Phytochemical Content and Efficacy Profiling of Plant-Based Materials and Preparations

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Growing demand for high-quality, safe, health-promoting or disease risk reducing plant-based medicinal products as consumers turn to increasingly healthy lifestyles.

Such products display high consumer acceptance, and high potential because of a series of advantages, i.e. perceived efficacy and safety, and relatively low costs.

Critical questions remain unanswered for many plants e.g. have consumption patterns (i.e. intake levels) changed compared to historical use? How comprehensive have toxicology studies been? Interactions? Adequate study sample power? Etc.....

Regulatory challenges also exist e.g. differences across regions that can impede trade and growth where not all products developed in SA can be exported to EU countries; certain plants used in e.g. Belgium and Italy shouldn’t be consumed in Austria.

Increasingly integrated approaches needed to ensure safe, quality product development which could also bring market growth and opportunities for job creation.
The EU PlantLIBRA Project Framework
(PLANT food supplements: Levels of Intake, Benefit and Risk Assessment)

- A large multinational collaboration: 25 partners spanning the EU, Asia, Latin America and South Africa

- Strong scientific knowledge-base of research groups and institutes aimed at improving science-based decision making and the use of safer quality products
CSIR’s Involvement

- Underscored by South Africa’s unique biodiversity
  - 24 000 plant species; high representation (up to 10%) of world’s plants; unique biomes

- Wide-spread use of plant based preparations
  - Significant proportion bear potential health benefits or are already used medicinally

- Empirical evidence often lacking to scientifically support benefit claims

- PlantLIBRA - an opportunity to contribute to, and to acquire state of the art approaches, protocols, and methodologies employed by leading collaborators to support local research strategies and initiatives

- A key entry-point to acquiring first hand information on the evolution of international (EU) regulatory requirements in the field
Phytochemical Content Profiling of Botanical Ingredients and Plant Food Supplements

Key questions

- How applicable are existing technologies to analysing the phytochemical complexities of plant material across the value chain i.e. raw ingredients through to products?

- To what extent do plant preparations from e.g. different regions processed by different methods vary in terms of overall and /or specific biomarker chemical composition?

- What are the current challenges and gaps?
## Methods for Botanical Ingredients and Plant Food Supplements

### 25 prioritised plant species

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Other Plant Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aloe ferox Mill.</td>
<td>Citrus limon (L.) Burm. F.</td>
</tr>
<tr>
<td>Borago officinalis L.</td>
<td>Peumus boldus Molina</td>
</tr>
<tr>
<td><strong>Boswellia serrata</strong> Roxb. Ex Colebr</td>
<td>Citrus sinensis (L.) Osbeck</td>
</tr>
<tr>
<td>Camellia sinensis (L.) Kuntze</td>
<td>Plantago ovata Forsk.</td>
</tr>
<tr>
<td>Senna alexandrina P. Mill.</td>
<td>Cynara cardunculus L.</td>
</tr>
<tr>
<td>Senna obtusifolia (L.) Irwin &amp; Barneby</td>
<td>Frangula purshiana (D.C.) Cooper</td>
</tr>
<tr>
<td>Senna tora (L.) Roxb</td>
<td>Foeniculum vulgare Mill.</td>
</tr>
<tr>
<td>Cinnamomum verum J. Presl</td>
<td>Serenoa repens (Bartr.) Small</td>
</tr>
<tr>
<td>Citrus aurantium L.</td>
<td>Silybum marianum (L.) Gaertn.</td>
</tr>
<tr>
<td></td>
<td>Harpagophytum procumbens (Burch.) DC Ex Mei</td>
</tr>
<tr>
<td></td>
<td>Valeriana officinalis L.</td>
</tr>
<tr>
<td></td>
<td>Glycine max (L.) Merr.</td>
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<tr>
<td></td>
<td>Silybum marianum (L.) Gaertn.</td>
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</table>

### Phytochemical profiling
- DNA analysis
- U(HPLC) mass spectrometry

### Contaminant residue determination
- GC mass spectrometry
- HPLC tandem mass spectrometry
- Biosensors
- Thermoluminescence
- ELISA
Methods for Botanical Ingredients and Plant Food Supplements

MS-based phytochemical profiling approach

- **Compound extraction method development**: Produce a representative extract of plant material containing most of the marker compounds.

- **UPLC-based chromatography**: Chromatographic methods for plant extract separation into the individual chemical components detectable with the available detectors.

- **Mass spectrometry identification**: Identify known compounds via exact mass and annotate the chromatogram for key compounds.
Harpagophytum procumbens (Burch.) DC. Ex Meisn.

