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Differential Extraction and GC-MS based Quantification of Sesquiterpenoids from Immature Heartwood of East Indian Sandalwood Tree

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Abstract
The East Indian sandalwood tree yields the costliest heartwood and essential oil that are used in traditional medicine, aromatherapy and in cosmetic and fragrance industries. Steam distillation is the traditional method employed for extraction of the sesquiterpenoid rich essential oil from chips of matured heartwood. However, there is no information available on the comparative extractability of sesquiterpenoids when different solvents are employed. Thus we used four different solvents to extract, detect and quantify fourteen major sesquiterpenoids from immature heartwood, by gas chromatography- mass spectrometry (GC- MS) method employing an ion trap quadruple (ITQ) mass analyzer. Results suggest that, with increasing solvent polarity the diversity of sesquiterpenoids decreased, but the quantities of santalols increased. Moreover, n-hexane remained the best extraction solvent for santalols, i.e., yielding up to 92.6 % of total sesquiterpenoids quantified. Furthermore, Z-α-trans-bergamotol, Z-epi-β-santalol and Z-β-santalols were found to be the most abundant constituents of immature heartwood.
Keywords: GC-MS, heartwood, *Santalum album*, sesquiterpenoid, solvent

1. Introduction

Santalum album L., the East Indian Sandalwood tree is a tropical woody member of Santalaceae. Sandalwood is the major source of costliest wood and essential oil extracted from it, a mixture of 90 % sesquiterpenoid alcohols, i.e., santalols. The heartwood of a 50 year old matured tree yields 2.5- 6 % of essential oil upon steam distillation, and is influenced by several intrinsic and external factors. The global annual requirement is about 10,000 tons of wood, equivalence of 200 tons of oil, involving a trade of more than $ 360 million, of which only 10 % is met from natural sources. The use of sandalwood oil in fragrances in USA is estimated to be approximately 48,000 lbs. / year (Burdock and Carabin, 2008). Sandalwood oil finds numerous applications in traditional medicine system Ayurveda (Dikshit and Hussain, 1984) while the heartwood powder displays anti-remorogenic, anti-inflammatory, anti-mitotic, anti-hypertensive, anti-pyretic and sedative properties (Desai *et al.*, 1991). Additionally, the santalols possess antiviral (Benencia and Courreges, 1999), anti-*Helicobacter pylori* (Takaishi *et al.*, 2005) and anti-cancer (Bommareddy *et al.*, 2012) properties.

The yield of any essential oil varies depending on the age of the tree, color of heartwood, individual tree under study, location within the tree and the environment of growth of the tree. Traditionally, as an age-old practice the steam distillate of the heartwood is sold as marketable sandalwood essential oil. Major constituents of commercially available sandalwood oil are sesquiterpene alcohols like, α- and β-santalols, bergamotols and several of their stereoisomers, whereas minor constituents includes lanceol, nuciferol, bisabolol and the sesquiterpene hydrocarbons such as α-and β-santalenes, bergamotenes, α-, β- and γ-curcumenes and β-bisabolene (Adams *et al.*, 1975; Christenson *et al.*, 1981; Demole *et al.*, 1976; Howes *et al.*, 2004; Jones *et al.*, 2006) and usually, α-santalol is more abundant (~46%) than β-santalol (~20%) (Anonis, 1998).

Previous reports indicate that, there are quantitative and compositional differences in oils obtained from young and mature sandalwood trees and across heartwood sampled at different levels in the tree (Shankaranarayana and Parthasarathi, 1987). It is also noteworthy, that, yields of secondary metabolites depends on the intrinsic characteristics of plant material, environmental, and genetic aspects, or by extrinsic aspects such as extraction solvents used (Muzika *et al.*, 2006).

This investigation was undertaken with two objectives, i.e., it primarily focused on the identification, quantification of the numerous sesquiterpenoid constituents from immature heartwood samples using GC -MS analyses and to show the differential extractability of sesquiterpenoid constituents by different solvents.

