KFRI Research Report No. 307

FINAL REPORT

Identification of *Santalum album* and *Osyris lanceolata* through morphological and biochemical characteristics and molecular markers to check adulteration

(Final Report of the project KFRI 509/06)

K. V. Bhat
Forest Utilization Division

M. Balasundaran
SNPFM Divison

M. Balagopalan
Instrumentation Division

Kerala Forest Research Institute
Peechi – 680 653, Thrissur District

Project title	:	Identification of *Santalum album* and *Osyris lanceolata* through morphological and biochemical characteristics and moleclular markers to check adulteration
Investigators	:	K. V. Bhat, M. Balasundaran and M. Balagopalan
Project Fellows	:	M. C. Anisha, C. Anupama and K. Sheena
Duration of the project	:	1 year (extended further for 10 months)
Source of funding	:	KSCSTE
Total budget outlay	:	Rs. 5,74,000/- (for 1 year)

Objectives

The broad objective of the study is to develop reliable methods to distinguish between *S. album* and *O. lanceolata* accurately. The specific objectives are:

1. To carry out microscopic studies and identify structural features suitable for distinguishing the woods of *Santalum album* and *Osyris lanceolata*
2. To compare santalol percentage in the wood dust of *Santalum* and *Osyris* for species identification
3. To develop and standardize DNA-based marker techniques suitable for distinguishing *S. album* and *O. lanceolata*

Methodology proposed to be followed

Standard methods of microtechnique will be followed for the study of wood structure of both *S. album* and *O. lanceolata*. Attempts will also be made to distinguish the woods in chip and powder forms through the study of cell morphology.

Chromatographic techniques will be used for comparison of α and β santalol levels in both species.

The methodology proposed for DNA studies is DNA fingerprinting using RAPD and microsatellite markers

Aspects of study and responsible investigator(s)

1. Physical /and anatomical characteristics – K. V. Bhat
2. Chemical aspects – M. Balagopalan
3. DNA fingerprinting - M. Balasundaran

CONTENTS

ABSTRACT

The study undertaken to elucidate the means and criteria to differentiate the woods of *Santalum album* and *Osyris lanceolata* showed the possibility of distinguishing the woods reliably on the basis of anatomical structure, colour of the hot water extract, chemical constituents and their proportion in the oil, and DNA fingerprinting.

Wood anatomical characteristics useful for distinguishing *S. album* from *O. lanceolata* were seriation of rays, type of crystalliferous cells and abundance of extractives. Wood of *S. album* had 1 to 2 seriate rays, crystals in axial parenchyma cells and scanty extractives; whereas, *O. lanceolata* had 1- to 3-seriate rays, crystals in ray cells and relatively abundant extractives. The hot water extract of S. *album* was pale yellowish without traces of red colour while that of *O. lanceolata* was reddish. Similarly, the oil extracted from *O. lanceolata* had a faint reddish hue as compared to *S. album* which was yellowish. The wood of *S. album* yielded thrice as much oil as that of *O. lanceolata*. Oil from *S. album* contained 46 to 57% and 0.42 to 1.56% α-Santalol and cis Lanceol respectively, while that from *O. lanceolata* contained 24 to 25% α-Santalol and 28% cis Lanceol. Chemical constituents such as α-Bergamotene, (Z)-β-Farnesene, β–Bisabolene, α-Bisabolol, Z-α-trans-Bergamotol were present only in *O. lanceolata,* while 2-Carene, α-Curcumene, Teresantalol and trans-β-santalol were found only in *S. album*. As DNA was unavailable from the dry specimens of *O. lanceolata,* DNA extracted from samples of locally found *O. wightiana,* reported as synonym of *O. lanceolata,* was used for the study. The genomic DNA extracted from the wood and leaf samples of *S. album* and *O. wightiana* was PCR-amplified using specific primers designed to amplify the 18S and 26S rDNA units. The variations in restriction patterns (RFLP) of these amplified products when digested with restriction endonuclease *Bam* HI served as tools to distinguish the two species. The 18S rDNA of *S. album* and *O. wightiana* contained 1695 and 1668 nucleotides and 26S rDNA contained 3204 and 3264 nucleotides respectively. Nucleotide sequence dissimilarities between the rRNA genes of the two species were also sufficient to distinguish *S. album* from *Osyris* species.

INTRODUCTION

The sandalwood genus *Santalum* (Santalaceae) is distributed in southern India, Sri Lanka, Malaysia, Indonesia, Hawaii, Australia and Pacific islands. Although there are about 25 species of *Santalum* in the world, the two main species used to produce commercial sandalwood are *Santalum album* (East Indian sandalwood) and *S. spicatum* (Australian sandalwood). In India, *S. album* is distributed mainly on the Deccan Plateau in the states of Karnataka and Tamil Nadu. In Kerala, the species occurs in Marayoor on the eastern side of the Western Ghats. The fragrant wood is highly prized and is the source of sandalwood oil used for incense, soaps, creams, perfume, and fine carving. With a history of use of over 4000 years, East Indian sandalwood is one of the oldest known perfume materials. Only within the last century has it been found in the American and European perfume industries.

The high price of true sandalwood oil in both domestic and international market has always been a reason behind frequent adulteration of this valuable material with other cheaper essential oils and resinous substances. Although this practice has been in vogue for nearly a century in the country and elsewhere, the trend of extensive use of other 'substitute sandalwoods' for distillation of oil is a recent trend. For this purpose recently a cheaper substitute, the 'African sandalwood' (*Osyris lanceolata*) imported from Tanzania, has started flooding into our sandal oil industry. This has led to a variety of problems in the age-old sandal oil extraction and trade. In a number of cases registered in this regard, there has been a dispute with regard to the correct identity of the material, which is not easy to ascertain.

Both *S. album* and *O. lanceolata* (*O. tenuifolia*) belong to the family Santalaceae and have many similarities in their physical characteristics. Being members of the same family, they are strikingly similar in most wood anatomical characteristics. Therefore, distinguishing one from the other anatomically is not very easy. Often the sandalwood involved in forest offences is in the form of small chips or powder in which case it is impossible to apply routine anatomical methods for identification. Therefore, it is necessary to look for some reliable means of identification of these species whereby they can be distinguished from each other accurately.

Besides anatomical methods, another useful method to distinguish the woods of *S. album* and *O. lanceolata* is chemical analysis particularly when the wood material is in particulate form. The oil content obtained from *S. album* and other sandalwoods is found to differ in their physical and chemical properties (Iyengar, 1968). The percentage yield of oil from Indian sandalwood and the high santalol content in it can be regarded as some important features for distinguishing the woods of *S. album* and *O. lanceolata.*

Nucleotide sequences of rDNA coding for rRNA, is highly conserved and the sequences are more or less specific for each species. Though, *S. album* and *Osyris* spp. belong to the same family, they belong to different genera. Hence, dissimilarities are likely to exist in the rDNA sequences between these two species. The sequences of 18S and 26S units of rDNA of *S. album* and *O. lanceolata* have been worked out. Though the nucleotide sequences in these two species are closely similar, differences exist at certain locations. Hence, in order to to distinguish *Santalum* and *Osyris,* attempts are necessary to DNA fingerprint these species through PCR amplification of 18S and 26S rDNA units and sequence these two genes and compare their sequences.

