Zambian Honey Analysis of Two Samples of Polyflora Blossom Honey (BH) & Forest Honey Honeydew (FB)

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Introduction

Analysis of two honey samples, BH and FB, was carried out using an untargeted metabolomics workflow (LC-ESI-QTOF-MS/MS) incorporating data processing and feature recognition with MZmine3 followed by spectral matching and molecular networking using GNPS, as well as *in silico* molecular formula identification, structure prediction and structure classification using SIRIUS5 (plugins CANOPUS, CSI:FingerID and ZODIAC).

This procedure relies on the chromatographic separation of components within the analysed samples, their ionization and mass determination, followed by isolated fragmentation and determination of the fragment masses. For each ion this results in a so-called fragmentation spectrum, which is characteristic (akin to a fingerprint) and holds structural information which allows structure prediction using modern *in silico* tools and matching to database spectra of known compounds. Employing such tools allowed to tentatively identify various components contained within the samples including abscisic acid, p-menthane-1,2,8-triol, other terpenoids, as well as several flavonoids and amino-acid glycosides among many other compounds.

SPECTRAL MATCHING AND MOLECULAR NETWORKING USING GNPS, AS WELL AS *IN SILICO* MOLECULAR FORMULA IDENTIFICATION,

Figure 1 shows an overview of the entire molecular network created using GNPS, consisting of more than 750 features (features refer to distinct ions observed during the experiments, several ions can emanate from the same compound, e.g. adducts etc., but this is usually limited to 2 or 3 features per compound and the majority of compounds only account for a single feature).

In the molecular network, features are represented by nodes and these are connected by edges based on the pair-wise similarity of their fragmentation spectra. Moreover, increasing edge width indicates greater spectral similarity. In addition to pair-wise comparison of all fragmentation spectra within a dataset, GNPS also facilitates comparison of the experimental spectra to public repository database spectra, allowing for potential identification of known compounds or derivatives of known compounds.

Figure 2 shows selected clusters (ensembles of connected nodes) of interest. The largest cluster (A) represents features matched to terpenoids, most notably:

1. abscisic acid, a common honey component with anti-inflammatory activity, as well as antidiabetic and anticancer potential;
2. vomifoliol, a neuroprotective compound [Tan et al. 2020] with anticancer potential [US NIH, https://drugs.ncats.io/drug/P86438KC5J] that has been reported from honey before [Jerkovic et al. 2010];
3. **p-menthane-1,2,8-triol**, a menthol derivative with potent antioxidant activity [Srivastava et al. 2012],

4. **an unidentified terpenoid** - The unidentified terpenoid of $C_{30}H_{40}O_8$ could be excoecariatoxin from *Gnidia kraussiana* (best search result when looking for terpenoid of said formula and this plant may occur in the gathering range of the bees).

5. betulin, an ubiquitous plant natural product with many health benefits [Amiri et al. 2020].

The nodes are represented as pie charts with proportions in these charts representing the relative contribution to each feature per sample (red=FB, blue=BH). For all major features the relative abundances are similar for both samples. Various related minor features were also detected. Node sizes correspond to relative feature abundance, however, some compounds ionise better than others so greater node size does not necessarily correspond to greater concentration. Nevertheless, related features, which are expected to occur in the same cluster, usually show similar ionisation efficiencies.

Cluster B and C represent features annotated as **amino-acid glycosides, including glucopyranosyl tryptophan (B) and N-(1-deoxy-fructosyl)phenylalanine**. Abundances for most of these features are greater in sample FB, particularly for glucopyranosyl tryptophan.

Cluster D represent flavonoids and includes annotations for **QUERCETIN**, **LUTEOLIN AND 5,7,3'-TRIHYDROXYFLAVONE**. These are antioxidants with various health benefits that are present in a wider range of plants and commonly found in honey as well. Luteolin, which appears to be the most abundant flavonoid, shows higher levels in sample BH whereas quercetin and 5,7,3'-trihydroxyflavone show similar levels in both samples. Flavonoids are known to not ionise very efficiently in positive mode, hence the small node size is likely misleading in judging the relative abundances of this compound class.

The features in cluster E were matched to **KYNURENIC ACID**, **a neuroprotective [Walczak et al. 2020] and antimicrobial compound commonly found in multifloral honeys** [Beretta et al. 2007] and **penicinoline**, a rare insecticidal derivative with potential anticancer effects that is known from mangrove endophytic fungus [Shao et al. 2010]. While kynurenic acid was detected at similar levels in both samples, penicinoline showed much greater relative abundance in sample FB.

