CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF FRENCH BFA PROPOLIS EXTRACTS

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Complex resinous product collected by honeybees from buds, leaves and exudates from various trees and plants mixed with beeswax and salivary secretions.

Two major classes* of propolis:
- European-type (poplar type): rich in flavonoids as well as phenolic acids and their esters (Europe, China and other countries)
- Tropical-type (Brazilian-type): rich in terpenic and prenylated derivatives

Used inside the hive:
- to seal the walls, to reduce the entry of the hive
- as a « chemical weapon » against microorganisms

Used in folk medicine as:
- antioxidant, antifungal, antibacterial, antiviral, anti-inflammatory...

* Bankova et al. 2000, Kumazawa et al. 2004
INTRODUCTION
BALLOT-FLURIN APICULTEURS

Ballot-Flurin Apiculteurs (BFA)

- Company (SME) located in the South-West of France
- Pionneer in France in organic beekeeping
- Expert in natural health
- Inventor of the Apiculture douce® (Gentle beekeeping) based on the language of bees and a deep respect for nature
- More than 60 effective products (Apitherapy and Apicosmetic products) of the highest quality with NO side effects:
  - 100% harmless, proved under medical control
  - Efficient
  - With 0% contaminant, proved by analysis

Collaboration with the faculty of pharmacy of Angers, France:
  - PhD : Chemical caracterization and biological activities of propolis extracts
SUMMARY

I. Chemical composition
   1) Propolis sample
   2) Extractions
   3) Identification
   4) Quantification

II. Antioxidant activity
   1) Total polyphenol content
   2) DPPH assay
   3) ORAC assay

Conclusion
I. Chemical Composition
I. CHEMICAL COMPOSITION

1) PROPOLIS SAMPLE

Mixture of 24 propolis samples from:
- different regions of France, predominantly in the South-West
- 2010 and 2011

Collection sites of the French BFA propolis samples
I. CHEMICAL COMPOSITION

2) Extractions

Five extractions with different solvents:

**Protocol n° 1**
Water

- \( H_2O, 15\text{min}, 100° C \)
- Aqueous extract
- Residue + waxes

7% yield

**Protocol n° 2**
70% EtOH, MeOH

- 70% EtOH or MeOH
- 3x2h, 25° C
- Alcoholic extract + waxes
- Residue

- -18° C over night
- Waxes

70% EtOH or MeOH extract
65-68% yield

**Protocol n° 3**
DCM, Mixed solvents*

- Cyclohexane
- 3x2h, 25° C
- Residue

- Waxes

Cyclohexanic extract: waxes

- DCM or mixed solvents
- 3x2h, 25° C
- Residue

DCM or Mixed solvents extracts
50-59% yield

*DCM: dichloromethane; Mixed solvents: DCM/MeOH/H\(_2\)O 31/19/4
I. CHEMICAL COMPOSITION

3) HPLC/DAD PROFILES

Two types of HPLC/DAD profiles:

- **Aqueous type**

  ![Graph](image)

  **1) Water - 280 nm**

- **Alcoholic, DCM and mixed solvents type**

  ![Graph](image)

  **2) DCM - 280 nm**

**Conditions:** HPLC system: Waters 2695 with DAD detector 2996, Phenomenex Luna C18 (150x4.6 mm) 3µm column, injection 10 mg/mL, flow rate 0.4 mL/min, mobile phase: H₂O + 0.1% formic acid (A) et MeOH + 0.1% formic acid, gradient elution: 40%B (0-10min), 40-50%B (10-25min), 50-60%B (25-55min), 60-90%B (55-70min), 90%B (70-80min)
I. CHEMICAL COMPOSITION

3) IDENTIFICATION

Chemical characterization

- Fractionation by Flash chromatography
- HPLC/UV
- HPLC/MS
- Scientific literature
- Commercial standards
- $^1$H and $^{13}$C NMR (1 and 2 dimensions)
I. CHEMICAL COMPOSITION

3) IDENTIFICATION

1) Aqueous extract

- Benzaldehyde and benzoic acid derivatives:
  1. 3,4-dihydroxybenzaldehyde
  2. 4-hydroxybenzaldehyde
  3. Vanillic acid
  4. 4-Hydroxyacetophenone
  5. Benzoic acid

- Cinnamic acid derivatives:
  2. Caffeic acid
  3. p-coumaric acid
  4. Ferulic acid
  5. Isoferulic acid
  6. 3,4-dimethoxycinnamic acid

