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Section 2 **Test Methodology and Assessment**

Chemotaxonomy of the genus Aquilaria (Thymelaeaceae)

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ABSTRACT

The genus *Aquilaria* Lam. (Thymelaeaceae) comprises 21 tree species and is mostly found in Southeast Asia. When the tree is infected (fungi, bacteria, etc.), its wood turns brownish or blackish (called agarwood) due to the secretion of an oleoresin in reaction to the infection. The resin is very fragrant and has been sought after and used for centuries by Buddhists, Hindus and Muslims to make incense for religious ceremonies. This oleoresin is mainly found in species of the genus Aquilaria, but also in a few species of the genera *Gyrinops* Gaertner and *Gonystylus* Teijsmann & Binnendijk. It is difficult to distinguish between these species and this lack of taxonomic knowledge has led to over-use of the trees, endangering these endemic species listed in CITES Appendix II. We used chemotaxonomy as a discrimination tool to analyse polyphenolic molecules, secondary metabolites, which are known to act as taxonomic markers in other plants.

This work showed the possibility of using some very old herbarium samples, which revealed good conservation of its phenolic profile. It is interesting to note that mangiferin, through its high concentration and constant presence in all the species of the genus Aquilaria analysed, constitutes a chemical marker for this genus. Comparative analysis of these phenolic molecules can therefore be very useful when seeking chemotaxonomic markers.

Keywords: *Aquilaria, Gyrinops, Gonystylu,* chemotaxonomy, polyphenolic molecules, secondary metabolites, mangiferin, taxonomic markers.

1. INTRODUCTION

The chemical composition of the oleoresin has already been analysed (see overview by Naef 2011), but it cannot be used to sufficiently distinguish between the species. Oleoresin is mostly produced by species of the genus *Aquilaria* but also by some species of the genera *Gyrinops* and *Gonystylus*. The species of the last two genera are also difficult to distinguish from each other, but they might be a substitute for *Aquilaria* agarwood and form part of a preservation policy. This lack of taxonomic knowledge for these Asian species producing agarwood has led to over-use of the trees, endangering the 17 endemic species of *Aquilaria* (Mabberly 2008), which are all listed in CITES Appendix II.

As commercial use of these oleoresins is only possible with reliable knowledge of the species and with strict traceability, we used a chemotaxonomy analysis as a discrimination tool. To that end we opted to study polyphenols, which are secondary metabolites found throughout the Plant Kingdom, but in highly varied forms (Bate-Smith 1962). A composition difference in these metabolites is less often due to a fluctuation in the surrounding environment than to a genotypic difference, hence the taxonomic value of these substances (Bell 1981). Polyphenol molecules are mainly synthesized in leaves and their role as chemotaxonomy markers has been demonstrated in numerous plants (Harbone *et al.* 1971, Bate-Smith and Richens 1973, Cooper-Driver and Swain 1977, Andary *et al.* 1988 b, Williams *et al.* 1988, Andary *et al.* 1992).

Earlier research showed the existence of several types of polyphenols in *Aquilaria spp* leaves: benzophenones (e.g. riflophenone, Feng *et al.* 2009), xanthone (e.g. mangiferin Qi *et al.* 2009), flavonoids (e.g. genkwanin, derivatives of luteolin and apigenin, Hara *et al.* 2008, Qi *et al.* 2009).

We conducted this study by comparing a batch of 40 specimens belonging to the genus Aquilaria (but requiring analysis) collected and dried in Laos and Cambodia, and some reference samples from the herbaria of the Museums of Paris (MNHN) and Leiden. The latter samples corresponded to voucher species belonging to some close genera: *Aquilaria*, *Gyrinops*, *Gonystylus* and *Wikstroemia*. *Aquilaria* and *Gyrynops* are classed in the sub-family of the *Aquilarioideae*, *Gonystilus* in that of the *Gonystiloideae* and *Wikstroemia* in that of the *Thymelaeoideae*. These three sub-families, with that of the *Gilgiodaphnoideae*, make up the family of the *Thymelaeaceae* (Hou 1960). These sub-families, apart from the *Gilgiodaphnoideae*, are endemic to southern, tropical Asia.