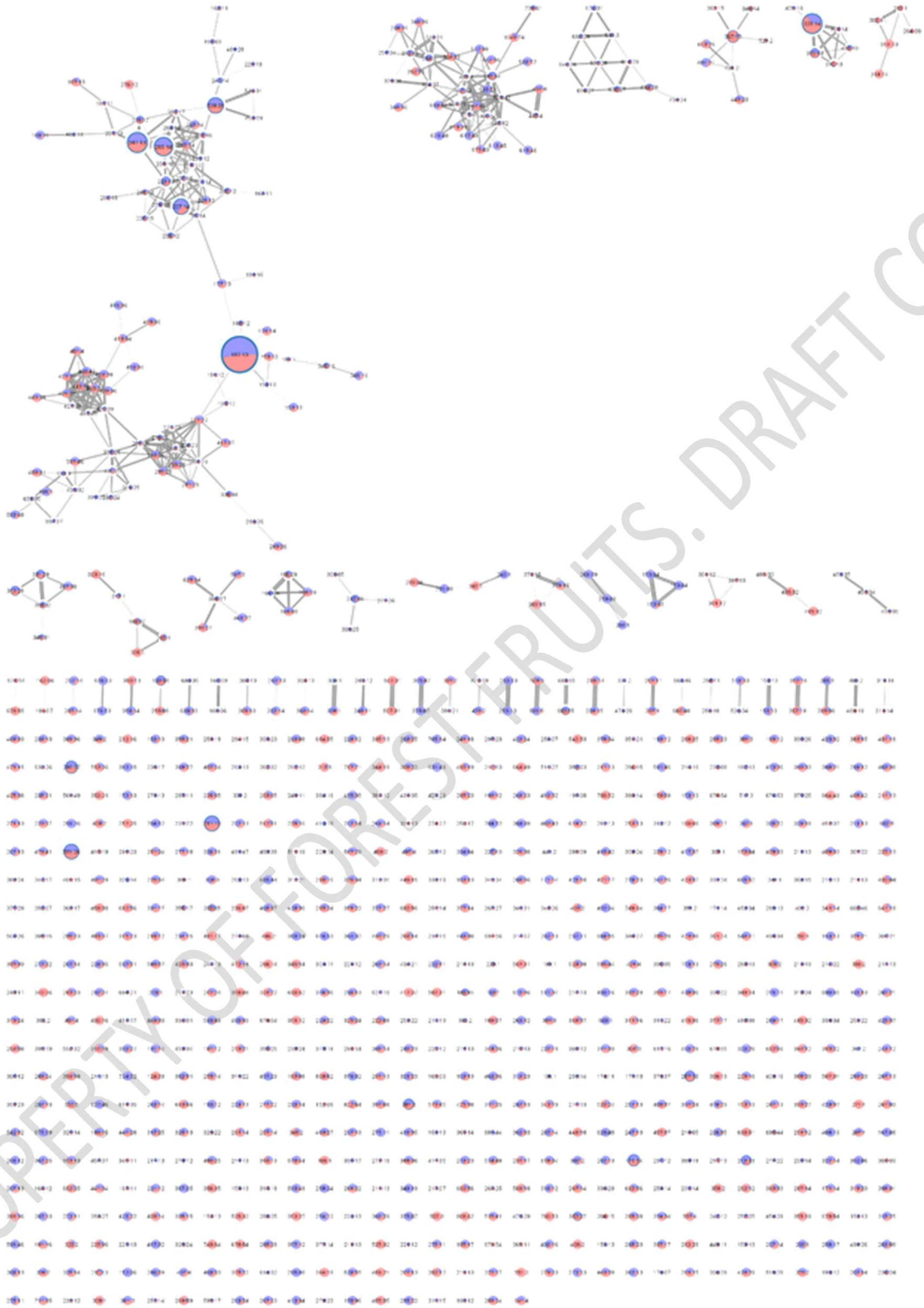


Fig.1 Full molecular network of sample BH (blue) and sample FB (red).

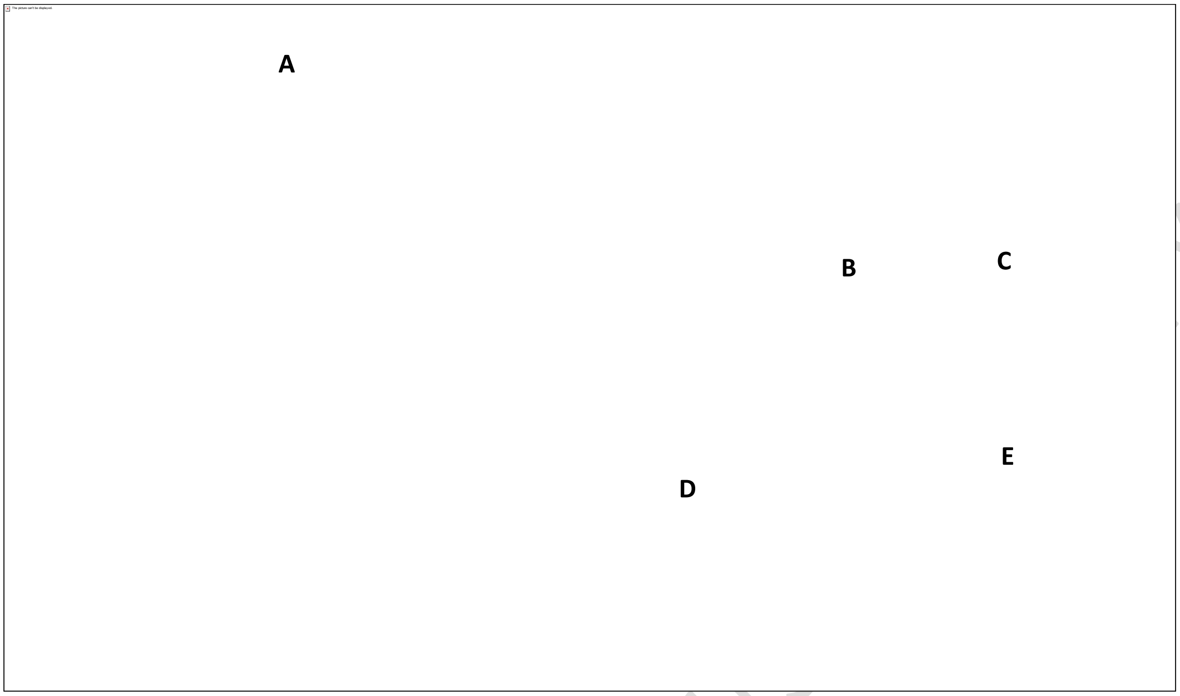


Fig.2 Selected molecular network clusters of sample BH (blue) and sample FB (red).

