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## **Advances in Pharmacological Activities and Chemical Composition of Propolis Produced in Americas**

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### **Abstract**

Propolis is a resinous material produced by bees from the selective collection of plant exudates that are subsequently mixed with beeswax and salivary bee secretions. Propolis has been used in folk medicine, and certainly, several studies have validated its biological properties. The chemical composition and pharmacological activities of propolis collected through North (including Central America and Caribbean) and South America have been studied in the last years, and several papers have reported differences and similarities among the analysed geographical samples. Propolis has been classified according to its aspect and plant source; however, the ecological diversity present along the Americas provides a plethora of botanical resins. Herein, we summarize and discuss most of the studies performed at present on this profitable product for apiculture, attempting to compare the bioactivity, phytochemical diversity and botanical sources of honeybee propolis produced in Americas.

**Keywords:** Propolis from Americas, biological properties, chemical constitution, *Apis mellifera*, botanical sources

## 1. Introduction

Bees are the most ecologically important pollinators for flowering plants, a coevolutionary activity they have been performing for more than 100 millions of years. In particular, eusocial bees have reached an evolutionary success by living in perennial colonies (approximately 50,000 individuals) and developing sophisticated recruitment communication mechanisms to foraging and profit the chemistry of plants via manufacture and application beehive products for their own benefit [1–5]. According to their significant role as vectors of pollen in agricultural crops and the impact of beehive products for human societies, bees have earned an important position in different civilizations through history and geographies. Indeed, the bee management practice has been described since ancient times, including two types of beekeeping: apiculture (Apinae) and meliponiculture (Meliponinae). The first includes the Asian honeybees (*Apis cerana*) and western European honeybees (*Apis mellifera*), while the second refers to the native tropical and subtropical stingless bees (*Melipona* sp., *Oxytrigona* sp., *Scaptotrigona* sp., *Tetragonisca* sp. and *Trigona* sp., among others). At present, the beekeeping practice with western European honeybee is geographically widespread as a consequence of human migrations; thus, *A. mellifera* have settled down in all the lands that men have done, reaching a wide phytogeographical distribution range, including almost every vegetated place on earth [2, 3, 6–10].

Inside the hive, the cooperative behaviour of eusocial bees is reflected by the contribution of each individual into colony maintenance, resulting in a suitable community health termed social immunity, which is characterized by hygienic practices, accompanied with the removal of diseased brood to avoid the persistence of pathogens and parasites inside the hive. However, one of the expensive consequences of social living is the disease transmission due to high interaction among individuals, and in order to supply additional immunological benefits, bees collect antimicrobial natural resins to produce a substance called propolis [4, 11–13]. Propolis, such as honey, beeswax and royal jelly, is one of the beehive products that have been valuable for human societies through the ages. In general, honeybees (*A. mellifera*) produce propolis on the basis of a selective harvesting of resins present in leaves, buds, sap flows, trichomes and other actively exuding plant structures that are subsequently mixed with beeswax and salivary gland secretions, yielding a chemically complex resinous material [1, 14–18]. The gathered exudates are mostly incorporated into propolis without chemical modifications; however, some glycosides are subjected to enzymatic action by salivary hydrolases from bees [19, 20]. Unlike honeybees, stingless bees produce different resinous materials by adding soil and clay particles to the final mixture of plant exudates and beeswax, resulting in a particular matrix often called geopropolis, which differs from propolis of *A. mellifera* by the presence of minerals in addition to the absence of plant trichomes [6, 20–22].

Propolis varieties produced by honeybee and stingless bee possess mechanical and biological properties, and these materials are used inside the hive in order to seal cracks, to prevent structural damage and to act as a thermoregulatory resource; in addition, those products are used as chemical weapons to protect the colony from diseases, acting as antimicrobial and as embalming substances that avoid putrefaction of killed intruders [1, 14–18, 23, 24]. Moreover,

in several human traditional medicine systems, propolis has been used as a remedy due to its properties. In fact, Ancient Egyptians, Greeks and Romans employed this sticky material mainly as a wound healing and as an antiseptic agent. In addition, in Central and South America, Maya and Inca civilizations used cerumen and geopropolis produced by stingless bees as a folk remedy [15, 20, 25–28]. Nowadays, propolis is used in alternative medicine in Japan, and as a remedy to treat wounds, burns, sore throat and stomach ulcer in the Balkan states, meanwhile geopropolis is employed by the population of some tropical countries in Americas as empirical remedy for wound healing, gastritis, infections among others. In this context, propolis and geopropolis represent a promising source of bioactive compounds for pharmacological research [1, 14–18, 21, 28, 29].

Propolis has been extensively studied, and in the last decades, propolis has aroused scientific attention and many reports have been published concerning its broad spectrum of pharmacological activities and its bioactive components [14, 15, 18, 28]. At present, the biological activities reported for propolis include antibacterial [30–32], antioxidant [31, 33], antiparasitic [34–38], antifungal [39–41], antiviral [42], local anaesthetic [43], anti-inflammatory [44, 45], immunomodulatory [46, 47], antitumor [48–50], and antiproliferative activity on cancer cell growth [50–54], among others. There are remarkable differences in the biological activities of propolis from dissimilar geographical origin, and those mainly depend on the qualitative and quantitative variations of its characteristic chemical constituents, which are provided by botanical sources. Thus, the chemical diversity of propolis is dictated by the phytogeographical conditions and the climatic characteristics, and finally by the honeybee species involved in its production [55–57]. In that sense, the chemical composition of propolis from temperate zones (Europe, North America, Southern South America, and West Asia) differs from those of tropical zones (Central and South America, South and Southern Asia and Africa), as several studies have reported it in recent years.

Exudates from poplar buds (*Populus* spp.) are described as the main botanical source of propolis from temperate regions, as well as birch (*Betula alba* L.), horse chestnut (*Aesculus hippocastanum* L.), alder (*Alnus glutinosa* Medik), beech (*Fagus sylvatica* L.), and some conifers. At present, over 300 chemical compounds have been identified in different temperate propolis, including phenolic acids and esters, flavonoids, terpenes, lignans, aromatic aldehydes and alcohols, fatty acids, stilbenes and steroids [14, 15, 18, 58]. Otherwise, over 250 compounds have been identified in propolis samples from different tropical regions, including prenylated benzophenones, organic acids, prenylated organic acids, terpenes, alcohols and isoflavonoids [58, 59], where the main plant origins are *Baccharis dracunculifolia*, *Araucaria angustifolia*, *Clusia minor*, *Clusia rosea*, *Dalbergia ecastophyllum*, *Macaranga tanarius*, *Hyptis divaricata* and *Eucalyptus citriodora*, among others [1, 58–61]. Nevertheless, it has been reported that despite the plant origin of propolis produced by *A. Mellifera*, its overall percent composition remains at certain point preserved, comprising 50% botanical resins, 30% waxes, 10% essential and aromatic oils, 5% pollen, and 5% other organic substances. However, as a result of chemical diversity present in propolis, its organoleptic properties may vary considerably, including its physical aspect,

consistency, aromatic smell, and its colour that fluctuates from dark-brown to yellow [22, 58, 62–64].

Propolis must be purified in order to proceed into pharmacological and chemical investigations. The removal of inert material, which is mainly wax, is generally performed by preparing alcoholic or hydro-alcoholic extracts of the macerated raw material, where ethanol, ethanol 70% and methanol are often used. Several analytical techniques have been used to identify and characterize the chemical constituents of propolis samples from different geographical origins, including chromatographic and spectroscopic methods, such as thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and their coupled techniques to mass spectrometry (MS) [1, 58]. GC–MS analysis has been used for the identification of volatile and semi-volatile components of propolis; however, many propolis constituents are not sufficiently volatile for GC–MS analysis, even with derivatization procedures. In that sense, electrospray ionization (ESI) has been extensively used to ionize nonvolatile, thermally unstable, heavy and polar molecules; therefore, ESI-MS and its tandem version ESI-MS/MS have been applied for analyse propolis. ESI ionizes more efficiently polar compounds with acid [negative ion mode (-)] or basic sites [positive ion mode (+)], an important characteristic for the chemical study of propolis, since most of the pharmacologically active constituents identified in propolis possess certain polarity, commonly acidic or phenolic moieties [65, 66]. In addition, nuclear magnetic resonance (NMR) techniques have been used to fully characterize the isolated compounds from propolis [1, 58].

The chemical composition and pharmacological activities of propolis samples collected throughout North America (including Central America and Caribbean) and South America have been studied in the last years, and several papers have reported differences and similarities among the analysed geographical samples. At present, propolis from Americas has been classified according to its aspect and plant source, due to the ecological diversity present along the continent that provides different botanical resins. The major classes include the North and Southern South American propolis described as ‘poplar’ type, as well as Cuban, Brazilian and Mexican ‘red’ propolis type, in addition to the Brazilian “green” type, and finally the Cuban and Venezuelan “Clusia” type, reflecting a propolis diversity produced as consequence of adaptive responses of European honeybees to the beehive necessities and vegetation present in Americas, both in tropics and in temperate zones. Moreover, propolis produced by the stingless bees in Americas has gain attention and different samples have been chemically and biologically analysed in recent years, exhibiting in some cases similarities in bioactivity and composition with propolis of honeybees from the tropical areas. However, in this chapter, we were exclusively focused on honeybee propolis.

Some authors have reviewed and presented an extensive number of reports in propolis research with different approaches [1, 14, 15, 18, 21, 28, 55, 58, 62], although the discussion of analyses carried out in propolis from Americas in an attempting to compare the bioactivity, phytochemical diversity and botanical sources has not been approached. Herein, we summarize and discuss for the first time; to the best of our knowledge, most of the studies on honeybee

propolis produced in Americas, including recent advances in research for this profitable product for apiculture. Most of the studies performed on propolis from Americas are depicted in **Figure 1**.



**Figure 1.** Chemical and pharmacological studies on propolis produced by *Apis mellifera* on Americas. The black dots on the Map indicate the geographical origin of studied propolis.

## 2. North American propolis

North America comprises the geographical region occupied by the countries of Greenland, Canada, United States and Mexico, in addition to the countries included in Central America and the Caribbean Islands. As a continent, North America is a terrestrial portion of the

Americas, which is considered rich in biodiversity and a land with a plethora of natural resources that are distributed along its extension. Moreover, this landmass has many topographical variations, from valleys to extensive chains of tall mountains, and is characterized by possessing a wide range of climates and biomes starting from tundra, subarctic forest, followed by temperate forest, plains, deserts and tropical forests [67, 68].

Although the honeybee is not native from North and South American continents, it is wide distributed throughout the Americas. European colonists have brought *A. mellifera* to the Western Hemisphere at the beginning of seventeenth century, by virtue of being a very adaptable bee species and for its management for human benefit [9, 69]. Afterwards, the African honeybee (*A. mellifera scutellata*) was initially introduced by scientists into Southeastern Brazil at mid-1950s, and since the accidental escape of African queen bees, a gradual process of Africanization of feral and managed *A. mellifera* colonies has reached the tropical and subtropical areas of North and South America [70–72]. Prior and after the Africanization process, propolis collected from Canada, United States, Mexico, Cuba, Honduras and El Salvador has been chemically and pharmacologically analysed, and here, we summarized the main results.

## 2.1. Canada

Chemical analysis on Canadian propolis by chromatographic technics, such as GC–MS and HPLC–MS, has identified the presence of poplar characteristic compounds. To the best of our knowledge, one of the initial studies on chemical composition of Canadian propolis was performed on a sample collected from Sydenham region in Ontario State [73]. Fifteen flavonoids, including pinobanksin-3-*O*-alkanoates and methyl ether derivatives of quercetin were detected. The major constituents in this Canadian propolis were the flavanones pinocembrin and pinobanksin-3-*O*-acetate and the flavones chrysin and galangin, and according to the presence of high amounts of these compounds, the botanical origin was associated to American poplars *P. deltoides*, *P. fremontii* and *P. maximowiczii* from section *Aigeiros*, which are characterized by the biosynthesis of these compounds [73].

In North America, poplars from section *Tacamahaca* and *Leuce* are as wide distributed as poplars from *Aigeiros* section. With the aim to determine the chemical composition of propolis from Canada collected outside the geographic zone of distribution of *Aigeiros* poplars, two samples from different regions the Boreal forest (Richmond, Québec) and Pacific coastal forest (Victoria, British Columbia) have been analysed by GC–MS. The major constituent in Victorian propolis was *p*-hydroxyacetophenone, followed by benzyl hydroxybenzoate, cinnamic acid and significant amounts of five dihydrochalcones. The presence of these compounds suggested that a poplar characteristic from the section of *Tacamahaca*, *P. trichocarpa* Torr. et Gray, is the plant source of propolis from the Canadian Pacific coast [74]. Otherwise, the main constituents found in propolis from Richmond region were *p*-coumaric and cinnamic acids and their derivatives, wherein the high presence of these compounds and the low amount of flavonoids are characteristic of resins of *Populus* spp. from section *Leuce*, which suggested that *P. tremuloides*, a widespread poplar in the Canadian Boreal forest, could be the botanical source of Richmond propolis. The antioxidant or antiradical activity of Canadian propolis collected from

British Columbia and Québec was determined by DPPH assays. Both samples presented a potent-free radical scavenging (FRS) activity (at  $\sim 26 \mu\text{g/mL} = 79 \pm 5$  and  $65 \pm 7\%$ , respectively), and those results were related to the presence of aforementioned diverse phenolic compounds [74].

More studies have been done regarding the chemical composition of commercialized extract formulations of Canadian propolis, such as Herstat® propolis extract ACF® (antiviral complex of flavonoids), which is patented and manufactured with propolis (at 3% concentration) collected from a specific area in Western Canada (probably Manitoba), which is rich in poplar trees. Bankova et al. [75] have found by GC–MS that the chemical composition of the marketed ointment formulation was mainly comprised by benzoic and *p*-coumaric acid, benzyl *p*-coumarate and a group of dihydrochalcones, in addition to pinocembrin chalcone and pinostrobin chalcone. Interestingly, the presence of all these compounds suggested a mixed botanical origin, the exudates of both *P. balsamifera* and *P. tremuloides* [75]. The antiviral activity of Herstat® propolis extract, specifically against both types of Herpes simplex infection: HSV-1 and HSV-2, was determined by clinical studies conducted with the application of this ointment, which resulted to be more effective than acyclovir treatment by presenting a significant shortening of healing time and a reduction of the local symptoms of cold sores [76, 77]. Additionally, with the aim to understand the basis of the antiviral activity of Herstat® Canadian propolis extract, Bankova et al. [75] determined by *in vitro* studies the virucidal effect of this ointment on HSV-1 and HSV-2 and the adsorption suppression of virus HSV-1 on MDBK bovine kidney cells. The *in vitro* results were in accordance with those obtained by structured clinical studies with the topical ointment, supporting the usefulness of propolis extract against herpes virus lesions [75].

