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Composition and Antibacterial Activity of the Lipophilic Fraction of Honeybee Pollen from Native Species of Montesinho Natural Park*

[1,2]Sandra I.T.R. Barbosa, [1]Armando J.D. Silvestre,
[1]Mário M.Q. Simões and [2]Maria L.M.F. Estevinho
[1]Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal
[2]Department of Biology, Escola Superior Agrária,
Instituto Politécnico de Bragança, 5300 Bragança, Portugal

Abstract: The lipophilic composition of honeybee pollen from *Cistus ladanifer*, *Castanea sativa* and *Rubus* sp. was analysed by GC-MS. The extracts are mainly composed by saturated and unsaturated fatty acids, sterols, long chain aliphatic alcohols, alkanes and alkenes. The profiles of chemical composition allow the chemical differentiation of pollen species. The biological activity of the lipophilic fractions against several Gram positive bacteria was demonstrated and related to the resistance of beehives to certain diseases when feed with such pollens.

Key words: Lipophilic extractive, honeybee pollen, *Cistus ladanifer*, *Castanea sativa*, *Rubus* sp., GC-MS analysis, antibacterial activity

Introduction

Honeybee pollen is one of the most prominent beehive's products. Due to its therapeutic properties and nutritional value, it has long been used not only in folk medicine but also as a dietary supplement.

Bee pollen is promoted as a dietary supplement with a wide range of nutritional and therapeutic properties. In fact, a wide number of promising properties of this natural product have been reported in the literature: for example, its protective action against side effects of radiotherapy in cancer patients (Wenning, 2003), its use in the treatment of allergic rhinitis (Boye *et al*., 1990; Staff *et al*., 1990) and its activity as an hepatoprotector (Juzwiak *et al*., 1992) along to its antiatherogenic effect (Ji *et al*., 1989; Zhao *et al*., 1990); in the treatment of some cases of benign prostatitis (Denis, 1966; Askupmar, 1967; Samochowiec *et al*., 1992; Rugendorff *et al*., 1993) and in the desensitization of children allergic to pollen (Wortmann, 1981; Krell, 1996). The regular consumption of honeybee pollen contributes to reduce the seric levels of triglycerides and cholesterol (Wójcicki *et al*., 1983). On the other hand, some studies reveal that the pollen's extract reduces not only the lipidic levels of the serum, but also the intensity of the atherosclerotic plaque (Wójcicki *et al*., 1986), contributing to a lower platelet aggregation, both *in vitro* and *in vivo* (Wójcicki and Samochowiec, 1984; Kosmider *et al*., 1983).

Despite the earlier mentioned properties, there is still some lack of knowledge on the relationship between the chemical composition of honeybee pollen and the reported biological properties. On the other hand, the chemical composition of honeybee pollen composition is variable and strongly dependent on the edafoclimatic conditions and on the botanical biodiversity present in the region where the beehives are located.

Corresponding Author: Armando J.D. Silvestre, Associate Professor, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal Tel: 351 234 370 711 Fax: 351 234 370 084

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Pollen is a natural source of proteins, fats, minerals and vitamins, which are necessary elements for the normal development of a bee colony (grubs and bees) and, likewise, are important for human nutrition (Cobo, 1984).

Concerning the lipophilic fraction of honeybee pollen extractive, published results demonstrate that they are mainly composed by fatty acids and hydrocarbons (Bianchi *et al*., 1990; Bonvehí and Jordà, 1997; Saa-Otero *et al*., 2000). However, in most studies the number of identified compounds is quite limited due to the detection/identification techniques used (mainly based on GC-FID and comparison with standards). A special attention has been devoted to this fraction because fatty acids play an important role in honeybee nutrition as they do for humans and additionally, these compounds also play another extremely important role: their antimicrobial activity. For example, linoleic and linolenic acids are bacterial growth inhibitors. In humans, for example, tests have showed that linoleic acid inhibited the etiologic agent of chronic gastritis and peptic ulcer, *Helicobacter pylori* (Petschow *et al*., 1996).

