	NR	1699
5 (C=C)	1582	1590	1581
6 (C=C)	1445	NR	1446
7 (C-O-H)	1365	NR	1372
8 (C-O-H)	1224	NR	1220
9 (C-O-H)	1187	NR	1186

Notation: NR=Not reported

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