

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

Biswapriya B. Misra<sup>1,2,\*</sup> Satyahari Dey<sup>1</sup>

1. Plant Biotechnology Laboratory, Department of Biotechnology, Indian Institute of Technology Kharagpur, Kharagpur 721302, WB, India
2. Centre for Chemical Biology, Universiti Sains Malaysia, 1st Floor, Block B, No.10, Persiaran Bukit Jambul, 11900 Bayan Lepas, Pulau Pinang, Malaysia

\* E-mail of the corresponding author: [bbmirsaccb@gmail.com](mailto:bbmirsaccb@gmail.com)

## 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 quadrupole (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- $\alpha$ -trans-bergamotol, Z-epi- $\beta$ -santalol and Z- $\beta$ -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,  $\alpha$ - and  $\beta$ -santalols, bergamotols and several of their stereoisomers, whereas minor constituents includes lanceol, nuciferol, bisabolol and the sesquiterpene hydrocarbons such as  $\alpha$ - and  $\beta$ -santalenes, bergamotenes,  $\alpha$ -,  $\beta$ - and  $\gamma$ -curcumenes and  $\beta$ -bisabolene (Adams *et al.*, 1975; Christenson *et al.*, 1981; Demole *et al.*, 1976; Howes *et al.*, 2004; Jones *et al.*, 2006) and usually,  $\alpha$ -santalol is more abundant (~46%) than  $\beta$ -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 from the ground, up to 10 cm depths. The chips and powders obtained were immediately processed for solvent extraction with four different solvents in eluotropic 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  $25 \pm 5$  °C, 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  $\text{Na}_2\text{SO}_4$  (HiMedia, India), concentrated *in vacuo*, 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  $\mu\text{m}$  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 °C to 220 °C, by ramping at 3 °C, and held at 220 °C 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  $\mu\text{A}$ ). The acquisition was made in full scanning mode (mass range 50–900 m/z; 3 scans/ second). Maximum ionization time was 25 miliseconds. 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  $\alpha$ -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  $39.3 \pm 6$ ,  $53.7 \pm 2.3$ ,  $44 \pm 4.6$  and  $37.5 \pm 5.2$  mg  $\text{g}^{-1}$  fresh weight heartwood material as determined gravimetrically after evaporation of solvents. Initially,  $\alpha$ -santalol purified from sandalwood oil was used as an authentic standard prepare a standard curve [equation:  $y = (2\text{E}+07) x + 30063$ ;  $R^2=0.998$ ] 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, bicylogermacrene,  $\alpha$ -cedrane and  $\alpha$ -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*- $\alpha$ -santalol and epi- $\beta$ -santalol, as reported earlier (Jones et al., 2006). However, this study indicates that, *Z*- $\alpha$ -trans-bergamotol (32.52- 43.2 ng g<sup>-1</sup> fresh weight of heartwood) is the most abundant sesquiterpene alcohol in immature heartwood, followed by *Z*-epi- $\beta$ -santalol and *Z*- $\beta$ -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*- $\alpha$ -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.

#### 5. Acknowledgements

The authors thank Mr. Shibendu Sekhar Das for data extraction and analysis. BBM received the Junior and Senior Research Fellowships of the Council of Scientific and Industrial Research (CSIR), New Delhi and Research Associateship conferred by Department of Biotechnology (DBT), Government of India. The experimental work in *S. album* in SD's laboratory is being supported under the project- Prospecting of novel genes and molecules of *Santalum album* L. [NGM], sponsored by DBT, Government of India.