In an earlier work it has been observed that, in beehives with an incidence of the pathogenic fungus *Ascophaera apis*, there was a decrease in the incidence of European foulbrood caused by *Melissococcus pluton* and it has been demonstrated that this inhibition was caused by the presence of linoleic acid produced by such fungus (Feldlaufer *et al*., 1993a). It was also demonstrated that linoleic acid inhibited the formation of American foulbrood spores caused by *Paenibacillus larvae larvae* (Feldlaufer *et al*., 1993b), a bacteria with the capability of forming spores which remain viable for long periods and survive to adverse conditions.

Since the studied pollen samples were shown to have considerably high amounts of fatty acids with high antimicrobial activity, this might indicate that we could be in the presence of pollens with biocide action, just like canola (*Brassica napus*) pollen (Manning, 2001).

For the last 30 years, thanks to the drive for a more natural diet in Europe and especially in France, a development of apiculture to produce pollen has been observed, due to its nutritional and medicinal properties (Liebelt, 1994). As a result, regions where traditionally only honey was produced, became potential producers of high quality bee-collected pollen. This is the case of the region of Montesinho Natural Park (MNP), the biggest Portuguese region with origin denomination in honey production.

Although many plant species, relevant as honeybee pollen providers, have been intensively studied, to our knowledge the chemical composition of the pollen of *Cistus ladanifer*, *Castanea sativa* and *Rubus* sp., the main pollen sources in MNP, a natural park in the north of Portugal, has not been fully studied yet.

Within the scope of a project aiming to study beehives' products from MNP, in this research we report the study of the chemical composition of the lipophilic fraction of honeybee pollen, namely *Cistus ladanifer*, *Castanea sativa* and *Rubus* sp., in the period investigated, from May to July 2002. The lipophilic extracts were also submitted to antibacterial activity tests against three Gram positive bacteria namely *Bacillus subtilis* CECT 498, *Bacillus cereus* CECT 148 and *Paenibacillus larvae* and two Gram negative bacteria, namely *Escherichia coli* CECT 101 and *Pseudomonas aeruginosa* CECT 108, envisioning beehive's sanitary contributions.

Materials and Methods

Samples

Bee pollen was collected from two distinct areas in Montesinho Natural Park during the flowering months (May to July 2002). Pollen pellets were kept in a pollen trap for no more than 24 h. The collected samples were lyophilised, colorimetricaly separated and identified by palinologic analysis according to the Erdtman (1969) method.

Extraction

Each sample (50 g) of finely grounded pollen from predominant species, i.e., *Cistus ladanifer*, *Castanea sativa*, *Rubus* sp., were Soxhlet extracted with *n*-hexane for 12 h. The solvent was evaporated to dryness and the extracts were weighed. The results were expressed in mg compound/g dry pollen.

Alkaline Hydrolysis

In order to detect the presence of fatty acids, alcohols and sterols in the esterified forms, 20 mg of each extract were dissolved in 10 mL of 1 M KOH in 10% aqueous methanol. The mixtures were heated at 100°C, under nitrogen atmosphere, during 1 h. The reaction mixtures were cooled, acidified with 1 M HCl to pH~2 and then extracted three times with dichloromethane. The solvent was then evaporated to dryness.

CG-MS Analysis

The GC-MS analysis and quantification of the major lipophilic pollen extractive (before and after alkaline hydrolysis) were performed as described elsewhere (Freire *et al*., 2002). Nearly 20 mg of each dried sample were trimethylsilylated and injected three times. GC-MS analyses were performed using a Trace Gas Chromatograph 2000 Series equipped with a Finnigan Trace MS mass spectrometer, using helium as the carrier gas (35 cm s^{-1}), equipped with a DB-1 JW capillary column (30 m×0.32 mm i.d., 0.25 μm film thickness). The chromatographic conditions were as follows: initial temperature: 80°C for 5 min; temperature rate: 4°C per min; final temperature: 285°C for 10 min; injector temperature: 290°C; transfer-line temperature: 290°C; split ratio: 1:100.