- Native to South Africa, Namibia, Botswana
- Known as Devil’s Claw, grapple plant or wood spider
- Medicinal uses ascribed to secondary tuberous roots which are harvested, sliced and dried before use; the Khoisan are documented to use the plant as an analgesic; other uses – treating digestive disorders, hypertension, exhibits diuretic properties, sedatory uses
- Key compounds include: iridoid glycosides, particularly harpagoside including small amounts of trans-coumaroyl harpagide, procumbide and sterols
Harpagophytum procumbens (Burch.) DC. Ex Mei

Annotated XIC mass chromatograms of the pure reference standards

Annotated XIC mass chromatogram of the test *H. procumbens* extract
Harpagophytum procumbens (Burch.) DC. Ex Mei

- Analysed 8 replicates/sample using CSIR and PlantLIBRA sourced raw material
- Data analysis mined mass spectra for biomarkers that can be used to evaluate similarities or differences
- High similarity (>95%) observed between the two samples; differences largely quantitative
- Approach suited for batch to batch screening of crude as well as processed plant material

BPI mass chromatograms of the two MeOH/ACN extracts

Harpagoside
Harpagophytum procumbens (Burch.) DC. Ex Mei

- Despite similarities, significant differences seen between the two samples
- Samples were similar (i.e. grouped within the ellipse), but at the same time clustered as unique groups

Scores Plot evaluation of the two MeOH/ACN extracts
Boswellia serrata Roxb. Ex Colebr

• Indian frankincense tree or Indian olibanum tree

• Commonly found in Rajasthan, Madhya Pradesh and Andhara Pradesh in India

• Main product is frankincense or olibanum

• Used in Ayurvedic medicine to treat arthritis, and various other inflammatory diseases such as bronchial asthma, osteoarthritis, and ulcerative colitis

• Primary constituents: volatile oil, polysaccharides, monoterpenes, diterpenes and lipophilic pentacyclic triterpene acids of the oleanane – (α-boswellic acids), ursane-(β-boswellic acids) and lupeolic acids

Boswellia serrata Roxb. ex Colebr (Source: Woodville, W., Hooker, W.J., Spratt, G., Medical Botany, 3rd edition, vol. 5: t. 32 (1832)
ESINeg mass chromatograms of the three *Boswellia* samples evaluated

- Data shown: a commercial capsule, a coarse resin (PL 090), and a medium fine resin (TPA 70-12-1)
- 16 key biomarker compounds could be tentatively detected, subsequently confirmed with reference standards
- Cembrenol, incensole, 11-keto-β-boswellic acid and acetyl-11-keto-β-boswellic acid; 12-ursen-2-diketone, 12-ursene-3,24-diol, 9,11-dehydro-α-boswellic acid, 9,11-dehydro-β-boswellic acid, lupeolic acid, α-boswellic acid, β-boswellic acid, acetyl-9,11-dehydro-α-boswellic acid, acetyl-9,11-dehydro-β-boswellic acid, acetyl-α-boswellic acid and acetyl-β-boswellic acid
Boswellia serrata Roxb. Ex Colebr

- Notable differences e.g. between TPA 70-12-1 and PL090
- Differences both concentration (quantitative) based, and the type (qualitative) of compounds

Hotellings T2 analysis of the ESINeg mass spectral data of 5 B. serrata samples
Valeriana officinalis L.

- Used since ancient Greece and Rome time as a medicinal herb; used in Ayurvedic medicines for treating various indications linked e.g. to the nervous, digestive and respiratory systems
- Valerenic acid and its derivates, and the valepotriates considered the most important for the plants sedative effects
- Roots and rhizomes contain two main groups of compounds, (1) the sesquiterpenes which include valerenic acid and its derivatives, valeranone, valeranal and kessyl esters and (2) the valepotriates which include valtrate, didrovaltrate, acevaltrate and isovaleroxyhydroxyvaltrate. Other compounds in the plant are flavonoids, triterpenes, lignans and alkaloids

Valeriana officinalis L. Common Valerin
(Source: Kohler, F.E., Medizinal Pflanzen, vol. 1: t. 47 (1887))
ESIPos and ESINeg XIC annotated chromatograms: *Valeriana officinalis*
**Valeriana officinalis** L.