- Flavanones/Dihydroflavonols:
  11. Pinobanksin-5-methyl ether
  14. Pinobanksin
  15. Naringenin

Water - 280 nm
I. CHEMICAL COMPOSITION

3) IDENTIFICATION

2) Alcoholic, DCM and mixed solvents extracts

- **Benzaldehyde and benzoic acid derivatives:**
  1. 3,4-dihydroxybenzaldehyde
  2. 4-hydroxybenzaldehyde
  3. Vanilline
  4. 4-Hydroxyacetophenone
  5. Benzoic acid

- **Cinnamic acid derivatives:**
  6. Caffeic acid
  7. p-coumaric acid
  8. Ferulic acid
  9. Isoferulic acid
  10. 3,4-dimethoxycinnamic acid
  11. Cinnamic acid
  12. 4-methoxycinnamic acid
  13. Cinnamylidene acetic acid

![Graph showing DCM - 280 nm analysis of extracts](image)
I. CHEMICAL COMPOSITION
3) IDENTIFICATION

2) Alcoholic, DCM and mixed solvents extracts

**Cinnamic ester derivatives:**
- **24** Isopent-3-enyl caffeate
- **25** Benzyl caffeate
- **28** Prenyl caffeate
- **30** Caffeic acid phenylethyl ester (CAPE)
- **32** Benzyl p-coumarate
- **33** Cinnamyl caffeate
- **36** Cinnamyl isoferulate
- **37** Cinnamyl p-coumarate
- **40** Benzyl cinnamate
- **41** Cinnamyl cinnamate
- **42** Cinnamyl cinnamylidene acetate

**Glycerol derivatives:**
- **16** 1,3-dicoumaroylglycerol
- **26** 2-acetyl-1,3-dicoumaroylglycerol

**Flavan-3-ol:**
- **34** New flavan-3-ol
I. CHEMICAL COMPOSITION

3) IDENTIFICATION

2) Alcoholic, DCM and mixed solvents extracts

- **Flavanones/Dihydroflavonols:**
  - 11 Pinobanksin-5-methyl ether
  - 14 Pinobanksin
  - 15 Naringenin
  - 22 Pinocembrin-5-methyl ether
  - 23 Pinocembrin
  - 27 Pinobanksin-3-acetate
  - 35 Pinostrobin
  - 38 Alpinone-3-acetate

- **Flavones/Flavonols:**
  - 17 Kaempferol
  - 18 Apigenin
  - 19 Luteolin methyl ether
  - 21 Quercetin-7-methyl ether
  - 29 Chrysin
  - 31 Galangin
  - 39 Tectochrysine

![Graph](image-url)
I. CHEMICAL COMPOSITION

4) QUANTIFICATION

Quantification of 12 major components (HPLC/DAD)

![Graph showing quantification of 12 major components with peak numbers and corresponding chemical structures below each peak.]

- **2** Caffeic acid
- **6** p-coumaric acid
- **7** Ferulic acid
- **8** Isoferulic acid
- **10** 3,4-dimethoxy cinnamic acid
- **23** Pinocembrin
- **27** Pinobanksin-3-acetate
- **28** Prenyl caffeate
- **29** Chrysin
- **30** CAPE
- **31** Galangin
- **35** Pinostrobin
## I. CHEMICAL COMPOSITION

### 4) QUANTIFICATION

Quantification of 12 major components (HPLC/DAD)

<table>
<thead>
<tr>
<th>Component</th>
<th>Water (mg/g)</th>
<th>70% EtOH (mg/g)</th>
<th>MeOH (mg/g)</th>
<th>DCM (mg/g)</th>
<th>Mixed solvents (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic acid</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
<tr>
<td>Isoferulic acid</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
<tr>
<td>3,4-dimethoxycinn acid</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
<tr>
<td>Pinocembrin</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
<tr>
<td>Pinobankin-3-acetate</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
<tr>
<td>Prenyl caffeate</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
<tr>
<td>Chrysin</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
<tr>
<td>CAPE</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
<tr>
<td>Galangin</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
<tr>
<td>Pinostrobin</td>
<td>70% EtOH (mg/g)</td>
<td>50% EtOH (mg/g)</td>
<td>MeOH (mg/g)</td>
<td>DCM (mg/g)</td>
<td>Mixed solvents (mg/g)</td>
</tr>
</tbody>
</table>