For quantitative analysis a measured amount of internal standard (tetracosane) was added to each sample. The respective multiplication factors needed for quantification of the peak areas were calculated as an average of three GC-MS runs.

Compounds were identified, as TMS derivatives, by comparing their mass spectra with the GC-MS spectral library, with data from the literature and in some cases, by the injection of standards.

Determination of Antibacterial Effect by Agar Well Diffusion Method

Suspensions of three Gram positive bacteria namely *Bacillus subtilis* CECT 498, *Bacillus cereus* CECT 148 and *Paenibacillus larvae* and two Gram negative bacteria, namely *Escherichia coli* CECT 101 and *Pseudomonas aeruginosa* CECT 108, were prepared in order to contain approximately 10^8 cfu mL^{-1} and the plates were inoculated. The lipophilic extracts were incorporated in a cavity (3 mm depth, 4 mm diameter) made in the center of the Petri plates (9 cm diameter) with nutrient agar. The plates were incubated at 37°C for 24 h in duplicate. The inhibition zones were measured and expressed in cm.

Results and Discussion

The pollen samples were identified on the basis of palynological analysis as monofloral *Cistus ladanifer*, *Castanea sativa* and *Rubus* sp. pollen.

Analysis of the Lipophilic Extracts

The extraction yields of the dichloromethane extract account for 1.1, 2.0 and 3.6% (on a dry basis) of *C. sativa*, *Rubus* sp. and *C. ladanifer*, respectively.

The composition of the lipophilic fraction of the studied extracts, as well as the abundances of individual components are shown in Table 1.

In general, the major lipophilic components of the three extracts are fatty acids (saturated and unsaturated), followed by alkanes, alkenes, sterols and alcohols (Fig. 1). The substantial increase in the amounts of extractives after alkaline hydrolysis and particularly in the amount of fatty acids, but also

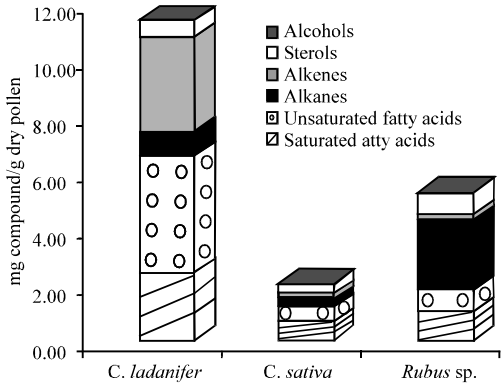

Fig. 1: Major families of compounds identified in the *n*-hexane extracts of bee pollen, before alkaline hydrolysis

Table 1: Chemical composition and abundances (mg/g of dry pollen) of the lipophilic fraction of *C. ladanifer*, *C. sativa* and *Rubus* sp. (BH: before alkaline hydrolysis; AH: after alkaline hydrolysis)