Furthermore, another commercial Canadian propolis acquired from Tropic™ products was analysed with the aim to determine the composition and to assess its antioxidant properties. Raw commercial propolis was extracted using a two-step sequential process with ethanol and water, wherein the higher antioxidant activity (by FRAP and DPPH methods) was exhibited by the ethanolic extracts, and a higher polyphenol and flavonoid content. Furthermore, the ESI-MS fingerprints revealed a resemblance with Brazilian brown propolis, in addition to the presence of chrysin and pinocembrin, among other flavonoids [78]. All these studies concerning to Canadian propolis provide enough phytochemical evidence about the botanical origin of those bioactive resins, which are mainly collected from different poplars species along the country, confirming the chemical composition of a propolis characteristic of temperate zones.

## 2.2. United States

The arrival of *A. mellifera* to the United States from England has been described circa 1622, initially in the Colony of Virginia and later in other Colonies in the Eastern region of North America [69]. In recent years, a significant number of studies have been done in regard to propolis collected throughout the United States, and one of the first studies was carried out with the aim to evaluate the inhibitory activity of propolis (collected in Illinois state) against *Paenibacillus larvae* ssp. *larvae* (formerly *Bacillus larvae*), the etiological agent of American foulbrood. A propolis solution exhibited an *in vitro* toxic effect on *P. larvae* (at  $10 \mu\text{g/mL}$ );

however, on later *in vivo* experiments, propolis administered to infected colonies only showed a short-term bacteriostatic effect that allowed *P. larvae* proliferation on the hive after treatment [79, 80].

To our knowledge, the subsequent analyses performed in propolis from the United States were focused on the identification of main chemical constituents of samples from Western Ohio, and both North and South Georgia (Athens and Claxton, respectively), where some similarities to the flavonoid profile of European propolis were found in Ohio and North Georgia samples, in addition to the identification of kaempferol, galangin, 3,3'-dimethoxyquercetin and 3-methoxykaempferol in Ohioan propolis [81]. Those flavonoids occur naturally in some species of *Populus*, *Pinus*, *Betula*, *Alnus* and *Aesculus* (horse chestnut), plant sources that are visited by honeybees for resins [16] and were proposed by the authors as possible botanical origin of Ohioan propolis. Interestingly, none of the aromatic acids (ferulic, caffeic and cinnamic acid) commonly present in European propolis were found in Ohioan sample, which established a difference among this propolis [81].

Moreover, in a comparative study with different geographical samples around the world, Kumazawa et al. [82] determined by HPLC analyses that a propolis sample from United States (supplied by Api corporation and Tamagawa University, Japan) was mainly composed by aromatic acids, flavonoids, and their ester and methyl ether derivatives. In particular, the high amounts of *p*-coumaric acid, pinobanksin-5-methyl ether, pinobanksin, chrysin, pinocembrin, galangin, pinobanksin-3-*O*-acetate and tectochrysin suggested a mixed botanical origin of exudates from *P. tremuloides* and poplars from section *Aigeiros* (Central and Eastern America): *P. fremontii* or *P. deltoides* or *P. maximowiczii* [18, 74, 75, 82]. In addition, this United States propolis presented a moderate FRS activity (DPPH assay:  $\geq 50\%$  at 20.0  $\mu\text{g/mL}$  and by  $\beta$ -carotene–linoleic acid system:  $\geq 30\%$  at 10.0  $\mu\text{g/mL}$ ) in comparison with samples from China Australia, New Zealand and Hungary ( $\geq 70\%$  at 20.0  $\mu\text{g/mL}$  and  $\geq 60\%$  at 10.0  $\mu\text{g/mL}$ , respectively) [82]. In another comparative study, the effective antimicrobial concentration of propolis from United States (at 20% concentration; the site collection is not specified), Turkey and Australia against oral pathogen microorganisms (*P. gingivalis*, *P. intermedia*, *C. rectus*, *F. nucleatum*, *C. albicans*, *C. parapsilosis*, *C. krusei*) without cytotoxicity induction on human gingival fibroblasts was determined. United States propolis, and as well as Australian, exhibited an effective growth-inhibitory activity against the tested microorganisms; however, at the same concentrations (dil. 1:256 for bacteria and 1:2048 for *Candida* species), a cytotoxic effect to gingival fibroblasts was observed [83].

In another comparative analysis, propolis from Eastern United States (Indiana and New York), Europe (Bulgaria, Finland and England) and Brazil was studied by ESI-MS/MS. Chrysin, pinocembrin and *p*-coumaric acid were identified in all those propolis samples, suggesting a similar botanical origin in poplar species in Eastern United States, South Brazil and Europe propolis [84]. Furthermore, propolis samples collected from the states of Oregon and Northern California (three samples from each one) were studied by GC-MS and HPLC analyses, displaying these samples a high content of *p*-hydroxyacetophenone, *p*-coumaric acid, *t*-cinnamic acid and flavonoids, such as galangin, chrysin, pinocembrin and pinobanksin derivatives, in addition to the presence of terpenes, terpenoids, chalcones and dihydrochal-

cones, which are chemical compounds characteristic of resins of balsam poplars of *Tacamahaca* section in the western part of the North American continent [85]. Interestingly, ferulic acid and caffeic acid esters were detected in samples from Oregon and only in one specimen from California; these compounds typically occur in resins from poplars of *Aigeiros* section (cottonwoods) and are absent in resins from poplars of the *Tacamahaca* section [86]. Taken together, these results indicated that two samples from California possessed a pure plant origin on *Tacamahaca* poplars, while the other propolis specimen from California and the three samples from Oregon have a mixed plant origin from both *Tacamahaca* and *Aigeiros* poplars due to the presence of caffeic acid esters and ferulic acid [85].

Recently, a comprehensive analysis by GC–MS of ten geographically distinct propolis samples collected throughout the United States, including the cold North, the wet Southeast and the dry Southwest Regions, has provided a classification system by applying a chemometric approach principal component analysis (PCA) based on the relative amounts of main chemical classes found in the samples. Propolis from New York (NY-2,3,8,10), Pennsylvania (PN-6), Louisiana (LA-1), Minnesota (MN-9), Nebraska (NE-4), Nevada (NV-5) and North Carolina (NC-7) was analysed, and over 60 chemical constituents grouped in main compound types (benzoic and cinnamic acid derivatives, chalcones, flavanones and dihydroflavonols, flavones and flavonols, phenolic glycerides, and terpenes) were identified [87]. As expected, different geographical samples presented distinct chemical profiles. In all samples, poplar-type propolis compounds were found (aromatic acids and their esters, flavonoids and chalcones). Three main groups were obtained: group I) propolis rich in cinnamic acid derivatives (samples NY-2, NY-3, MN-9 and NY-10), such as benzoic, cinnamic, *p*-coumaric, and ferulic acids and benzyl-*p*-coumarate, is characteristic of *P. tremuloides* Michx. (American aspen) exudates, which would be the botanical source of these samples [87].

Group II) propolis with high concentrations of flavonoids (NE-4, PA-6 and NY-8), such as pinocembrin, pinobanksin, pinobanksin-3-*O*-acetate, chrysin, galangin and pinocembrin chalcone, chemical profile typical for poplar bud resins from section *Aigeiros* (*P. fremontii* resins were considered the main plant source). Group III) propolis rich in triterpenes (LA-1, NV-5, and NC-7), such as 3-oxo-6 $\beta$ -hydroxy-lup-20(29)-en-28-oic acid [87], a triterpenic acid previously identified in Honduran propolis, which suggested the participation of additional botanical resins to poplar exudates, essentially the tree *Liquidambar styraciflua* L. (main botanical source of Honduran propolis) [88], a distributed plant in east and southeast regions of the United States and characterized by the occurrence of this triterpene, among benzyl *p*-coumarate, and cinnamic acid derivatives. The presence of triterpenes is a new finding for North American propolis, since triterpenes have been only reported in samples from tropical and subtropical regions [58, 87, 88]. Moreover, the quorum sensing inhibitory (QSI) activity of the ten United States propolis was evaluated using the acyl-homoserine lactone (AHL)-dependent *Chromobacterium violaceum* strain CV026, with the aim to identify potential antivirulence capacity in those samples. The group II exhibited the highest QSI effect. This classification provided an insight on the mixed plant origin of some United States propolis, in addition to the already-known poplar-type propolis [87].

In another study, with the aim to characterize the antimicrobial activity of propolis against beehive pathogens, 12 samples were collected from different geographical regions of the United States (Chaska, MN; Baton Rouge, LA; Ithaca, NY; Jamestown, ND; Lincoln, NE; Raleigh, NC; Wakinville, GA; Tucson, AZ; Aspen, CO; Vacaville, CA; Beaumont, TX; Fallon, NV) and were evaluated against the bee pathogens *P. larvae* and *Ascosphaera apis*. The chemical composition profile of propolis samples was analysed by LC-MS-based metabolomic methods, revealing differences on chemical patterns and, as well, different ability of propolis samples to inhibit the growth of both pathogens. The highest activity on *P. larvae* and *A. apis* was exhibited by propolis from Nevada (IC<sub>50</sub>: 41.6 and 8.6 µg/mL, respectively), followed by Texas (IC<sub>50</sub>: 46.9 and 10.0 µg/mL, respectively) and California samples (IC<sub>50</sub>: 74.1 and 7.4 µg/mL, respectively) [13].

In order to track the botanical origin of antimicrobial resins (against *P. larvae*) gathered by *A. mellifera* in Northeastern United States, an analysis by HPLC and UPLC-TOF-MS of plant material collected by individual honeybees from an apiary located in Minnesota was carried out. Afterwards, using metabolomic methods (principal component analysis), the phytochemical patterns were analyzed and compared to those of resinous material collected from 6 North American *Populus* spp. and 5 hybrids, in addition to other plants in the surrounding areas. The results showed that honeybees only foraged resins from *P. deltoides* and *P. balsamifera* among many other plant sources available, including the chemotaxonomically related ones. From 26 individual resin foraging bees, 10 resulted to transport resin from *P. deltoides* and 15 from *P. balsamifera*. Moreover, phytochemicals present on *P. deltoides* and *P. balsamifera* resins did not showed to be influenced by regional or seasonal effects. These data suggested that honeybees discern among closely related *Populus* species to collect resinous material, in addition to a foraging behaviour maintained by individuals inside the hive to exclusively one plant source. Finally, the antimicrobial effect of *Populus* spp. resins against *P. larvae* presented differential inhibition as consequence of variations in secondary metabolites present in those resins [4].

Further chemical studies have been done in propolis collected outside the temperate poplar zone of North America, specifically in the Sonoran Desert (Southwestern United States). Wollenweber and Buchmann [89] have analysed several propolis samples from managed and feral honeybee colonies located in Arizona State with the aim to determine the botanical origin of propolis from desert zones. The widespread vegetation in Sonoran Desert is comprised by xeromorphic shrubs and cacti rather than poplars, which are scarcely found in some water-course zones. Wollenweber and Buchmann determined by TLC and GC-MS that some propolis samples presented the fingerprint pattern of *P. fremontii*, and as well, some others exhibited a mixed plant origin of *P. fremontii* and *Ambrosia deltoidea* (Torrey) Payne; meanwhile, samples out of flight reach of poplars contained flavonoid and other phenolic compounds characteristic to specific plants in this area, such as *Ambrosia deltoidea* and *Encelia farinosa* A. Gray [89].

In addition to the mainland United States, the Hawaiian Islands chain is included in the political territory of this country, and it represents a totally different ecological scenario with tropical characteristics. In that sense, poplars are not available for propolis production in Hawaii; thus, honeybees must use other botanical sources. In a recent study, Inui et al. [90]

have chemically investigated Hawaiian propolis by HPLC–ESI-MS in order to identify its botanical origin. A family of prenylflavonoids was identified in this propolis sample [90], chemical compounds that are additionally present in the Pacific propolis type (Okinawa, Taiwan, Indonesia and Myanmar propolis) [61]. By comparison of the chemical profile *Macaranga tanarius* resulted to be the main plant origin [90]. Previous studies suggested that exudates from buds and bark of *Plumeria acuminata*, *P. acutifolia*, *Schinus terebinthifolius* and *Psidium guajava* could be gathered by honeybees [18].

All these studies on United States propolis revealed that *A. mellifera* visits mainly *Populus* species in order to gather their prized antimicrobial resins. Although the phytochemical evidence additionally established that different complementary plants provide attractive resins to honeybees or even they represent the mainly chemical source of propolis collected from diverse climatic regions of the continental United States, plants such as *L. styraciflua*, *A. deltoidea* and *E. farinosa* and even *M. tanarius* in the tropical Hawaiian islands. Moreover, the great input provided by Wilson et al. [4] regarding to the foraging fidelity of one single individual of the colony to collect resins from exclusively one plant source, which suggests organization and specialization of individuals to particular plants in order to provide chemical diversity inside the hive. All these studies emphasize the variety of propolis types produced in the ecological regions of United States, suggesting mixed botanical origins for particular samples, yielding in a wide spectrum of pharmacological activities.

### 2.3. Mexico

Mexico is included among the five countries of the world with a great richness of endemic species, and this consideration is mainly related to the wide range of topographical diversity and the variety of climatic zones that lie between North American deserts and Mesoamerican forests [91]. Propolis collected from different ecological regions of Mexico has been analysed, including samples from North American deserts (Sonoran Desert), tropical forests, southern semi-arid highlands and temperate sierras.

