Chapter 5

Essential oil constituents derived from different organs of a relictual conifer *Wollemia nobilis*

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Abstract

The chemical composition of the essential oil of leaves (0.9%, w/v) and twigs (0.3%, w/v) of *Wollemia nobilis* (Araucariaceae) – a remnant species thought to have been extinct for 65 million years – was investigated by GC/MS. The main constituents of both leaf- and twig-derived oil samples were: 16-kaurene (61.8% and 38.2% respectively) and germacrene D (9.9% and 22%). The principal difference was a considerably more pronounced sesquiterpene presence in the twig-oil, amounting to 33.5%, than in its folial counterpart (23.4%). On the contrary, while remaining the dominant group in both oil samples under investigation, diterpenoids were relatively more abundant in leaf-derived oil constituting 65.3%, versus 41.7% detected in twigs. To our knowledge, this is the first report dealing with the essential oil composition of Wollemi pine twigs, as opposed to the leaf-derived volatiles.

Introduction

The discovery of Wollemi pine in 1994 made international headlines, hailed as the 'botanical find of the century'. The unique conifer was found in a deep Triassic sandstone gorge in Wollemi National Park within 150 km of Sydney. Interestingly, the aboriginal meaning of the word 'wollemi' translates to 'watch out, look around you' (Hill, 1996). Yet, a rugged Australian wilderness managed to keep its secret for millions of years.

Subsumed, on a morphological basis, to the 200 million year old conifer family Araucariaceae, *Wollemia* constitutes a whole new monotypic genus (Jones *et al*., 1995). While the evolutionary relationships within the 'monkey puzzle tree' family are poorly known, *Wollemia* was confirmed to be distinct from the related genera *Araucaria* and *Agathis* by Gilmore and Hill (1997) through DNA sequencing of the plastid gene *rbc*L. The sequence data, combined with different ranges of other conifer taxa, imply that *Wollemia* derived prior to its taxonomic counterparts and may be the earliest derived genus in Araucariaceae (Setoguchi *et al*., 1998). An alternative hypothesis suggests that *Wollemia* be a sister group to *Agathis* with these two forming a clade that is sister to *Araucaria* (Gilmore & Hill, 1997; Stefanovic *et al*., 1998). Despite the origin of the relictual pine remaining an evolutionary enigma, the preliminary investigation of the chemistry of *W. nobilis* is consistent with that of other araucaroids, with the abundance of diterpenoids in their leaf oils being a seemingly characteristic trait (Brophy *et al*., 2000).

As surveyed at allozyme, amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) loci, the genetic diversity of Wollemi pine proved to be exceptionally low. It is considered, in fact, the most extreme case known in planta accounted for by the combination of such probable contributing factors as small population effects (less than a hundred trees occurring in the wild, in an inaccessible canyon), clonality and below-average genetic variation within the botanical family (Peakall *et al*., 2003). Thus, each genetic individual seems to play a key role in the future evolution of the monotypic conifer, as the genetic diversity within this species constitutes the full genetic range of the genus. Moreover, microflora and other species associated with the microhabitat provided by the relictual pine are vital and unique elements contributing to the biodiversity of Wollemi National Park, New South Wales and, indeed, the Australian continent. It is noteworthy that *Pestalotiopsis guepini* – a paclitaxel producing fungus, was found to be a representative of the endophytic flora harboured by *W. nobilis* (Strobel *et al*., 1997).

The aforementioned considerations, combined with the immense popularity of the 'green dinosaur' keenly sought after as a horticultural plant, reinforce the necessity of reducing the threat to the population in the wild from illegal collectors. For this reason, the Australian government has undertaken an unprecedented conservation programme, making *Wollemia nobilis* commercially available as a potted plant (NSW Department of Environment and Conservation, 2006). Thus, in early 2006 first Wollemi specimens left 'the land down under', opening a window into an unimaginably ancient past – a great opportunity of studying this extraordinary conifer worldwide.

The present study aims at a detailed analysis of the chemical composition of essential oil samples derived from different plant organs: leaves and twigs. Since the only report on the chemistry of Wollemi pine available to date (Brophy *et al*., 2000) deals with the analysis of leaves obtained from mature trees indigenous to Australia, we also hope to draw parallels and point out possible discrepancies, as our samples originate from a juvenile Wollemi specimen cultivated *ex situ*.