	C. ladanifer		*C. sativa*		*Rubus* sp.	
	BH	AH	BH	AH	BH	AH
Octanoic acid	0.0661	0.828	0.0273	0.0184	0.0118	0.0358
Nonanoic acid	0.0732	0.0715	0.2095	0.1714	0.0271	0.0552
Decanoic acid	0.2233	0.4992	0.0048	0.0122	0.0110	0.0242
Undecanoic acid	0.0145	0.0318	-	-	-	-
Dodecenoic acid	tr	-	-	-	-	-
Dodecanoic acid	0.1076	-	-	-	0.0045	0.0172
Octanedioic acid	-	-	-	-	0.0061	0.0106
Nonadioic acid	0.0164	0.0470	0.0090	0.0098	0.0171	0.0236
Tetradecanoic acid	0.0334	0.1119	0.0040	0.0109	0.0055	0.0457
Decanedioic acid	0.0072	0.0949	0.0059	tr	-	-
Nonadecane	0.0895	0.1206	0.0046	-	-	-
1-Hexadecanol	-	-	-	-	0.0055	0.1307
Hexadecenoic acid	0.0103	tr	-	-	-	-
Hexadecanoic acid	1.4038	2.7408	0.1171	0.2656	0.2462	1.0292
Heneicosene	0.0142	0.1888	-	-	-	-
Heneicosane	0.1674	0.2018	0.0081	0.0098	0.0514	0.0514
Z-9-Octadecen-1-ol	-	-	-	-	-	0.0296
Heptadecanoic acid	0.0075	-	-	-	0.0054	0.0370
1-Octadecanol	-	-	-	-	0.0119	0.0971
Linoleic acid	2.5245	4.6843	0.1478	0.3808	0.0536	0.2863
Linolenic acid	0.6988	1.6278	-	-	0.2758	1.3868
Oleic acid	0.5231	1.4381	0.2195	0.8626	0.4838	1.7656
Octadecanoic acid	0.1307	0.5625	0.0268	0.0630	0.0661	0.3520
Tricosene	0.0261	-	0.0126	0.0141	0.0724	0.0945
11-Octadecenoic acid	-	-	-	-	0.0166	0.0357
Tricosane	0.0848	0.1051	0.0948	0.0934	1.5908	1.6191
1-Eicosanol	0.0765	0.0635	0.0045	0.0144	0.0083	0.0642
2-Hydroxy-9,12-octadienoic acid	0.0594	0.1590	-	-	-	-
Eicosanoic acid	0.0280	0.0658	0.0332	0.0477	0.1165	0.1461
Pentacosene	0.0514	0.0604	0.0118	0.0112	0.0708	0.0686
Pentacosane	0.1982	0.3234	0.0727	0.1001	0.8247	0.9307
Hexacosane	-	-	-	-	0.0164	0.,0264
Docosenoic acid	0.2418	0.5295	0.0066	0.0274	-	-
Docosanoic acid	0.0836	0.1138	0.0226	0.0296	0.4006	0.4254
Heptacosene	0.0430	0.0278	0.0112	0.0104	0.0227	0.4254
Heptacosane	0.1255	0.3954	0.0809	0.4606	0.2736	-
1-Docosanol	-	-	0.0103	0.0222	-	-