One of the most investigated Mexican propolis types is Sonoran Desert propolis. Since almost a decade, several studies have been reporting the biological activities and main chemical constitution of samples collected from arid and semi-arid lands in the Sonora State [Ures (UP), Pueblo de Alamos (PAP) and Caborca (CP)]. Hernandez et al. [51] reported that the chemical composition of Sonoran propolis was mostly comprised by phenolic acids, flavonoids and their ester derivatives; moreover, pinocembrin, chrysin and pinobanksin-3-*O*-acetate were the main constituents in these three samples [51]. In particular, the presence of rutin, naringenin and hesperetin was exclusively found in propolis from PAP; meanwhile, xanthomicrol was found in the samples of PAP and CP, and 3'-desmethoxysudachitin compound was only detected in CP [51]. According to Wollenweber and Buchmann [89], the presence of xanthomicrol and 3'-desmethoxysudachitin is characteristic of *A. deltoidea* exudates, a plant that could be implied in the botanical source of CP and PAP since its widespread distribution along the Sonoran Desert. Otherwise, the presence of caffeic acid phenethyl ester (CAPE) was restricted only to UP, which is a chemical compound found in propolis from temperate zones, in addition to the higher amounts of pinocembrin, chrysin and pinobanksin-3-*O*-acetate found in this sample

that supported this resemblance to temperate propolis, suggesting that poplars from section *Aigeiros*, such as *P. fremontii* could be the botanical source of this propolis [14, 55, 73].

Additionally, the chemical constitution of propolis from CP was further investigated in another study carried out by Li et al. [54], which resulted in the NMR characterization of three new flavonoids: (2*R*,3*R*)-3,5-dihydroxy-7-methoxyflavanone 3-(2-methyl)butyrate, (7''*R*)-8-[1-(4'-hydroxy-3'-methoxyphenyl)prop-2-en-1-yl]chrysin, and (7''*R*)-8-[1-(4'-hydroxy-3'-methoxyphenyl)prop-2-en-1-yl]galangin, and as well other 41 isolated chemical compounds characteristic of exudates from the genus *Populus*, including aromatic acids, flavonoids and its esters. In addition, the *in vitro* cytotoxicity of the 44 chemical compounds was evaluated on PANC-1 human pancreatic cell line, and (7'*R*)-8-[1-(4'-hydroxy-3'-methoxyphenyl)prop-2-en-1-yl]galangin showed to possess the most potent preferential cytotoxicity (PC<sub>50</sub>: 4.6 μM) [54]. Lately, two phenylallylflavanones, (2*R*,3*R*)-6-[1-(4'-hydroxy-3'-methoxyphenyl)prop-2-en-1-yl]pinobanksin and (2*R*,3*R*)-6-[1-(4'-hydroxy-3'-methoxyphenyl)prop-2-en-1-yl]pinobanksin 3-acetate were identified in CP by first time and were new for propolis in general. These phenylallylflavanones additionally displayed a cytotoxic effect against PANC-1 (PC<sub>50</sub>: 17.9 and 9.1 μM, respectively) [92]. Moreover, the cytotoxic evaluation of 39 of those 44 compounds, isolated from CP, was carried out on a panel of six different cancer cell lines: murine colon carcinoma (colon 26-L5), murine melanoma (B16-BL6), murine Lewis lung carcinoma (LLC), human lung adenocarcinoma (A549), human cervix adenocarcinoma (HeLa) and human HT-1080 fibrosarcoma (HT-1080). The compounds (2*R*,3*S*)-8-[4-Phenylprop-2-en-1-one]-4',7-dihydroxy-3',5-dimethoxyflavan-3-ol, cinnamyl *p*-coumarate and 2-acetyl-3-caffeoyl-1-*p*-coumaroylglycerol exhibited the most potent cytotoxic effect in comparison with the tested flavonoids, phenolic acid derivatives and glycerides from CP [53].

More studies have been done in order to determine the biological activities of Mexican propolis samples collected in semi-arid and arid zones (UP, PAP and CP). Sonoran propolis showed a strong antiproliferative effect on human and murine cancer cell lines A549 (IC<sub>50</sub>: 58.6 μg/mL), HeLa (IC<sub>50</sub>: 31.7–49.8 μg/mL), LS-180 (IC<sub>50</sub>: 53.3–84.9 μg/mL), RAW 264.7 (Abelson murine leukemia virus transformed macrophages; IC<sub>50</sub>: 0.8–5.2 μg/mL) and M12.C3.F6 (murine B-cell lymphoma cells; IC<sub>50</sub>: 3.1–6.8 μg/mL). Moreover, CAPE, galangin, xanthomicrol and chrysin induced a significant antiproliferative effect on most of the cancer cell lines evaluated (IC<sub>50</sub>: 3.2–95.4 μM) [51]. Since DNA harvested from cancer cells treated with UP exhibited a ladder of internucleosomal DNA cleavage pattern characteristic of apoptosis, in addition to the morphological changes observed in treated cells, a study conducted with the aim to determine biochemical events produced at earlier stages of apoptosis has been done. By annexin V-FITC/Propidium iodide double labelling, it has been demonstrated that Sonoran propolis treatment induced antiproliferative effect on M12.C3.F6 cells through apoptosis induction, and this apoptotic effect resulted to be mediated by modulations in the loss of mitochondrial membrane potential and through activation of caspases signalling pathway (3, 8 and 9). Additionally, some of the constituents of Sonoran propolis that induce apoptosis in cancer cells were characterized by an HPLC–PDA–ESI-MS/MS analysis, followed by isolation procedures and NMR spectroscopy that yield eighteen flavonoids, commonly described in poplar-type propolis, including two esters of pinobanksin, pinobanksin-5-methylether-3-*O*-propanoate

and pinobanksin-5-methylether-3-*O*-butyrate were described by first time in propolis samples in general. Moreover, pinobanksin, pinobanksin-3-*O*-propanoate, pinobanksin-3-*O*-butyrate, pinobanksin-3-*O*-pentanoate, galangin, chrysin and CAPE induced antiproliferative activity on M12.C3.F6 cells through apoptosis induction [52].

The antibacterial and FRS activities of Sonoran propolis (UP, PAP and CP) have been tested by broth microdilution method and by DPPH assay, respectively. Sonoran propolis exhibited antibacterial activity against only Gram-positive bacteria, and UP presented the highest inhibition against *Staphylococcus aureus* (MIC: 100 µg/mL), followed by CP. CAPE, an exclusive constituent of UP, showed high growth inhibitory activity towards Gram-positive bacteria, particularly against *S. aureus* (MIC: 0.1 mM). CP presented the highest FRS activity (86% at µg/mL). The chemical constituents CAPE and rutin presented a high antioxidant activity (90.4 and 88.5% at 70 µM, respectively) in comparison with ascorbic acid control (95.0% at 70 µM). These results suggested that the presence of CAPE and rutin could be implied in the biological activities induced by Sonoran propolis [31]. Furthermore, the anti-*Vibrio* activity of those propolis samples collected in North-western Mexico was evaluated by broth microdilution method. UP presented the highest antibacterial effect against *Vibrio cholerae* O1 serotype Inaba, *V. cholerae* non-O1, *V. vulnificus* (MIC<sub>50</sub>: <50 µg/mL), and *V. cholerae* O1 serotype Ogawa (MIC<sub>50</sub>: 100 µg/mL). The constituents CAPE and galangin presented a potent growth inhibitory activity (MIC<sub>50</sub>: 0.05–0.1 mM) against *V. cholerae* strains (non-O1 and serotype Ogawa) [93]. Additionally, the *in vitro* antiparasitic activity of Sonoran propolis against *Giardia lamblia* has been tested. UP showed the highest growth inhibitory effect (IC<sub>50</sub>: 63.8 µg/mL) in comparison with CP and PAP (IC<sub>50</sub>: >200 µg/mL). Among the chemical constituents of Sonoran propolis evaluated, CAPE had the highest growth inhibitory activity (IC<sub>50</sub>: 222.1 µM), followed by naringenin (IC<sub>50</sub>: 461.8 µM), hesperetin (IC<sub>50</sub>: 494.9 µM) and pinocembrin activity (IC<sub>50</sub>: 680.6 µM) [34].

Since UP showed to be one of the most biologically active of the Sonoran propolis tested, the evaluation of the seasonal effect on the chemical composition and biological activities (anti-proliferative, antiparasitic and antioxidant activities) of UP has been done. The collected seasonal samples [spring (sp), summer (s), fall (f) and winter (w)] were analysed by an HPLC–DAD–UV method, wherein from the qualitative point of view, the chemical profile of the seasonal samples was similar; however, the results for antiproliferative effect on M12.C3.F6 cell line [sp (IC<sub>50</sub>: 11.6 µg/mL) > w (IC<sub>50</sub>: 26.6 µg/mL) > s (IC<sub>50</sub>: 49.7 µg/mL) > f (IC<sub>50</sub>: 54.5 µg/mL)] and antiparasitic activity on *G. lamblia* [s (IC<sub>50</sub>: 23.8 µg/mL) > w (IC<sub>50</sub>: 59.2 µg/mL) > sp (IC<sub>50</sub>: 102.5 µg/mL) > f (IC<sub>50</sub>: 125.0 µg/mL)] presented significant differences, which suggested that slightly quantitative variations on the bioactive constituents could be implicated in the seasonal effect of biological activities of UP. All propolis samples had weak FRS activity (<25% at 100 µg/mL) [34, 94].

Recently, the immunomodulatory properties of UP were tested (0.2–20.0 µg/mL) in a comparative study with Brazilian (Botucatu, Sao Paulo) and Cuban (Havana) propolis on pro- and anti-inflammatory cytokine production [tumor necrosis factor (TNF)-α and interleukin (IL)-10, respectively] by human monocytes. Brazilian propolis stimulated both TNF-α and IL-10 production by monocytes; meanwhile, Cuban propolis stimulated TNF-α and inhibited IL-10

production. UP exerted the opposite effect, inhibited TNF- $\alpha$  and stimulated IL-10 production. These results are due to qualitative and quantitative differences in the chemical constitution of the three samples, since different constituents that may exert pro- and anti-inflammatory activity depending on concentration. It is reported that the major compounds found in Brazilian, Cuban and Mexican propolis samples used in this study are artepillin C, isoflavonoids and pinocembrin, respectively, [46].

In a recent chemical comparative study performed with the aim to develop and validate a suitable RP-HPLC method to determine and quantify flavonoid markers in Mexican propolis, 11 samples collected at different ecological regions in six states (Estado de Mexico, Puebla, Chiapas, Zacatecas, Tlaxcala and Guanajuato) were analysed. Acacetin, 4',7-dimethyl naringenin and 4',7-dimethyl apigenin were used as marker components in this study, and the method was applied to establish some quantitative variations related to seasonal and geographical conditions of the propolis samples. 4',7-Dimethyl apigenin was selected as an appropriate marker of Mexican propolis, followed by 4',7-dimethyl naringenin. Both chemical compounds were considered useful for quality control procedures in the geographical origin validation of Mexican propolis [95].

The chemical constitution of a Mexican red-type propolis collected from Champoton at Southern Mexico (Campeche State) was analysed. Three new compounds 1-(3',4'-dihydroxy-2'-methoxyphenyl)-3-(phenyl)propane, (Z)-1-(2'-methoxy-4',5'-dihydroxyphenyl)-2-(3-phenyl) propene and 3-hydroxy-5,6-dimethoxyflavan were identified [56], in addition to seven known flavanones, isoflavans and pterocarpans that have been described in Cuban and Brazilian propolis. The occurrence of these compounds is related to the chemical profiles of plant exudates from the genus *Dalbergia*, which suggested the botanical relation of red Mexican propolis and *Dalbergia* species [56, 58].

Moreover, in a comparative study about the volatile constituents of propolis from honeybees and stingless bees collected in the Yucatan peninsula, ninety-nine compounds were identified by GC-MS, wherein common compounds were present in both types of propolis. However, styrene, phenylacetaldehyde, trans-sabinene hydrate, nonanal, decanal, 2-undecanone, cyperen, cis- $\alpha$ -bergamotene, massoia lactone, ar-curcumene, cis-calamenene, cardina-1,4-diene,  $\alpha$ -cadinene,  $\beta$ -eudesmol,  $\alpha$ -bisabolol, neryl linalool, geranyl linalool, manoil oxide, kaur-16-ene, pentacosane and heptacosane were identified only in honeybee propolis [96].

In general, these studies confirm the chemical diversity present in propolis produced by *A. mellifera* in Mexico, suggesting the participation of exudates from different plant species as consequence of the ecological diversity present throughout the country, some of the chemical compounds could be useful as taxonomic markers to differentiate the type of Mexican propolis. In addition, the plethora of biological properties, including antioxidant, antibacterial, antiproliferative, cytotoxicity, antiparasitic and immunomodulatory effects, has demonstrated that Sonoran propolis is a source of bioactive constituents for pharmacology research.

## 2.4. Cuba

The isle of Cuba is the largest in the Caribbean Sea, and together with Island of Youth and over 4000 islands comprise the Cuban Archipelago. The moderate tropical climate together with the exposure to different wind currents, topographical variations, diversity in moisture levels and types of soil produce heterogeneous ecological regions, such as the wetlands in the southern coast, tropical desert-like conditions in eastern coast, and pine forest at mountains, which make Cuba an example of almost every ecosystem present throughout the Antilles Islands [97]. Cuban propolis is the most investigated propolis type from North America (including Caribbean and Central America) and several studies have been published in regard to its composition and properties.

The first studies carried out on Cuban propolis were mainly focused on its biological properties, since it is considered as a traditional homemade remedy in this country. However, most of these studies were performed without a certain information on the chemical constitution of Cuban propolis, which makes it difficult to draw a direct correlation between bioactivity reported 20 years ago and the chemical constituents identified at present [58]. The antioxidant effect of ethanolic extracts of Cuban propolis from Baracoa and Pinar del Rio province was analysed by their scavenging action against different species of oxygen radicals (superoxide and alkoxy) using luminol-sensitized chemiluminescence, both propolis preparations showed a high antioxidant activity against superoxide ( $IC_{50}$ : 5.0 and 9.5  $\mu\text{g/mL}$ , respectively) and alkoxy ( $IC_{50}$ : 0.5 and 0.6  $\mu\text{g/mL}$ , respectively) radicals [98].

Moreover, the hepatoprotective effects of Cuban red propolis (CRP) from Havana region were evaluated in different models of acute liver injury induced by paracetamol (600 mg/kg) [99, 100], and by allyl alcohol (64 mg/kg) [101], both in mice. The intraperitoneal administration of ethanolic extract of propolis (25, 50 and 100 mg/kg) showed to decrease significantly the enzymatic activity of alanine aminotransferase (ALAT), the levels of reduced glutathione (GSH), and as well showed to reduce liver damage, these protective effects of propolis were produced both, before (30 min.) and after (2 h) paracetamol hepatotoxicity induction [99, 100]. Similar results were obtained with CRP administration before allyl alcohol (30 min.) [101].