Thus, the present investigation envisages exploration of the possibility of developing some reliable methods to distinguish between *S. album* from *O. lanceolata*, and possibly other species of *Osyris.* To achieve this objective, the study envisages generating information/criteria on physical and anatomical characteristics, chemical nature of the oil and also DNA fingerprinting and gene sequencing.

MATERIALS AND METHODS

For a comparative study of wood structure, chemical characteristics and DNA studies, the wood material of *S. album* was obtained from mature trees growing locally at Peechi and at Marayoor (Kerala). Wood samples of *O. lanceolata* were obtained from consignments of African sandalwood imported from Tanzania by different local firms and sandal oil distillation units of Kerala. Besides, the Indian *Osyris* species namely, *O. wightiana* was collected from Chinnar, Kerala.

Anatomical studies

Standard procedures of microtechnique were followed for the study of wood structure of *S. album* and *O. lanceolata.* Transverse and longitudinal sections of 20-30 micron thickness cut on a sliding microtome were stained with different staining methods such as Tannic acid-ferric chloride and Saffranin (double staining) and also toluidine blue 'O' (O'Brien *et al*., 1964). The morphology of cell types was also studied through maceration of the wood tissue. For histochemical localization of extractives, ferric chloride and benzidine tests were used.

Chemical analysis

For chemical analysis of the oil from *S. album* and *O. lanceolata* the protocol followed by Mwang'ingo *et al*.(2003) was adopted. 10g of air-dry heartwood was finely chopped, treated with liquid nitrogen and crushed and then extracted with dichloromethane for three days in Soxhlet apparatus. After remotion of the solvent under reduced pressure, the extract was distilled with odour-free water for two hours to obtain 350 ml of distillate. The distillate was saturated with NaCl and extracted with fresh distilled diethyl ether (3x100 ml). The ether solution was dried under Na_2SO_4 and concentrated through rotary evaporator to obtain yellow oil used for gas chromatography-mass spectrometry (GC-MS) analysis (Mwang'ingo *et al.,* 2003). The resulting oil was weighed and expressed as percentage of the weight of wood powder used for extraction.

The composition of different constituents was determined on a Shimadzu GCMS-QP 2010S system. Chromatography was performed on a 30 m x 0.25 mm i.d.x 0.25 μm DB-5MS column using an oven program of 40-220°C at 4°C/min. Helium was used as carrier gas with constant flow of 1.78 mL/min. Sample injection of 1 μL was used with a split ratio of 1:10. The injection temperature was 300°C. Ion source and transfer line temperatures were held at 200°C and 260°C. The MS was fitted with an EI source operated at 0.7 kV, and mass spectra were recorded in the range m/z 40-400 at 1 scan/0.5 s. The software used was GC MS solution version 2.40.

Oils were diluted to 1% with diethyl ether prior to analysis. Different constituents separated were identified by parallel comparison of their retention times and mass spectra with NIST, PEST and PESTIC mass spectral database.

DNA Studies

Extraction of DNA was done from heartwood and sapwood of *S. album* and *O. lanceolata*. DNA was also extracted from *O. wightiana*, another species (supposed to be a synonym of *O. lanceolata*) (Sha Zhen Shu, 2003; CES, 2006), growing in Munnar, Marayoor and Chinnar Wildlife Sanctuary. *O. wightiana* also provides fragrant heartwood similar to sandal and *O. lanceolata*. The samples were chipped into small pieces, and about 100 mg of chips were ground into a fine powder using liquid nitrogen for DNA extraction. Besides heartwood and sapwood, DNA was also extracted from leaf samples of the species (Table 1). DNA extraction and purification from all the samples were done using QIAGEN DNeasy Plant Mini Kit following the manufacturer's protocol.

Precise DNA bands were obtained from all samples when extracted, except dry heartwood of *S. album* and *O. lanceolata*. DNA was electrophoresed on 1.5% agarose gel indicating presence of pure DNA as shown in Table 1. The samples obtained were used for PCR amplifications of 18S and 26S rDNA units of *S. album* and *O. wightiana* using primers specifically developed for sandal and *Osyris* species. PCR amplification reactions were performed using FINNZYMES High Fidelity PCR Kit.

Table 1. Plant species and tissues used for DNA extraction

Sl. No.	Tissues used	Species		
		S. album	*O. lanceolata*	*O. wightiana*
1	Leaves	+	Sample Unavailable	+
2	Dry heartwood	-	-	Sample Unavailable
3	Heartwood (up to 6 months after felling the tree)	+	Sample Unavailable	+
4	Sapwood (up to 1 year after felling the tree)	+	Sample Unavailable	+

+ DNA extracted - DNA unavailable

The PCR amplified 18S and 26S rDNA from sandal and *Osyris* samples were digested with restriction endonuclease *BamH*I. The digests were separated electrophoretically on 2% agarose gel, stained in aqueous solution of ethidium bromide, visualized and documented using Vilber Lourmat Gel Documentation system. The RFLP fingerprints of sandal and *Osyris* species were compared for distinguishing the species. The PCR amplified products of 18S and 26S rDNA from sandal and *Osyris,* characterized by single band were further subjected to nucleotide sequencing at MWG Biotech Pvt. Ltd., Bangalore. The nucleotide sequences of 18S and 26S rDNA of sandal and *O. wightiana* were compared using bioinformatics tool CLUSTAL W and NCBI-BLAST for distinguishing the species.

RESULTS AND DISCUSSION

Wood anatomical features

Woods of both *S. album* and *O. lanceolata* are fine-textured and nearly straight-grained. Heartwood of the former is yellowish brown and the latter has a pale reddish hue. *S. album* has a distinctive sweet fragrance while *O. lanceolata* has a relatively feeble fragrance somewhat similar to the former.

Both *S. album* and *O. lanceolata* are diffuse porous woods with small vessels not visible even under a hand lens. Growth rings are indistinct in both the species although faint, closely spaced, concentric markings are discernible on the transverse surface of the wood.

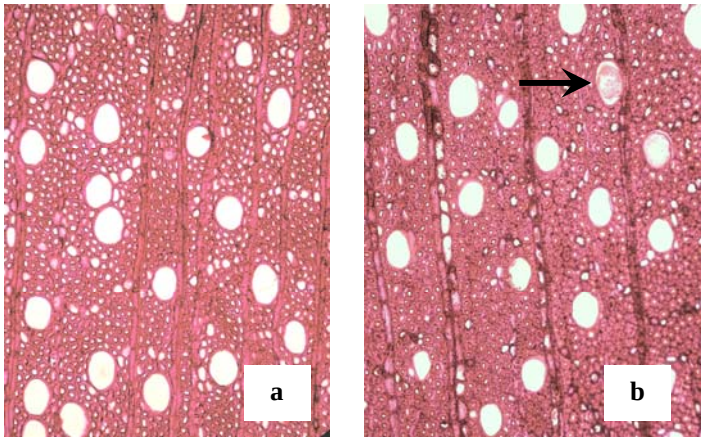

Fig. 1. Transverse sections of the wood of (a). *Santalum album* and (b). *Osyris lanceolata* x90.