Table 1: Continued

	C. ladanifer		*C. sativa*		*Rubus* sp.	
	BH	AH	BH	AH	BH	AH
Linoleic acid 1-monoglyceride	0.0308	-	-	-	-	-
Lanost-8-en-3-one	-	-	-	-	0.0436	-
1-Tetracosanol	-	-	-	-	0.0089	0.0033
Tetracosenoic acid	0.1115	0.2963	0.0171	0.0251	-	-
Tetracosanoic acid	0.0434	0.0560	0.0318	0.0304	0.6574	1.0685
2-Pentacosanol	-	-	0.0863	0.2053	-	-
Nonacosene	0.5012	0.6662	-	-	-	-
Nonacosane	0.1046	0.2165	-	-	0.0359	0.0317
Pentacosanoic acid	-	-	0.0116	0.0109	-	-
1-Hexacosanol	-	-	-	-	0.0096	0.0474
γ-Tocopherol	-	-	-	-	tr	-
22-Hydroxydocosanoic acid	-	-	0.0164	0.0133	-	0.4934
Hexacosanoic acid	0.0504	0.0420	0.0972	0.0809	0.2351	0.1232
Hentriacontene	1.9274	2.5411	0.0120	0.0379	-	-
Heptacosan-2-ol	-	-	0.0179	-	-	-
Hentriacontriene	0.2490	0.2911	-	-	-	-
23-Hydroxytetracosanoic acid	-	-	0.0653	0.0594	-	-
24-Hydroxytetracosanoic acid	-	-	0.0044	0.0035	-	-
1-Octacosanol	-	-	-	-	-	0.1190
α-Tocopherol	tr	tr	-	-	0.0071	0.0237
$\Delta^{5,7}$-Colestadienol	-	-	-	-	0.3727	0.6061
Brassicasterol	-	-	-	-	0.1016	0.2444
Campesterol	0.2227	0.2788	-	-	-	-
Octacosenoic acid	-	-	0.0452	0.0432	-	-
Octacosanoic acid	0.0203	0.0532	0.0132	0.0200	-	-
Stigmasterol	0.0778	0.0960	-	-	-	-
Tritriacontene	0.8440	1.0723	-	-	-	-
β-Sitosterol	0.0770	0.1051	0.0968	0.1083	0.1279	0.3656
25-hydroxy-hexacosanoic acid	-	-	tr	tr	-	-
Sitostanol	0.2260	0.2961	-	-	-	-
Spinasterol	-	-	-	-	0.0846	0.1036
1-Triacontanol	-	-	-	-	-	0.1294
2-Triacontanol	-	-	0.0443	0.0442	-	-
α-Amirin	-	-	-	-	0.0215	0.0432
Cycloartenol	-	-	-	-	0.0311	0.1390
Triacontenoic acid	0.0575	tr	0.0422	0.0290	-	-
Z-24-Tritriaconten-2-one	-	-	0.0060	-	-	-
2-Tritriacontenol	-	-	0.0436	0.0384	-	-
Dotriacontenol	0.0339	0.2593	-	-	-	-
Tritriacontenoic acid	-	-	0.0161	0.0124	-	-
Tetratriacontenol	0.0260	0.1654	0.0873	0.0737	-	-
2-Tetratriacontanol	-	-	0.2189	0.1791	-	-
Total identified	11.74	20.86	2.14	3.7	5.51	12.87
Unidentified	1.61	1.22	1.13	1.21	2.94	7.94
Total	13.35	22.08	3.27	4.91	8.45	20.815

in sterols and alcohols (Fig. 2) is due to the fact that a fraction of these components is present in the extracts in the esterified forms.

Alkanes and alkenes were also found in the three extracts, coming probably from plants epicuticular waxes.

The pollen from *C. ladanifer* is the one with higher contents of lipophilic components, followed by *Rubus* sp. and *C. sativa* pollen (Fig. 1 and 2).

As far as the individual composition is concerned, *C. ladanifer* extract is characterized by its high content of fatty acids (before and after alkaline hydrolysis). Among unsaturated fatty acids, linoleic, linolenic, oleic and docosenoic acids are the most abundant. Among saturated fatty acids, palmitic

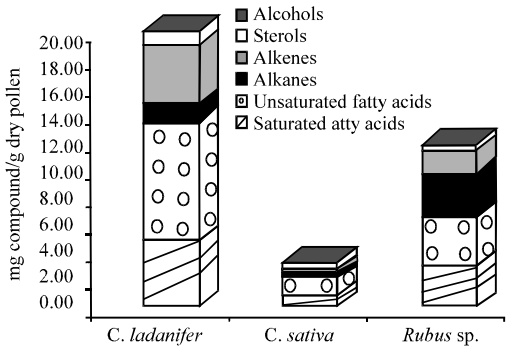

Fig. 2: Major families of compounds, identified in the *n*-hexane extracts of bee pollen, after alkaline hydrolysis

acid is the most abundant, which is in agreement with previous studies (Bonvehí and Jordà, 1997; Saa-Otero *et al*., 2000). Significant amounts of decanoic and dodecanoic acids were also found. Octanoic, nonanoic and decanedioic acids were found as well, but in lower quantities. Although undecanoic acid exists in small amount, its presence is important, since besides not existing in the other studied species, it is referenced as being an antimicrobial agent (Hornitzky, 2003). Finally, 2-hydroxyocta-9,12-dienoic acid was identified for the first time in the pollen of *C. sativa*.