Additionally, CRP was evaluated in other two models of acute hepatotoxicity induced by carbon tetrachloride ( $\text{CCl}_4$ ) and by galactosamine (1000 mg/kg) in Sprague–Dawley rats [102–104]. The treatment with CRP (5, 10 and 25 mg/kg) in rats with hepatotoxicity induced by  $\text{CCl}_4$ , showed to reduce ALAT and hepatic malondialdehyde (MDA) levels in blood serum, and as well decreased the triglyceride (TG) levels in liver in comparison with control group [102]. Furthermore, histopathological evaluation revealed rats treated with CRP (25, 50 and 100 mg/kg) exhibited a significant reduction in liver injury, according to the low count of affected cells and the limited extension of steatosis area in comparison with those rats with control treatment [102, 103]. Similar biochemical histopathological results were obtained by CRP against the hepatotoxicity induced by galactosamine [104]. After these experiments, it was suggested that CRP probably exerted its hepatoprotective effects by antioxidant properties (scavenging action against oxygen radicals) [100–104].

The antipsoriatic, anti-inflammatory and analgesic effects of CRP were additionally assessed in another study, wherein CRP induced the formation of granular layer in a mouse tail model, reflecting its antipsoriatic activity; meanwhile, its anti-inflammatory effect was observed using three different test models [Cotton-pellet granuloma assay in rats Sprague–Dawley (dose: 50 mg/kg i.g.), croton oil-induced edema (dose: 25%; 2.5  $\mu$ L) and the peritoneal capillary permeability test in Swiss albino mice (dose: 10 mg/kg)]. CRP exhibited an analgesic activity using the models of acid-acetic-induced writhing (25 mg/kg i.g.) and hot plate test in Swiss albino mice (40 mg/kg) [105]. Moreover, the sensitizing properties of CRP were assessed, and CRP did not induce erythema, edema, and the study also revealed the absence of dermal and ocular toxicity in guinea pigs and New Zealand rabbits at 24 h. However, a moderate contact allergy potential was identified in CRP treatment by slight induction of erythema [105].

Since Cuban propolis presented several biological activities, subsequent studies came as consequence of the renewed interest in exploring the chemical composition of propolis for drug development [61, 106]. An ethanolic extract of Cuban propolis collected from Nuevitas, Cuba has yielded the isolation and structural determination of propolone A, the first polyisoprenylated benzophenone isolated from tropical propolis [106]. Although the presence of polyisoprenylated benzophenones in propolis from the tropical areas has been previously proposed, the full characterization of individual benzophenone molecules has not happened until this study [107]. In addition, propolone A showed significant antimicrobial and fungicidal activities against several Actinomyces, yeasts and Gram-positive and Gram-negative bacteria [106] that encouraged the study and isolation of other bioactive prenylated benzophenone derivatives present in Cuban propolis.

The presence of polyisoprenylated benzophenones in Cuban propolis suggested that resins from genus *Clusia* could be implied as botanical source [108]. Copey tree (*Clusia rosea*) is widespread distributed throughout Cuba, and nemorosone, a prenylated benzophenone, is one of the major compound present in floral resins of copey tree [108, 109]. In that sense, a phytochemical study of 21 propolis samples collected from different locations along the country was carried out with the aim to track the presence of nemorosone and other benzophenones characteristic of *Clusia* species. Nemorosone was identified as major compound in propolis from the western, eastern and central Cuba. Additionally, a mixture of xanthochymol and guttiferone E, present in resins secreted by fruits but not on floral resins of *C. rosea*, was detected in a lesser proportion in those propolis samples. This study has also provided evidence about the cytotoxic effect of nemorosone on human cancer cell lines HeLa, Hep-2, PC-3 and U251 ( $IC_{50}$ : 1.9–7.2  $\mu$ M) and FRS activity ( $IC_{50}$ : 44.1  $\mu$ M). Nemorosone showed to be more biologically active than a mixture of two its methyl derivatives on cytotoxicity ( $IC_{50}$ : 29.4–94.5  $\mu$ M) and antioxidant ( $IC_{50}$ : >200) evaluations [109]. Later, comprehensive chemical composition analysis of propolis collected from Guantánamo region led to the structural determination of three new polyprenylated benzophenones derivatives (propolones B-D), in addition to the presence of garcinielliptone I and hyperibone B [110].

By virtue of the differences of endemic plants in tropical and temperate regions and also the ecological diversity of Cuba, further investigations on the chemical composition of propolis from different regions of Cuba were carried out. A scrupulous chemical analysis of a CRP from

Pinar del río provided a cutting edge input on composition of propolis in general by reporting for the first time the presence and structural determination of isoflavonoids (isoflavones, isoflavans and pterocarpan) in a propolis sample, in addition to gallic acid, isoliquiritigenin, and (-)-liquiritigenin [57]. The occurrence of isoflavonoids is pretty restricted in nature and is characteristic of Leguminosae family, and these findings yield two different Cuban propolis types, suggesting the participation of at least two diverse botanical sources, one that provides prenylated benzophenones and another the isoflavonoids [57].

With the aim to study the chemical similarities and differences on Cuban propolis and to establish a classification system according to the presence of secondary metabolites, 65 propolis samples from different regions of Cuba were chemically analysed by HPLC–PDA, HPLC–ESI/MS and NMR. Cuban propolis types were grouped in three groups, including CRP rich in isoflavonoids; Cuban brown propolis (CBP), characterized by a high amount of polyisoprenylated benzophenones; and Cuban yellow propolis (CYP) that contains aliphatic compounds, which was sub-classified depending on its content on triterpenic alcohols (type A) and polymethoxylated flavonoids (type B) [58, 111–113]. Once CBP was identified as the major type of propolis produced in Cuba, biological assays were performed, especially on its capacity to inhibit *in vitro* cancer cell proliferation, since nemorosone, its main constituent, showed a potent cytotoxic effect on human cancer cells [109, 111]. CBP exhibited anti-metastatic effect in mouse mammary carcinoma Ehrlich's ascites tumour (EAT) cells in NMRI immunocompetent mice (5–23 µg/mL), in addition to cytotoxicity on diverse cancer cell lines, suggesting the potential of Cuban propolis as a source of possible anticancer agents [114].

Other studies showed that BCP induced significant antiproliferative activity on human breast cancer cell lines, preferentially on MCF-7 (estrogen receptor positive; ER  $\alpha$ +) rather than MDA-MB-231 (estrogen receptor negative ER  $\alpha$ -) in a dose-dependent manner. The antiproliferative effect on MCF-7 was partially related to apoptosis induction after an arrest in G1 phase of cell cycle was detected. Moreover, the co-administration of 17- $\beta$ -estradiol and an antagonist (ICI 182,780) allowed to hypothesize that BCP possesses an estrogen-like activity, although the effect would not be exclusively considered ER-dependent because the mortality also induced on MDA-MB-231 by BCP [115].

Otherwise, the treatment with nemorosone on MCF-7, MDA-MB-231 and LNCaP cells induced a selective antiproliferative effect on MCF-7 by arresting the cell cycle in G0/G1 phase, in addition to a reduction in the expression of pERK1/2 and PAkt. Nemorosone did not induce antiproliferative effect on MDA-MB-231 nor human prostate cancer cells LNCaP (which express ER $\beta$  but not ER $\alpha$ ), which suggested that nemorosone is the main responsible for the antiproliferative effect of BCP on ER $\alpha$ + breast cancer cells, and it could have therapeutic applications in breast cancer treatment since its activity on ER $\alpha$ + cells [116]. Moreover, nemorosone exhibited cytotoxicity in neuroblastoma cell lines, including their clone selected for resistance to chemotherapeutic compounds. It induced a G0/G1 arrest on cell cycle that yields a reduction in S-phase population, in addition to the detection of an upregulation of p21<sup>Cip1</sup>, presence of apoptotic DNA laddering, the activation of caspase 3 activity, dephosphorylation of ERK1/2 in LAN-1 and the inhibition of Akt/PKB [114]. Due to the reported correlation of nemorosone cytotoxicity on cancer cell lines to direct action on estrogen receptor

(ERs), other studies have been done. By *in vitro* tests [recombinant yeast assay (RYA) and E-screen assay], the antiestrogenic activity of nemorosone was demonstrated, exhibiting this benzophenone a reduction on the cell proliferation induced by 17- $\beta$ -estradiol (E2). Additionally, the treatment with nemorosone did not induce DNA damage in breast cancer cells MCF-7 BUS or in normal breast cells MCF10A. These results suggested that nemorosone could be a promising adjuvant for ER antagonists [117].

In addition to the presence of nemorosone in CBP, there have been identified mucronulatol and plukenetione A as cytotoxic and antiproliferative compounds that could contribute to the potent inhibitory activity of CBP on cancer cell proliferation. The occurrence of plukenetione A has previously been identified in *Clusia plukenetii*, and in later studies in CBP. Plukenetione A is reported to exert considerably cytotoxicity in a panel of cancer cell lines, including colon, ovarian, prostate carcinomas and neuroblastoma cells ( $IC_{50}$ : between 1.7 and 16.3  $\mu\text{g/mL}$ ); in addition, it induced in HCT8 cells that the depletion of S phase transitory cells as consequence of a G0/G1 arrest in cell cycle, followed by the presence of apoptotic DNA laddering, changes in gene expression patterns of genes required for cell replication and maintenance, accompanied by the inhibition of the enzymatic activity of both topoisomerase I and DNA polymerase [118]. The isoflavonoid mucronulatol has been described as one of the most cytotoxic constituents for Caribbean propolis. In general mucronulatol showed cytotoxicity MDR1-/MDR3+ cells (2.7–10.2  $\mu\text{g/mL}$ ), but not on MDR1+ cells (> to 100  $\mu\text{g/mL}$ ), which resulted as consequence of an interruption of cell cycle progression, by blocking at G1, accompanied by an upregulation of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> and a downregulation of cyclin E and CDK4, interfering in general with the cell cycle machinery [119]. The presence of mucronulatol has also been reported in Brazilian and Mexican red propolis [56].

Other different biological properties of nemorosone have been evaluated, including the mutagenic, antimutagenic and estrogenic effects. The mutagenic and antimutagenic activity of nemorosone were assessed by the Ames test on *Salmonella typhimurium* strains (TA97a, TA98, TA100 and TA102), wherein nemorosone did not induce any mutagenic activity; meanwhile, nemorosone exhibited a moderate to strong protective effect (31 and 53% of inhibition, respectively) in association with mutagens in strains TA100 and TA102. Nemorosone induced estrogenic activity detectable by recombinant yeast assay at various concentrations (EEq of  $0.41 \pm 0.16$  nM), concluding with those results that nemorosone could have a chemotherapeutic application in breast cancer research [120].

Red propolis has also been described in other tropical countries, including Brazil, Mexico and Venezuela. Brazilian red propolis (BRP) is a propolis type produced by honeybees from the resinous exudates of *Dalbergia ecastophyllum* in Northeastern Brazil [121, 122], and its chemical composition is mainly comprised by isoflavonoids, neoflavonoids, flavonoids and polyisoprenylated benzophenones (PPBs) [123, 124], which suggested some similarities to Cuban red propolis (CRP). In order to investigate the chemical composition, the botanical source and to draw a relation between different red propolis from Americas, CRP and BRP were analysed in a comparative study by HPLC–DAD–MS, in addition to *D. ecastophyllum* exudates (DEE) [125]. The presence of flavanones, isoflavones, isoflavans, pterocarpanes and a chalconoid was identified in both red-type propolis (BRP and CRP) and DEE. In addition, guttiferone E/

xanthochymol and oblongifolin A were exclusively detected in BRP. The flavans retusapurpurin A and the new retusapurpurin B were found to be the pigments responsible of the red colour in those samples. Indicating these results similarities in phytochemical composition of propolis collected from different tropical zones in Americas, since they apparently share exudates from *Dalbergia* species (probably DEE) as main plant source. However, the presence of PPBs in BRP suggested a complementary botanical origin in this sample. This study provided valuable information to attempt a more appropriate propolis classification [125].

All these studies contribute to understand the chemical diversity present in propolis from tropical zones, wherein *Populus* species are not present and honeybees have to gather bioactive exudates from other plant sources. In addition, these studies provide the pharmacological characterization of prenylated benzophenones and isoflavonoids, chemical constituents that represent a promissory source of therapeutic agents and could be used as chemical markers in future standardization of Cuban propolis. Subsequent studies are necessary in order to understand the mechanism of hepatoprotective effects of CRP, and as well to determine the chemical compounds involved.

## 2.5. El Salvador

As observed, honeybees had to find other different plant sources of bioactive resins in the tropics to those commonly visited in the temperate zones; thus, the chemical diversity present in tropical propolis, and the pharmacological properties of its constituents has attracted so much interest. In tropical propolis, it has been reported a variety of compounds, including the presence of polyprenylated benzophenones, isoflavonoids, and triterpenes in Cuban propolis [57, 111, 113], the occurrence of isoflavonoids and polyisoprenylated benzophenones in Brazilian propolis and, and 1,3-diarylpropane derivatives, flavonoids, and isoflavonoids from Mexican propolis [56].

With the aim to continue the studies of propolis from tropical Central America, a sample collected in the vicinities of Usulután, El Salvador, was studied and two new chalcones were isolated and characterized by NMR (2',3'-dihydroxy-4,4'-dimethoxychalcone and 2',3'-4-trihydroxy-4'-methoxychalcone) by first time in propolis. Both compounds presented a good antibacterial activity on *S. aureus* ( $29 \pm 3$  and  $23 \pm 1$  mm of inhibition zone, respectively) but not on *E. coli*. In addition, those chalcones inhibited *C. albicans* growth ( $19.3 \pm 0.6$  and  $29 \pm 1$  mm, respectively); thus, better antibacterial and antifungal activities than original propolis extract were presented ( $12 \pm 1$  and  $11 \pm 1$  mm, respectively). However, none of them exhibited a more effective cytotoxicity in brine shrimp (*A. salina* nauplii) lethality bioassay than propolis extract ( $LC_{50}$ :  $39 \pm 9$  mg/mL) [126].

