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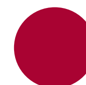

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Identification of Fungal H⁺-ATPase Inhibitors by Microfractionation and HPLC-HRMS-SPE-NMR

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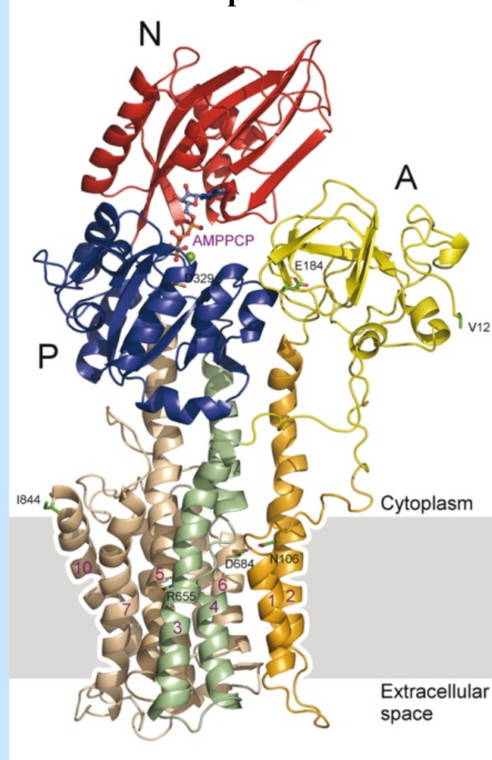
Background - *Fungal Fight*

A large number of fungal proteins have been proposed as potential targets for novel antifungal agents,[1] however, current available antifungal agents are primarily targeting the intracellular membrane biosynthesis[1] and thus need to enter the fungus to act. In our search for novel and more efficient antifungal compounds, we are focusing on the plasma membrane (PM) H^+-ATPase enzyme as target.

Plants are exposed to a wide array of phytopathogenic fungi in their natural habitat, and have been forced to develop antifungal metabolites in order to survive.[2,3] Hence, as previously suggested by Monk and coworkers,[4] it is reasonable to assume that some plants have the PM H^+-APTase enzyme as target for the antifungal metabolites. However, plant extracts are very complex mixtures, and the traditional bioassay-guided fractionation used for identification of individual bioactive components are very time-consuming and suffers from inherently low resolution during the fractionation process. To circumvent this, we have developed a bioanalytical platform that combines high-resolution microplate bioassays[5] with HPLC-SPE-NMR.[6] In the present work, we report crude extract screening of 48 plant-extracts for fungal PM H^+-ATPase inhibitors - followed by high-resolution bioassay and HPLC-SPE-NMR analysis for identification of individual active constituents.

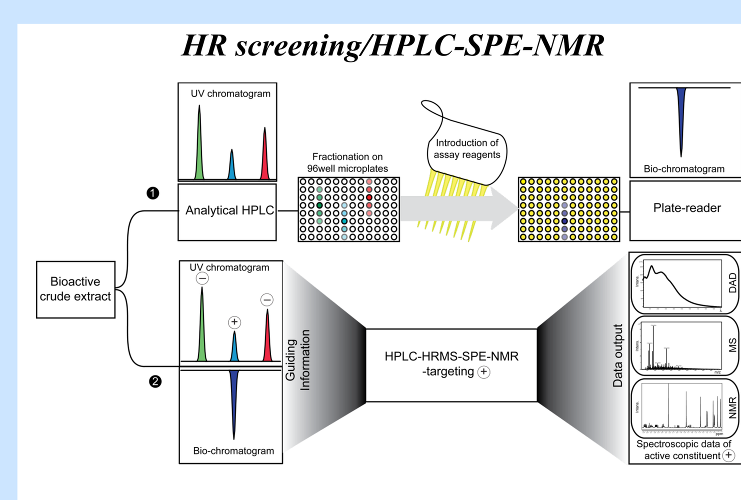
Crystal structure of the H^+-ATPase in plants.