STRUCTURE PREDICTION AND STRUCTURE CLASSIFICATION USING SIRIUS5 (PLUGINS CANOPUS, CSI:FINGERID AND ZODIAC

In addition to the molecular networking analysis, the same raw data which was analyzed through molecular networking, was processed using CANOPUS within SIRIUS5. This served to classify the detected features according to compound classes, providing an overview with more comprehensibility regarding the potential structures of detected compounds. While molecular networking and spectral matching through GNPS relies on comparison of experimental spectra from the analysed samples and spectra from repository databases, analysis through CANOPUS employs a deep neural network to predict the compound class a feature belongs and the features are then categorized using ClassyFire.

Such categorized data can be visualized as a sunburst chart using plotly in python (see Fig.3, the .html files for these charts are attached and can be opened in a web browser, they are interactive). Moreover, SIRIUS5 contains the plugin CSI:FingerID which predicts structures by rationalising fragmentation spectra and assigning them to the best matching structures from structural databases, so no direct comparison of experimental to repository spectra is involved.

1. *The tentative compound identifications which are indicated on the outermost level of the sunburst charts, were based on a direct spectral match when present, or else on the predicted structure from CSI:FingerID.*
2. *The annotations derived from spectral matches are indicated by an excalmation mark preceding the compound name. If no structure prediciton was possible, the feature ID is shown instead.*

Figures 4 and 5 represent depictions of the sunburst charts focussed on prenol lipids and cinnamic acid derivatives/flavonoids, respectively.

When focussing on the prenol lipids (Fig.4) one compound is notable which was not highlighted through molecular networking (Fig. 1+2), namely 11,13-dehydroeriolin, which is a component of *Carpesium abrotanoides* (traditional medicinal herb) [Kudumela et al. 2019].

Figure 5 shows the sunburst charts focussed on flavonoids and cinnamic acid derivatives. Two of the major components were not highlighted during molecular networking and these include **tricoumaroyl spermidine**, a known plant and pollen component [Madeo et al 2018] with antiviral potential [Hattori et al. 2001] and **cinnamate**, an antidiabetic compound [Adisakwattana et al. 2017] known to occur in propolis [Mishima et al. 2005].