In another study, two diterpene glycosides (*ent*-8(17)-labden-15-*O*- $\alpha$ -L-rhamnopyranoside and *ent*-8(17)-labden-15-*O*-(3'-*O*-acetyl)- $\alpha$ -L-rhamnopyranoside) were isolated from a propolis sample from the Eastern region of El Salvador, and both were reported by the first time in propolis. These new labdenol glycosides exhibited good antibacterial activity against *S. aureus* ( $21.0 \pm 1$  and  $20.3 \pm 0.6$  mm at 4 mg/mL, respectively) and showed to be more effective than the propolis ethanolic extract ( $12 \pm 1$  mm at 4 mg/mL), but those glycosides did not induce any effect on *E. coli* and *C. albicans*. Additionally, *ent*-8(17)-labden-15-*O*-(3'-*O*-acetyl)- $\alpha$ -L-

rhamnopyranoside exhibited a better cytotoxicity ( $LC_{50}$ :  $15 \pm 7$  mg/mL) than the extract ( $LC_{50}$ :  $39 \pm 9$  mg/mL) in brine shrimp lethality bioassay [127].

## 2.6. Honduras

In Central America, there are a high richness of endemic species, and since chalcones and diterpene glycosides were found in Salvadoran propolis [126, 127], and not in other tropical propolis analysed (Cuban, Brazilian and Mexican), the chemical investigation of other sample from Central America was carried out in order to establish differences and similarities in tropical propolis from Americas. To the best of our knowledge, only one study of Honduran propolis has been reported, wherein a sample collected in Marcala was fractionated and led to the isolation of cinnamic ester derivatives, including a new (*E,Z*)-cinnamyl cinnamate, in addition to flavanones, triterpenes, aromatic acids and one chalcone. Honduran propolis inhibited the ATPase activity of Pdr5p (70% at 100  $\mu$ g/mL), a protein responsible for a multidrug resistance phenotype in *Saccharomyces cerevisiae*, and four of its most abundant constituents (*E*-Cinnamyl-(*E*)-cinnamate ( $IC_{50}$ : 2.58  $\mu$ M), (*E*-Cinnamyl-(*E*)-*p*-coumarate ( $IC_{50}$ : 1.54  $\mu$ M), 6 $\beta$ -Hydroxy-3-oxo-lup-20(29)-en-28-oic acid ( $IC_{50}$ : 1.03  $\mu$ M) and sakuranetin ( $IC_{50}$ : 1.20  $\mu$ M) were also potent inhibitors [88].

The presence of these cinnamic ester derivatives has been described in exudates and volatile fractions of *L. styraciflua* (Honduras styrax, Hamamelidaceae). Interestingly, high amount of the triterpene compound 6 $\beta$ -hydroxy-3-oxolup-20(29)-en-28-oic acid have been reported in the cones of *L. styraciflua*, which suggested that *L. styraciflua* would be the botanical source of this Honduran propolis sample, in addition to the relative abundance of this plant in the surrounding areas to the beehives [88]. Cinnamic ester derivatives and 6 $\beta$ -hydroxy-3-oxolup-20(29)-en-28-oic acid were later described in North Carolina propolis, in addition to the presence of characteristic poplar flavonoids. Therefore, *L. styraciflua* exudates were proposed as secondary botanical source of North Carolina propolis. In that sense, Honduran and North Carolina propolis share a common plant origin, changing in some way the paradigm of poplar-type propolis in temperate areas, such as North Carolina, by additional incorporation of resins gathered from plants present in tropical and temperate zones of North America [87, 88].

## 3. South American propolis

South America is characterized by possessing the highest plant diversity of any other region in the world, which is due to several aspects, including its continental size and location (latitude and longitude), the presence of the largest extension of tropical forest and finally the Andes Mountains that form the biggest mountain system in the world, representing a linkage between tropical and temperate latitudes across South America [128, 129]. Propolis samples from different regions of South America have been studied. Brazilian propolis is by far the most analysed propolis in South America, followed by Argentinean, Chilean, Uruguayan and Venezuelan. Recently, propolis from Colombia and Bolivia has been studied; meanwhile,

Peruvian propolis has only been included in biological comparative studies. In this section, most relevant and recent advances in South American propolis research topic are summarized.

### 3.1. Colombia

Colombian vegetation possesses a great biodiversity with a high number of endemic plant species distributed in a variety of tropical forests, steppe and grasslands, representing a source of great variety in phytochemical substances [130]. Propolis is used in Colombia as a folk remedy, and in cosmetic and food industries, however, few studies in Colombian propolis have been done [131]. In a recent study, propolis collected from Medellin region, and subsequently extracted with n-hexane/methanol and fractionated with dichloromethane, exhibited an inhibitory effect on mycelial growth against *Botryodiplodia theobromae* (23.5% at 1 mg/mL) and two different strains of *Colletotrichum gloeosporioides* (38.1 and 47.6% at 1 mg/mL, respectively), which are important postharvest fungi that affect tropical and subtropical fruits. Through antifungal bioassay-guided fractionation of dichloromethane fraction, three labdan-type diterpenes were characterized: isocupressic acid [15-hydroxyabda-8(17),13E-dien-19-oic acid], (+)-agathadiol [abda-8(17),13-diene-15,19-diol] and epi-13-torulolol [8(17),14-abda-dien-13S, 19-diol], chemical compounds that could be responsible of the antifungal effect of Colombian propolis [131].

The presence of isocupressic acid and other labdan diterpenes has been described in Brazilian green propolis, a propolis type that has its plant sources in the resins of *Baccharis* spp. and *Araucaria heterophylla* [23]. In addition, the occurrence of agathadiol, isocupressic acid and turulolol has been reported in Algerian propolis, where its main botanical origin seems to be *Cistus* spp. (Cistaceae) [132]. In temperate regions of America, including Central America, inhabit three genera of Cistaceae (*Crocantemum* spp., *Hudsonia* spp. and *Lechea* spp.), however, in order to draw a phytochemical origin of those compounds, the botanical origin has to be investigated. Moreover, in another study, a propolis sample collected from La Union (Antioquia), showed a weak to moderate antifungal activity (MIC<sub>50</sub>: >1 mg/mL) on *C. acutatum*, *C. gloeosporioides*, *Aspegillus* sp. and *Penicillium* sp., in addition to a bacteriostatic (1.0 mg/mL) and bactericidal (10.0 mg/mL) effect against *B. subtilis* (Gram+). By GC-MS were detected fatty acids, and their esters, sesquiterpenes, pentacyclic tripterpenes and bicyclic labdan-type diterpenes, such as isocupressic acid, agathadiol and 13-epi-turulolol [133]. The presence of terpenes in both analysed samples of Colombian propolis suggested a possible common botanical source that has to be investigated in order to understand the origin of those phytochemicals and the biological activities of Colombian propolis.

### 3.2. Venezuela

As Colombia, Venezuela is a tropical region in South America that represents an enormous source of endemic plants and a great variety of bioactive compounds. One of the first chemical compositional analysis in tropical propolis was performed with 38 samples collected in different tropical areas of Venezuela, including Bolívar, Nueva Esparta, Cojedes, Yaracuy, Monagas, Mérida, Táchira, Portuguesa, Sucre, Baringas, Aragua and Carabobo, with the aim to identify phytochemical evidence for the botanical origin of Venezuelan propolis. Most of

these propolis samples contained similar chromatographic patterns characteristic of phenolic compounds, specifically of prenylated benzophenones, since flavonoids were identified only in few samples, and they were methylated 6-oxygenated flavones. The HPLC profile of resins excreted by the flowers of *Clusia minor* provides a very similar phenolic profile, suggesting that the botanical source of polyprenylated benzophenones present in Venezuelan propolis was the exudates from *C. minor* and other *Clusia* spp. [107].

Moreover, in a later study on Venezuelan propolis from Trujillo State, the presence of the already reported polyisoprenylated benzophenones scrobiculatones A and B was identified, together with their derivatives 18-ethyloxy-17-hydroxy-17,18-dihydroscrobiculatone A and 18-ethyloxy-17-hydroxy-17,18-dihydroscrobiculatone B (first time described in this study). Additionally, a mixture of scrobiculatones A-B exhibited a significant antibacterial activity against *S. aureus* (MIC: 125  $\mu\text{g}/\text{mL}$ ), a moderate toxicity towards *A. salina* nauplii ( $\text{LC}_{50}$ :  $14 \pm 6 \mu\text{g}/\text{mL}$ ) and low FRS activity by DPPH assay (10%) [134]. The chemical composition and the antioxidant effect of other three samples (V-1, V-2 and V-3) collected in San Antonio de los Altos, Venezuela, was subsequently analysed. The presence of prenylated benzophenone derivatives, diterpenes and triterpenes was detected by GC-MS, suggesting again the participation of *Clusia* spp. resins in Venezuelan propolis collected from different regions. Additionally, propolis samples (0.04% wt.) increased the oxidation stability of triacylglycerol molecules in comparison with control, and the effect of V-2 and V-3 samples was higher than V-1 (approximately 1.5-fold) [135].

### 3.3. Peru

Peruvian propolis has not yet been analysed from the chemical compositional point of view; however, there is a comparative report wherein propolis from Peru, Brazil, Netherlands and China was studied to determine the cytotoxic, hepatoprotective and FRS capacity (DPPH method) of the collected samples. Methanol (MeOH ext.) and water (Wt ext.) extracts were prepared for each propolis samples. Peruvian propolis showed the lowest FRS activity among the other propolis samples ( $\text{ED}_{50}$ : MeOH ext. 82.3  $\mu\text{g}/\text{mL}$  and Wt ext. 94.9  $\mu\text{g}/\text{mL}$ ), although it exhibited moderate cytotoxicity on murine colon 26-L5 carcinoma (51.1  $\mu\text{g}/\text{mL}$ ) and HT-1080 fibrosarcoma cells (76.4  $\mu\text{g}/\text{mL}$ ). The hepatoprotective activity of propolis was tested on primary cultures of mouse hepatocytes induced to cell death with D-galactosamine (D-GalN) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), wherein the MeOH extract of Peruvian sample showed toxicity at 200  $\mu\text{g}/\text{mL}$ ; meanwhile, the water extract exhibited significant hepatoprotective activity at 200  $\mu\text{g}/\text{mL}$  (approximately 45%) [136].

### 3.4. Bolivia

Bolivia is located at the centre of South America, and its territory comprises a transition into humid tropical and dry subtropical climatic zones. The geographical characteristics result in a great biodiversity present along the country; however, scarce phytochemical studies of Bolivian flora have been done. Propolis is used in Bolivia as antimicrobial remedy to treat infections and respiratory illnesses [137]. Bolivian propolis has been recently studied, and according to the chemical profile of ten samples collected from the main centres of beekeeping

in Bolivia, two main types of propolis have been identified. The first correspond to propolis from the valley regions (Cochabamba, Chuquisaca and Tarija) which were characterized by the presence of prenylated phenylpropanoids in a high amount, including *p*-coumaric and caffeic acid derivatives, such as drupanin (3-prenyl-*p*-coumaric acid) and artepillin C (3,5-diprenyl-*p*-coumaric acid) that were previously reported on Brazilian green propolis resulting from *Baccharis dracunculifolia* exudates [137, 138]. Meanwhile, the second type of Bolivian propolis came from La Paz and Santa Cruz regions, and their chemical composition was mainly comprised by cycloartane and pentacyclic triterpenes, including cycloart-24-en-3 $\beta$ ,26-diol, cycloart-24-en-3-one, cycloart-24-en-26-ol-3-one, mangiferonic acid methyl ester and lup-20(29)-en-3-one, which were triterpenes identified for first time in propolis in general. The antioxidant capacity of these ten samples was determined by DPPH, FRAP and ABTS assays, propolis samples rich in phenolic compounds presented moderate to strong antioxidant activity, while propolis rich in triterpenes showed a weak active [137].

Moreover, the antimicrobial properties of those ten Bolivian propolis samples and their main constituents were tested against 11 bacterial pathogenic strains of clinical relevance (*S. aureus* ATCC 25923, methicillin-resistant *S. aureus* ATCC 43300, *Escherichia coli* ATCC 25922, the clinical isolated *E. coli* 121, *E. coli* 122, *E. coli* LM2, *Salmonella enteritidis* MI, *Salmonella* sp. (LM), *Yersinia enterocolitica*-PI, *Pseudomonas* sp. and *Proteus mirabilis* 94-2) using micro-broth dilution method. Propolis samples exhibited different effects, from inactive (MICs > 1000  $\mu$ g/mL) to low (MICs 250–1000  $\mu$ g/mL), moderate (62.5–125  $\mu$ g/mL) and high antibacterial activity (MIC 31.2  $\mu$ g/mL). The samples that were rich in phenolics showed the high antibacterial activity; in comparison, terpene-rich samples were mostly inactive and some presented low activity. Kaempferol-3-methyl ether and drupanin were the most active constituents of Bolivian propolis. Additionally, the activity of propolis samples towards promastigotes of *Leishmania amazonensis* and *L. braziliensis* was evaluated and the results obtained were similar to those of antibacterial assays. The most active samples against *L. amazonensis* and *L. braziliensis* were from Cochabamba (IC<sub>50</sub>: 12.1 and 7.8  $\mu$ g/mL, respectively) and Tarija (IC<sub>50</sub>: 8.0 and 10.9  $\mu$ g/mL, respectively) [139]. These studies provided an insight in phytochemical variations of propolis from different regions devoted to beekeeping in Bolivia, suggesting the participation of at least two different botanical resins, which should be characterized in order to associate the pharmacological activities of those propolis samples to a certain plant origin. These results suggested that Bolivian phenolic-rich propolis could be a promissory source of antibacterial and antiparasitic therapeutic agents.

### 3.5. Brazil

Brazil has an extensive territory that comprises the richest flora in the world, a huge plant biodiversity with over 56,000 species, which represents approximately 19% of the total flora of the world [140]. Thus, Brazilian propolis possess a particular chemical diversity, involving different plant resins from dissimilar eco-regions, resulting in a variety of propolis types, including those with characteristic chemical composition of both tropical and temperate regions. At present, Brazilian propolis has been by far one of the most investigated propolis types, and a significant number of chemical compounds have been identified in samples

collected throughout the country. Due to the large amount of chemical and pharmacological studies of Brazilian propolis, herein, we present the most significant reports in an attempt to summarize hundreds of studies.