Vessels are exclusively solitary and are circular to oval in cross sectional view (Fig. 1). Diameter of vessels ranges from 50 to 70 μm in *S. album* and 60 to 90 μm in *O. lanceolata*. Perforation of vessels is simple and pits alternate. Pits to parenchyma and rays with minute border. Heartwood vessels more commonly open without much deposits. However, some vessels contain tyloses (Fig. 1b, at arrow; Fig. 2b).

Axial parenchyma in both *S. album* and *O. lanceolata* is diffuse, scattered among fibres as single cells and also in aggregates or fine lines. Parenchyma not visible under hand lens. In *O. lanceolata* parenchyma cells contain dense accumulation of extractives In *S. album* extractives are scanty. Chambered crystalliferous cells enclosing rhomboidal crystals are occasionally found in *S. album* (Fig. 2d, at arrows) but not in *O. lanceolata.*

Tracheids are present as distinct strands or in association of vessels (Fig. 2a, at arrow). They have pitting similar to that found on vessel walls. Fibres in both the species have thick walls and are non-septate. They have distinctly bordered pits found on both radial and tangential walls.

Rays are short and fine; of heterogeneous type III in both the species. One-to-two seriate in *S. album* and one-to-three seriate in *O. lanceolata*. Uniseriate rays are composed of square cells whereas multiseriate rays have a single marginal row of square cells.

Extractives are relatively less in ray cells of *S. album* while abundant extractives are found within ray cells in *O. lanceolata*. In addition, ray cells frequently contain rhomboidal crystals in *O. lanceolata* whereas crystals are absent in ray cells in *S. album*

Wood anatomical characteristics of diagnostic value

The wood anatomical characteristics that are helpful in distinguishing *S. album* from *O. lanceolata* include structure of rays, particularly their seriation, occurrence and distribution of crystals and abundance of extractives within parenchyma cells. Samples of solid wood of *S. album* and *O. lanceolata* can thus be distinguished from each other based on microscopic features as follows:

Sl. No.	Wood anatomical feature	*Santalum album*	*Osyris lanceolata*
1	Ray structure	Rays 1 to 2 seriate	Rays 1 to 3 seriate
2	Rhomboidal crystals	Confined to chambered axial parenchyma; never found in ray cells	Crystals abundant and found usually in ray cells; rare in chambered axial parenchyma
3	Heartwood extractives	Extractives scanty in parenchyma cells	Extractives abundant in parenchyma cells

As found in the present study, rays in *S. album* are reported to be uni- to biseriate (Pearson and Brown, 1932; Metcalfe and Chalk, 1950; Kulkarni, 1995). However, there are some reports of occurrence of triseriate rays in *S. album* (Rao *et al*., 1998). The present study has not been able to find triseriate rays in several *S. album* samples examined. With regard to

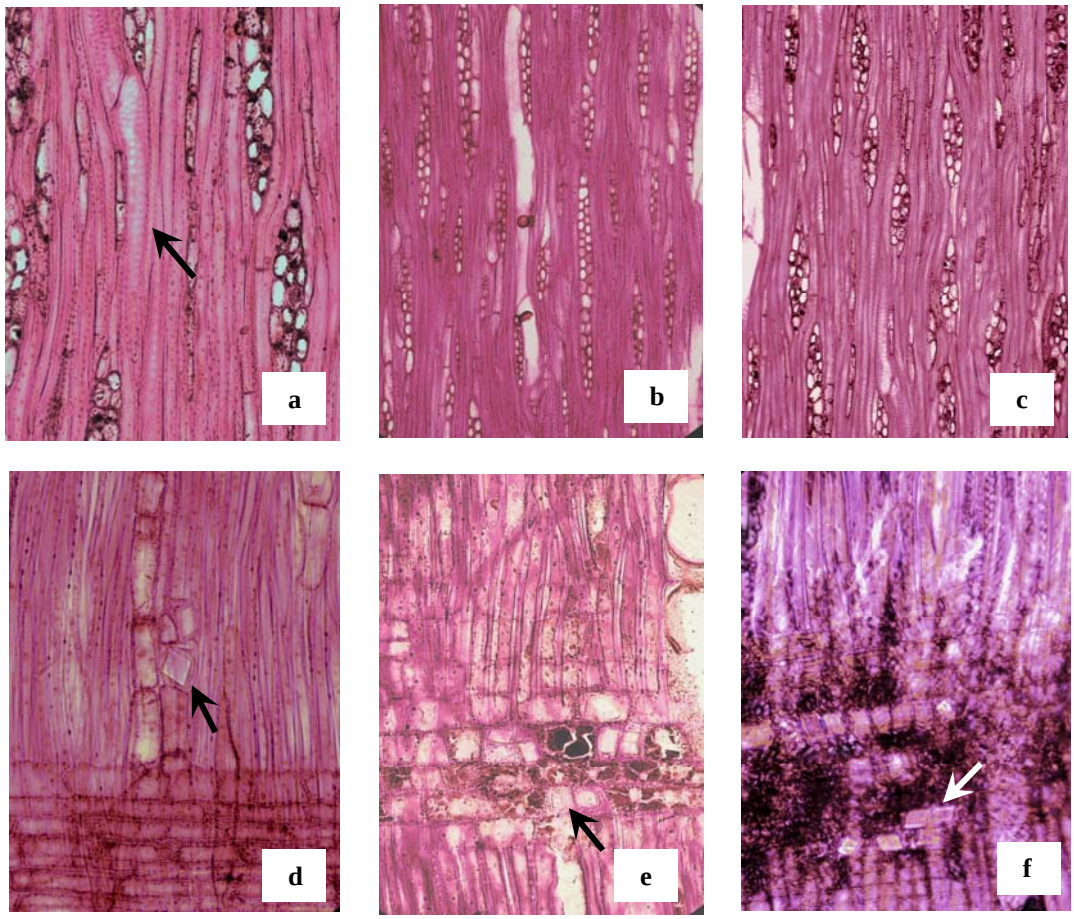

Fig. 2. a .TLS of *O. lanceolata* showing tracheids (at arrow). X150. **b.** TLS of *S. album* showing uni- and biseriate rays. x90 **c.** TLS of *O. lanceolata* showing 1 to 3-seriate rays. x90 **d.** RLS of *S. album* showing rhomboidal crystals in chambered axial parenchyma cells. x180 **e, f.** RLS of *O. lanceolata* showing rhomboidal crystals in ray parenchyma cells (at arrow). X180

the presence of crystals also there are some discrepancies. Metcalfe and Chalk (1950) have mentioned occurrence of crystals in chambered cells in most species of Santalaceae except in the genus *Osyris*. Crystals have not been observed in ray cells among the different genera of this family. However, observations from the present study show that crystals occur in chambered cells in *S. album* but never in ray cells, whereas in *O. lanceolata*, crystals occur in ray cells, often very abundantly. Thus if the ray seriation fails to distinguish these woods, they can be readily differentiated on the basis of distribution of crystals. The abundance of reddish brown extractives accumulated in parenchyma cells of *O. lanceolata* in contrast to *S. album* can also be taken as a distinguishing feature. The heartwood extractives of *O. lanceolata* stain black with ferric chloride solution, indicating the presence of tannins.