Materials & methods

Plant material

A juvenile (1-year-old, 30 cm in length) Wollemi pine bearing the registered Wollemi pine™ logo was purchased in 2008 from Arboretum Kalmthout, Belgium – a representative of Wollemi Australia exclusively licensed by the Royal Botanic Gardens Sydney (RBGS) through NSW National Parks and Wildlife Service (NPWS) to propagate and market the Wollemi pine in Australia and internationally. The conifer was additionally authenticated at the Botanical Garden 'De Kruidhof', Buitenpost, the Netherlands. A voucher specimen (gro-ASOK-02) is deposited in our department.

Isolation procedure

The oil samples were separately isolated from 14.2 g of air-dried and freshly ground (1 mm) leaves as well as 6.3 g of air-dried and coarsely ground twigs by hydrodistillation for 3 h in 300 mL water, according to the determination of the essential oil content in vegetable drugs, using the apparatus described in the *Nederlandse Farmacopee* (1966). Xylene (100 µL) was used as the collection liquid, and the oil was stored at -20°C until analyzed. The oil was diluted 50 times with cyclohexane prior to GC/MS analysis.

Gas Chromatography-Mass Spectrometry

A Shimadzu GCMS QP5000 system was used (Shimadzu Corporation, Japan) equipped with a GC-17A gas chromatograph, an AOC-20i autoinjector, and GCMS Solution version 1.10 software. The GC conditions were: column, Zebron Capillary GC Column, ZB-5 MS (15 m x 0.25 mm; film thickness 0.1 μm); oven temperature programme, 50-310°C at 5°C/min; injector temperature, 260°C; carrier gas, He; total flow, 59.3 mL/min; split ratio, 21:1; injected volume, 2.0 μL. MS conditions: ionization energy, 70 eV; ion source temperature, 250°C; interface temperature, 300°C; scan speed, 4 000 scans/s; mass range, 34-600 u.
The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{29} *n*-alkanes, with mass spectral databases and from literature (Adams, 2001; Joulain & König, 1998; Flavor & Fragrance Library Shimadzu Benelux, 's-Hertogenbosch, the Netherlands, 2003). The percentages of the components were calculated from the GC peak areas, using the normalization method.

Results & discussion

Hydrodistillation of the leaves and twigs of *Wollemia nobilis* yielded 0.9%, w/v and 0.3%, w/v oil, respectively. In total 42 components could be identified in the leaves and 40 in twigs, corresponding to 95.9% and 77.8% of the oil derived from the respective organs. To our knowledge, this is the first report dealing with the essential oil composition of Wollemi pine twigs, as opposed to the leaf-derived volatiles.

Table 1. Composition of the leaf and twig oils of *Wollemia nobilis*

compound	RI[a]	leaves [%]	twigs [%]
β-pinene	975	0.8	0.5
β-myrcene	995	0.4	0.3
3-methylene-1,7-octadiene	1033	2.2	1.5
4-carene	1058	nd[b]	tr[c]
octen-1-ol acetate	1119	tr[c]	0.2
6-camphenol	1122	0.4	0.2
(*E*)-3(10)-caren-4-ol	1135	0.9	0.3
verbenol	1143	0.2	0.1
6,6-dimethyl-2-methylene-bicyclo[2.2.1]-heptan-3-one	1154	0.1	nd[b]
Z-*β*-terpineol	1170	0.2	0.2
8-oxo-*cis*-ocimene	1184	nd[b]	0.1
2-acetyl-2-carene	1185	0.4	nd[b]
myrtenol	1190	0.1	tr[c]
verbenone	1198	0.2	nd[b]
(*E*)-3(10)-caren-2-ol	1214	0.1	nd[b]
carvone	1232	tr[c]	nd[b]
2,2-dimethylvaleroyl chloride	1238	tr[c]	tr[c]
bergamol	1249	tr[c]	tr[c]
(*Z*)-2-decenal	1253	tr[c]	nd[b]
bornyl acetate	1267	0.2	0.3
p-mentha-6,8-dien-2-ol acetate	1317	0.1	nd[b]
p-menth-8-en-2-ol acetate	1325	0.4	0.5
cyclobuta[1,2:3,4]dicyclopentene, decahydro-3a-methyl-6-methylene-1-(1-methylethyl)-[1S-(1.*α*,3a.*α*,3b.*β*,6a.*β*,6b.*α*)]	1345	tr[c]	0.1
sativene	1348	nd[b]	tr[c]
1-ethenyl-1-methyl-2,4-bis(1-methylethenyl) cyclohexane	1356	0.2	0.2
7,11-dimethyl-3-methylene-1,6,10-dodecatriene	1373	0.4	0.5
unknown, possible sesquiterpene[d]	1388	0.1	tr[c]
β-*cis*-ocimene	1399	0.2	0.2
germacrene D	1450	9.9	22.0
germacrene B	1460	0.8	1.2