One of the first studies, focused on chemical characterization of bioactive constituents of Brazilian propolis, was carried out with a sample collected in Sao Paulo state, which yielded the isolation of three new compounds: 3,5-diprenyl-4-hydroxycinnamic acid, 3-prenyl-4-dihydrocinnamoyloxycinnamic acid and 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran [141]. These Brazilian propolis constituents presented antimicrobial activity against *Bacillus cereus* (MIC 15.6, 31.3 and 125 µg/mL, respectively), *Enterobacter aerogenes* (31.3, 62.5 and 125 µg/mL, respectively) and towards *Arthroderma benhamiae* (15.6, >250 and 62.5 µg/mL, respectively). Although the occurrence of cinnamic acid and other phenylpropanoid acid derivatives has been previously reported in propolis from temperate regions, the finding of prenylated derivatives was new for propolis [141].

In other studies, the occurrence of fifteen more cinnamic acid derivatives, in addition to nine *p*-coumaric acid derivatives, was identified from two different samples collected in Minas Gerais, together with other compounds, including six caffeoyl quinic acid derivatives, nine flavonoids, one prenylated phenolic acid, four diterpenoic acids and one lignin [142, 143]. Additionally, benzofuran derivatives (A and B) together with two known isoprenylated compounds were isolated from Brazilian propolis (not specified region). A moderate cytotoxicity was showed by benzofurans A and B against highly liver-metastatic murine colon 26-L5 carcinoma (12.4 and 13.7 µg/mL) and human HT-1080 fibrosarcoma cells (13.9 and 43.2 µg/mL) [144]. At present, hundreds of studies have been performed in order to chemically characterize the different samples of Brazilian propolis, revealing the great variety of compounds in those samples, including more than 145 constituents that have been identified and some of them were additionally tested to characterize their pharmacological activities [28, 58].

In order to establish the basis of Brazilian propolis classification, 500 samples were collected from different regions, including southern (Paraná and Rio Grande do Sul), Southeastern (Sao Paulo, Minas Gerais) and Northeastern (Bahía, Piauí, Ceará and Pernambuco) of Brazil. According to their chemical profile, the samples were classified into 12 groups, wherein five groups were identified in the Southern, one from Southeastern and six from Northeastern Brazil, suggesting a greater plant diversity on Northeastern and Southern than Southeastern region [145, 146]. The antimicrobial activity against *S. aureus* and *S. mutans* was tested, and propolis from group 6 originated from Bahia state (Northeastern Brazil) showed the highest inhibitory effect on both microorganisms (inhibition zone: 6 and 9 mm, respectively) [146]. Subsequently, the botanical origin of one representative group of each region was investigated. The foraging behaviour of honeybees that produced Brazilian brown propolis (BBP) from the South (Paraná, group 3), Brazilian red propolis (BRP) collected in Northeastern (Bahia, group 6) and Brazilian green propolis (BGP) collected from Southeastern (Sao Paulo, group 12) was observed to identify which plant is visited to gather the respective resins. The plant buds or unexpanded leaves were cut off to extract the resinous material, and using reversed-phase high-performance thin-layer chromatography (RPHPTLC), reversed-phase high performance liquid chromatography (RPHPLC), and GC-MS, the plant origin was revealed [145].

The main compounds identified in propolis from group 3 were flavonoids and organic acids, and the chemical profile of that sample was similar to that of poplar exudates, in particular to *Populus alba*, which were assumed as the main botanical source of this propolis from Paraná state. Interestingly, poplar trees are not native in Southern South America; nevertheless, European immigrants planted poplar trees in the temperate Southern Brazil [71, 147]. In propolis from group 6, the presence of some aromatic compounds, terpenoids and fatty acid esters was recognized and exudates from *Hyptis divaricata* Pohl were suggested to be the main botanical source according to the chromatographic profile [71]. However, since some disparities were observed in the chemical profile of *H. divaricata* and propolis of group 6, in addition to the identification of prenylated benzophenones (hyperibone A) in a later study, both facts suggested the participation of resins from some species of *Clusia* genus as supplementary botanical source [58, 148].

In group 12, the presence of prenylated derivatives of *p*-coumaric acid, the characteristic artepillin C (3,5-diprenyl-4-hydroxycinnamic acid), among other aromatic acids (dihydrocinnamic, *p*-coumaric, ferulic, caffeic and caffeoylquinic acids) and some flavonoids, including kaempferid, 5,6,7-trihydroxy-3,4'-dimethoxyflavone and aromadendrine-4'-methyl ether, and other compounds were found. Based on chemical evidence, *Baccharis dracunculifolia* resins resulted to be the main botanical source of this propolis type, in addition to *Araucaria angustifolia* and *Eucalyptus citriodora* exudates [59, 71]. The antibacterial and antifungal effect of propolis from group 12 against *S. aureus* and *C. albicans* (10.5 and 15 mm of inhibition zone, respectively) allowed to determine that the most bioactive resins came from *B. dracunculifolia* leaf exudates (9 and 16 mm, respectively) [59]. Subsequent studies on BGP and *B. dracunculifolia* exudates (Minas Gerais, group 12) by HPLC-APCI-MS and GC-MS allowed to identify 126 constituents present in those samples, including mainly cinnamic acid and its derivatives, flavonoids, benzoic acid and a few benzoates, non-hydroxylated aromatics, and aliphatic acids and esters, in addition to prenylated compounds, alkanes and terpenoids [138]. Moreover, particular triterpenoids have been identified in BGP, including lupeol, esters of lupeol,  $\alpha$ - and  $\beta$ -amyrins, cycloartenol, lanost-7,24-dien- $\beta$ -ol [58].

With the aim to establish a more precise correlation among Brazilian propolis types, thirty-eight samples were collected, from Minas Gerais, Sao Paulo, Paraná, Mato Grosso do Sul, Bahia and Alagoas, and were analysed by ESI-MS and tandem mass spectrometry (ESI-MS/MS). A principal component analysis (PCA) has been applied to determine similarities and differences in the secondary metabolite fingerprint of propolis samples. Brazilian propolis was divided into groups, according to their chemical profile and geographical origins. BRP from Alagoas and Bahia was divided into two main groups (R1 and R2), and as well propolis from South and Southeastern Brazil were divided in one BGP group, which contained the greatest number of samples, and two groups of BBP (B1 and B2) from Paraná state. Seven compounds were used as markers and allowed the classification of Brazilian propolis into those five groups according to PCA analysis, wherein the presence of intersection of constituents among samples was detected. Pinocembrin was in BRP R2 and BBP B1, and chsysin in BBP B1, 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran in BBP B2, 3-prenyl-4-hydroxycinnamic acid, 3,5-diprenyl-4-hydroxycinnamic acid and dicaffeoylquinic acid were identified in BGP and BBP B2, *p*-

coumaric acid in BGP and BBP (B1 and B2), and 3-methoxy-4-hydroxycinnamaldehyde in BBP (B1 and B2). As observed, there are chemical crossing in the five groups [84].

In regard to the chemical composition of BRP, recent studies have reported the presence of prenylated benzophenones, such as guttiferone E/xanthochymol mixture, the isoflavonoids isosativan and medicarpin, the triterpenoid ketone 20(29)-lupen-3-one, and also a naphthoquinone epoxide (isolated for the first time from a natural source) in a sample collected from the state of Alagoas [124]. The presence of these compounds suggested a mixed plant origin, since prenylated benzophenones are described in floral resins of *Clusia* species (Clusiaceae); in particular, the mixture of guttiferone E/xanthochymol and the triterpenoid have been reported in Cuban propolis [58]. Meanwhile, isoflavonoids have been related to *Dalbergia* species (Leguminosae). Additionally, isosativan, medicarpin and the guttiferone E/xanthochymol mixture presented antibacterial activity by inhibiting *S. aureus* (14, 23 and 19 mm), *E. coli* (0, 14 and 12 mm) and *C. albicans* (15, 26 and 0) growth. Moreover, the mixture guttiferone E/xanthochymol exhibited FRS activity by DPPH assay (49% at 48  $\mu$ M) [124]. It is also reported that the presence of retusapurpurins A and B (C30 isoflavanes) provides the red pigment to those red propolis in different regions of Americas [125].

More studies on chemical composition of BRP collected from Alagoas have been done, identifying methyl o-orsellinate, methyl abietate, medicarpin, 2,4,6-trimethylphenol, and the isoflavonoids, homopterocarpin, 4',7-dimethoxy-2'-isoflavonol and 7,4'-dihydroxyisoflavone. Biological tests have been carried out for the BRP extract, including antimicrobial against *S. aureus* ATCC 25923 and *S. mutans* UA159 (MIC: 25–50  $\mu$ g/mL), antioxidant (57% at 90  $\mu$ g/mL) and cytotoxic activity against HeLa cell line (IC<sub>50</sub>: 7.45  $\mu$ g/mL) [149]. In another study of BRP, the cytotoxicity of a sample collected from the South coast of Paraiba State was evaluated on Human pancreatic cancer cells (PANC-1), and since Paraiba BRP exhibited a 100% cytotoxicity at 10  $\mu$ g/mL, a subsequent phytochemical analysis was carried out and led to the isolation of 43 compounds, mainly flavonoids, including pterocarpanes, flavanonols, isoflavanones, chalcones, isoflavans, isoflavones, flavanones, lignans, a flavonol, a isoflavanonol and a neoflavonoid. Three novel compounds: (6aS,11aS)-6a-ethoxymedicarpan, 2-(2',4'-dihydroxyphenyl)-3-methyl-6-methoxybenzofuran and 2,6-dihydroxy-2-[(4-hydroxyphenyl)methyl]-3-benzofuranone, in addition to (6aR,11aR)-3,8-dihydroxy-9-methoxy-9-methoxypterocarpan, exhibited the most potent effect (100% cytotoxicity at 12.5  $\mu$ M) on PANC-1 cells [123].

The chemical composition of an essential oil extract obtained from Brazilian propolis collected in Rio de Janeiro was characterized, and 26 constituents were identified, wherein  $\beta$ -caryophyllene (12.7%), acetophenone (12.3%) and  $\beta$ -farnasene (9.2%) were the major constituents, in addition to the new compounds found linalool (6.47%), -elemene (6.25%), methyl hydrocinnamate, ethyl hydrocinnamate,  $\alpha$ -ylangene and valencene. This essential oil extract presented antimicrobial activity against *S. aureus* (14 mm, inhibition zone), *S. epidermidis* 25/04 (10 mm), *S. epidermidis* 194/02 (10 mm), *S. pyogenes* 93007 (14 mm), *S. pyogenes* 75194 (18 mm) and *E. coli* (17 mm) by agar diffusion method [150].

Since Brazilian propolis is continuously produced all over the year, it was important to determine the seasonal effect on propolis composition and evaluate the influence of season on its biological activities. By GC and GC-MS, the seasonal variations in chemical composition of

BGP collected from Sao Paulo state were investigated. All the seasonal samples contained phenolic compounds, mainly cinnamic acid derivatives as major constituents; however, the presence of diterpenes appeared in summer sample and reached a predominate percentage in the autumn sample, but being absent during the other seasons, which suggested the participation of at least two plants as botanical source [59]. These chemical variations could be important for the practical application of propolis, and in order to evaluate the effect of these slight changes on Brazilian propolis composition, antibacterial assays on bacterial strains isolated from human infections were carried out. The growth of Gram-positive bacteria, *S. aureus*, was inhibited at low concentrations of seasonal propolis samples (0.4% v/v), whereas Gram-negative, *E. coli*, bacteria were less susceptible to propolis treatment. It was concluded that there was no significant difference on the inhibitory action of propolis samples, discarding a seasonal effect on antibacterial activity of propolis from Sao Paulo, Brazil [30].

Propolis possesses immunomodulatory activities, and it has been reported that its immunostimulant effect is produced via macrophage activation, which enhances macrophage phagocytic capacity [28, 151, 152]. The effect of BGP on activation by reactive oxygen ( $H_2O_2$ ) and nitrogen (NO) species was determined on peritoneal macrophages obtained from male BALB/c mice. Both *in vivo* and *in vitro* experiments were carried out, and propolis stimulation resulted to induce a slight augmentation in  $H_2O_2$  releasing and a moderate inhibition of NO generation in a dose-dependent manner, suggesting that propolis acts on host non-specific immunity by macrophage activation [153]. Moreover, the effects of BGP on fungicidal activity of macrophages against *Paracoccidioides brasiliensis* were evaluated. Considering that cell-mediated immunity plays a significant role in host defence against this pathogen, peritoneal macrophages from BALB/c mice were stimulated with BGP and subsequently challenged with *P. brasiliensis*. This study suggested an increase in fungicidal activity of macrophages after propolis stimulation [151].

The role of toll-like receptors (TLR) in microbial pattern recognition and as well as the action of pro-inflammatory cytokines are important to trigger the initial events of immune response. Thus, the immunomodulatory action of BGP has been evaluated in regard to those mechanisms of innate immunity, and BGP treatment resulted to upregulate TLR-2 and TLR-4 expression, together with the production of pro-inflammatory cytokines (IL-1 $\beta$  and IL-6) in peritoneal macrophages and spleen cells of Male BALB/c mice treated with BGP of Sao Paulo State (200 mg/kg) for 3 days, suggesting the favourable action of BGP by enhancing immune responses in macrophages and spleen cells of treated mice [154]. Moreover, the action of BGP on antibody production was studied in rats immunized with bovine serum albumin, wherein BGP (10%), independently of the season of the year, stimulated antibody production, which was concluded to be a consequence of synergic effects, since isolated compounds and *B. dracunculifolia* exudates did not induced the antibody production [155].

Other studies in regard to the effects of BGP on immune response of acutely and chronically challenged stressed mice have been also investigated. After 3 days treatment under acutely stress conditions, BGP (200 mg/kg) restored TLR-2 and TLR-4 expression and increased IL-4 production in mice, in comparison with control [156, 157]. Meanwhile, BGP (200 mg/kg) during 7 days treatment in stressed mice increased the production of  $H_2O_2$  in macrophages and

reduced the alterations found in spleen [158]. Moreover, the BGP treatment (200 mg/kg) for 14 days in mice submitted to chronic stress exerted similar effects, demonstrating these experiments the immunomodulatory activity of BGP under *in vivo* experimental stress conditions [28, 152, 159, 160].