#### References

- Adams, D.R., Bhatnagar S.P., & Cookson, R.C. (1975), "Sesquiterpenes of *Santalum album* and *Santalum spicatum*", *Phytochemistry* 14, 1459-1460.
- Alonso-Amelot, M.E., Perez-Mena, M., Calcagno, M.P., Jaimes-Espinoza, R., & Castillo, U. (1992), "Ontogenic variation of biologically active metabolites of *Pteridium aquilinum* (L. Kuhn), pterosins A and B, and ptaquiloside in a bracken population of the tropical Andes", *Journal of Chemical Ecology* 18, 1405-1420.
- Anonis, D.P. (1998), "Sandalwood and sandalwood compounds", *Perfumer and Flavorist* 23, 19-24.
- Benencia, F., & Courreges, M.C. (1999), "Nitric oxide and macrophage antiviral extrinsic activity", *Immunology* 98, 363-370. doi: 10.1046/j.1365-2567.1999.00864.x
- Bommareddy, A., Rule, B., VanWert, A.L., Santha, S., & Dwivedi C. (2012), " $\alpha$ -Santalol, a derivative of sandalwood oil, induces apoptosis in human prostate cancer cells by causing caspase-3 activation", *Phytomedicine* 19, 804-811.
- Brunke, E.J., Schatkowski, D., Struwe, H., & Tumbrink, L. (1988), "Bergamotol and spirosantalol--new constituents of East Indian sandalwood oil", *Developments in Food Science* 18, 819-831.
- Burdock, G.A., & Carabin I.G. (2008), "Safety assessment of sandalwood oil (*Santalum album* L.)", *Food and Chemical Toxicology* 46, 421-432.
- Christenson, P.A., Secord, N., & Willis, B.J. (1981), "Identification of trans- $\beta$ -santalol and epi-cis- $\beta$ -santalol in East Indian sandalwood oil", *Phytochemistry* 20, 1139-1141.
- Demole, E., Demole, C., & Enggist, P. (1976), "A chemical investigation of the volatile constituents of East Indian Sandalwood Oil (*Santalum album* L.)", *Helvetica Chimica Acta* 59, 737-747.
- Desai, V.B., Hiremath, R.D., Rasal, V.P., Gaikwad, D.N., & Shankarnarayana, K.H. (1991), "Pharmacological screening of HESP and sandal oils", *Indian Perfumer* 35, 69-70.
- Dikshit, A., & Hussain, A. (1984), "Antifungal action of some essential oils against animal pathogen", *Fitoterapia* 55, 171-176.

- Endo, T., & Suga, T. (1992), "Demonstration of geranyl diphosphate synthase in several higher plants", *Phytochemistry* 31, 2273-2275.
- Figueiredo, A., Barroso, J., Pedro, L., & Scheffer, J. (2008), "Factors affecting secondary metabolite production in plants: volatile components and essential oils", *Flavour and Fragrance Journal* 23, 213-226.
- Hillis, W.E. (1987), *Heartwood and Tree Exudates*, Berlin: Springer-Verlag, pp. 268.
- Howes, M.J.R., Simmonds, M.S.J., & Kite, G.C. (2004), "Evaluation of the quality of sandalwood essential oils by gas chromatography–mass spectrometry", *Journal of Chromatography A* 1028, 307-312.
- Jennings, W., & Shibamoto, T. (1980), *Quantitative analysis of flavor and fragrance volatile by glass capillary column gas chromatography*, New York: Academic Press.
- Jones, C.G., Ghisalberti, E.L., Plummer, J.A., & Barbour, E.L. (2006), "Quantitative co-occurrence of sesquiterpenes; a tool for elucidating their biosynthesis in Indian sandalwood, *Santalum album*", *Phytochemistry* 67, 2463-2468.
- Marriott, P.J., Shellie, R., & Cornwell, C. (2001), "Gas chromatographic technologies for the analysis of essential oils", *Journal of Chromatography A* 936, 1-22.
- Muzika, R.M., Campbell, C.L., Hanover, J.W., & Smith, A.L. (2006), "Comparison of techniques for extracting volatile compounds from conifer needles", *Journal of Chemical Ecology* 16, 2713-2722.
- Oprean, R., Tamas, M., Sandulescu, R., & Roman, L. (1998), "Essential oils analysis. I. Evaluation of essential oils composition using both GC and MS fingerprints", *Journal of Pharmaceutical and Biomedical Analysis* 18, 651-657.
- Sangwan, N.S., Farooqi, A.H.A., Sabih, F., & Sangwan, R.S. (2001), "Regulation of essential oil production in plants", *Journal of Plant Growth Regulation* 34, 3-21.
- Shankaranarayana, K.H., & Parthasarathi, K. (1987), "On the content and composition of oil from heartwood at different levels in sandal", *Indian Perfumer* 31, 211-214.
- Takaishi, Y., Ochi, T., Shibata, H., Higuti, T., Kodama, K.H., & Kusumi, T. (2005), "Anti- *Helicobacter pylori* compounds from *Santalum album*", *Journal of Natural Products* 68, 819-824.