We also took as an example of the use made of the genus *Aquilaria*, other than for its oleoresin, a quality study involving the analysis of a herbal tea comprising *Aquilaria spp* leaf fragments, marketed as “Agarwood Tea”.

This chemotaxonomy analysis was carried out by two-dimensional thin-layer chromatography (quick and inexpensive method). The nature of the studied molecule was suggested by the position of each spot on the chromatogram, confirmed by a colorimetric analysis of the amount of colour in the Red, Green and Blue channels. This approach was totally original.

2. METHODOLOGY

For further details about the methodology, see Andary *et al.* (2019).

2.1 Plant material

Forty specimens of Aquilaria spp. from Laos (24) and Cambodia (16) were collected and dried. We also studied a sample of *Aquilaria crassna* collected and dried in 2010 by Professor Kiet Le Cong in Vietnam.

Ten voucher specimens were provided by the French National Natural History Museum in Paris (MNHN) and 12 specimens were provided by the National Herbarium of Leiden, the Netherlands.

Altogether, we analysed 6 *Aquilaria* species and 10 species belonging to three other genera: *Gyrinops* Gaertner, *Gonystylus* Teijsmann and Binnendijk, and *Wikstroemia* Endl.

2.2 Chromatographic analyses

For these chemotaxonomy studies, we chose to analyse polyphenol compounds from dried leaves. All the solvents were of "analysis" quality (VWR, France) and were as follows: acetic acid, hydrochloric acid, dichloromethane, ethanol and methanol.

Analyses steps:

- Preparation of plant extracts
- Two-dimensional thin-layer chromatography (2D TLC)
- Reference extract and molecule depositing
- Visualization and interpretation of the chromatograms
- Chromatogram photography technique after visualization

2.3 Colour analysis method for fluorescence spots

See Andary *et al.* (2019).

3. CONCLUSION

This work showed the possibility of using some very old herbarium samples, since the analysis of a herbarium sample from the French National Natural History Museum in Paris, which was 140 years old, revealed good conservation of its phenolic profile when compared to a sample of the same species collected and dried just a few months earlier, despite the leaves having undergone chemical decontamination. Comparative analysis of these phenolic molecules can therefore be very useful when seeking chemotaxonomic markers.

A chemical analysis of leaves from species belonging to four genera of the Thymelaeaceae revealed a large number of polyphenols (mangiferin, coumarins, derivatives of luteolin and apigenin and caffeic acid, etc.) making up a specific phenolic profile for each of these genera. It is interesting to note that mangiferin, through its high concentration and constant presence in all the species of the genus *Aquilaria* analysed, constitutes a chemical marker for this genus, which is not the case for the genera *Gonystylus* and *Wickstroemia*, from which it was totally absent. However, the presence of mangiferin at very different concentrations in the three species of the genus *Gyrinops* studied confirmed the strong phylogenetic relation that is known to exist between *Aquilaria* and *Gyrinops*.

In addition, these results remove the uncertainty surrounding herbarium sample No. 30000 of the Poilane collection, deposited at MNHN in Paris and labelled "*Aquilaria baillonii* or *Gyrinopsis baillonii".* In fact, as this sample does not contain any mangiferin, it cannot belong to the genus *Aquilaria*, but may belong to the genus *Gyrinops*.

The chromatography method we used in this analysis has the merits of being simple, practical and inexpensive. In addition, the colorimetric analysis of spots on the chromatograms belonging to various molecules gave an original result that improves the accuracy of the physico chemical authentification of chromatographed molecules contained in a plant extract.

This chromatographic analysis is not only an aid for the systematics of these species, but may also help in the in situ conservation of their genetic diversity. It may also be a traceability tool with a view to seeking species authenticity and quality in the various fields of use, as we were able to show by analysing dried leaf fragments sold in Asia as herbal tea (Agarwood Tea). This herbal tea is appreciated for its sedating, anti-inflammatory, anti-microbial and anti-diabetic qualities.

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