Distinguishing the woods based on physical methods

As mentioned earlier, the wood of *O. lanceolata* has a faint reddish hue as compared to *S. album* which is yellowish without any traces of red. It is understandable that the difference

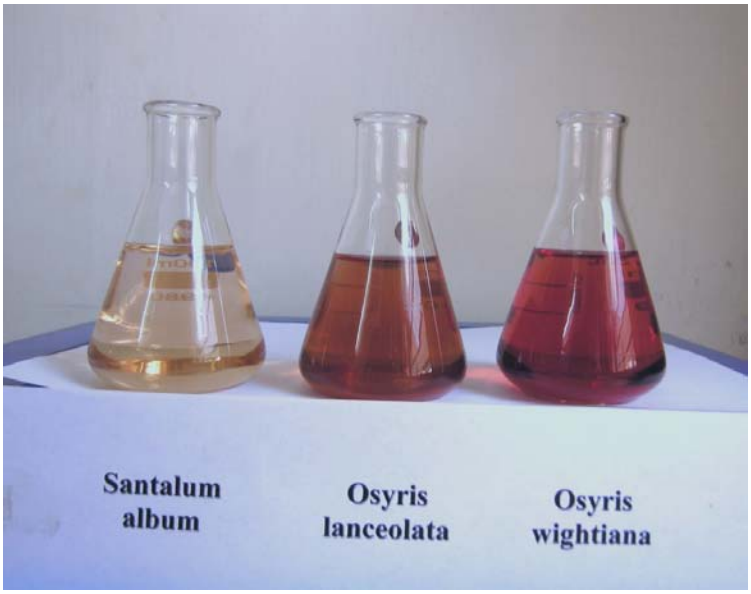

Fig. 3. Water extracts of *S. album* and *O. lanceolata and O. wightiana*

is attributable to the extractives contained in the heartwood. The difference becomes very obvious when samples of these woods are boiled in water for 30 to 60 minutes. While the extract of S. *album* is pale yellowish without any traces of red, that of *O. lanceolata* is reddish. Obviously, the difference in colour is due to type of water soluble extractives

present in the woods. The extracts did not show any marked difference in pH. The difference in colour of the extract can be used as a very simple physical test for distinguishing woods of *S. album* and *O. lanceolata.*

Chemical analysis

The extracted oil of *O. lanceolata* has a faint reddish hue as compared to *S. album* which is yellowish without any trace of red colour. The difference in colour can be used as a very simple physical test for distinguishing the species.

Difference in oil yield

There was marked difference between *S. album* and *O. lanceolata* in the quantity of oil produced; the former had invariably higher oil content (Table 2). Also, the oil yield from different samples of S. *album* varied to a great extent. The observed difference could be due to several factors including environmental, genetic and tree age. It has been found that the proportion of heartwood has direct influence on oil yield (Iyengar, 1968; Coppen, 1995). They also noted that the oil content is higher in basal portion of the tree which decreases towards the top. Similar observations were also made by Shankaranarayana and Parthasarathi (1986). The commercial value of sandalwood depends on its heartwood oil content and the quantity of heartwood per tree.

Table 2. Variation in quantity of oil produced from *S. album* and *O. lanceolata*

Species	Sample no.	Oil quantity (%)
S.album	1	5.73
	2	6.07
	3	8.16
	4	7.62
	5	6.03
O. lanceolata	1	1.88
	2	3.37
	3	1.15

Chemical constituents

The chromatograms obtained from the extracted oils of *S. album* and *O. lanceolata* are shown in Figs. 4 and 5, respectively. The chromatogram of *S. album* (Fig. 4) was nearly

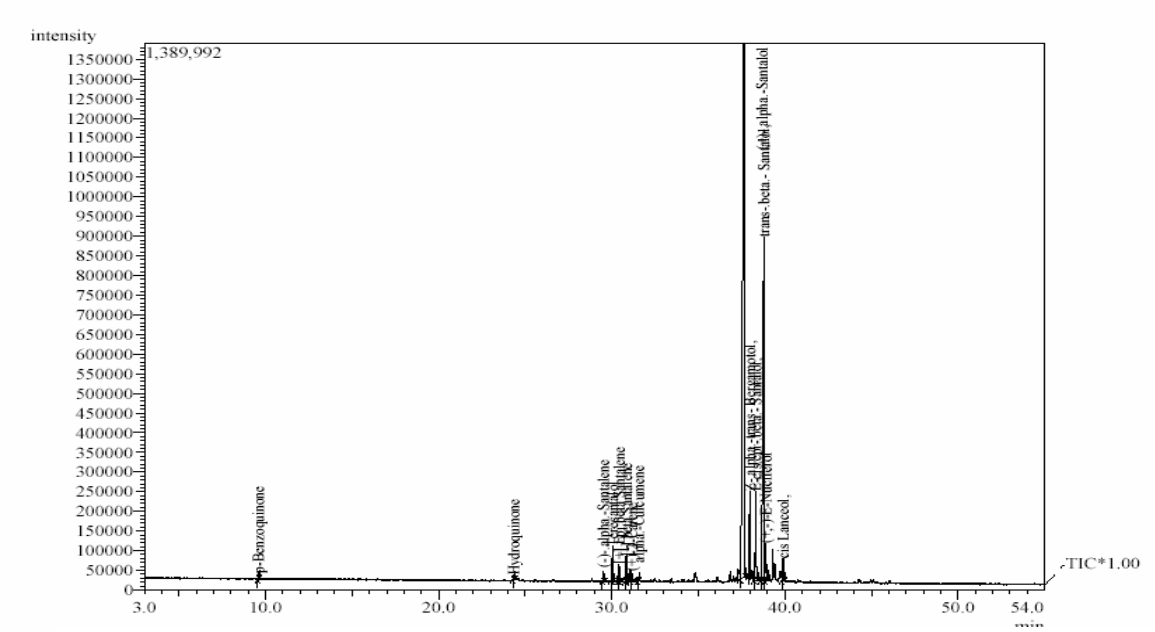

Fig.4. GC-MS total ion chromatogram of *S. album*

Peak identification: 1. p-Benzoquinone; 2. Hydroquinone; 3. α-Santalene; 4. Teresantalol; 5. Epi-β-Sntalene; 6. β-Santalene; 7. 2-Carene; 8. α-Curcumene; 9. α-Santalol; 10. Z-α-trans-Bergamotol; 11. E-cis,epi-β-Santalol; 12. β-Santalol; 13. E-Nuciferol; 14. trans-β-Santalol; 15.cis Lanceol.