2. Materials and Methods

2.1 Plant material and extraction of sesquiterpenoids

The immature heartwood of a 7 year old field grown tree, growing in the Indian Institute of Technology Kharagpur campus, was bored at chest height form the ground, up to 10 cm depths. The chips and powders obtained were immediately processed for solvent extraction with four different solvents in eluotrophic series, i.e., n-pentane, n-hexane, diethyl ether, ethyl acetate (all spectroscopy grade, from E. Merck, Germany), in four independent sets, in several batches. Solvent extraction was performed in Erlenmeyer flasks of 250 ml volume, for 2 h each, at 25 ± 5 ºC, with intermittent shaking in a 10 % (w/v) ratio of tissue to solvent. During extraction 0.01 % (w/v) BHT (butylated hydroxytoluene) (Sigma-Aldrich, St. Louis, MO, USA) was added as a synthetic antioxidant to protect the phytochemicals from auto oxidation, as well as an internal standard. Following this, extracts were dried over Na_2SO_4 (HiMedia, India), concentrated *in vaccuo*, in an N, N- series rotary evaporator (Eyela, Tokyo) at 40 °C. The extracts were reconstituted in pyridine when required and proceeded with GC-ITQ-MS analysis.

2.2 Gas chromatography ion trap–mass spectrometry (GC-ITQ-MS) analysis

The solvent extracts were analyzed by GC-ITQ-MS using a Thermo Trace GC Ultra™ gas chromatograph system (Thermo Scientific, USA), equipped with a 30 m x 0.25 mm i.d., 0.25 µm film thickness, non-polar TR-5MS fused silica capillary column, connected to a ion trap quadrupole (ITQ) mass selective detector (unit mass resolution). Split was 1: 50, with helium as carrier gas at a flow rate of 1 ml/ min, while the damping gas flow was 0.3 ml/ min. The initial oven temperature was set to 40 °C for 1 min. The GC oven temperature program was as follows: 40 ºC to 220 ºC, by ramping at 3 ºC, and held at 220 ºC for 20 minutes. The injector temperature was 220 °C and the transfer line was held at 220°C. The detection was performed by a Thermo ITQ 900™ mass spectrometer in the EI mode (ionization energy of 70 eV, ion source temperature of 180°C, emission current of 220 µA). The acquisition was made in full scanning mode (mass range 50–900 m/z; 3 scans/ second). Maximum ionization time was 25 milisecs. A solvent delay time of 5 min (set off) was used to avoid overloading the mass spectrometer with hexane. Data collection, analysis and integration were performed using the software XCalibur™ (version 2.0.7). Areas were recorded for all detectable peaks, and percent composition was calculated by taking area of peak divided by total chromatogram area x 100. For identification of the compounds, a solution of n-alkanes (n-octane to n-hexadecane) was injected in the GC-MS system after and the analysis was performed using the same instrumental conditions. This allowed the calculation of Kováts/ linear/ -retention indices (KI/ LRI/ RI) for each compound and compare with the authentic standards (spiking and co-elution experiments) and literature in order to ensure the correct identification, allowing variability in retention times up to < 0.05 min. The sesquiterpenoid constituents were identified according to the National Institute of Standards and Technology (NIST) and Wiley standard mass spectral libraries (spectral fit value of > 90) supplied with the instrument. Web resources such as Dr. Duke's Phytochemical and Ethnobotanical Database (http://www.ars-grin.gov/duke/) and Flavornet (http://www.flavornet.org/flavornet.html) were used for confirmation of KI and RI values. The sesquiterpenoid content was calculated from a standard curve made from α-santalol using similar set up and was expressed as nanograms per gram of fresh weight material.

3. Results and Discussion

GC and GC-MS are preferred methods for analyses of essential constituents from plants where the identification is based on comparison of retention indices against reported literature and by comparison of their mass spectra in published libraries or databases (Jennings and Shibamoto, 1980). Constituents of sandalwood oil from commercially available steam distillates have been exhaustively analyzed (Howes *et al.,* 2004). Similarly, GC-MS analyses of heartwoods from matured trees were reported (Jones *et al.,* 2006). However, there are no available reports on the constituents from immature heartwood of sandalwood tree and the effect of solvents on differential extractability of sesquiterpenoids from such samples.