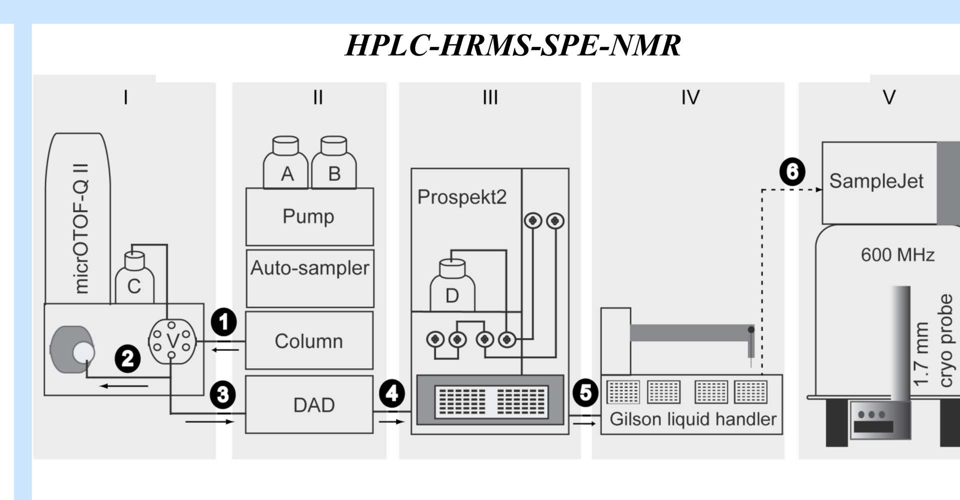
The setup - *HR screening/HPLC-SPE-NMR*

Empowered by the separation capabilities of analytical HPLC, high-resolution bioassay-guided HPLC-SPE-NMR has been shown to provide a platform that can substitute the traditional bio-guided fractionation with considerable reduction of workload and improved efficiency.[7] In this approach, chromatographically separated fractions will be subjected to a given bio-activity test to afford the so-called biochromatogram. The resulting biochromatogram will then be used as a tool for targeted analysis of active constituents. Such biochromatogram can be acquired through micro-fractionation followed by offline assay.

HR screening/HPLC-SPE-NMR



HPLC-HRMS-SPE-NMR



Results - *Crude extract screening*

▪ From 48 plants to 20 plants

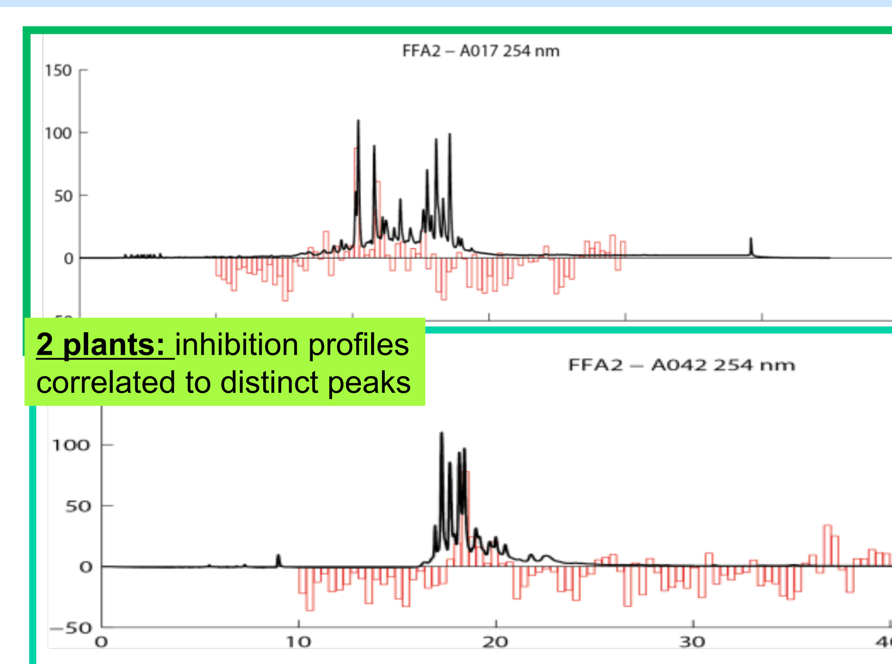
Extracts were tested in three different concentrations and those showing inhibition higher than 95% for all concentrations or a concentration-dependent activity profile were selected for semi-high-resolution screening.