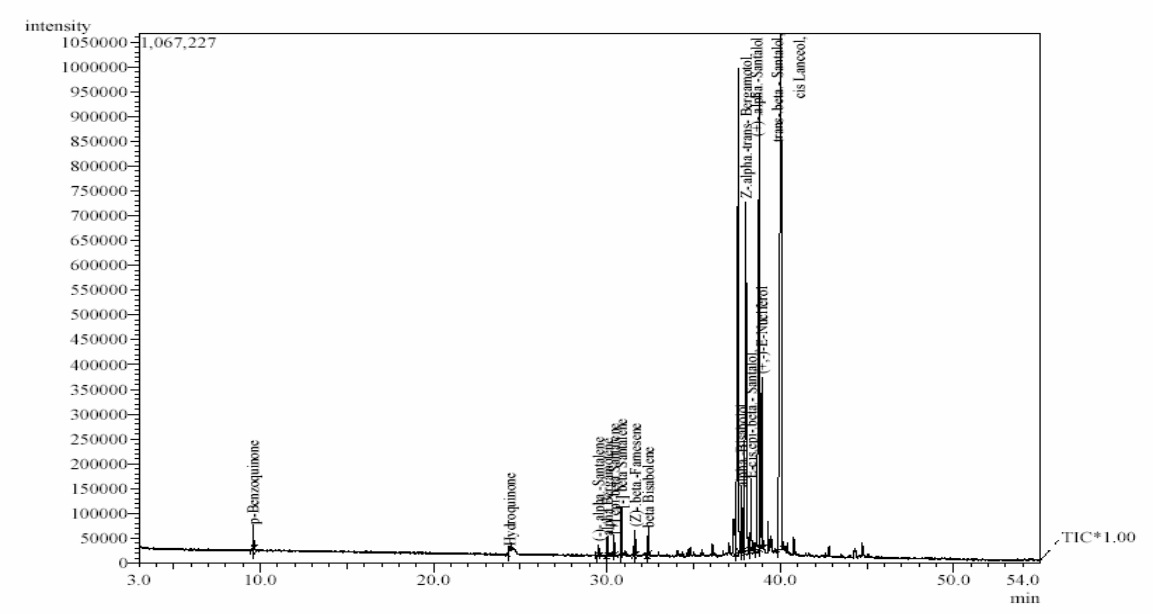

Fig.5. GC-MS total ion chromatogram of *O. lanceolata*
Peak identification: 1. p-Benzoquinone; 2. Hydroquinone; 3. α-Santalene; 4. α-Bergamotene; 5. Epi-β-Santalene; 6. β-Santalene; 7. (Z)-β-Farnesene; 8. β–Bisabolene; 9. α-Santalol; 10. α-Bisabolol; 11. Z-α-trans-Bergamotol; 12. E-cis,epi-β-Santalol; 13. β-Santalol; 13. E-Nuciferol; 14. cis Lanceol.

identical with that published by Howes *et al*. (2004). The different compounds identified in the chromatogram of *O. lanceolata* were in consonance with that reported by Mwang'ingo

et al. (2003), except for a few compounds. It is seen from the chromatograms that, certain constituents were common to *S. album* and *O. lanceolata* while some were seen only in *O. lanceolata* and few were observed in *S. album* alone. The constituents present in both species with varying concentrations were para Benzoquinone, α-Santalene, Epi-β-Santalene, β-Santalene, α-Santalol, Z-α-trans-Bergamotol, E-cis,epi-β-Santalol, β-Santalol, E-Nuciferol and cis Lanceol. The constituents α-Bergamotene, (Z)-β-Farnesene, β–Bisabolene, α-Bisabolol, Z-α-trans-Bergamotol were present only in *O. lanceolata* while 2-Carene, α-Curcumene, Teresantalol and trans-β-santalol were found only in *S. album*. Altogether 19 constituents were identified which are listed in Table 3.

Table 3. Compounds in the oils isolated from *S. album* and *O. lanceolata*.

Compounds	Retent ion time	Per cent composition							
		S. album (samples)					*O. lanceolata* (samples)		
		1	2	3	4	5	1	2	3
p-Benzoquinone	9.59	0.30	0.40	0.17	0.28	0.30	0.54	0.39	2.40
Hydroquinone	24.40	---	---	---	0.21	---	0.34	0.27	2.23
α -Santalene	29.51	1.26	0.54	0.74	0.49	0.17	0.25	0.23	---
α-Bergamotene	30.03	---	---	---	---	---	0.51	---	---
Teresantalol	30.04	1.23	1.37	2.84	1.64	0.93	---	---	---
Epi-β-Santalene	30.42	1.33	0.69	1.15	0.87	0.31	0.45	0.40	---
β-Santalene	30.84	1.93	0.89	1.51	1.27	0.50	1.15	0.97	---
2-Carene	31.15	0.45	0.60	---	0.46	0.30	---	---	---
α-Curcumene	31.54	0.49	0.61	0.25	0.17	---	---	---	---
(Z)-β-Farnesene	31.63	---	---	---	---	---	0.60	0.52	0.85
β –Bisabolene	32.39	----	---	---	---	---	0.67	0.61	1.12
α-Santalol	37.68	50.73	46.05	52.28	54.15	57.06	24.04	25.06	20.28
α-Bisabolol	37.76	---	---	---	---	---	2.22	1.89	0.46
Z-α-trans-Bergamotol	37.98	2.71	2.15	4.67	4.16	4.05	13.36	12.45	13.12
E-cis,epi-β-Santalol	38.36	7.29	11.17	5.16	4.94	5.15	2.00	2.21	4.66
β-Santalol	38.84	28.14	30.45	26.52	27.60	27.95	20.27	21.31	21.44
E-Nuciferol	38.94	1.84	2.17	1.95	2.42	1.55	5.47	5.49	3.96
trans-β-Santalol	39.32	1.08	1.44	1.21	---	1.29	---	---	---
cis Lanceol	39.99	1.23	1.48	1.56	1.34	0.42	28.14	28.21	29.48

The composition of different constituents was expressed as peak area percent. *S. album* contained relatively lower values for 2-Carene (0.3-0.6%) and α-Curcumene (0.17-0.61%). Teresantalol (0.93-2.84%) and trans-β-santalol (1.08-1.44%) were also detected only in *S. album*.

