Transforming the tools of tannin analysis

Professor Juha-Pekka Salminen explains why tannins represent such a challenging subject and how marrying chemistry and ecology can dramatically expand knowledge of plant-herbivore interactions

Could you provide an introduction to the main research themes of your laboratory and summarise its current objectives?

Our main research theme is plant polyphenols, focusing on their identification, quantification and bioactivity measurements. At present we are trying to evaluate how different types of polyphenol groups are distributed in the plant kingdom and how this has affected plant species bioactivity. At the same time we are studying the evolution of polyphenol-based bioactivity in plants. All our research is connected to plant-herbivore interactions (tannins as plant defensive compounds), human nutrition (tannins as beneficial antioxidants or food preservatives) and ruminant nutrition (tannins as anti-parasitic agents).

What is the scientific significance of tannins and why are they a challenging subject for study?

Tannins are one of the most ubiquitous groups of plant secondary metabolites. They are found in most, but not all, plant species. Tannin-containing plants are consumed on a daily basis by humans, insects, ruminants, etc. and function differently for each. Typically, these biomolecules are erroneously considered as a homogenous group of compounds and are mainly quantified as so-called 'total tannins'. This is totally false, since individual tannins differ greatly in structure and activity. Moreover, one compound can be harmful to insect herbivores and ruminant parasites, but beneficial for humans. There are many hundreds of tannin structures, many of which are quite complicated chemically. The challenges, therefore, come from complex macromolecule structures that would require complicated chemical analyses instead of simplified ones.

How did you come to develop an interest in natural chemistry, particularly tannins?

I have always had a keen interest in nature and its phenomena. In 1998, I graduated as an environmental chemist and was then asked to carry out a PhD investigating birch leaf gallotannins. Contrary to expectations, I found no gallotannins in birch but a lot of ellagitannins. At that time, tools for ellagitannin quantification were underdeveloped and their bioactivity was poorly understood. With these challenges and the exceptionally fascinating ellagitannin structures in mind, I decided to make a contribution to this little known area of study.

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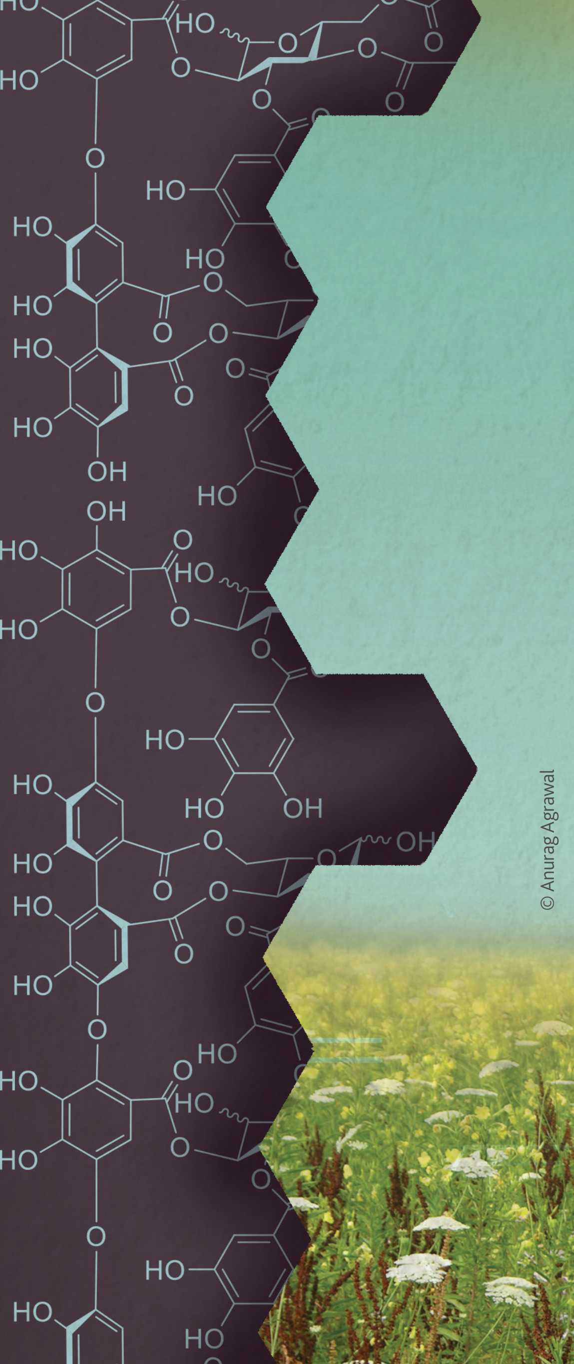
Can you define ellagitannins, and explain why they are one of the least studied and underestimated classes of bioactive plant polyphenols?