The four solvents used for extraction of sesquiterpenoids, i.e., n-hexane, n-pentane, diethyl ether and ethyl acetate yielded 39.3 ± 6, 53.7 ± 2.3, 44 ± 4.6 and 37.5 ± 5.2 mg g^{-1} fresh weight heartwood material as determined gravimetrically after evaporation of solvents. Initially, α-santalol purified from sandalwood oil was used as an authentic standard prepare a standard curve [*equation*: y= (2E+07) x + 30063; R^2=0.998] based on the peak area noted in the GC chromatogram. Upon GC-ITQ-MS analyses, the signature mass fragments of m/z 161 and 204 aided in investigation of most consistently occurring constituents across all four extracts. We found out that the sesquiterpenoid alcohol content to be higher than the sesquiterpene hydrocarbon content in the immature heartwood, even though the diversity of hydrocarbon constituents is evident and is significantly different from a typical sandalwood oil sample (Howes *et al.,* 2004). In contrast, it has been shown that mature plants frequently exhibited much lower sesquiterpene (hydrocarbon) content than younger plants (Alonso-Amelot *et al.,* 1992). Many sesquiterpene hydrocarbons and their derivatives i.e., patchoulene, trans-caryophyllene, bicyclogermacrene, α-cedrane and α-bisabolene, were mostly over represented in the n-hexane extract. Although

in trace, we could detect and quantify an unknown oxygenated sesquiterpenoid [R*t*, 16.87, $C_{17}H_{26}O_2$, MW: 262] in all but the diethyl ether extract. The major constituents obtainable from matured heartwoods are Z-α-santalol and epi-β-santalol, as reported earlier (Jones et al., 2006). However, this study indicates that, Z-α -trans-bergamotol (32.52- 43.2 ng g^{-1} fresh weight of heartwood) is the most abundant sesquiterpene alcohol in immature heartwood, followed by Z-epi-β-santalol and Z-β-santalols (Table 1). In fact, bergamotol is known to be a minor constituent of sandalwood oil (Brunke *et al.,* 1988).

Complexities of constituents, their sheer numbers, and similarities in mass spectra and difficulties in peak identification (Oprean *et al.,* 1998) have rendered the temperature-programmed conditions as popular choice for essential oil analyses (Marriott *et al.,* 2001). Moreover, plants do synthesize and catabolize a plethora of terpenoids ranging from C_5 to C_{40} and higher, in a spatio-temporal manner for different purposes throughout the course of plant development (Endo and Suga, 1992). The sesquiterpenoids extracted using different solvents yield different profile during the growth of heartwood of the tree, whereas it is difficult to infer the biological functions they confer. Signaling and biochemical events and processes associated with secondary metabolism during heartwood formation are poorly known (Hillis, 1987). Moreover, it is well known that the yields and compositions of essential oils are strongly influenced by organ maturation, environmental cues and the plant's genetic factors (Sangwan *et al.,* 2001; Figueiredo *et al.,* 2008). Thus, GC-MS based profiling of various solvent extracts and developmental phases of the heartwood, would provide insight into the diversity and biological roles of sesquiterpenoids in this commercially important tree species.

4. Conclusion

From this study, it is evident that solvents affect the extractability of sesquiterpenoids from samples in a differential manner. We observed that the immature heartwood of sandalwood tree deposits many previously unreported sesquiterpenoid constituents and their derivatives and is rich in Z- α -trans-bergamotol. The sesquiterpenoid diversity is found to be higher for n-hexane extract, while higher amounts are obtainable in case of n-pentane extracts. Depending on the targeted constituent for a particular study or application, an extraction solvent might be chosen for the purpose, especially in sandalwood research.

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Table 1. GC-MS based quantification profiles of sesquiterpenoids obtained from the four solvent extracts of heartwood chips of matured sandalwood tree.