α-muurolene	1493	nd[b]	tr[c]
spathulenol	1539	2.5	2.2
filipendulal	1546	0.4	0.2
(*iso*)-aromadendrene epoxide	1555	2.1	1.7
[1ar-(1a.*α*,4.*β*,4a.*β*,7.*α*,7a.*β*,7b.*α*]-decahydro-1,1,4,7-tetramethyl-1*H*-cycloprop[e]azulen-4-ol	1560	0.4	0.5
aromadendrene (2)-oxide	1605	1.4	1.9
α-cadinol	1613	0.4	0.5
(*iso*)-geraniol	1637	0.5	nd[b]
trans-longipinocarveol	1639	0.7	0.8
caryophyllene oxide	1650	nd[b]	tr[c]
trans-Z-α-bisabolene epoxide	1662	0.1	tr[c]
tetrahydrogeranyl acetate	1816	0.7	0.2
phtalic acid, butyl-2-ethylhexyl ester	1833	tr[c]	tr[c]
unknown, possible sesquiterpene[e]	1857	3.2	1.7
sandaracopimar-15-en-8.*β*.-yl acetate	1875	0.8	0.6
phylocald-15-ene	1920	1.0	1.6
kaur-16-ene	2023	61.8	38.2
4*β*,17-(acetoxy)-kauran-18-al	2052	1.7	0.4
kaur-16-en-18-oic acid, methyl ester	2123	nd[b]	1.0
total amount identified [%]		95.9	77.8
essential oil v/w [%]		0.9	0.3
grouped components			
monoterpene hydrocarbons		1.3	1.0
oxygenated monoterpenes		5.9	3.7
sesquiterpene hydrocarbons		14.6	25.7
oxygenated sesquiterpenes		8.8	7.8
diterpene hydrocarbons		62.8	39.8
oxygenated diterpenes		2.5	2.0

[a] retention index relative to C_9-C_{29} *n*-alkanes on a ZB-5 MS column
[b] not detected
[c] trace (<0.1%)
[d] MS, 70 eV, *m/z* (rel. int.): 204 $[M]^+$ (6.5), 189(6.8), 161(23.6), 147(8.4), 121(32.8), 105(28.9), 91(40.9), 41 (100)
[e] MS, 70 eV, *m/z* (rel. int.): 272$[M]^+$ (6.7), 257(22,0), 230(3.7), 202(2.5), 187(5.1), 175(5.7), 161(5.7), 148(12.7), 137(35.6), 119(20.4), 105(30.4), 91(40.6), 81(45.8), 41(100)