α-Bergamotene was found only in one sample of *O.lanceolata* (0.51%). Also, (Z)-β-Farnesene (0.52-0.85%) and β–Bisabolene (0.61-1.12%) were detected only in *O.*

lanceolata. (Z)-β-Farnesene is widely distributed in essential oils and is a valuable fixative for perfumes and the typical sandalwood aroma makes it fresher and greener (Kerr, 2000). Bisabolene is known to be important in medicine as an antiulcer active principle compound (Yamahara *et al*., 1992). It is also a component in some insecticides that forms part of the defence response targeted to control insect pests and possibly fungal pathogen attacks (Bohlmann *et al*., 1998). The α-Bisabolol, found only in *O. lanceolata,* constituting 0.46-2.22% of the total oil, forms an important component in a wide range of cosmetic formulations (Madhavan and Andersen, 1999; Tatsu and Noriaki, 1996; Kadir and Barry, 1991). The soothing property of α-Bisabolol has led the oil to be in high demand and have sensitive skin and hair-care applications.

Hydroquinone was found in one sample of *S. album* and in all samples of *O. lanceolata.* p-Benzoquinone was higher in *O. lanceolata* (0.39-2.40%) than in *S. album* (0.17-0.4%). α -Santalene, β-Santalene and Epi-β-Santalene were higher in *S. album* (0.17-1.26%, 0.50-1.93% and 0.31-1.33%) compared to *O. lanceolata* (0.23-0.25%, 0.97-1.15% and 0.40-0.45%). Santalenes could not be detected in one sample of *O. lanceolata.*

Quantification of different constituents in *S.album* and *O. lanceolata* showed considerable difference in the composition of α-Santalol, β-santalol, E-cis,epi-β-Santalol, Z-α-trans-Bergamotol, E-Nuciferol and cis Lanceol. The quantity variation of the above constituents in both species is shown in Fig. 6.

Of the compounds, α-Santalol forms the highest proportion in *S. album* (46.05-57.06%), almost two times higher than that in *O.lanceolata* (20.28-25.06%). β-Santalol (26.52-30.45%) and E-cis,epi-β-Santalol (4.94-11.17%) content were found to be higher in *S. album* while in *O. lanceolata,* the contents ranged from 20.27-21.44% and 2.0-4.66%, respectively. The samples of *S. album* met with the ISO/FDIS 3518 (2002) specifications for both α- and β-santalols. The oils of *O. lanceolata* had lower α-Santalol level than that allowed by the standard for *S. album* (ISO, 2002). Verghese *et al.* (1990) examined santalol content in sandalwood oil and met with a range of 40-55% for α-Santalol and 17-27% for β-santalol. Santalol per cent has been often used to represent the quality of sandalwood. Santalol is a sesquiterpene alcohol which adds to the fine woody notes of East Indian

sandalwood, and is used in perfumery. α-santalol is however somewhat weak and cedarwoody. The distinguished perfumer Arcadi Boix Camps (2000) regards that the material has a weaker, less floral and more resinous odour. β-santalol is considered the finer sandalwood odoured material, which Arcadi Boix Camps (2000) considers is floral-radiant. Brunke (1983) describes β-santalol as the santalol isomer having greater olfactory significance, or superior fine woody note.

Per cent composition of Z-α-trans-Bergamotol, E-Nuciferol and cis Lanceol was found to be much higher in *O. lanceolata*, and was 12.45-13.36%, 3.96-5.49% and 28.14-29.48%, respectively. But *S. album* was composed of 2.15-4.67% Z-α-trans-Bergamotol, 1.55-2.42% E-Nuciferol and 0.42-1.56% cis Lanceol. Essential oil of *S. album* contained only relatively lower levels of cis Lanceol while *O. lanceolata* contained a large amount of cis Lanceol. Z-α-trans-Bergamotol possessing a strong milky oriental aroma and which adds the fatty-nutty and milky odour aspects of the sandalwood oil (Brunke and Schmaus 1995). Natural volatile wood oil E-Nuciferol is a terpenic natural perfumery, which is also one of the five

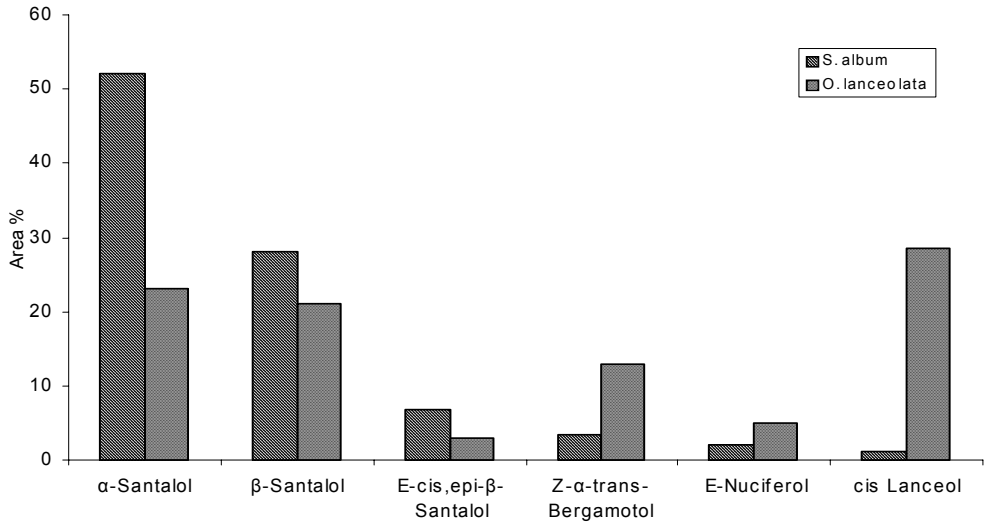

Fig. 6. Mean value of percentage composition of α-Santalol, β-santalol, E-cis,epi-β-Santalol, Z-α-trans-Bergamotol, E-Nuciferol and cis Lanceol (constituents with considerable variation) in *S.album* and *O. lanceolata*

major sesquiterpenic alcohol, generally found in *Santalum spicatum* oil (Piggot *et al*., 1997). cis Lanceol is a smooth somewhat sweet creamy woody note, much less crude and more pleasant.

The composition estimates of different constituents showed that this is appropriate for detecting the quality of *S. album* and *O. lanceolata*. This study has shown that the chemical profile obtained using GC-MS analysis, and in particular the constituent levels in both species, is valuable in assisting the quality variation of *S. album* and *O. lanceolata* and hence the species identification. Two major constituents in *S. album* were α- and β-santalol which is the prime determinant in the quality of sandalwood oil. GC-MS profiles may provide information regarding the species of origin; for example, preliminary investigations in this study suggest that the oils of *S. album* comprised of 46.05-57.06% and 0.42-1.56% α-Santalol and cis Lanceol respectively, but the oils of *O.lanceolata* contained 24.04-25.06% α-Santalol and 28.14-28.21% cis Lanceol.