No	Species	Family	Plant part	Percent inhibition					
				7.5 mg/mL		15 mg/mL		30 mg/mL	
1	*Alafia barteri*	Apocynaceae	Leaves + twigs	27	11	33	19	33	27
2	***Alchornea cordifolia***	**Euphorbiaceae**		**99**	**98**	**98**	**98**	**97**	**97**
6	***Baissea leonensis***	**Apocynaceae**	**Leaves + twigs**	**99**	**99**	**99**	**99**	**99**	**100**
7	***Baissea leonensis***	**Apocynaceae**	**Fruit**	**98**	**98**	**98**	**98**	**97**	**97**
11	***Caloncoba gilgiana***	Achariaceae	**Leaves**	**99**	**99**	**98**	**97**	**99**	**97**
12	*Croton longiracemosus*	Euphorbiaceae	Branches	-8	-13	3	9	19	7
13	*Croton longiracemosus*	Euphorbiaceae	Root bark	20	12	27	19	34	18
14	*Croton membranaceus*	Euphorbiaceae	Leaves + twigs	40	18	40	28	53	41
15	*Dovyalis macrocalyx*	Salicaceae	Stem	-4	-13	21	1	35	18
16	*Euadenia eminens*	Capparaceae	Aerial parts	33	15	43	25	49	32
17	*Euadenia eminens*	Capparaceae	Root	2	-47	4	-36	13	-7
18	*Gymnema sylvestre*	Apocynaceae	Aerial parts	34	19	38	26	59	46
19	***Haplocoelum foliolosum***	**Sapindaceae**	**Aerial parts**	**99**	**99**	**100**	**99**	**100**	**100**
20	***Hubertia ambavilla***	**Asteraceae**	**Leaves + twigs**	**27**	**29**	**56**	**58**	**84**	**86**
21	***Hubertia tomentosum***	**Asteraceae**	**Leaves**	**36**	**19**	**50**	**31**	**75**	**71**
22	***Hypericum scabrum***	**Hypericaceae**	**Whole plant**	**59**	**51**	**96**	**75**	**97**	**96**
23	***Lophira alata***	**Achnaceae**	**Leaves**	**41**	**38**	**60**	**56**	**96**	**90**
24	*Momordica charantia*	Curcurbitaceae	Aerial parts	31	27	33	35	42	39
25	***Mussaenda tristigmatica***	**Rubiaceae**	**Leaves + twigs**	**99**	**98**	**99**	**98**	**99**	**98**
47	***Sphenocentrum jollyanum***	**Menispermaceae**	Leaves + twigs	**39**	**24**	**55**	**36**	**76**	**68**
48	***Sauvagesia erecta***	**Ochnaceae**	**Whole plant**	**98**	**98**	**99**	**98**	**97**	**98**

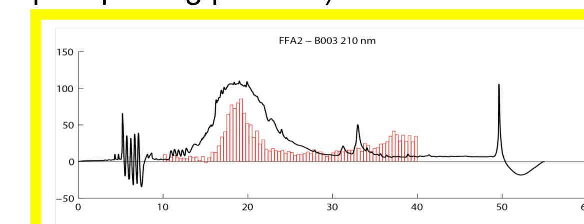
Results – *Semi-HR-screening*

▪ From 20 plants to 2 plants

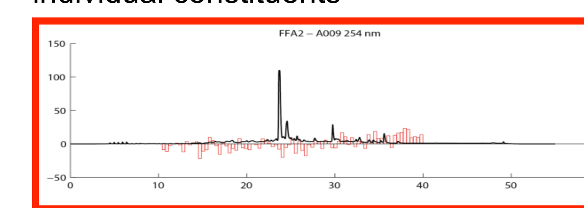
The 20 samples selected for semi-high-resolution screening (assay resolution: 2.66 data points per min) were assayed for their ability to inhibit the PM H^+-ATPase.



8 plants: Inhibition correlated to tannins (large polyphenols with inherent ability of precipitating proteins)