Comparative analysis of the investigated oil samples, as presented in Table 1, indicates a more pronounced monoterpene content in Wollemi foliage, reaching 7.3%, while the twig-derived oil contains 4.5%. The principal monoterpenoid constituents of both oils are β-pinene (0.8% and 0.5%, for leaves and twigs, respectively) and β-myrcene (0.4% and 0.3%). Sesquiterpene presence is considerably more pronounced in the twig-derived oil sample, amounting to 33.5%, than in its folial counterpart containing 23.4% of the C_{15} terpenes. On the contrary, while remaining the dominant group in both oil samples under investigation, diterpenoids are relatively more abundant in leaf-derived oil constituting 65.3%, as opposed to 41.7% detected in twigs. One might speculate that this observation could be accounted for by the formation of 'active isoprene units' – basic C_5 terpene building blocks, isopenthenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), proceeding via two alternative pathways. While the long known mevalonate (MVA) pathway (McGarvey & Croteau, 1995; Ruzicka, 1953), localized in the cytosol, provides the isoprene units for sesquiterpene biosynthesis, the plastid-localized methylerythritol phosphate (MEP) pathway, discovered and investigated only in recent years by Rohmer *et al*. (known, therefore, also as the *Rohmer pathway*) (Flesch & Rohmer, 1988; Rohmer *et al*., 1993; Rohmer *et al*., 1996; Rohmer, 2008) is thought to feed the biosynthesis of mono- and diterpenoids. The aforementioned cell-compartmentation (Trapp & Croteau, 2001) seems to be in accord with the postulated direct light interference with the non-mevalonate pathway (Seemann *et al*., 2006), and consequently with the obviously prevalent cross-talk between the cytoplasmic and plastidial biosynthetic routes (Hemmerlin *et al*., 2003; Schuhr *et al*., 2003). Hence the significantly higher amounts of C_{15} terpenoids observed in the plant organs less photosynthetically active and less exposed to the light than the foliage. Germacrene D is the most prevalent sesquiterpenoid identified in both analyzed samples (9.9% in the leaf- and 22% in the twig-oil), accompanied by such hydrocarbons as germacrene B (0.8% and 1.2%), as well as an oxygenated C_{15} representative – spathulenol (2.5% and 2.2%). But one twig-specific sesquiterpenoid component – α-muurolene could be detected. As previously noted, diterpenoids constitute the most quantitatively significant fraction of both oil samples, yet qualitatively – the least versatile. Next to the most abundant 16-kaurene (61.8% and 38.2%, for leaves and twigs, respectively), only three C_{20} representatives were present in both samples, namely: phyllocald-15-ene (1% and 1.6%), an oxygenated derivative of sandaracopimarene (0.8% and 0.6%) and 4β,17-(acetoxy)-kauran-18-al (1.7% and

0.4%), while an ester of kaur-16-en-18-oic acid could additionally be detected in the twig-derived oil.

An alternative goal of the hereby presented study was to draw conclusions as to the presumed disparities between our observations and previously published data concerning the chemistry of *W. nobilis* foliage (Brophy *et al*., 2000). The first notable difference seems to be the overall yield of the discussed experimental procedures. While Brophy *et al*. report 29 structures, accounting for 88.2% of the leaf oil sample recovered in 0.5%, our study detects 42 terpenoids corresponding to 95.9% of the hydrodistillate retrieved in 0.2% from the raw plant material. Thus the sensitivity of the gas-chromatographic separation technique coupled to the mass spectrometry based detection method utilized herein seems to exceed that of the previously reported methodology. As far as the particular terpene fractions, discrepancies of both quantitative and qualitative nature could be observed. Monoterpene content proposed in the previous study is significantly higher than the amount of C_{10} terpenes found in our sample (12% vs. 7.6%, relative to the entire amount of chemical structures identified in respective experiments). Moreover, while α-pinene was the most prevalent representative reported before (8.8%), our investigation showed no presence of this monoterpene, with its β isomer detected in a significantly lower amount (0.8%). Both studies suggest that germacrene D be the most abundant amongst leaf sesquiterpenoids. They differ, however, on the total percentage of sesquiterpenes present in folial oils – 15.4% proposed by Brophy *et al*., as opposed to 24.4% detected herein. The most consistent results were obtained while investigating the C_{20} terpene fractions, with the total amount of diterpenes reaching around 70% of the structures identified in both samples, and 16-kaurene being the most prominent representative.

Several reasons accounting for the aforementioned disparities could be put forth. First of all, the plant material put to analysis herein was dried prior to distillation, which in turn could explain the overall higher content of oxygenated terpenes in our oil sample, as opposed to fresh foliage studied in Australia – significantly more abundant in isoprenoid hydrocarbons rather than their oxidized metabolites. Indeed, while revealing no other differences, be it of qualitative or quantitative nature, the analysis of oil derived from fresh leaves of our Wollemia specimen confirmed the decreased influence of oxygenation processes (data not shown). What is more, while the group of Brophy investigated leaves of wild mature Wollemi trees obtained from their natural habitat – Wollemi National Park, our sample was derived from a ju-

venile pot-cultivated specimen propagated in Arboretum Kalmthout, Belgium. Thus environmental, ecological and developmental considerations could be postulated.