Ellagitannins are compounds constructed of a central polyol moiety – most typically glucose – that is first esterified with gallic acid(s) before forming covalent carbon bonds to develop the characteristic hexahydroxydiphenoyl moieties of ellagitannins. After these steps multiple intra- and inter-molecular oxidation steps bring about complexity to monomeric and oligomeric ellagitannin structures.

They have not been well studied for two major reasons: convenient analysis tools for these compounds have not been available, whereas condensed tannins have been very simple to measure with the specific acid-butanol assay; and they show fairly poor protein precipitation capacity in comparison to other tannin types – and are therefore considered a less efficient plant defence.

To what extent does your team work with other research groups? Is a collaborative, interdisciplinary approach essential to your success?

Collaboration is one of the key factors when you work at the confluence of two branches of science. Success is not possible if chemists try to be biologists, or vice versa. Typically, the most significant and reliable scientific results are obtained when each research problem is tackled by utilising experts from all scientific disciplines involved. For instance, it is very difficult to think of high-quality studies in the chemical ecology of tannins where both chemists and biologists were not involved. Of course, chemists can carry out chemical ecology of tannins on their own, but it is possible that at some crucial point the quality of the research could be compromised, and one can then ask 'is this worth continuing on my own or should I consider collaboration with biology experts?' In my opinion one should always aim for high quality results first, and think of other aspects, such as gaining credit for the work, later.



Show a little **sensitivity**

Redressing the lamentable state of the tools currently available for studying plant phenolics, researchers at the **University of Turku** are developing highly sensitive methods that take tannin analysis right to the cutting edge

DESPITE THEIR POSITION as one of the most studied groups of plant secondary metabolites, tannins suffer from a history of oversimplified analyses. This is mainly due to their structural complexity, which can be an obstacle to ready quantification as individual and well-characterised plant components. Thus the analytical tools available to chemical ecologists have often failed to take this complexity into account, resulting in misinterpretations and conclusions sometimes in marked contrast to those of more detailed chemical analyses. It is recognised, however, that a proper understanding of tannins and their distribution in the plant kingdom would significantly help elucidate a variety of relationships between plants and their herbivorous predators, whether insect, mammalian or human.

Perhaps the most tantalising question of all for chemical ecologists is the nature of anti-herbivore activity and the mechanisms of plant self-defence. In order to change assumptions surrounding tannins and the difficulty in addressing their complexity, researchers at the University of Turku, Finland, aim to improve modern methods of chemical analysis and use these methods to help map true tannin diversity in the plant kingdom; a foundation from which many questions surrounding plant activity can begin to be explored in detail.

At the University's Department of Chemistry, Professor Juha-Pekka Salminen specialises in natural compound chemistry and since 2009 has led the Natural Chemistry Research Group, a collective of chemists and ecologists who primarily focus on the quantitative and qualitative analysis of tannins. A joint international effort, Salminen's team receives support and funding from the Academy of Finland and the EU's Seventh Framework Programme (FP7). In working towards the evolution of today's analytical approaches, the group aims to uncover the types of tannins produced by various plant species, their biosynthetic pathways and biological activities, and how these are affected by changes in tannin structure.

OVER SIMPLIFICATION

The Natural Chemistry Research Group has been operational since 2009, yet Salminen's own work in the field of tannins first began in 1998 and has remained his primary professional interest. Most concerning of all has been the lack of compound-specific tools for analysing tannins, where instead they are traditionally quantified as a lump of compounds, as Salminen states: "In the past, people thought all tannins shared similar bioactivities; we now know that this is not the case". This simplification of tannin complexity means surprisingly little is known about their molecule-specific bioactivities. This is less surprising, however, when considering that analysis can only go as far as the technology will allow.

The Group's most recent project – PolyphenOx – intends to capitalise on the advances brought about by Salminen's investigations into the possibilities of compound-specific and polyphenol group-specific tools – research that is inextricably linked to discoveries regarding the anti-herbivorous effects of plants. Generally thought to function as a plant's means of self-defence, it is assumed that tannins make a plant innutritious and foul tasting due to protein precipitation. Tannins differ throughout the various parts of a plant, and between

INTELLIGENCE

NATURAL CHEMISTRY RESEARCH GROUP

OBJECTIVES

To develop new techniques for tannin analyses, and use these to map the distribution of tannin complexity in the plant kingdom. Together with measured structure-activity relationships this knowledge can be utilised to create new bioactive feeds for ruminants and to understand how well different plant species are defended against mammalian and insect herbivores. The group's database will be useful for others interested in utilising plant tannins for various other types of applications.

