

Full Length Research Paper

Agarwood resin production and resin quality of *Gyrinops walla* Gaertn

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Agarwood is a resin produced by certain species of family Thymalaeaceae due to a self-defence mechanism. Most species of *Aquilaria* and a few species of *Gyrinops*, *Aetoxylon* and *Gonystylus* are capable of producing agarwood. *Gyrinops walla*, a member of the family Thymalaeaceae is recorded only in the wet zone of Sri Lanka and very rarely in southwest India, has not been previously studied to identify its ability of producing agarwood. Therefore the present study was the first ever to conduct and identify the production of agarwood in *G. walla* and the quality of its resins. Six *G. walla* trees growing in two distinctive areas of the wet zone of Sri Lanka were used for the present study. All six trees had natural wounds occurred sometime before the sample collection due to abrasions or fallen branches. The dark coloured tissues of the affected areas were carefully collected without cutting the trees and resins were extracted by solvent extraction method. The extracted resins were analysed using gas chromatography to identify the different compounds. Finally these compounds were compared with that of selected *Aquilaria* species. The results revealed a strong similarity of resin compounds of *G. walla* with that of *Aquilaria* species which are commercially used to collect agarwood. Further studies should be conducted to identify the effects of artificial resin induction methods on *G. walla* that are already used on *Aquilaria* species.

Key words: *Gyrinops walla*, *Aquilaria*, agarwood, retention indices.

INTRODUCTION

Agarwood, a highly valuable and fragrant resin, is used as incense for religious ceremonies, perfumes in the Arab world, ornamental materials and medicinal components in oriental medicine (Chen et al., 2011). This resin impregnated woody tissues produced in the heartwood area are mainly found in certain species of *Aquilaria* which has been a highly priced commodity for more than 2000 years (Nor Azah et al., 2008). It mainly comes from the damage caused to healthy trunks or branches of the trees of those *Aquilaria* species in the family Thymalaeaceae by mold. In a natural environment, it often takes several years for a wild damaged *Aquilaria* species plant to perform agarwood (Gerard, 2007). *Aquilaria* is an evergreen tree that grows up to 40 m high and 60 cm in diameter. Leaves are 5 to 9 cm long and

oblong lanceolate in shape. It bears white flowers that are sweetly scented. In addition to *Aquilaria*, agarwood products have also been recorded from species of the closely related genus *Gyrinops* (Eurlings and Gravendeel, 2005) and more distantly related *Aetoxylon* and *Gonystylus* (Airy, 1954; Compton and Zich, 2002; Blanchette, 2003). *Aquilaria* and *Gyrinops* belong to sub-family Aquilarioideae (Domke, 1934) and are separated by the number of stamens only. Species of the genus *Gyrinops* are grown as trees or shrubs. Leaves of these species are alternate and coriaceous. Its inflorescence is sessile or shortly pedunculate, terminal or axillary, of fascicles or few-flowered. Flowers are pentamerous and hermaphrodite. The pedicels articulate at the base (Dassanayake and Fosberg, 1981).

From the recorded eight species, *Gyrinops ledermanii* and *G. versteegii* are known to produce agarwood resins. In addition to that, agarwood resin production has been recorded in *Gonystylus macrophyllus*, *G. bancanus* (Compton and Zich, 2002) and *Aetoxylon sympetalum*

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(Airy, 1954).

Hou (1960) refers to 12 species of *Aquilaria* and 8 species of *Gyrinops* while Mabberly (1997) refers to 15 species of *Aquilaria*. According to Nor Azah et al. (2008), throughout the countries where it is distributed, there are 25 species of *Aquilaria* and out of that, 15 species are reported to form agarwood. However, according to Ng et al. (1997), nine species out of 15 recorded *Aquilaria* species, that is, *A. beccariana*, *A. crassna*, *A. filaria*, *A. hirta*, *A. khasiana*, *A. malaccensis*, *A. microcarpa*, *A. rostrata*, and *A. sinensis* produce agarwood (Compton and Zich, 2002). *Aquilaria* species naturally distribute in Asia from northern India to Vietnam and Indonesia (Blanchette and van Beek, 2005). According to Gunn et al. (2003), *Aquilaria* and *Gyrinops*, the two important agar producing genera are normally distributed in at least 12 countries: Bangladesh, Bhutan, Cambodia, India, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Thailand, Vietnam and Papua New Guinea. However, China also has a few *Aquilaria* species and *A. sinensis* is the dominant species among them (Zhang et al., 2010).