References

Adams RP. 2001. Identification of essential oil components by gas chromatography/mass spectroscopy. Allured Publishing Corporation, Carol Stream, IL

Anonymous. 1966. Nederlandse Farmacopee, 6th ed., 2nd printing, p 72, Staatsuitgeverij, 's-Gravenhage

Brophy JJ, Goldsack RJ, Wu MZ, Fookes CJR, Forster PI. 2000. The steam volatile oil of *Wollemia nobilis* and its comparison with other members of the Araucariaceae (*Agathis* and *Araucaria*). Biochem Syst Ecol 28:563-578

Flavor & Fragrance Library Shimadzu Benelux, 's-Hertogenbosch, the Netherlands

Flesch G, Rohmer M. 1988. Prokaryotic terpenoids. The biosynthesis of the bacteriohopane skeleton: formation of isoprenic units from two distinct acetate pools and a novel type of carbon/carbon linkage between a triterpene and D-ribose. Eur J Biochem 175:405-411

Gilmore S, Hill KD. 1997. Relationships of the Wollemi pine (*Wollemia nobilis*) and the molecular phylogeny of Araucariaceae. Telopea 7:275-291

Hemmerlin A, Hoeffler J-F, Meyer O, Tritsch D, Kagan I, Grosdemange-Billiard C, Rohmer M, Bach TJ. 2003. Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells. J Biol Chem 278:26666-26676

Hill KD. 1996. The Wollemi pine: discovering a living fossil. Nat Res 32:20-25

Jones WG, Hill KD, Allen JM. 1995. *Wollemia nobilis*, a new living Australian genus and species in the Araucariaceae. Telopea 6:173-176

Joulain D, König WA. 1998. The atlas of spectral data of sesquiterpene hydrocarbons. E.B.-Verlag Hamburg, Germany

McGarvey DJ, Croteau R. 1995. Terpenoid metabolism. Plant Cell 7:1015--1026

NSW Department of Environment and Conservation. 2006. Wollemi Pine (*Wollemia nobilis*) Rocovery Plan. NSW Department of Environment and Conservation, Hurstville NSW. DEC 2006/519, ISBN 1 920 887 63 6

Peakall R, Ebert D, Scott LJ, Meagher PF, Offord CA. 2003. Comparative genetic study confirms exceptionally low genetic variation in the ancient and endangered relictual conifer, *Wollemia nobilis* (Araucariaceae). Mol Ecol 12:2331-2343

Rohmer M. 2008. From molecular fossils of bacterial hopanoids to the formation of isoprene units: discovery and elucidation of the methylerythritol phosphate pathway. Lipids 43:1095-1107

Rohmer M, Knani M, Simonin P, Sutter B, Sahm H. 1993. Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochem J 295:517-524

Rohmer M, Seemann M, Horbach S, Bringer-Meyer S, Sahm H. 1996. Glyceraldehyde 3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate pathway for terpenoid biosynthesis. J Am Chem Soc 118:2564-2566

Ruzicka L. 1953. The isoprene rule and the biogenesis of terpenic compounds. Experientia 9:357-367

Schuhr CA, Radykewicz T, Sagner S, Latzel C, Zenk MC, Arigoni D, Bacher A, Rohdich F, Eisenreich W. 2003. Quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. Phytochem Rev 2:3-16

Seemann M, Tse Sum Bui B, Wolff M, Miginiac-Maslow M, Rohmer M. 2006. Isoprenoid biosynthesis in plant chloroplasts via the MEP pathway: direct thylakoid/ferredoxin-dependent photoreduction of GcpE/IspG. FEBS Lett 580:1547-1552

Setoguchi H, Osawa TA, Pintaud J-C, Jaffré T, Veillon J-M. 1998. Phylogenetic relationship within Araucariaceae based on *rbc*L gene sequences. Amer J Bot 85:1507-1516

Stefanovic S, Jager M, Deutsch J, Broutin J, Masselot M. 1998. Phylogenetic relationships of conifers identified from partial 28S rRNA gene sequences. Amer J Bot 85:688-697

Strobel GA, Hess WM, Li J-Y, Ford E, Sears J, Sidhu RS, Summerell B. 1997. *Pestalotiopsis guepini*, a taxol-producing endophyte of the Wollemi pine, *Wollemia nobilis*. Aust J Bot 45:1073-1082

Trapp SC, Croteau R. 2001. Genomic organization of plant terpene synthases and molecular evolutionary implications. Genetics 158:811-832