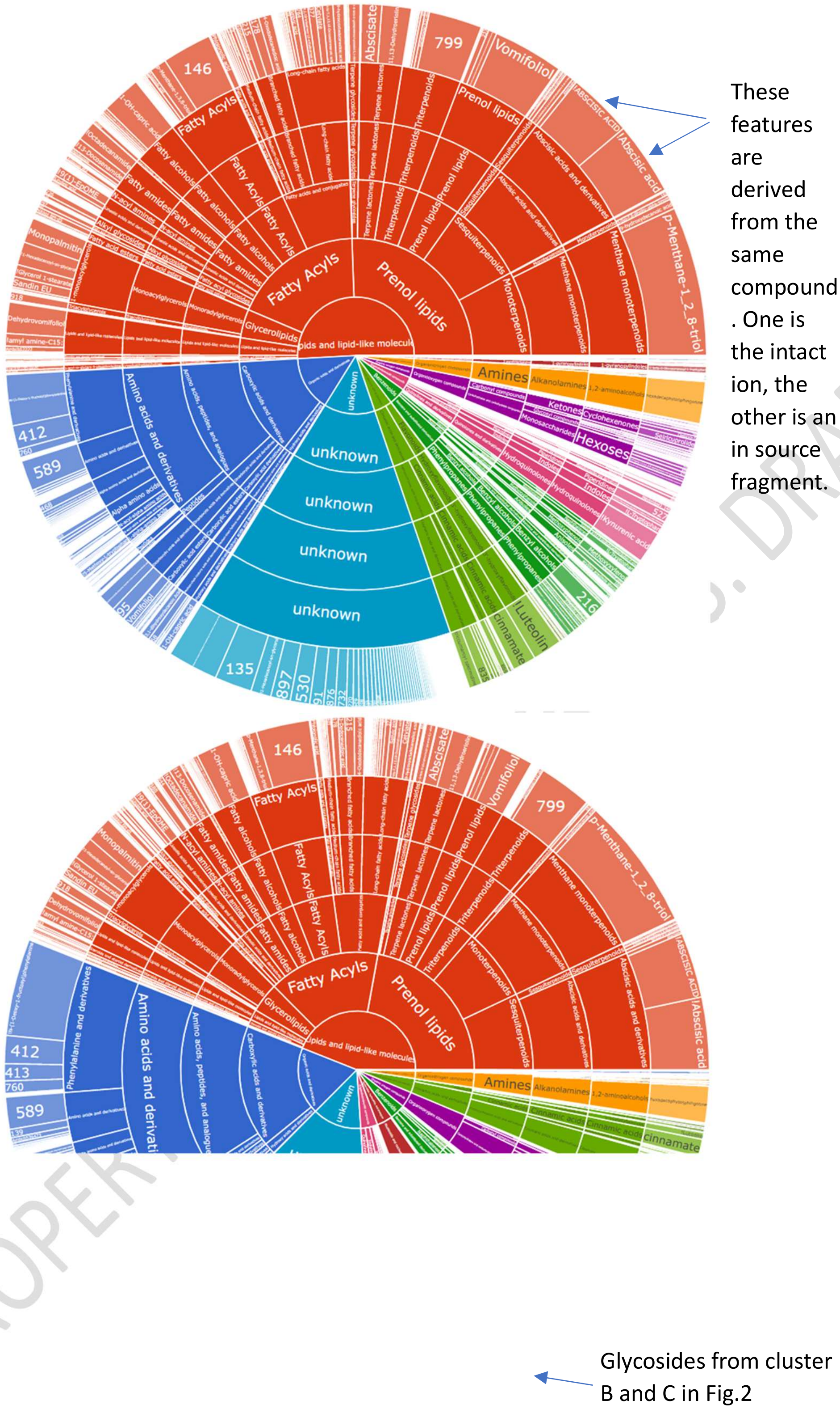


Fig.3 Sunburst charts depicting the CANOPUS output for LC-MS/MS data of sample BH (top) and sample FB (bottom).

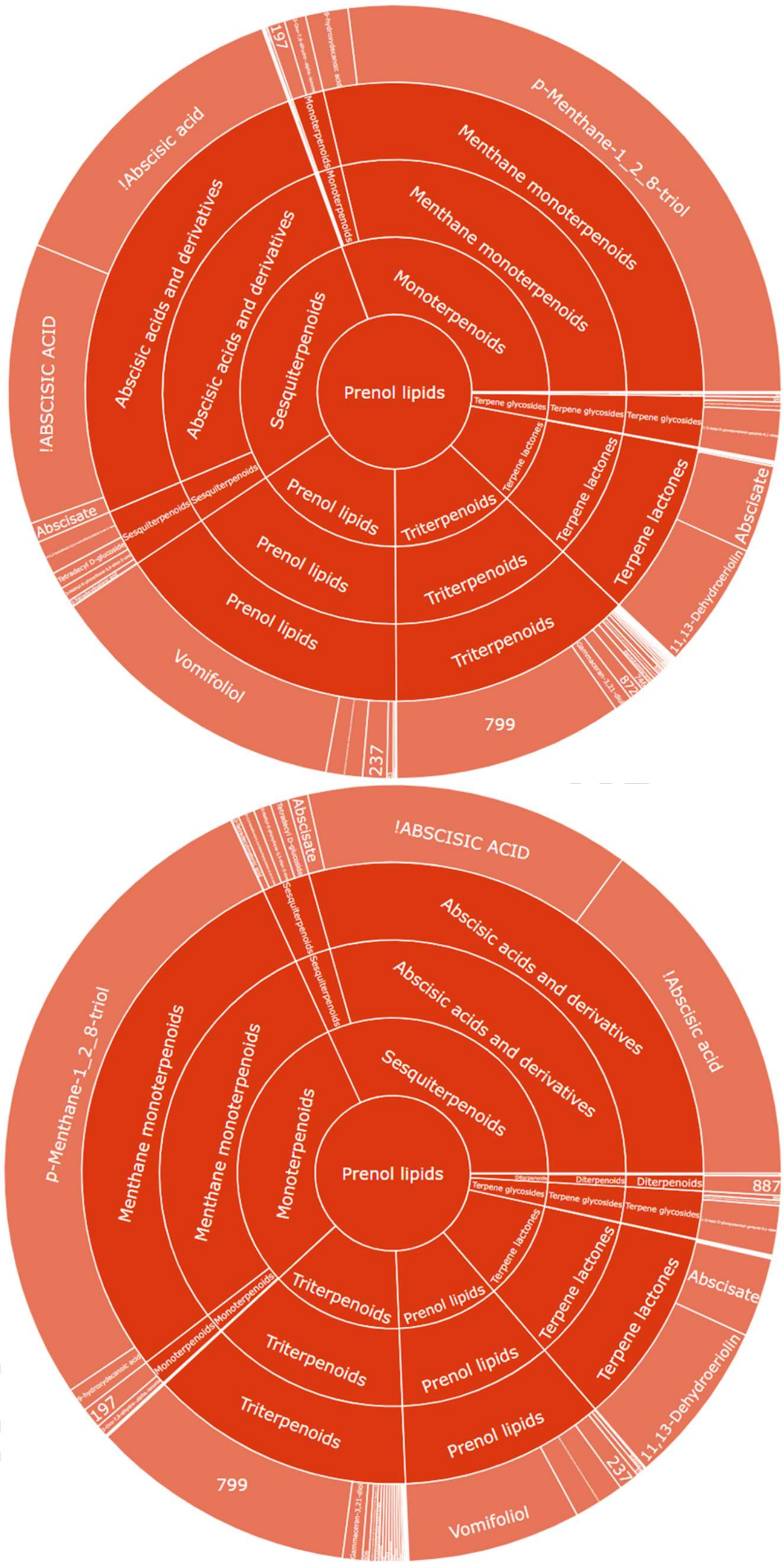


Fig.4 Sunburst charts depicting the CANOPUS output focussed on prenol lipids. Sample BH (top) and sample FB (bottom).