Campesterol and sitostanol are the most abundant sterols in *C. ladanifer* extract, followed by smaller amounts of stigmasterol and β-sitosterol. α-Tocopherol was also identified in the *C. ladanifer* extract.

In the *C. sativa* pollen extract the major components are also fatty acids. Oleic acid, followed by linoleic acid, are the most abundant unsaturated fatty acids.

It is worth mentioning that linolenic acid is not present in this pollen extract whereas it is present in considerable amounts in the other two species pollen extracts. This observation is rather important, as it might point out to a specific characteristic of *C. sativa* pollen from the studied region. In fact, in previous studies on this same species from other geographic locations, linolenic acid is referenced as being one of the most abundant unsaturated fatty acids (Bonvehí and Jordà, 1997; Saa-Otero *et al*., 2000).

Nonanoic acid is the most abundant saturated fatty acid of *C. sativa* pollen, unlike the preceding species, in which palmitic acid is the most abundant.

Several ω-hydroxyacids, namely 23-hydroxytetracosanoic acid, 24-hydroxytetracosanoic acid and 25-hydroxytetracosanoic acid were, to our knowledge, identified for the first time.

In the *C. sativa* pollen extract, long-chain alcohols and mainly 1-docosanol, 2-tetratriacontanol and 2-tritiacontenol represent a significant fraction, particularly after alkaline hydrolysis. Oleic acid is the most abundant unsaturated fatty acid in *Rubus* sp. pollen extract, followed by linolenic and linoleic acids whereas docosanoic acid is the most abundant saturated fatty acid, followed by palmitic acid.

β-sitosterol and brassicasterol are the most abundant components of *Rubus* sp. pollen extracts. It is worth mentioning that brassicasterol, lanost-8-en-3-one, cycloartenol, β-amyrin, stigmasta-3, 5-dien-7-one, $\Delta^{5,7}$-colestadienol and spinasterol were, to our knowledge, identified for the first time in the *Rubus* sp. pollen.

α-Tocopherol and γ-tocopherol were also identified in *Rubus* sp. pollen extracts. Since this type of compounds is only found in very small quantities in food and because of its well-known importance, the presence of these vitamins in bee pollen - a product whose potentiality as a dietary supplement is fairly significant-becomes particularly interesting.

When comparing the studied samples (Fig. 1 and 2), it is obvious that the extract of *C. ladanifer* is the richest in lipophilic compounds, especially in unsaturated fatty acids. This makes this species particularly interesting from the biological activity point of view, since these acids prevent platelet aggregation (Erasmus, 1996), contributing to the anti-atherosclerotic activity of bee pollen.

On the other hand, the presence of phytosterols makes bee pollen even more interesting, since these compounds are being increasingly used as phytonutrients due to its ability to reduce the levels of blood cholesterol (LDL-C) (Tapiero *et al*., 2003).

Antimicrobial Activity Essays of Bee Pollen Extracts

To evaluate the antimicrobial activity of the studied extracts, they were tested towards two Gram negative bacteria-*Escherichia coli* (CECT 101) and *Pseudomonas aeruginosa* (CECT 108)-and three Gram positive bacteria-*Bacillus cereus* (CECT 148), *Bacillus subtilis* (CECT 498) and *Paenibacillus larvae*-the latter as an example of causal agent of bees' diseases (American foulbrood).

The results shown refer to the utilization of 300 μg of linoleic acid + linolenic acid. We have weighed the adequate amount of extract of each species necessary to obtain an amount of the two acids of 300 μg, since in the analysed samples these acids represent the major fraction.

The lipophilic extract of *C. ladanifer* pollen has shown the most pronounced inhibitory effect in what concerns the growth of *P. larvae* species, followed by the lipophilic extract of *C. sativa* pollen and then by the extract of *Rubus* sp. pollen (Fig. 3).