- **Aqueous extract:** predominantly caffeic and p-coumaric acids (total 185 mg/g)
- **Other extracts:** same profiles (pinobanksin-3-acetate, pinocembrin, chrysin, galangin…)
- **Alcoholic extracts:** Similar values for the 12 components (total 194 mg/g)
- **DCM extract:** best contents (total 272 mg/g)
II. ANTIOXIDANT ACTIVITY
II. ANTIOXIDANT ACTIVITY

1) POLYPHENOLIC CONTENT

Total polyphenol content (Folin-Ciocalteu method)

- Polyphenol contents between **239 and 292 mg GAE/g**\(^1\)
  (European type propolis: 200-300 mg GAE/g)\(^2\)
- Contents of aqueous, DCM and mixed solvents extracts slightly higher than alcoholic ones

\(^1\) GAE/g: milligram of Gallic Acid Equivalent per gram
\(^2\) Laskar et al. 2010, Kumazawa et al. 2004
II. ANTIOXIDANT ACTIVITY
2) DPPH ASSAY

DPPH (diphenylpicrylhydrazyl) colorimetric assay

Principle:

- Method based on singulet electron transfer (SET)
- Standard: Trolox, a water-soluble analog of \( \alpha \)-tocopherol (vitamin E)
- Results expressed as micromole of trolox equivalent per gram of extract (\( \mu \)mol TE/g)
II. ANTIOXIDANT ACTIVITY

2) DPPH ASSAY

DPPH (diphenylpicrylhydrazyl) colorimetric assay

Results:

- Activity of propolis extracts 2 to 4 times higher than E392
- Higher activities observed for mixed solvents, aqueous and 70% EtOH extracts

II. ANTIOXIDANT ACTIVITY

3) ORAC ASSAY

ORAC (Oxygen Radical Absorbance Capacity) fluorimetric assay

**Principle:**

- Fluorescein + AAPH free radicals → Fluorescein oxidation
- Fluorescein oxidation (loss of fluorescence) is delayed

Fluorescein + AAPH free radicals
(antioxidant compounds)

- Method based on hydrogen atom transfer (HAT)
- Same standard: Trolox
II. ANTIOXIDANT ACTIVITY
3) ORAC ASSAY

ORAC (Oxygen Radical Absorbance Capacity) fluorimetric assay

Results:

- Activity of propolis extracts 3 to 5 times higher than E392
- Higher activities observed for mixed solvents, aqueous and 70% EtOH extracts
Chemical composition:

- **Aqueous extract:**
  predominantly cinnamic acid derivatives (caffeic and \( p \)-coumaric acids...)

- **Alcoholic, DCM and mixed solvents extracts:**
  - Cinnamic acid derivatives (caffeic and \( p \)-coumaric acids...)
  - Cinnamic ester derivatives (prenyl cafeate, CAPE...)
  - Flavanones/dihydroflavonols (pinobanbsin-3-acetate, pinocembrin...)
  - Flavones/flavonols (chrysin, galangin...)

> Poplar-type (*Populus nigra* L.)
Antioxidant activity:

- All extracts showed a very good antioxidant activity associated with a high total polyphenol content
- Activity slightly higher for mixed solvents, aqueous and 70% EtOH extracts
## CONCLUSION

### Comparison of the five extracts

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Water</th>
<th>70% EtOH</th>
<th>MeOH</th>
<th>DCM</th>
<th>Mixed solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yield (%)</strong></td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><strong>Total content of 12 components (mg/g) TC12</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Total polyphenol content (mgGAE/g) TPC</strong></td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>DPPH (µmol TE/g)</strong></td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td><strong>ORAC (µmol TE/g)</strong></td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

### Positive points
- Best TPC
- Good DPPH, and ORAC
- Green solvent: WATER
  - Good DPPH, ORAC
  - Medium TPC, DPPH, ORAC
- Best TC12
- Good TPC
- Best DPPH and ORAC

### Negative points
- Low yield
- Alcohol
- MeOH: toxic alcohol
- DCM: toxic organic solvent
- Presence of DCM: toxic organic solvent

**Water extract** Very interesting in pharmaceutical, cosmetic and food additive products
Ballot-Flurin Apiculteurs

All Ballot-Flurin Team

The beekeepers beside us who work every day to make the bees stronger and harvest the best propolis and bee products

University of Angers (SONAS Lab)

Pr Pascal Richomme
Dr Anne-Marie Le Ray
Marie-Christine Aumond

Thank you for your attention