- Multiple (N=16) samples from across different regions analysed
  - FPM=Fine plant material
  - FLPM=Fine lumpy plant material
  - CPM=Coarse plant material
  - TSE=Tarry solid extract
  - LIQ=Liquid
  - CAP=Capsule
  - TAB=Tablet

<table>
<thead>
<tr>
<th>NAME</th>
<th>SAMPLE FORMAT</th>
<th>STUDY NAME</th>
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<tbody>
<tr>
<td>Valeriana officinalis</td>
<td>FPM</td>
<td>PL REF</td>
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<td>PL 152</td>
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<td>CPM</td>
<td>PL 264</td>
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<td>PL 320</td>
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<td>LIQ</td>
<td>PL 076</td>
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<td>Valeriana off.</td>
<td>LIQ</td>
<td>PL 210</td>
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<tr>
<td>Valerian root extract</td>
<td>CAP</td>
<td>COM1</td>
</tr>
</tbody>
</table>

*V. officinalis* samples subjected to analysis
Valeriana officinalis L.

- Visual comparison clearly complex; computational methods key to delineating detailed multiplex profiles
- Two samples in particular, PL210 (liquid) and COM1 (capsule) differed significantly from the rest, as confirmed through PCA

ESINeg BPI mass chromatograms of the 16 V. officinalis samples
**Valeriana officinalis** L.

- PL210 (liquid) and COM1 (capsule) differed distinctly and significantly from the other samples i.e. outliers beyond the ellipse.

- Both excluded in subsequent PCA analysis of the original raw data to assess differences and/or similarities between the remaining samples.

ESINeg PCA Scores Plot of all 16 **V. officinalis** samples
Valeriana officinalis L.

- Underlying composition of the remaining groups similar, but distinct groups observed
- Differences between samples from e.g. group A/B (PLREF) and C/D (PL076) revealed similarities between the samples linked to Valeriana cpds present in both
- Differences between PLREF and PL076 caused by the compounds originating from other plant extracts added to the formulation of PL076
- Technology relevant to identifying admixtures/adulterations e.g. Hoodia gordonii vs Opuntia spp.

Melissa officinalis
Tilia platyphyllos
Passiflora incarnate
Eschscholtzia californica
Crataegus oxyacantha

Revised ESINeg PCA Scores Plot (PL210 / COM1 excluded)
Pesticide Residue Analysis

- Material was extracted using dispersive solid phase extraction, known as the “QuEChERS” (Quick Easy Cheap Rugged Safe) approach.
- Analysis performed by gas chromatography – mass spectrometry (GC-MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS).
- Examples of trace amounts of dichlorvos, and azoxystrobin (at 0.072 mg.kg) identified in *Boswellia serrata* and *Valeriana officinalis*.
- Contaminant residues such as pesticides highlight the added complexity of analysing plant material used in health-related applications.
Key Points for Consideration

- Technologies such as LC-MS/MS can highlight very clearly the compound complexities of plant-based materials and final products.

- Wide variability can exist in the compound content (qualitative and quantitative) of identical plant species obtained from different sources:
  - Such methods useful for identifying material adulterations.

- Compositional variability presents a major challenge to QA/QC, unlike e.g. synthetic compounds produced by a single ascribed process.

- How does variability affect the intended use of such products (e.g. final efficacy, health benefits, non-intended effects)?
Benefit Assessment of Plant Food Supplements

- *In vitro* assays for PFS assessment using advanced and novel approaches

  - Digestive tract enzyme function e.g. amylase, sucrase, isomaltase, and lipase
  - Cell signalling and protective mechanisms e.g. ((anti) hormonal, antioxidant- and chemo preventive activity)
  - Oxidative and inflammatory enzymes e.g. cyclooxygenase-2, NADPH oxidase, human recombinant cGMP-phosphodiesterase 5 and 6 activities
  - Antimicrobial and antiviral assays e.g. gram-positive bacteria, gram-negative bacteria, acid-fast bacilli, yeast, RNA and DNA viruses
  - Lipid metabolism e.g. cAMP-phosphodiesterase inhibition in 3T3-L1 adipocytes
  - Biotransformed extracts e.g. using beta-glucosidase, UDP-glucuronosyl transferases, sulfotransferases and catechol-O-methyl transferase

- 96-well micro-dilution technique using tetrazolium salts (Eloff, 1998)

  - 1mg/ml extract diluted two-fold to 1:1024

- Based on the antiviral plaque assay (Kuo et al., 2005; Schimidtke et al., 2001; Abou-Karam and Shier, 1990; Welsh et al., 1978).