KEY COLLABORATORS

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JUHA-PEKKA SALMINEN is Professor of Natural Compound Chemistry and the leader of the Natural Chemistry Research Group at the University of Turku, Finland. His group specialises in the chemical analysis of plant polyphenols, especially tannins. They were the first team to find ellagitannins larger than pentamers in plants.

species, increasing the complexity of plants' chemical warfare machinery together with alkaloids, terpenes, cyanogenic glycosides and glucosinolates. Without this complexity, the planet's vegetation would most likely have been consumed long ago. Salminen's work, however, offers vital information regarding tannins. Alongside protein precipitation, it is now hypothesised that tannin oxidation contributes to plant defence by inducing oxidative stress in those attempting to make a meal of it.

Previous studies using so-called 'total tannin' methods were able to detect tannins possessing good protein precipitation capacity. These analyses, however, neglected C-glycosidic ellagitannins (ETs), which are actually the most active pro-oxidant tannins found in plants. Furthermore, Salminen's group has also been able to identify undecameric ETs – rare tannins 100 per cent more complex than the prior pentamer threshold. The ability to detect their presence has allowed for the development of compound-specific tandem mass spectrometry (MS/MS) methods, allowing the quantification of ETs in any plant sample. From this, a more complete picture of the relationship between plants and herbivores has successfully been confirmed, with field studies definitively showing rapid plant evolution in real-time. The evolution was driven by the presence of herbivorous insects that encouraged plants to produce more complex defensive ETs. All this was shown at the molecular level only, since there were no evolutionary changes in the total tannin concentration – again emphasising the need for careful chemical investigation of tannins.

AIMING HIGH

The tannin oxidation hypothesis, initially proposed by Dr Heidi M Appel in 1993, postulates tannins may derive the majority of their anti-herbivore properties from their 'oxidatively active' state at high pH as opposed to hydrogen bonds acquired through protein precipitation at low to neutral pH. Although this changed the conceptual role of tannins in plant defence, widespread testing of this hypothesis has been hindered because many laboratories do not have suitable facilities and equipment to perform assays. To address this, Salminen has been developing a simplified method for estimating the oxidative capacity of any plant sample at high pH, using common and inexpensive laboratory equipment. This method yields two entirely new types of data – oxidative capacity in both milligrams per gram, and in the percentage of total phenolics, allowing researchers to correlate their findings with herbivore performance, even in less specialised laboratories.

To raise the quality of tannin analysis further, Salminen's lab has recently developed a sensitive group-specific methodology. This is achieved by utilising ultraperformance liquid chromatography – tandem mass spectrometry (UPLC–MS/MS), making new methods available to more modern phytochemistry laboratories.

The new rapid quantitative and qualitative analyses methods are used to achieve the PolyphenOx project. "At present, we are able to reveal and quantify all tannin classes and other common polyphenol groups in any plant extract within 10 minutes," Salminen reports. Covering more than 3,000 species of plant, this rapid analysis will allow PolyphenOx to uncover a great deal about varying polyphenol groups, their constituent compounds and their distribution in the plant kingdom. "I am personally astonished by the capacity of these instruments to reveal such a diversity of information within one run," Salminen admits. "For instance, the sizes and sub-types of proanthocyanidin tannins are now revealed directly from the crude extract whereas previously, this singular task demanded two or more time-consuming steps. Now one rapid step is enough to measure the whole tannin diversity of the sample, including ellagitannins and gallotannins as well."

SELECTING WITH SPEED

Harnessing this kind of knowledge will be immensely valuable for researchers wishing to apply an expanded understanding of plant secondary metabolites into other areas. Although chemical ecologists are greatly interested in the anti-herbivore effects of tannins, their beneficial role in ruminant nutrition and human health is also being pursued. It has been shown, for example, that some tannins are able to reduce the volume of intestinal parasites in several species of ruminant and even reduces greenhouse gas emissions of ruminants. If breeders want to select plant varieties displaying beneficial traits such as these, they will need rapid, sensitive analysis methods like UPLC-MS/MS to realise their goals. The pool of information Salminen is busy generating will be a hugely valuable source from which many other applications will no doubt evolve as chemical ecology develops a more profound understanding of the interactions between plants and their predators.

Proper understanding of tannins and their distribution in the plant kingdom would significantly help elucidate a variety of relationships between plants and their herbivorous predators