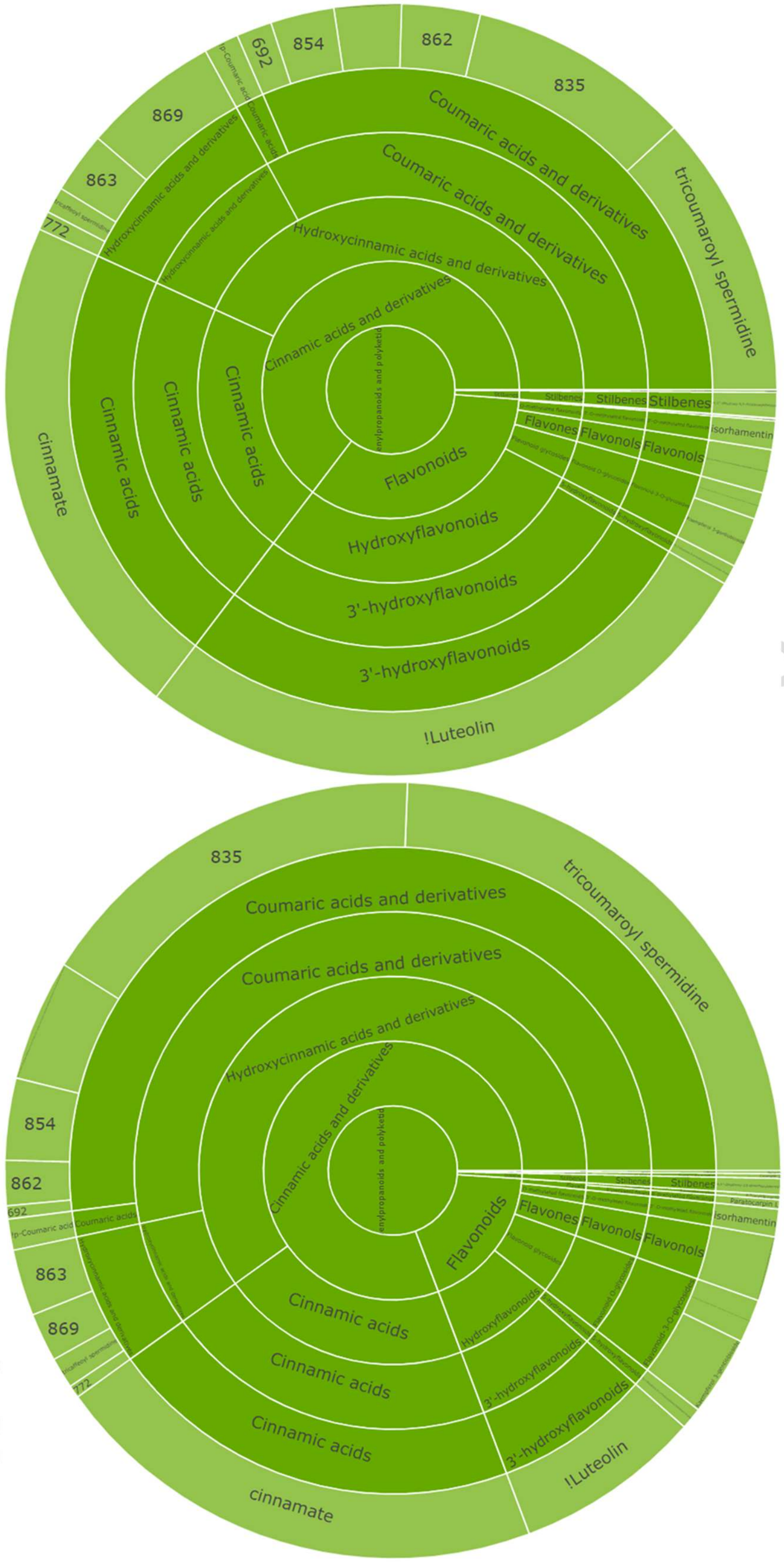
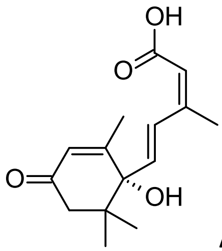


Fig.5 Sunburst charts depicting the CANOPUS output focussed on flavonoids and cinnamic acid derivatives. Sample BH (top) and sample FB (bottom).

Notes on compounds



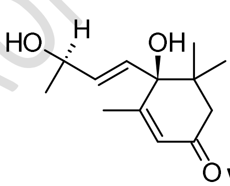
ABSCISIC ACID (ABA), plant hormone, anti-inflammatory, *in vitro* anticancer activity, antidiabetic acitivity. {The phytohormone abscisic acid (ABA) is present in nectar, honey and adult honey bees.} Negri et al

{It has been demonstrated that ABA plays a key role in glucose metabolism, inflammation, memory and tumor growth………At the functional level, it has been demonstrated that ABA plays a key role in resistance against different microbial pathogens [43,44,45]. Zhou and coworkers, 2016; studied ABA effects on brain gliomas. This data support a key role of ABA in promoting apoptosis of cancer cells [46]. Moreover, a protective role on type 2 diabetes has been demonstrated [47,48]. On this respect, plasmatic ABA (ABAp) levels, correlate with different glucose dysregulation conditions.} Balino et al.

{Abscisic acid is naturally present in fruits and vegetables, and it plays an important role in managing glucose homeostasis in humans. According to the latest U.S. dietary survey, about 92% of the population might have a deficient intake of ABA due to their deficient intake of fruits and vegetables…….Moreover, emerging evidence suggests that the ABA response might be dysfunctional in diabetic subjects. Follow on intervention studies in healthy individuals show that low-dose dietary ABA administration exerts a beneficial effect on the glycemia and insulinemia profiles after oral glucose load. These recent findings showing benefits in humans, together with extensive efficacy data in mouse models of diabetes and inflammatory disease, suggest the need for reference ABA values and its possible exploitation of the glycemia-lowering effects of ABA for preventative purposes.} Zocchi et al.