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. Weight	Formula/ Mol.	Peak Area (AU)	Quantity (ng/ g FW Tissue)
<b><i>n-hexane extract</i></b>						
1	$\alpha$ -Cedrane	11.26	C <sub>15</sub> H <sub>26</sub> , 206		120832.7	0.14
2	$\alpha$ -Santalene	11.81	C <sub>15</sub> H <sub>24</sub> , 204		1232831	1.8
3	Z- $\alpha$ -Santalol	12.41	C <sub>15</sub> H <sub>24</sub> O, 220		1700298	2.51
4	$\alpha$ -Bisabolene	12.93	C <sub>15</sub> H <sub>24</sub> , 204		113938.7	0.13
5	Patchoulene	14.1	C <sub>15</sub> H <sub>24</sub> , 204		39912.57	0.01
6	Cedrane-8, 13-diol	14.44	C <sub>15</sub> H <sub>26</sub> O <sub>2</sub> , 238		218663.5	0.28
7	Bicyclogermacrene	15.95	C <sub>15</sub> H <sub>24</sub> , 204		582164.9	0.83
8	trans-Caryophyllene	16.46	C <sub>15</sub> H <sub>24</sub> , 204		839435	1.21
9	Z-epi- $\beta$ -santalol	16.62	C <sub>15</sub> H <sub>24</sub> O, 220		21061654	31.55
10	Unknown Sesquiterpenoid	16.87	C <sub>17</sub> H <sub>26</sub> O <sub>2</sub> , 262		189462.6	0.24
11	Z- $\beta$ -Santalol	17.01	C <sub>15</sub> H <sub>24</sub> O, 220		13138430	19.66
12	E- $\beta$ -Santalol	21.57	C <sub>15</sub> H <sub>24</sub> O, 220		3070377	4.56
<b><i>n-pentane extract</i></b>						
1	$\alpha$ -Santalene	11.81	C <sub>15</sub> H <sub>24</sub> , 204		7284601	10.88
2	Z- $\alpha$ -Santalol	12.41	C <sub>15</sub> H <sub>24</sub> O, 220		9473986	14.17
3	$\alpha$ -Bisabolene	12.93	C <sub>15</sub> H <sub>24</sub> , 204		1890748	2.79
4	Bicyclogermacrene	15.95	C <sub>15</sub> H <sub>24</sub> , 204		2454743	3.64
5	Z- $\alpha$ -trans-Bergamotol	16.37	C <sub>15</sub> H <sub>24</sub> O, 220		28832782	43.2
6	trans-Caryophyllene	16.46	C <sub>15</sub> H <sub>24</sub> , 204		195693	0.25
7	Z-epi- $\beta$ -Santalol	16.62	C <sub>15</sub> H <sub>24</sub> O, 220		7949336	11.88
8	Unknown Sesquiterpenoid	16.87	C <sub>17</sub> H <sub>26</sub> O <sub>2</sub> , 262		131557.5	0.15
9	Z- $\beta$ -Santalol	17.01	C <sub>15</sub> H <sub>24</sub> O, 220		8382828.3	12.52

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



This academic article was published by The International Institute for Science, Technology and Education (IISTE). The IISTE is a pioneer in the Open Access Publishing service based in the U.S. and Europe. The aim of the institute is Accelerating Global Knowledge Sharing.

More information about the publisher can be found in the IISTE's homepage:

<http://www.iiste.org>

The IISTE is currently hosting more than 30 peer-reviewed academic journals and collaborating with academic institutions around the world. **Prospective authors of IISTE journals can find the submission instruction on the following page:**

<http://www.iiste.org/Journals/>

The IISTE editorial team promises to review and publish all the qualified submissions in a fast manner. All the journals articles are available online to the readers all over the world without financial, legal, or technical barriers other than those inseparable from gaining access to the internet itself. Printed version of the journals is also available upon request of readers and authors.

### **IISTE Knowledge Sharing Partners**

EBSCO, Index Copernicus, Ulrich's Periodicals Directory, JournalTOCS, PKP Open Archives Harvester, Bielefeld Academic Search Engine, Elektronische Zeitschriftenbibliothek EZB, Open J-Gate, OCLC WorldCat, Universe Digital Library, NewJour, Google Scholar