Once the effect of BGP on the initial steps of the immune response was evaluated in murine models, the immunomodulatory effect of BGP was evaluated on receptors expression, cytokine production and fungicidal activity of human monocytes. The BGP treatment (5, 10, 25, 50 and 100  $\mu\text{g/mL}$ ) resulted to upregulate TLR-4 and CD80 expression and decreased the production of TNF- $\alpha$  and IL-10 as concentration treatment increased. The fungicidal activity of human monocytes after incubation with BGP and challenged with *C. albicans* was increased in a dose-dependent manner. Moreover, cytokine production was reduced by blocking TLR-4, whereas the fungicidal activity was affected by blocking TLR-2, suggesting the involvement of these receptors in the mechanism of action of BGP [161].

Later, in an attempt to investigate the BGP constituents involved in the immunomodulatory effects on the innate immunity, caffeic and cinnamic acids (5, 10, 25, 50 and 100  $\mu\text{g/mL}$ ) have been assessed on human monocytes. Caffeic acid downregulated TLR-2 and HLA-DR expression. Otherwise, cinnamic acid downregulated TLR-2, HLA-DR and CD80; meanwhile, it upregulated TLR-4 expression depending on concentration. Both phenolic acids inhibited TNF- $\alpha$  and IL-10 production, whereas they increased the fungicidal activity of monocytes against *C. albicans*, without affecting cell viability. These data suggested that caffeic and cinnamic acid are partially involved in BGP effect on cell receptors expression and cytokine production, although the fungicidal activity of monocytes treated with those phenolic acids could be due to different mechanisms, possibly involving reactive oxygen and nitrogen species [162, 163].

In another study, the antioxidant and anti-inflammatory action of BGP and caffeic acid were determined. By DPPH assay, FRS activity of caffeic acid ( $\text{EC}_{50}$ : 2.5  $\mu\text{g/mL}$ ) resulted to be more effective than that of BGP ( $\text{EC}_{50}$ : 18.51  $\mu\text{g/mL}$ ), and the treatment with BGP and caffeic acid (5, 10, 25, 50 and 100  $\mu\text{g/mL}$ ) exerted anti-inflammatory action, by inhibiting nitric oxide (NO) production in RAW 264.7 cells. Moreover, both treatments suppressed LPS-induced signalling pathways, namely p38 MAPK, JNK1/2 and NF- $\kappa\text{B}$ , and did not induce hepatotoxicity at the tested concentrations, suggesting that caffeic acid may be involved in the antioxidant and anti-inflammatory effects of BGP [164]. BGP and its main botanical source, *B. dracunculifolia* exudates have been demonstrated to exert cytotoxic effect on several human cancer cell lines, including HEP-2, CaCo2, HCT116, HT-29 and SW480, and moreover on canine osteosarcome cells [165–168].

All these results obtained for Brazilian propolis reflect the enormous plant biodiversity of the country, obtaining different phytochemical patterns in samples collected from different geographical origins. Therefore, it is necessary to further investigate the chemical composition of samples produced in different zones in order to understand the plant origin of the bioactive compounds present in propolis from entire Brazil. The broad pharmacological activities tested for Brazilian green, red and brown propolis are a great example of biological interest in this beehive product, which makes it one of the most characterized propolis in the world. It is

important to continue the studies on Brazilian propolis to provide a more precisely insight about the possibilities of tropical, temperate and a mixture of both types of propolis from this biodiverse country.

### 3.6. Uruguay

Uruguay is covered by the temperate sub-humid grasslands, a biome extended through the vast plains of Southern South America, including part of Northeast and Central Argentina and Southern Brazil [169]. Uruguayan propolis has been investigated, and one of the first studies reported the identification of 22 different phenolic acids and flavonoids in six samples collected from different regions of the country. The presence of these constituents was detected by HPLC–PDA–UV, and resins from *Eucalyptus globulus*, *Populus* sp., *Betula* sp. and *Salix* sp. were suggested to be the botanical source of Uruguayan propolis. Those six propolis samples showed antibacterial activity against *B. subtilis* and *S. aureus* (MIC: 80–130 µg/mL); otherwise, the inhibitory effect of Uruguayan propolis against *E. coli* (MIC: 800–1000 µg/mL) was less efficient. Alkylperoxyl radical (ROO●) scavenging potential activity was additionally exhibited by those propolis samples (3.4–4.1 µg/mL) [170].

In another study, the chemical constitution of propolis collected from Montevideo was further investigated using HPLC–MS and NMR techniques and led to the isolation of eighteen flavonoids, four aromatic carboxylic acids and eleven phenolic acid esters, in addition to 3 new compounds elucidated: pinobanksin-3-*O*-isobutyrate and pinobanksin-3-*O*-(2-methyl)butyrate and 2-methyl-2-butenyl ferulate. The major constituents in that sample were pinobanksin-3-*O*-propanoate, pinobanksin-3-*O*-acetate, pinobanksin, pinostrobin, pinocembrin, techtochrysin, chrysin, galangin and cinnamyl *p*-coumarate, which suggested similarities to Southern Brazil, North American and European propolis [171]. Moreover, by RP–HPTLC, GC–MS and RP–HPLC analyses, it was determined that the phytochemical profile of propolis from Southern Brazil, Argentina and Uruguay was similar and correlated with that of *P. alba* resins; therefore, it was concluded that resins of poplar trees are the main plant origin of Uruguayan propolis [147].

Subsequently, a propolis sample from Montevideo was included in a comparative study with different geographical samples, wherein Uruguayan propolis was qualitatively similar to those from the United States, New Zealand, Hungary, China and other temperate samples included. Additionally, Uruguayan propolis presented a moderate antioxidant activity by a FRS method (DPPH assay: ≥30% at 20.0 µg/mL) and by β-carotene–linoleic acid system (≥45% at 10.0 µg/mL) [82]. The antioxidant properties of ten propolis samples collected in Southern Uruguay were further evaluated by *in vitro* (FRS by ORAC and inhibition of lipid and protein oxidation) and by cellular assays. Uruguayan propolis showed a high FRS activity by ORAC assay (800 µmol trolox equiv/g propolis). Uruguayan propolis inhibited LDL lipoperoxidation and protein nitration, and it was effective at cellular level by increasing endothelial nitric oxide synthase (eNOS) expression and inhibited endothelial NADPH oxidase, indicating a potential benefit by increasing nitric oxide bioavailability in the endothelium [172].

The volatile compounds of three samples from Central-southern Uruguay were analysed by static headspace technique coupled with a GC–MS, and compared with volatile content of

Brazilian, Estonian and Chinese propolis. Monoterpenes ( $\alpha$ - and  $\beta$ -pinenes) were predominant, and the volatile profile of Uruguayan and Brazilian propolis was composed mainly by  $\alpha$ -pinene and  $\beta$ -pinene (64.6–77.6%). Brazilian propolis was distinguished by a high amount of  $\beta$ -methyl crotonaldehyde (10.1%), and one of Uruguayan samples displayed the presence of limonene (15.6%). By principal component analysis of the volatile profile found in samples showed high differences, falling into separate clusters. In this study, the geographical origin of Uruguayan and Brazilian propolis is not specified; however, by the results obtained, it could be suggested that Brazilian propolis is from the southern region, since the similarities in volatile composition to Uruguayan propolis [173]. The results obtained in these studies for Uruguayan propolis confirm the chemical characteristics of propolis from temperate zones.

### 3.7. Argentina

Argentina has a vast territory and a great diversity of ecological regions, including subtropical rain forests and temperate sub-humid grasslands or “Pampas.” However, two thirds of Argentinean mainland is comprised by extensive arid and semi-arid region between the Andes and cold subantarctic zones. Natural arid and semi-arid plain regions include western Chaco, Monte and Patagonian steppe, and these are ecologically similar to those semi-arid regions of North America, present in Sonora, Sinaloa and Great Basin, respectively [174, 175]. Several studies have differentiated propolis into two main groups, one from temperate zones and the other from tropical regions; however, few studies have been done in regard to propolis from arid and semi-arid areas [89]. Propolis from different regions of Argentina has been investigated, twenty-five samples collected in temperate, arid and semi-arid lands in Northern Argentina (Santiago del Estero, Tucumán, Chaco, Salta, Catamarca, Jujuy and Misiones) were chemically and biologically analysed. By absorption spectra, RP-HPTLC and RP-HPLC analysis, it was found that 16 of the 25 samples presented a similar phenolic profile to that exhibited by samples from Southern Brazil and Uruguay. The main phenolic constituents identified in those 16 samples were pinobanksin, pinocembrin, chrysin, galangin, tectochrysin and 1,1-dimethylallylcaffeic acid [176]. Propolis samples from Catamarca and Tucumán presented the highest phenolic content, exhibiting greater amounts of pinocembrin, 1,1-dimethylallylcaffeic acid, ferulic acid and cinnamic acid in comparison with the other 20 samples analyzed by Isla et al. [176]. Additionally, these two propolis presented the higher antibacterial activity against *S. aureus* ATCC 25923 As well (inhibition zone: 4 and 5 mm, respectively) and presented the high FRS activity by DPPH assay (>40%) [176].

Moreover, other biological properties of propolis from Tucumán have been tested, including cytotoxicity with the lethality test of *A. salina* ( $LD_{50}$ : 100  $\mu$ g/mL), toxicity and mutagenicity on both *S. typhimurium* TA98 and TA100 (did not exhibit toxicity at 300  $\mu$ g/plate), genotoxicity on *Allium cepa* (not presented) and antimutagenicity, inhibiting the effect of isoquinoline (IQ) and 4-nitro-*o*-phenylenediamine (NPD) ( $ID_{50}$ : 40 and 20  $\mu$ g/plate, respectively). Furthermore, the chemical constituent 2',4'-dihydroxychalcone of Tucumán propolis showed cytotoxic activity ( $LC_{50}$ : 0.5  $\mu$ g/mL) and was able to inhibit the mutagenicity of IQ ( $ID_{50}$ : 1  $\mu$ g/plate), whereas genotoxic or mutagenic effects were not observed [177]. Additionally, the antimycotic activity of Tucumán propolis and its compounds, pinocembrin and galangin, has been tested against

*Trichoderma* spp., *Penicillium notatum*, *Aspergillus niger*, *Fusarium* sp., *Phomopsis* spp., *Saccharomyces carlsbergensis*, *Rhodotorula* spp. by bioautography, hyphal radial growth, hyphal extent and microdilution in liquid medium. Wherein propolis inhibited filamentous fungal growth and exhibited an antifungal moderate activity (MIC: 77–349  $\mu\text{g/mL}$ ). Pinocembrin, and galangin presented higher antifungal activity (MIC: 77 349  $\mu\text{g/mL}$ ), and their assumed as partially responsible for the fungitoxic activity of Tucumán propolis [178].

Similar results have been obtained from Tucumán propolis against human opportunistic and pathogenic fungi, specifically on dermatophytes and yeasts (*C. albicans* ATCC 10231, *C. tropicalis* C 131, *S. cerevisiae* ATCC 9763, *C. neoformans* ATCC 32264, *A. flavus* ATCC 9170, *A. fumigatus* ATCC26934, *A. niger* ATCC 9029, *T. rubrum* C 110, *T. mentagrophytes* ATCC 9972, and *M. gypseum* C 115). All the dermatophytes and yeasts were inhibited by three different Tucumán propolis samples (MIC: 16–125  $\mu\text{g/mL}$ ). The most susceptible fungi were *M. gypseum*, *T. mentagrophytes* and *T. rubrum*. A bioassay-guided isolation of Tucumán propolis and chemical characterization by NMR and HPLC–ESI-MS/MS yield two bioactive chalcones: 2',4'-dihydroxy-3'-methoxychalcone and 2',4'-dihydroxychalcone that displayed strong activity against clinical isolates of *T. rubrum* and *T. mentagrophytes* (MIC: 1.9–2.9  $\mu\text{g/mL}$ ). In addition, pinocembrin, galangin, 7-hydroxy-8-methoxyflavanone presented a moderate antifungal activity. By the presence of those compounds was identified in exudates of *Zuccagnia punctata* Cav. (Caesalpinieae), which was assigned as the botanical origin from Tucumán propolis samples [179].

Other studies focused on the characterization of propolis from Catamarca Province in Northwestern Argentina have been done. Propolis collected from El rincón presented FRS activity by ABTS assay ( $\text{SC}_{50}$ : 6.9  $\mu\text{g/mL}$ ) and by  $\beta$ -carotene–linoleic acid antioxidant assay ( $\text{SC}_{50}$ : 2.0  $\mu\text{g/mL}$ ), additionally exhibited antibacterial effect on methicillin resistant *S. aureus* (MRSA) by the microdilution method and bioautographic assays (MIC: 65  $\mu\text{g/mL}$ ). This propolis led to the isolation and characterization by NMR of twelve compounds, wherein the most bioactives were 2',4'-dihydroxy-3'-methoxychalcone, 2',4'-dihydroxychalcone, 2',4',4'-trihydroxy-6'-methoxychalcone, 5-hydroxy-4',7'-dimethoxyflavone, 4',5'-dihydroxy-3,7,8-trimethoxyflavone and 7-hydroxy-5,8-dimethoxyflavone [180].

FRS activity by DPPH assays of Argentinean propolis collected in the regions of Mendoza, Rio Negro, La Pampa, and Entre Rios was evaluated. Almost all of the propolis samples presented FRS activity (40–60% at 20  $\mu\text{g/mL}$ ), in exception to the samples from La Pampa and Entre Rios. Greater amounts of caffeic acid, ferulic acid, caffeic acid phenetyl ester were found by HPLC–PDA–UV analysis in propolis samples with the stronger FRS activity. In general, the presence of flavonoids, such pinocembrin, chrysin, pinobanksin, pinobanksin-3-O-acetate and galangin, was found in almost all samples [181]. Moreover, three samples collected from two different pythogeographical regions (Prepuna and Monte) exhibited FRS activity by DPPH assays ( $\text{IC}_{50}$ : 28–43  $\mu\text{g/mL}$ ) and by  $\beta$ -carotene–linoleic acid antioxidant assay ( $\text{IC}_{50}$ : 2.0–8.4  $\mu\text{g/mL}$ , respectively). The samples from Monte region showed the highest inhibitory effect on different strains of *S. aureus* and *E. faecalis* ( $\text{MIC}_{100}$ : 50 and 100  $\mu\text{g/mL}$ , respectively). Nine compounds were identified by HPLC–DAD–UV, and two of them were only identified in samples from Monte region 2',4'-dihydroxychalcone and 2',4'-dihydroxy 3'-methoxychalcone. These two

bioactive chalcones, present in propolis from El Rincon and Monte region, have been detected in propolis from Tucumán and in *Zuccagnia punctata*, a perennial shrub in Argentinean arid regions, which is identified as botanical source of this propolis from Argentinean arid lands [182].