{The present study showed that ABA is effective in improving glyco-metabolic and inflammation parameters in patients with IFG or IGT.

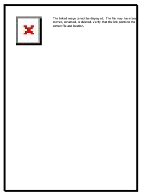
Recently, Magnone et al. demonstrated that chronic consumption of a supplement containing a low dose of ABA ameliorated the prediabetes markers (FPG −30.2% and HbA1c −8.1%) in subjects with borderline values of FPG (≥100 mg/dl) and HbA1c (≥5.7%) [24] defined in the American Diabetes Association (ADA) recommendations for prediabetes [2]. It has been also proven that the improvement was greater in these subjects than in healthy ones, suggesting the beneficial effect of low-dose ABA supplementation in prediabetics.] Derosa et al.



VOMIFOLIOL, *in vitro* anticancer activity

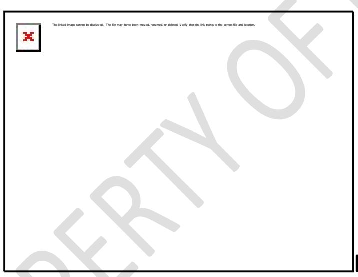
{The leaves of Antidesma ghaesembilla Gaertn (Phyllanthaceae) was subjected to phytochemical investigation. The powdered leaves were extracted with ethanol and then partitioned among petroleum ether, carbon tetrachloride and chloroform. Fractionation of the chloroform extracts affords the isolation and structural elucidation of vomifoliol.} Maria et al.

{Vomifoliol is a characteristic terpene from family Rubiaceae which may be useful as a chemotaxonomic marker….Vomifoliol and stigmasterol have anti-proliferative activity on human lung cancer cell lines} from NIH (US) https://drugs.ncats.io/drug/P86438KC5J

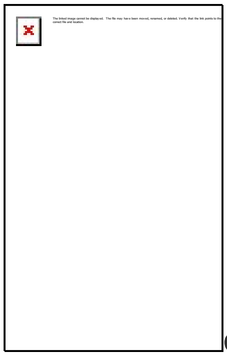


P-MENTHANE-1,2,8-TRIOL, syn.: 4-(2-hydroxypropan-2-yl)-1-methylcyclohexane-1,2-diol (HPMCD). Potent antioxidant, not much literature

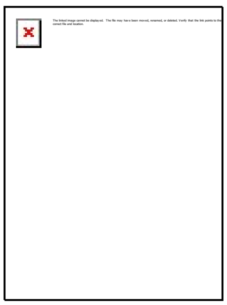
{In this paper, we report the antioxidant and cytoprotective properties of 4-(2-hydroxypropan-2-yl)-1-methylcyclohexane-1,2-diol (HPMCD) isolated from the aqueous extract of Decalepis hamiltonii roots. Our results show that HPMCD is a potent scavenger of superoxide , hydroxyl (radical dotOH), nitric oxide (radical dotNO), and lipid peroxide (LOOradical dot) physiologically relevant free radicals with IC50 values in nmolar (56–582) range. HPMCD also exhibited concentration dependent secondary antioxidant activities like reducing power, metal chelating activity, and inhibition of protein carbonylation. Further, HPMCD at nmolar concentration prevented CuSO4-induced human LDL oxidation. Apart from the in vitro free radical scavenging activity HPMCD demonstrated cytoprotective activity in primary hepatocytes and ehrlich ascites tumor (EAT) cells against oxidative stress inducing xenobiotics. The mechanism of cytoprotective action involved maintaining the intracellular glutathione (GSH), scavenging of reactive oxygen species (ROS), and inhibition of lipid peroxidation (LPO). Based on the results it is suggested that HPMCD is a novel bioactive molecule with health implications in both prevention and amelioration of diseases involving oxidative stress as well as in the general well being.} Srivastava et al 2012



BETULIN, ubiquitous natural product with wide range of biological activitie. {Betulin (B) and Betulinic acid (BA) are natural pentacyclic lupane-structure triterpenoids which possess a wide range of pharmacological activities. Recent evidence indicates that B and BA have several properties useful for the treatment of metabolic disorders, infectious diseases, cardiovascular disorders, and neurological disorders.} Amiri et al. 2020

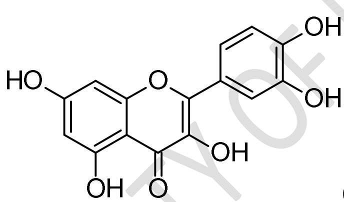
**GLUCOPYRANOSYL TRYPTOPHAN**, no reports found on bioactivity.

{Tryptophan-N-glucoside in Fruits and Fruit Juices} Diem et al. 2000



N-(1-DEOXY-1-FRUCTOSYL)PHENYLALANINE, no reports found on bioactivity

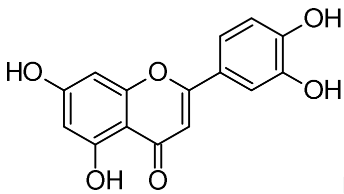
{According to quantitation via UHPLC-MS/MS, **FRU-PHE** was < 1.54 mg/kg in NMAH and > 10.00 mg/kg in AHAH, showing Fru-Phe is a potential indicator of artificial heating acacia honey.} Yan et al. 2022.---comment: So it is not unusual to detect this in honey but high levels may point to artificial heating, we cannot quantify the amounts based on the data at hand.