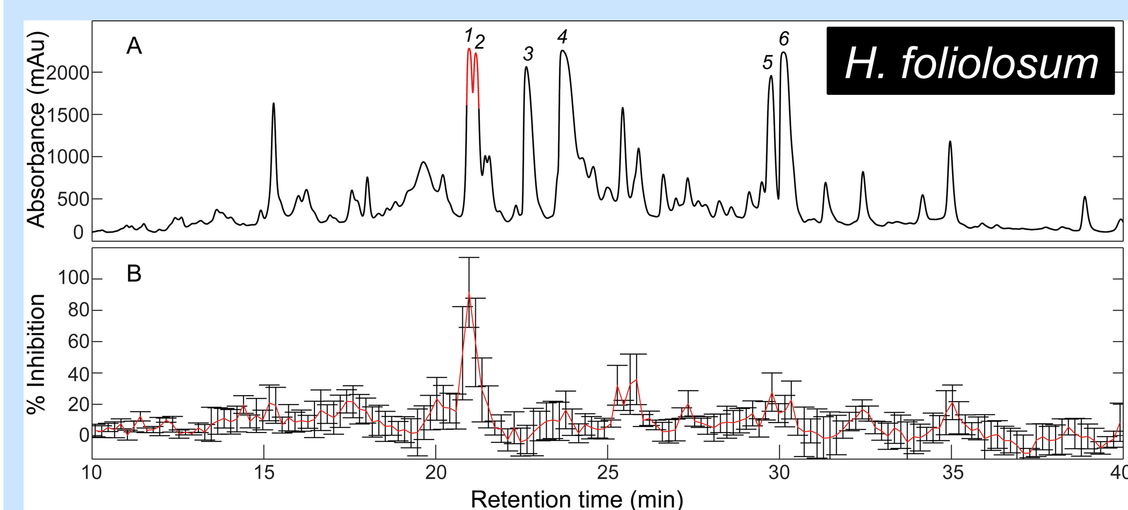
10 plants: No significant inhibition from individual constituents



Results - *HR-screening*

▪ From 2 pants to 2 compounds

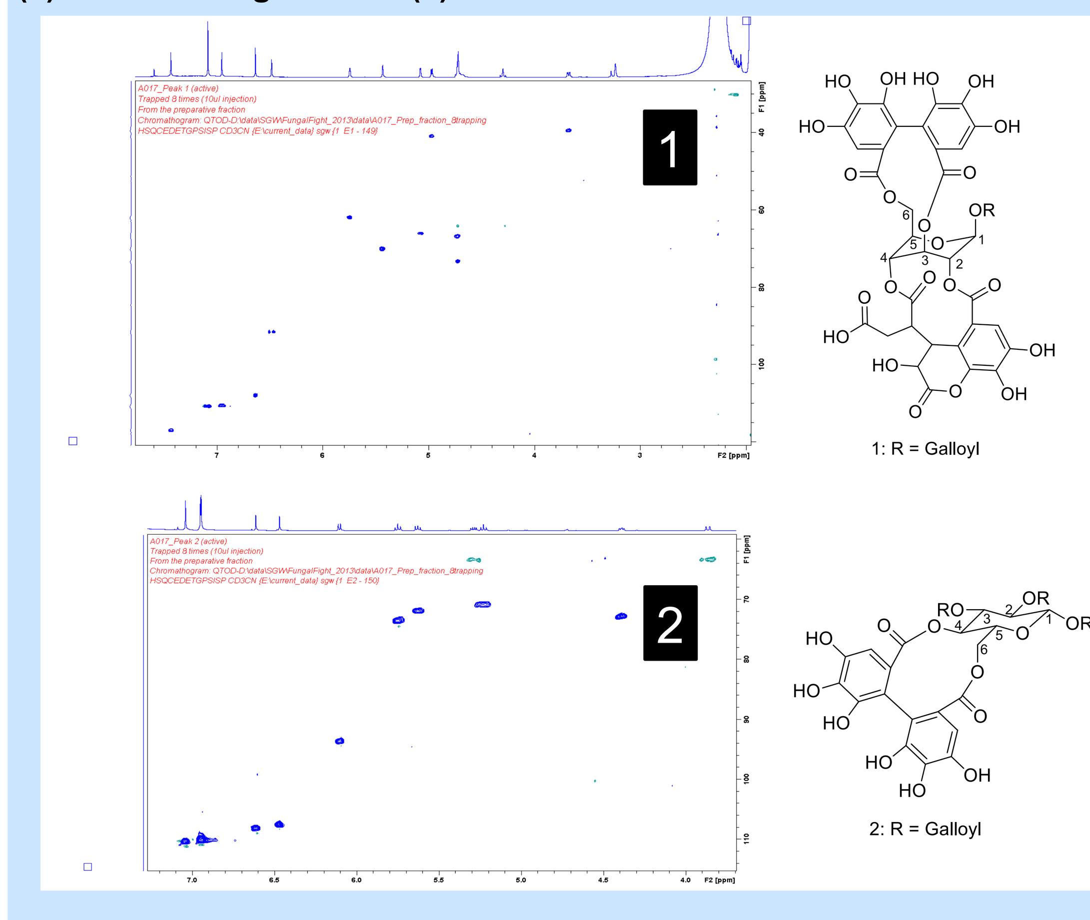
The two plants (*Haplocoelum foliolosum and Sauvagesia erecta)* showing distinct chromatographic peaks correlated with PM H^+-ATPase inhibition in semi-HR screening were subjected to high-resolution screening (assay resolution: 5.33 data points per min).