DNA Studies

Dry heartwood samples of *S. album* as well as of *Osyris* species did not give precise DNA amplification at the expected amplification product range. This may be probably because of the degraded DNA found in the non-living tissues of the heartwood. Since, leaf samples and fresh wood samples of *O. lanceolata* were unavailable, molecular studies were done using samples from sandal and *O. wightiana*. All the leaf samples gave amplifications at 1.7 kbps for 18S rDNA and at 3.3 kbps for 26S rDNA in respect of sandal and *O. wightiana.* However, DNA from heartwood samples less than 6 months and sapwood samples less than one year after felling the trees only amplified.

Restriction enzyme digestion using *Bam* HI

The PCR amplified products of 18S and 26S rDNA from sandal and *O. wightiana* leaf samples were digested with restriction endonuclease *Bam*HI to figure out the species variation between *S. album* and *O. wightiana*. Fig. 7 shows the restriction patterns, distinct for *S. album* and *Osyris.* There is one restriction site for *Bam* HI in both the species for 18S rDNA. Both the species gave two bands each, the smaller fragment of *Osyris* is 465 bp in size while the comparable size of sandal is 492 bp. This size difference, as seen in Fig.7 (a) can be used to distinguish *S. album* from *Osyris*. The difference in restriction pattern is also

seen in 26S rDNA fragments. There are two restriction sites in both the species that are expected to give three fragments each for each species. Fig.7(b) shows 3 fragments for each of the species and an artifact for sandal. The distinguishing bands are 680 bp and 1340 bp fragments of *S. album* and 668 bp and 1412 bp fragments of *Osyris.* The RFLP polymorphism visible as difference in band position of the two species is the distinguishing feature.

Based on the above findings, it is presumed that sandal can be distinguished from *Osyris* based on RFLP pattern of the two rRNA genes of the two species.

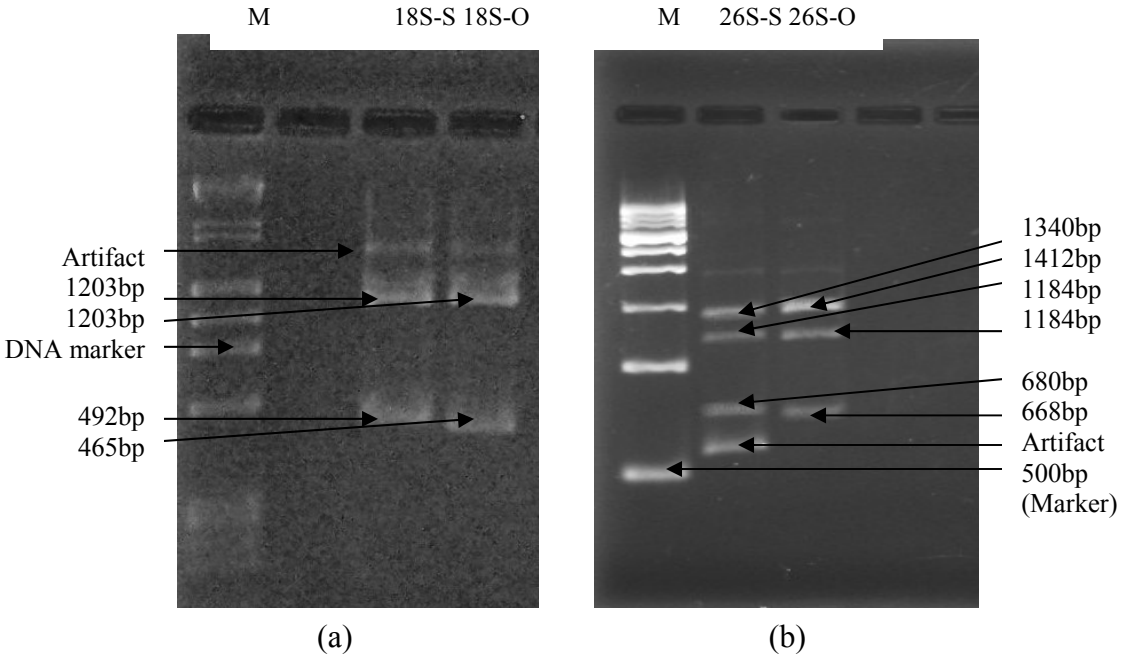

Fig. 7. Restriction digestion pattern of 18S (a) and 26S rDNA (b) obtained using *Bam* HI. 18S-S and 26S-S: *Santalum album*; 18S-O and 26S-O:*Osyris wightiana.* Note the difference in restriction profiles

Sequencing of PCR amplified rDNA genes

The PCR amplified products of 18S and 26S ribosomal DNA from sandal and *Osyris* leaf samples were sequenced to bring out the species variation between them. Amplified rDNA products characterized by a single band were subjected to sequencing at MWG Biotech (Bangalore) and the sequence data were further analyzed. The 18S ribosomal DNA of *S. album* was found to contain 1695 nucleotides and that of *O. wightiana* 1668 nucleotides. Similarly, the 26S ribosomal DNA of *S. album* was 3204 nucleotides long and that of *O.*

wightiana was 3264 nucleotides long. The 18S and 26S rDNA sequences of *S. album* and *O. wightiana* were further subjected to sequence similarity search using NCBI-BLAST. The nucleotide sequences of 18S rDNA genes of Marayur origin sandal and Munnar origin *O. wightiana* were compared with sequences of sandal and *O. lanceolata* available in the NCBI website.

Table 4. 18S and 26S rDNA NCBI-BLAST Sequence Identity Values

	Species	18S rDNA	
		Santalum album (NCBI accession Number = L24416)	*Osyris lanceolata* (NCBI accession number = U42803)
18S rDNA	*Santalum album* (Marayur)	99%	98%
	Osyris wightiana (Munnar)	98%	99%
		26S rDNA	
		Osyris lanceolata (NCBI accession number = AF389274)	
26S rDNA	*Santalum album* (Marayur)	97%	
	Osyris wightiana (Munnar)	98%	

18S rDNA sequence *O. lanceolata* deposited in the NCBI nucleotide library showed 99% similarity to the 18S rDNA sequence of *O. wightiana* from Munnar further supporting the report that *O. wightiana* and *O. lanceolata* could be synonyms; likewise, 98% sequence identity was revealed between the 18S rDNA sequence of *S. album* from Marayur and that of *O. lanceolata* sequence deposited in NCBI. Comparison of the nucleotide sequence similarity between sandal from Marayur and NCBI accession showed 99% identity for 18S rDNA.

Similarly the 26S rDNA sequence of *O. lanceolata* deposited in the NCBI nucleotide library showed 98% sequence similarity to the 26S rDNA sequence of *O. wightiana* from Munnar; also 97% sequence identity to the 26S rDNA sequence of *S. album* from Marayur.

Sequence comparison of Marayur sandal and Munnar *O. wightiana* through multiple sequence alignment using CLUSTAL W package showed a similarity score of 98 for 18S rDNA and 92 for 26S rDNA thus identifying dissimilar regions between the two. The

nucleotide sequence data further confirm the difference between the genome of *S. album* and *O. lanceolata* (*=O. wightiana*).