QUERCETIN, found in many plants

{Quercetin, a member of the flavonoids family, is one of the most prominent dietary antioxidants. It is ubiquitously present in foods including vegetables, fruit, tea and wine as well as countless food supplements and is claimed to exert beneficial health effects. This includes protection against various diseases such as osteoporosis, certain forms of cancer, pulmonary and cardiovascular diseases but also against aging. Especially the ability of quercetin to scavenge highly reactive species such as peroxynitrite and the hydroxyl radical is suggested to be involved in these possible beneficial health effects. Consequently, numerous studies have been performed to gather scientific evidence for these beneficial health claims as well as data regarding the exact mechanism of action and possible toxicological aspects of this flavonoid. The purpose of this review is to evaluate these studies in order to elucidate the possible health-beneficial effects of the antioxidant quercetin. Firstly, the definitions as well as the most important aspects regarding free radicals, antioxidants and oxidative stress will be discussed as background information. Subsequently, the mechanism by which quercetin may operate as an antioxidant (tested in vitro) as well as the potential use of this antioxidant as a nutraceutical (tested both ex vivo and in vivo) will be discussed.} *Boots et al. 2008*

{The most widely distributed flavonoid in foods, including honey, is quercetin.} Olas 2020.



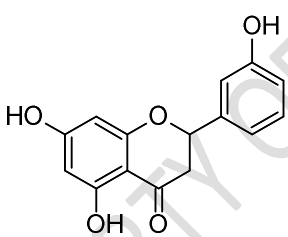
LUTEOLIN

Epidemiological evidence suggests that flavonoids may play an important role in the decreased risk of chronic diseases associated with a diet rich in plant-derived foods. Flavonoids are also common constituents of plants used in traditional medicine to treat a wide range of diseases. The purpose of this article is to summarize the distribution and biological activities of one of the most common flavonoids: luteolin.

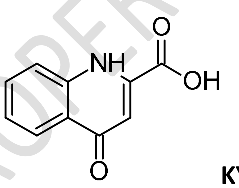
This flavonoid and its glycosides are widely distributed in the plant kingdom; they are present in many plant families and have been identified in Bryophyta, Pteridophyta, Pinophyta and Magnoliophyta. Dietary sources of luteolin include, for instance, carrots, peppers, celery, olive oil, peppermint, thyme, rosemary and oregano. Preclinical studies have shown that this flavone possesses a variety of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial and anticancer activities.

The ability of luteolin to inhibit angiogenesis, to induce apoptosis, to prevent carcinogenesis in animal models, to reduce tumor growth in vivo and to sensitize tumor cells to the cytotoxic effects of some anticancer drugs suggests that this flavonoid has cancer chemopreventive and chemotherapeutic potential.} Lopez-Lazaro 2009

{Some bioactive compounds, such as Galangin, kaempferol, quercetin, isorhamnetin, and luteolin, are present in all types of honey} Rahman et al. 2014



5,7,3'-TRIHYDROXYFLAVANONE, similar activity to the other flavonoids.

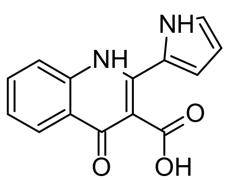


KYNURENIC ACID, may have anticancer potential

{Kynurenic acid (KYNA) is an endogenous tryptophan metabolite exerting neuroprotective and anticonvulsant properties in the brain.} Walczak et al. 2020

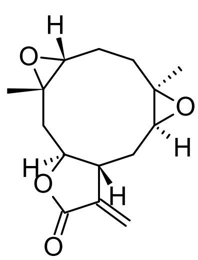
{KYNA, a Trp metabolite, shows neuroprotective activity against excitotoxic amino acids by antagonizing the NMDA receptor (glycine, glutamate). **Here we report the identification of KYNA by a combination of ESI-MS/MS and 1D- and 2D-NMR analyses in honey**

varieties of arboreal origin. KYNA are absent in single-flower honeys from herbal flowers. These different distribution patterns might possibly involve an indirect plant defence mechanism against fungal pathogens and herbivorous parasites, ever-present on wild trees. **The presence of KYNA in honey may explain its pain-relieving effects reported in the literature. The substance, acting in concert with honey flavonoids (COX-2 inhibitors), by antagonizing the NMDA receptor may contribute to the antinociceptive effect of honey. Moreover, kynureninates, owing to their antimicrobial properties, can favour the successful outcome of wounds and burns.} Beretta et al. 2007**



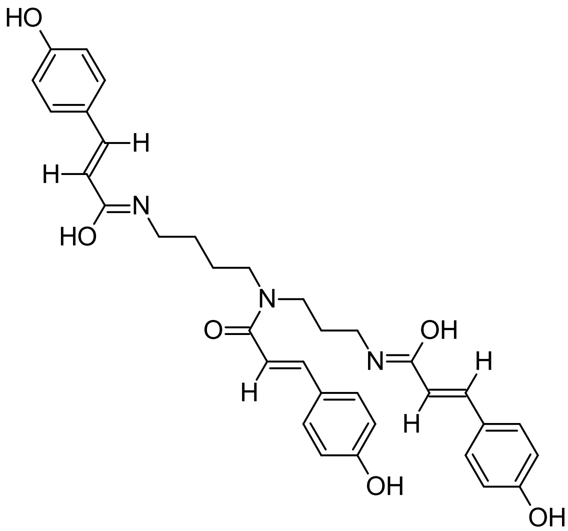
PENICINOLINE, discovered in 2010 from a mangrove endophytic fungus, cytotoxic, may have selectivity against cancer cells, never been reported from honey.