From *H. foliosum* two peaks (peak 1 and 2) were correlated with > 80% inhibition of the PM H^+-ATPase. However, despite the noticeable inhibition in both crude extract screening and semi-high resolution assay, *S. erecta* did not show any peak correlated to a defined inhibition profile in high-resolution assay. This can be attributed to the possible loss of aggregate activities of multiple constituents due to lower residual complexity of the tested compounds in the HR screening compared to both semi-HR and crude extract screening.

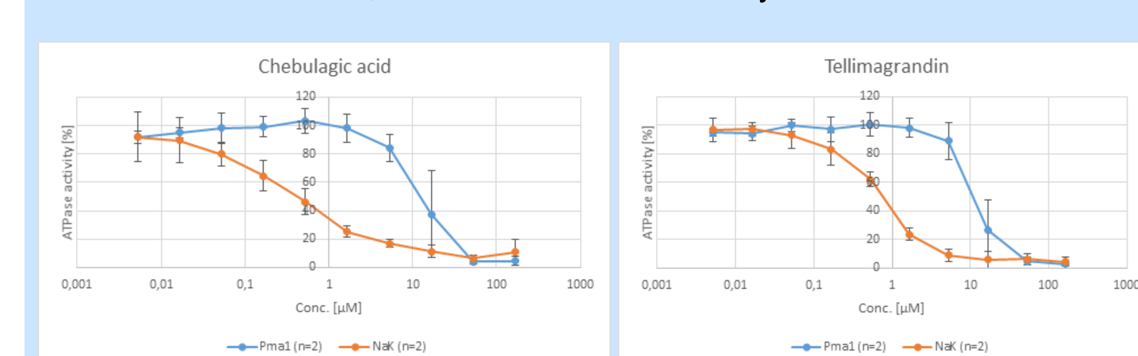
Results - *HPLC-HRMS-SPE-NMR*

Detailed analysis of HRMS and NMR data acquired via HPLC-HRMS-SPE-NMR, led to identification of the two active metabolites as **Chebulagic acid (1)** and **Tellimagrandin II (2).**



Results - *IC_{50} and MIC*

The two HITs (**Chebulagic acid (1)** and **Tellimagrandin II (2))** were purified by preparative scale HPLC and subjected to IC_{50} determination in PM H^+-ATPase as well as Na^+,K^+-ATPase inhibition assay.



Compound	IC50 [µM]	
	Pma1	NaK
Chebulagic acid	12	0,5
Tellimagrandin II	10	0,8

Moreover, minimum inhibitory concentrations of the two compounds were assessed against growth of two fungal strains (*Candida albicans* and *Saccharomyces cerevisiae).*

Fungal strain	Chebulagic acid	Tellimagrandin
S. cerevisiae (041)	2.3 µg/mL	2.3 µg/mL
C. albicans SC5314	>72 µg/mL	>70 µg/mL

Concluding remarks

- Thorough investigation of 48 plant extracts for fungal PM H^+-ATPase inhibitors led to identification of two active metabolites. *i.e.*, Chebulagic acid (**1**) and Tellimagrandin II (**2**).
- Systematic combination of crude extract screening, high-resolution screening and HPLC-HRMS-SPE-NMR analysis allowed optimized workflow.
- High-resolution PM H^+ ATPase inhibition assay allows subsequent HPLC-SPE-NMR analysis to be targeted bioactive constituents only
- Cryogenic probe detection (1.7 mm) allowed characterization of metabolites (with high PM H^+-ATPase inhibition) direct from analytical-scale HPLC of crude extract

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