The study showed that RFLP DNA finger printing and/or nucleotide sequence data of 18S rDNA and 26S rDNA genes can be used as molecular tools to distinguish fresh wood pieces and leaf tissues of *S. album* and *O. lanceolata (=O. wightiana)*.

Conclusions

The study undertaken to elucidate the means and criteria to differentiate the woods of *Santalum album* and *Osyris lanceolata* showed the possibility of distinguishing the woods reliably on the basis of anatomical structure, colour of the hot water extract, chemical constituents of oil (mainly santalol content), and DNA fingerprinting.

Acknowledgements

We are grateful to Dr. J. K. Sharma, former Director and Dr. R. Gnanaharan, Director, KFRI for their encouragement and support. Thanks are due to Ms. M. C. Anisha, Ms. C. Anupama and Ms. K. Sheena for their efficient work as Project Fellows in the project and also to Mr. K. Renjithkumar, worked for a brief period as Project Fellow in the project. We are also grateful to Dr. Jose Kallarackal , Dr. K. M. Bhat and Dr. T. K. Dhamodaran for the editorial scrutiny of the draft report.

REFERENCES

Arcadi Boix Camps 2000. Perfumery: Techniques in Evolution. Allured Publishing Corp., Carol Stream, USA 7 p.

Bohlmann, J., Crock, J., Jetter, R. and Croteau, R. 1988. Terpenoid-based defences in conifers: cDNA cloning, characterization, and functional expression of wound inducible (E)-alpha-bisabolene synthase from grand fir (*Abies grandis*). Proceedings of the National Academy of Sciences, 95: 6756-6761.

Brunke E.J. and Schmaus,G. 1995. New active odour constituents in Sandalwood Oil: part 2: Isolation, structural elucidation and synthesis of nor-α-trans-bergamotenone. Dragoco Report 6/1995 pp 245-257.

Brunke E.J. 1983. Woody Aroma Chemicals Dragoco Report 6/1983: 146 p

CES, 2006. Centre for Ecology Law, and Policy, Environment Department, The University of York, UK. www.york.ac.uk/res/celp/

Coppen, J. J. W. 1995. Flavors and Fragrances of Plant Origin. Food and Agriculture Organization of the United Nations, Rome, Italy. 101p.

Howes, M. R., Simmonds, S. J. M. and Kite, G. C. 2004. Evaluation of the quality of sandalwood essential oils by gas chromatography-mass spectrometry. Journal of Chromatography A 1028: 307-312.

International Standard ISO/FDIS 3518. 2002. Oil of sandalwood (*Santalum album* L.). ISO, Gneva (Final Draft).

Iyengar, A. V. V. 1968. The East Indian sandalwood oil. Indian For. 94:57-68

Kadir R. and Barry, B.W., 1991. Alpha-bisabolol, a possible safe penetration enhancer for dermal and transdermal therapeutics. International Journal of Pharmaceutics 70 (1-2): 87-94.

Kerr J., 2000. Essential Oil Profile – Australian Sandalwood Oil. Aromatherapy Today 15: 8-12.

Kulkarni, H.D. 1995. Sandal (*Santalum album* L.) descriptor. in R. A. Srimathi, H. D. Kulkarni and V. R. Venkatesan (eds.) Recent Advances in Research and Management of Sandal (*Santalum album* L.) in India. Associated Publishing Co., New Delhi. pp. 249-259

Madhavan, B.N. and Andersen, F.A. 1999. Final Report on the Safety Assessment of Bisabolol. International Journal of Toxicology, 18 (3): 33-40.

Metcalfe, C. R. and Chalk, L. 1950. Anatomy of the Dicotyledons. Vol. II. Clarendon Press, Oxford.

Mwang'ingo, P. L., Teklehaimanot, Z., Hall, J. B. and Lulandala, L. L. L. 2003. African sandalwood (*Osyris lanceolata*): Resource assessment and quality variation among populations in Tanzania. Southern Afr. For. J. No. 199: 77-88

O'Brien, T. P., Feder, N. and McCully, M. E. 1964. Polychromatic staining of plant cell wall by toluidine blue 'O'. Protoplasma 59:367-373

Pearson, R. S. and Brown, H. P. 1932. Commercial Timbers of India. Vol. II. Central Publication Branch, Calcutta

Piggott, M.J., Ghisalberti, E.L. and Trengove, R.D. 1997. Western Australian sandalwood oil: Extraction by different techniques and variations of the major components in different sections of a single tree. Flavour and Fragrance Journal 12(1): 43-46.

Rai, S.N. and Sarma, C.R., 1990. Depleting sandalwood production and rising prices. Indian Forester, 116: 348-355.

Rao, R.V., Hemavathi, T.R., Sujatha, M., Luxmi Chauhan and Raturi R.D. (1998). Stemwood and rootwood anatomy of *Santalum album* L. and the problem of wood adultration in Radomiljac, H. S. Ananthapadmanabha, R. M. Welbourm and K. S. Rao (eds.)Sandal and its Products : ACIAR Proceedings, 84: 93-102.

Ruffo, C.K, Birnie, A. and Tengnas, B. 2002. Edible wild plants of Tanzania. Region Land Management Unit Technical Handbook series 27. RELMA/(SIDA), Nairobi, Kenya. 766 p.

Shankaranarayana, K.H. and Parthasarathi, K. 1986. On the content and composition of oil from heartwood at different levels in Sandal. Indian Perfumer, 31: 211-214.

Sha Zhen Shu 2003. Osyris Linnaeus. Flora of China 5: 211.

Srinivasan, V.V., Sivaramakrishnan, V.R., Rangaswamy, C.R., Ananthapadmanabha, H.S. and Sankaranarayana, K.H., 1992. Sandal (*Santalum album*.). Indian Council of Forestry Research and Education, Dehra Dun, India. 233p.

Tatsu, M. and Noriaki, K. 1996. Skin cosmetics containing hydrogenated bisabolol. Kanebo Ltd, Japan.

Verghese, J., Sunny, T. P. and Balakrishnan. K.V. 1990. (+)– alpha Santalol and (-)-beta Santalol (Z) concentration, a new quality determinant of east Indian sandalwood oil. Flavour and Fragrance Journal, 5(4): 223-226.

Walker, H. 1966. The market for sandalwood oil. Tropical Product Institute, Ministry of Overseas Development. London. 13p.

Yamahara, J., Hatekeyama, S., Tanigushi, K., Kawamura, M., Yoshikawa, M. and Yakugaku, Z., 1992. Stomachic principles in ginger 2. Pungent and anti-ulcer effects of low polar constituents isolated from ginger, the dried rhizomes of *Zingiber-officinale* Roscoe cultivated in Taiwan-the absolute stereostructure of a new diarylheptanoid. Journal of the Pharmaceutical Society of Japan, 112: 645-655.