{Compounds 1 (penicinoline) and 1a were also tested for their insecticidal activity against Aphis gossypii, Plutella xylostella, Heliothis virescens, Septoria tritici, and Uromyces fabae using assays based on insect mortality and the area of leaf eaten by the methods described previously.15 Compound 1 exhibited strong activity against A. gossypii with 100% mortality on a mixed population at 1000 ppm, a very impressive result for a natural product.} Shao et al 2010



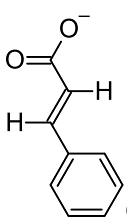
11,13-DEHYDROERIOLIN, plant natural product found in Carpesium abrotanoides and Schkuhria schkuhrioides, **not reported in honey before**.

{Schkuhria pinnata (Lam.) Kuntze ex Thell is used traditionally to treat a number of diseases linked with bacterial infections and inflammation such as eye infections, diarrhoea, diabetes and stomach problems. Despite these uses, there is still insufficient information on the isolation and evaluation of active compounds from this plant. Thus, the study was aimed at identification and characterisation of the antibacterial and anti-inflammatory active extracts of Schkuhria pinnata.} Kudumela et al 2019.


TRICOUMAROYL SPERMIDINE, Tricoumaroyl spermidine is a natural product found in Quercus dentata, Aphelandra tetragona, and other organisms. Pollen component. <u>Not much known about this conjugate but spermidine itself:</u> <u>**"Spermidine delays aging in humans" Madeo et al. 2018**</u>

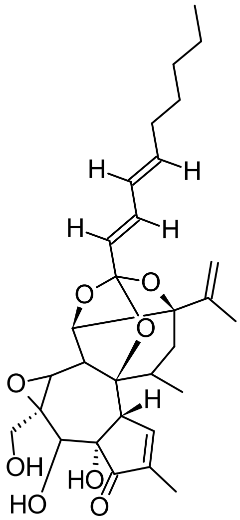
{Phenolamides such as those utilized by the gut microbiota (N1,N5,N10-tricoumaroyl spermidine, m/z 582.260; N1,N5,N10-tricaffeoyl spermidine, m/z 630.245; and p-coumaroylputrescine, m/z 233.129; Fig 3 and S2C Fig) are also compounds of the exine as they are deposited on top and into its cavities as part of the pollen coat [66]} "Disentangling metabolic functions of bacteria in the honey bee gut" Kesnerova et al 2017.

{From a methanol extract of Artemisia caruifolia, which showed a moderate inhibitory activity on HIV-1 protease in a preliminary screening, N1 ,N5 ,N10-tri-p-coumaroylspermidine and three dicaffeoylquinic acids were isolated. The former compound was found to appreciably inhibit HIV-1 protease} Hattori et al 2001


CINNAMATE,

{Cinnamic Acid and Its Derivatives: Mechanisms for Prevention and Management of Diabetes and Its Complications} Adisakwattana 2017

{Honey bee propolis is rich in cinnamic acid derivatives.} Mishima et al 2005



EXCOECARIATOXIN, antineoplastic- (Blocking the formation of neoplasms (growths that may become cancer). Borris et al 1984

EXPERIMENTAL DETAILS:

Honey samples were analysed using an on a Bruker Compact Qtof mass spectrometer connected to an Thermo Scientific Ultimate 3000 chromatograph equipped with a reversed-phase Acclaim 120 C-18 column (2.1x100mm, 2.6, 3µm). Solid-phase extraction was employed for sample preparation using Supelclean C-18 cartidges (1g). This was done to separate the highly water soluble components such as sugars within the honey from the less polar organic compound of interest, so as to elevate their concentration in the samples for greater depth of detection. Portions of honey (7.5 g each) were dissolved in 25 mL of deionised water and adsorbed onto the SPE cartridges. The loaded samples were washed with 15 mL deionized water, followed by elution with 10 mL methanol and drying *in vacuo*. This procedure was carried out in duplicate for each sample as well as for a blank consisting of pure deionised water. The samples were then reconstituted in 2mL methanol. Chromatographic separation was achieved using a gradient mobile phase program starting with 95/5 % (H_2O+0.1% formic acid / MeCN+0.1% formic acid) and changing to 0/100 % over 15 min with a two-minute hold at the end. Volumes of 5 µL were injected.

COMMENTS FROM MEETING

The most interesting molecule is P-MENTHANE-1,2,8-TRIOL, - not common in honey,

-We need to try to quantify its levels and further identify its use and source

We need to have some anti-oxidant levels assayed and compare them to others

Honey and digestion – do a literature review or contract someone to do it

Quantify the levels of terpenoids (and flavonoids?) and compare it to other honeys

Dr Jarmo will check with Professor to check on quantification capacity and whether the terpenoid P-Menthane can be purified and quantified.

Same study but of other honeys needs to be done – what other honeys should we test?

1. Comparative study on Anti-oxidant levels in various types of honey.
2. Quantify and compare levels of terpenoids and flavonoids in our honey and others.
3. How to sell our honey based on biodiversity?
4. Is there such a thing as MGO that can be added to honey?