Serial No.	Constituents	Retention time (min)	Mol. Formula/ Mol. Weight	Peak Area (AU)	Quantity (ng/ g FW Tissue)
n-hexane extract					
1	α-Cedrane	11.26	$C_{15}H_{26}$, 206	120832.7	0.14
2	α-Santalene	11.81	$C_{15}H_{24}$, 204	1232831	1.8
3	Z-α-Santalol	12.41	$C_{15}H_{24}O$, 220	1700298	2.51
4	α-Bisabolene	12.93	$C_{15}H_{24}$, 204	113938.7	0.13
5	Patchoulene	14.1	$C_{15}H_{24}$, 204	39912.57	0.01
6	Cedrane-8, 13-diol	14.44	$C_{15}H_{26}O_2$, 238	218663.5	0.28
7	Bicyclogermacrene	15.95	$C_{15}H_{24}$, 204	582164.9	0.83
8	trans-Caryophyllene	16.46	$C_{15}H_{24}$, 204	839435	1.21
9	Z-epi- β -santalol	16.62	$C_{15}H_{24}O$, 220	21061654	31.55
10	Unknown Sesquiterpenoid	16.87	$C_{17}H_{26}O_2$, 262	189462.6	0.24
11	Z-β -Santalol	17.01	$C_{15}H_{24}O$, 220	13138430	19.66
12	E-β-Santalol	21.57	$C_{15}H_{24}O$, 220	3070377	4.56
n-pentane extract					
1	α-Santalene	11.81	$C_{15}H_{24}$, 204	7284601	10.88
2	Z-α-Santalol	12.41	$C_{15}H_{24}O$, 220	9473986	14.17
3	α-Bisabolene	12.93	$C_{15}H_{24}$, 204	1890748	2.79
4	Bicyclogermacrene	15.95	$C_{15}H_{24}$, 204	2454743	3.64
5	Z- α -trans-Bergamotol	16.37	$C_{15}H_{24}O$, 220	28832782	43.2
6	trans-Caryophyllene	16.46	$C_{15}H_{24}$, 204	195693	0.25
7	Z-epi- β -Santalol	16.62	$C_{15}H_{24}O$, 220	7949336	11.88
8	Unknown Sesquiterpenoid	16.87	$C_{17}H_{26}O_2$, 262	131557.5	0.15
9	Z-β -Santalol	17.01	$C_{15}H_{24}O$, 220	8382828.3	12.52

10	E-β-Santalol	21.57	$C_{15}H_{24}O$, 220	83661.31	0.08
Diethyl ether extract					
1	α-Cedrane	11.26	$C_{15}H_{26}$, 206	68577.31	0.06
2	α-Santalene	11.81	$C_{15}H_{24}$, 204	60746.62	0.05
3	α-Bergamotene	11.94	$C_{15}H_{24}$, 204	924779.2	1.34
4	Z-α-Santalol	12.41	$C_{15}H_{24}O$, 220	5935639	8.86
5	α-Bisabolene	12.93	$C_{15}H_{24}$, 204	1158938	1.69
6	Bicyclogermacrene	15.95	$C_{15}H_{24}$, 204	1516995	2.23
7	Z-α-trans-Bergamotol	16.37	$C_{15}H_{24}O$, 220	21709316	32.52
8	Z-epi- β -Santalol	16.62	$C_{15}H_{24}O$, 220	10679410.98	15.96
9	Z-β -Santalol	17.01	$C_{15}H_{24}O$, 220	11803022.2	17.61
10	E-β-Santalol	21.57	$C_{15}H_{24}O$, 220	1837412	2.71
Ethyl acetate extract					
1	α-Cedrane	11.26	$C_{15}H_{26}$, 206	102865.96	0.09
2	α-Santalene	11.81	$C_{15}H_{24}$, 204	4589940.63	6.84
3	Z-α-Santalol	12.41	$C_{15}H_{24}O$, 220	5592370.7	8.38
4	Cedrane-8,13-diol	14.44	$C_{15}H_{26}O_2$, 238	220652.7	0.29
5	Z-α-trans-Bergamotol	16.37	$C_{15}H_{24}O$, 220	5293815.4	7.93
6	Z-epi- β -Santalol	16.62	$C_{15}H_{24}O$, 220	6176125.52	9.23
7	Unknown Sesquiterpenoid	16.87	$C_{17}H_{26}O_2$, 262	131557.49	0.15
8	Z-β -Santalol	17.01	$C_{15}H_{24}O$, 220	5438779.12	8.11