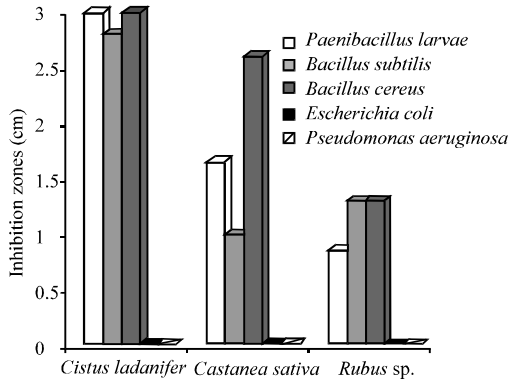

Fig. 3: Inhibition zones resulting from the utilization of the *Cistus ladanifer*, *Castanea sativa* and *Rubus* sp. pollen lipophilic extracts

These results are entirely supported by the quantitative analysis of the extracts, since the extract of *C. ladanifer* pollen contains the higher content of linoleic acid, besides containing also considerable amounts of other acids (e.g., undecanoic acid) referenced as antimicrobial agents.

Regarding the two other species (*C. sativa* and *Rubus* sp.), the high inhibition caused by the extract of *C. sativa* pollen might have been due to linoleic acid which, in this species' pollen, appears in significant amounts, while in *Rubus* sp. pollen the 300 μg of the sum of the two acids refers almost exclusively to linolenic acid.

The behaviour of the other bacteria studied was similar, since the extract of *C. ladanifer* has shown the higher activity, depending on the tested bacteria. In the case of *E. coli* and *P. aeruginosa* there was no growth inhibition, which might be related to the mechanism of action of the fatty acid *versus* the mechanism of defence of the Gram negative bacteria.

Given the high antimicrobial activity of the *C. ladanifer* extract, we might be in the presence of a biocide species for some bacteria, namely *Paenibacillus larvae*, *Bacillus subtilis* and *Bacillus cereus*.

Therefore, there might be a relationship between the existence of *C. ladanifer* in the region and the low incidence of American foulbrood. This observation suggests that the possible use of fatty acids as a control strategy for bees' diseases should be better studied given that, unlike antibiotics, no unwanted secondary effects are known. Thus, based on the described results, two strategies can be initially adopted to prevent beehives diseases: i) to displace affected beehives to locations where the native flora produces pollen rich in the fatty acids that have demonstrated significant antimicrobial activity; or ii) to feed the beehives with pollens that possess high concentration of those fatty acids.

Conclusions

The lipophilic fraction of the three pollen extracts studied were found to be composed essentially by fatty acids, sterols, long chain aliphatic alcohols and also smaller amounts of alkanes and alkenes.

Beyond well known pollen compounds, thirteen new compounds were detected for the fist time in the analysed samples, namely 2-hydroxyocta-9,12-dienoic acid in the *C. ladanifer* extract, 23-hydroxytetracosanoic acid, 24-hydroxytetracosanoic acid, 25-hydroxytetracosanoic acid and Z-24-tritriaconten-7-one in the *C. sativa* extract and Z-9-octadecen-1-ol, brassicasterol, lanost-8-en-3-one, cycloartenol, β-amyrin, stigmasta-3,5-dien-7-one, $\Delta^{5,7}$-colestadienol and spinasterol in the *Rubus* sp. extract.

The quantitative and qualitative differences found between the composition of the studied samples and published results are probably due to the geographic location allowing a clear distinction in the lipidic profile from region to region. Therefore, we pursue the chemical factors, present in monofloral samples, that discriminate accurately authentic pollen from certain region.

The biological activity results demonstrate an inhibitory action of the studied extracts, particularly of the *C. ladanifer* extract in the Gram positive bacteria's growth, namely in *Paenibacillus larvae, Bacillus subtilis* and *Bacillus cereus*. This might be an important contribution to reduce the risk, or even immunise beehives, to the American foulbrood disease.

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