- Plant extracts screened at 500ug/ml
Antimicrobial and Antiviral Assays

The panel screened included:

- gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*)
- gram-negative bacteria (*E. coli*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*)
- acid-fast bacilli (*Mycobacterium aurum*)
- yeast (*Candida albicans*)
- viruses (Coxsackievirus, herpes simplex virus type 1 and adenovirus)

Plant extracts

*Echinacea purpurea* ERB / RAD; *Andrographis paniculata* 12 / 65; *Pelargonium sidoides*; *Matricaria recutita L.*; *Calendula sp.*; *Vitis vinifera*
Antiviral Assays - Cytotoxicity

Cytopathic effects of herpes simplex virus 1 (A) untreated vero cells, (B) herpes treated cells (8dpi) exhibiting cell rounding, syncytia and detachment
Antiviral Assays - Viruses

Cytopathic effect inhibition (% CPE$_{50}$) of plant extracts at 500ug/ml and standards against herpes simplex (1)

Cytopathic effect inhibition (% CPE$_{50}$) of plant extracts at 500ug/ml and cidofovir against adenovirus

Cytopathic effect inhibition (% CPE$_{50}$) of plant extracts at 500ug/ml and guanidine against coxsackievirus
Antimicrobial and Antiviral Assays – Outcomes

- Several plant extracts exhibit apparent antiviral activity *in vitro* compared to conventional compounds e.g. *Matricaria spp* extracts *vs* herpes simplex virus (1)

- Small potential differences in antiviral activity observed against e.g. herpes simplex (1) for different extract preparations of the same plant

- Phytochemical analysis could provide insights to the chemical composition of such extracts, and the influence on biological activity of composition differences

- Interestingly, no discernible activity observed *in vitro* against microbial strains at 1mg/ml or less
Biomarkers in humans of exposure associated with bioactive components from plants

- The subjective nature of self-reported intake assessment methods presents numerous challenges to obtaining accurate dietary intake vs nutritional status / efficacy benefits.

- This may be overcome by using intake biomarkers, which are more objective in assessing consumption (or exposure) compared to e.g. self-reported dietary intake.

- Typically, blood or urinary samples are used to measure metabolite biomarkers.

- Significant knowledge gaps exist however, even for popular PFS, on the identity and use of suitable plant metabolite biomarkers to determine exposure e.g. for Ginkgo biloba, Panax ginseng, and Camellia sinensis.

- Consortia partners developing methods to identify and analyse plant metabolites to determine exposure e.g. for Ginkgo biloba, Panax ginseng, and Camellia sinensis.
LC-QQQ method developed to quantify ginkgo terpene lactones in urine, following ethyl acetate extraction.

Ginkgolide J (GJ; LOQ <500 nM)
Ginkgolide C (GC; LOQ<10 nM)
Ginkgolide A (GA; LOQ<50 nM)
Ginkgolide B (GB; LOQ<10 nM)
Bilobalide (BB; LOQ <500 nM)
Ethyl gallate (IS1)
Taxifolin (IS2)

First study to report intact GJ in human urine.
Dose dependent excretion of 5 gingko principals, following consumption of 7.2 and 14.4 mg terpene lactones (N=12, p<0.01 all compounds)

Total compound excretion ranged:
- 4 to 12 μmol (low dose)
- 10 to 22 μmol (high dose)

Explorative analysis did not identify classical phase 2 conjugates

Ginkgolides A & B, plus bilobalide excellent biomarkers to track and link consumption vs benefits and safety
- **Ginseng intervention** collected 24 hr urine (N=12) following 10 and 20 mg of oral **ginsenosides** from commercial supplement

- New LC-QQQ analytical method developed

- Un-metabolised native panaxdiol / panaxtriol ginsenosides were **not detected** in urine

Ginsenoside G-Rh1: a mono-glycosylated panaxtriol
Green tea intervention compares acute (1d) and long-term (6 and 12wk) supplementation at 1.35 and 2.7g/d GT extract.

- Have successfully utilized EC and EGC conjugates as GT biomarkers in a 50 person RCT.

- Currently completing data analysis, addressing the use of colonic metabolites (e.g. hydroxyphenyl valerolactones) as GT biomarkers.

**Camellia sinensis (Green Tea)**

**LC-QQQ analysis of EGC & EC Metabolites in Human Urine**
Summary

- Reliable accurate analysis of plant compositions remains challenging especially where biochemical constituents are complex/ low in conc.

- Comprehensive data still lacking on the extent to which compositional diversity influences biological activity, even for popular preparations

- Intake biomarkers increasingly relevant to accurately estimating dietary intake vs nutritional status / safety / efficacy benefits

- More widespread use of harmonised strategies that address such challenges, from phytochemical profiling through to *in vitro* and human studies, may reduce the inconsistencies in data reported across the field
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