The chemical composition of propolis collected from the Andean locality of Bauchaceta in San Juan province was characterized by HPLC–ESI-MS/MS and GC–MS techniques and revealed a lignan and volatile organic acid profile that matched with the exudates of *Larrea nitida*, which was suggested as its main botanical source. Andean propolis presented antifungal activity against Dermatophytes and yeasts (MIC: 31.3–125 µg/mL). A bioassay-guided isolation of the most active compounds yield two lignans characterized as 3'-methyl-nordihydroguaiaretic acid (NDGA) and nordihydroguaiaretic acid (NDGA) that showed high inhibitory activity against *T. mentagrophytes*, *T. rubrum*, *M. gypseum* (15.6–31.25 µg/mL) and clinical isolates of *Candida* spp., *Cryptococcus* spp., *T. rubrum* and *T. mentagrophytes* (MIC: 31.3 62.5 µg/mL). In addition, 4-[4-(4-hydroxy-phenyl)-2,3-dimethyl-butyl]-benzene-1,2-diol, 4'-methyl-nordihydroguaiaretic acid and two epoxyignans meso-(rel 7S,8S,7'R,8'R)-3,4,3',4'-tetrahydroxy-7,7'-epoxyignan and (7S,8S,7'S,8'S)-3,3',4'-trihydroxy-4-methoxy-7,7'-epoxyignan) were isolated and identified by their spectroscopic data in NMR experiments. These six compounds were isolated from propolis for the first time [183]. Similar antifungal activity results were obtained from other eleven samples collected in San Juan province, and the identification and isolation of MNDGA and NDGA. The flavonoids chrysin, pinocembrin and galangin were the most bioactive compounds [184].

A reverse phase LC–DAD–MS method has been developed to quantify phenolic acids and flavonoids present in propolis and found that the most abundant constituents from Argentinean, European and Chinese propolis were chrysin (2–4%), pinocembrin (2–4%), pinobanksinacetate (1.6–3%) and galangin (1–2%) [185]. Furthermore, the typical fingerprint of propolis from Argentina, Italy and Spain was determined by on-line HPLC–ESI/MS analysis, and those propolis samples showed the same total ion chromatogram (TIC) profile, in addition to a similar amount of pinocembrin (39–49% of the total identified flavonoids), suggesting a poplar-type origin for Argentinean propolis [186]. Interestingly, the subtropical montane forest of “El Siambón” in Tucumán region is characterized by the presence of native vegetation including members of Lauraceae, Myrtaceae, Fabaceae, Juglandaceae, Salicaceae and Nyctaginaceae families, as well as the introduced poplar, eucalyptus and pinus trees, which are described as the most visited trees by honeybees for resins in the Tucuman region [178].

However, in another study, propolis samples from 30 different regions of Santiago del Estero province were analysed and classified into three groups according to the main arboreal species in the region. Nineteen chemical constituents were identified in these 30 samples, pinocembrin, quercetin, kaempferol, chrysin, salicylic, 4-hydroxybenzoic, benzoic, ferulic and gallic acids were observed in most of the analysed samples. Some quantitative differences of each component were found in the three groups, and interestingly, no *Populus* species were found in apiary environments, where the most abundant plant species were *Geoffroea decorticans*, *Prosopis alba*, *Prosopis nigra*, *Schinopsis lorentzii*, *Acacia aroma*, *Cercidium praecox*, *Schinus fasciculatus*, and *Larrea divaricata*. These studies suggest that flavonoids characteristic of

temperate zones, which are present in Argentinean propolis, could be gathered from other plant species than poplar [187].

Furthermore, the antibacterial, antiradical and antioxidant activities of those 30 propolis samples from Santiago del Estero province were analysed, and variability in bioactivity was found. All samples presented a substantially similar inhibitory effect on *S. aureus* ATCC 25923 (8.5–11.4 mm), and about 77% of the samples showed an inhibitory zone with diameter longer than 9 mm. Additionally, there was observed a moderate correlation between antibacterial activity and total polyphenol and flavonoid contents. However, antimicrobial activity correlated better with pinocembrin content than with total polyphenol content. Results for the antioxidant activity by the  $\beta$ -carotene–linoleic acid assay presented high variability among samples (16.2–84.7%) as consequence of quantitative differences in chemical composition. Similar results were obtained by FRS activity on DPPH assay (20.4–89.9%) [188].

All these studies carried out to characterize the chemical composition and biological properties of Argentinean propolis have demonstrated that samples collected from different ecological regions throughout the country exhibited different chemical profiles; however, flavonoids are present in the most of the analysed samples, and chemical compounds such as lignans and chalcones could be used as markers to suggest the participation of a particular botanical source; thus, the phytochemical diversity found in Argentinean propolis has its botanical origin on several plants, including *Populus alba*, *Larrea nitida*, *Larrea divaricata* and *Zuccagnia punctata*.

### 3.8. Chile

Chile extends along the Southwestern coast of South America, between the geographical barriers of the Andes on the East and Pacific Ocean on the West, resulting in a unique flora developed as consequence of the environmental isolation that consists in many endemic plant species. Chile owns the Atacama Desert on the North, Chilean Mediterranean-type region in North-central, temperate forest in South-central region and subpolar forests at south [189, 190].

Some studies have been carried out in Chilean propolis collected from different regions. A propolis sample from Quebrada Yaquil in Santa Cruz (Region VI) in the Mediterranean semi-arid region of central Chile have led to the isolation and structural determination by NMR of five lignans, including 3 novel compounds: 1-(4-hydroxy-3-methoxyphenyl)-1,2-bis{4-[(E)-3-acetoxypropen-1-yl]-2-methoxyphenoxy}propan-3-ol acetate, and two different optical isomers ( $[\alpha]^{25}D + 8.8^\circ$  and  $[\alpha]^{25}D - 15.6^\circ$ , respectively) of 1-(4-hydroxy-3-methoxyphenyl)-2-{4-[(E)-3-acetoxypropen-1-yl]-2-methoxyphenoxy}propan-1,3-diol 3-acetate, in addition to the already reported 3-acetoxymethyl-5-[(E)-2-formylethen-1-yl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran, and 3-acetoxymethyl-5-[(E)-3-acetoxypropen-1-yl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran. The hives were located in a region with the dominant species sclerophyllous shrubs and herbaceous species, such as *Lithrea caustica*, *Quillaja saponaria*, *Cryptocarya alba*, *Kageneckia oblonga*, *Colliguaja odorifera*, *Trevoa trinervis*, *Baccharis linearis*, *Peumus boldus*, *Madia sativa*, *Helenium aromaticum* and *Pasithea caerulea*, which were considered as possible botanical sources of resins [190].

In another study, propolis was collected from hives located at Cuncumen (also Mediterranean-type climate), and fractionated to yield viscidone, vanillin, 3',4'-(methylenedioxy)acetophenone, 3-ethoxy-4-methoxybenzaldehyde, cinnamic acid and 3-methoxy-4-hydroxymethyl ester. These chemical compounds were already described; however, this was the first report on propolis composition of an arid, and a Mediterranean-type climate area. Additionally, the frequency of pollen grains observed in this Cuncumen propolis by optical and scanning electronic microscopy (SEM) allowed to suggest that resins of *Eucalyptus* spp. and *Salix humboldtiana* are the main botanical source of *A. mellifera* in central Chile. Pollen grains of *B. linearis*, *Q. saponaria* and *L. caustica* were also present in propolis sample in lower amounts [191]. Therefore, in order to understand more about the chemical composition and botanical sources available for honeybees in Central Chile, the same research group studied other sample from sclerophyllous shrubland coast (Colliguay), and by chromatographic procedures led to the isolation and characterization by NMR of seven phenolic compounds, including pinocembrin, acacetin, galangin, izalpin, kaempferide, prenyletin and diarylheptane. The botanical origin of Colliguay propolis was investigated by palynological analysis in optic microscopy, and the most abundant pollen grains and leaf fragments found in the sample were related to the plants *Escallonia pulverulenta*, *S. humboldtiana* and *Eucalyptus globulus*, which suggested their participation as botanical sources of this Chilean propolis. Probably honeybees obtain resins rich in pinocembrin from *E. pulverulenta*, since this flavanone was formerly reported from this source [192].

Other studies have been carried out in propolis from Central Chile. Six samples collected from the region of Santiago (Curacaví, Lo Cañas, Buin, Caleu, Cajón del Maipo and pireque) were analyzed by HPLC-ESI-MS/MS, and 30 chemical phenolic compounds were identified, including pinocembrin, pinobanksin, pinobanksin-3-O-alkanoates, caffeic acid phenethyl ester (CAPE), chrysin, galangin, kaempferol, kaempferide, ferulic acid, quercetin and quercetin methyl ether derivatives. The antioxidant properties of those samples were analyzed by FRAP, ORAC-FL, ORAC-PGR and DPPH radical methods. All samples exhibited a different FRS activity. The samples that presented CAPE (Curacaví, Buin and Cajón del Maipo) and quercetin (Buin and Lo Cañas) exerted the best antioxidant activity. Pinobanksin was found in all the samples, a compound that would be a suitable candidate for the standardization of propolis from the region of Santiago [193]. In a subsequent study, the anti-inflammatory effect of propolis from Caleu and Buin was tested through mice ear edema model, in addition to the *in vitro* assessment of nitric oxide (NO) production by RAW 264.7 cells stimulated with lipopolysaccharide (LPS). Buin propolis presented anti-inflammatory effect in the murine model (64%), and moreover, significantly decreased the NO release in RAW 264.7 cells in a dose-dependent manner [194].

In addition, a propolis sample collected from San Vicente de Tagua-Tagua (VI Region) was biologically assessed, and showed FRS activity by DPPH (100% at 80 µg/mL) and scavenger effect on superoxide anion (100% at 0.78 µg/mL), as well this Chilean propolis presented antiproliferative activity (at 80 µg/mL for 72 h incubation) by MTT assays on human tumour cell lines KB, Caco-2 and DU-145 (9, 45 and 23% cell viability, respectively). Galangin, caffeic acid, p-coumaric acid, ferulic acid and CAPE were identified by HPLC analysis in San Vicente

sample, and since the most abundant plant species in this region were *Peumus boldus*, *Q. saponaria*, *P. alba* and *Pinus radiata*, the botanical origin of this propolis according to the presence of those compounds would be the resins of *P. alba* [195].

Moreover, the chemical constitution, the botanical origin and antibacterial activity of twenty samples collected from Central and Southern regions of Chile (Valparaíso, Metropolitana, Libertador Bernardo O'Higgins and La Araucanía Regions) were investigated. Quercetin, myricetin, kaempferol, pinocembrin, coumaric acid, caffeic acid and CAPE were identified in propolis samples by HPLC–MS. All Chilean propolis samples presented a growth inhibitory effect on *S. mutans* and *S. sobrinus* (MIC: 0.9–8.2 µg/mL). By palynological analysis, the plant structures of native species, such as *Trevoa quinquenervia*, *Aristotelia chilensis*, *L. caustica*, *Retanilla trinervia*, *Q. saponaria*, and species of the genus *Escallonia* were found in propolis samples from central regions, whereas *Lotus uliginosus*, *Aextoxicon punctatum*, *B. linearis* and *Eucryphia cordifolia* were identified in samples from Southern Chile, additionally no structures of the genus *Populus* were detected in all the samples. These results suggested that honeybees could obtain CAPE and those flavonoids from other species rather than poplars [196].

With the aim to determine whether the bioactivity against *S. mutans* of Chilean propolis from “La araucanía” region was influenced by the year of collection, three different samples from spring of 2008, 2010 and 2011 were studied. The chemical composition of the annual samples presented qualitative differences by LC-MS analysis. Apigenin, genistein, kaempferol, myricetin, pinocembrin, quercetin, CAPE, caffeic acid, *p*-coumaric acid and ferulic acid were found in all the samples. Otherwise, the presence of daidzein, rutine, and chlorogenic and gallic acid was not constant. The antimicrobial activity of the annual samples did not presented significant variations (MIC: 0.91, 0.22 and 0.39 µg/mL); however, the biofilm formation in *S. mutans* cultures treated with Chilean propolis showed to be influenced by the year of collection [197].

These reports provide important information about chemical composition of Chilean propolis. At present, two main types of Chilean propolis are described, those with lignans as main compounds, and the others with flavonoids and phenolic acids related to propolis collected from temperate regions; however, the palynological evidence obtained indicated that native plants, such as *S. humboldtiana*, *E. globulus*, *E. pulverulenta*, *L. uliginosus*, *B. linearis*, *Q. saponaria* and *L. caustica* are related to the botanical origin of Central and Southern Chile. Moreover, the antibacterial, cytotoxic, antiproliferative and antioxidant activities of Chilean propolis make it a bioactive product for further investigations.

## 4. Conclusions

Propolis produced by honeybees in Americas represents an important source of diverse bioactive compounds, such as nemorosone and other prenylated benzophenones, artemillin C, CAPE, terpenes labdane type, chalcones, flavonoids, lignans, among others. These secondary metabolites are biosynthesized by different plants present in diverse ecological regions throughout the continent. Thus, propolis from Americas possess constitutional particularities,

and some samples share both temperate and tropical chemical constitution, and even characteristic compositional mixtures are present in some propolis from North and South America, resulting in a huge propolis diversity. In addition, propolis from temperate zones is not restricted to poplar, birch, pines and horse chestnut exudates as main and exclusive botanical source, since recent findings on propolis from United States and Honduras tracked liquidambar species as botanical source, providing a peculiar and transcontinental alternative source of resinous material.

At present, the chemical constitution of North American and South American propolis is partially characterized; however, more studies are needed to understand and identify the bioactive compounds found in those samples and to determine the mechanism of action through which they exert their pharmacological activities. Additionally, more studies should be done in order to understand the plant resin bee foraging behaviour, the role of propolis inside the hive and the benefits of botanical chemistry available along the ecological regions present in America, which imply interdisciplinary work to draw a relationship between bee health and the ecosystem implied. Finally, in accordance to the broad spectrum of biological activities, the high variability and complexity of North American and South American propolis, it becomes necessary to develop a more precise classification of this propolis diversity that combine the qualitative and quantitative plant origin fingerprint, and as well as the biological properties of this beehive product.

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