Aquilaria species have not been recorded in Sri Lanka and *G. walla* is the only member of the genus *Gyrinops* present in the country. It is a medium tall tree which grows up to 15 m in high with straight, slender trunk with a small, rounded crown. The bark is thin, smooth and strongly fibrous and brownish-grey in colour (Dassanayake and Fosberg, 1981). According to Dassanayake and Fosberg (1981) other than in Sri Lanka, *G. walla* had been recorded only in the extreme southwest of India. However, it appeared to be very rare in India and there are no documentary evidences found to prove its presence. Therefore most probably *G. walla* may present only in the wet zone of Sri Lanka. Although in his study, Hou (1960) mentioned about the distribution of *G. walla* in Sri Lanka, its agarwood producing ability was not recorded in the literature in the past. Therefore the objective of the present study was to identify the resin production ability of *G. walla* and the composition of agarwood resin. In order to achieve this objective, it was expected to compare the analysed compounds of the resins extracted from sampled *G. walla* with that of selected *Aquilaria* species.

Studies on the chemistry of agarwood have reported presence of sesquiterpenes, chromone derivatives, sesquiterpene furanoids, tetradecanoic acid and pentadecanoic acid (Djerassi et al., 1993; Ishihara et al., 1991; Ng et al., 1997; Tamuli et al., 2005). The heartwood of *Aquilaria* is fine, black or brown in colour and fragrant. The sesquiterpenoids and chromone derivatives are the main source of agarwood's particular aroma (Prachakul, 1989; Takemoto et al., 2008). However, agarwood resins vary in their composition and some resins contain a large amount of sesquiterpene compounds and others contain principally benzylacetone (Yang et al., 1989). The plant synthesises these aromatic terpenes when it is injured by insects, physical cuts,

bacterial infections and chemical simulations (Poain and Poain, 2001; Bunyapraphatsara and Chokchajareonporn, 1996).

The common methods used to induce agarwood make the deliberate wounding of trees with large knives and the hammering of nails into tree trunks. A chemical method has also been developed recently (Zhang et al., 2010).

METHODOLOGY

Sampling and data collection

Two distinctive areas of the Western Province of the low country wet zone of Sri Lanka were selected for sample collection. These two areas, named Labugama and Yagirala are located in Colombo and Kalutara Administrative districts respectively. Both sample locations are bordered to tropical wet evergreen natural forests.

Resin induction studies by using artificial methods have not been previously conducted for *G. walla*. Therefore samples were extracted from the trees which produced resins due to natural injuries such as abrasions or fallen branches. Among the large number of trees observed for the present study, agarwood resin formation was found only in a few due to the very low occurrence of natural injuries to the trees. Due to this reason, three trees were selected from each area and the amount of resinous tissues that could be collected was very low. The details of the selected two areas, the locations of trees and their sizes are given in Table 1.

In addition to that, authentic samples of *Aquilaria crassna* agarwood oil were obtained from Wescorp Agarwood Ltd. (Wescorp Group, WA, Australia) to compare the resin compounds of *G. walla*.

Resin extraction

The stem colour of *G. walla* is off white to pale yellow. However, the tissues which produced agarwood resins due to injuries become dark in colour. Those tissues can be seen when the wounds of the stem or the branches are closely observed. Figure 1 shows the cell structure and agarwood resins formed in the tree stem. The wounds of the trees occurred on the main stem from the ground level to 2 m were observed to collect the samples for the present study. Due to the low availability of the resinous areas, it was careful to extract the dark coloured tissues using a sharp chisel and a hammer without felling the trees. Collected tissues with resins were size reduced manually using a sharp edge cutter. Sample of 1 g equivalent was placed in a scintillation glass vial and 10 ml of dichloromethane was added. Extract was collected after 12 h. This process was repeated up to three times.

Table 1. Location details and sizes of the six trees used for the present study.

| Location | Divisional secretariat | District | Tree | Dbh cm | Height m | Latitude | Longitude |
|----------|------------------------|----------|------|--------|----------|-----------------|------------------|
| Labugama | Seethawaka | Colombo | L1 | 12.0 | 8.0 | 6° 48' 58.496'' | 80° 10' 25.352'' |
| | | | L2 | 21.5 | 12.0 | 6° 48' 28.699'' | 80° 10' 24.917'' |
| | | | L3 | 30.0 | 20.0 | 6° 48' 26.161'' | 80° 10' 26.612'' |
| Yagirala | Walallawita | Kalutara | Y4 | 22.5 | 10.5 | 6° 21' 40.064'' | 80° 10' 41.732'' |
| | | | Y5 | 15.0 | 7.5 | 6° 21' 39.967'' | 80° 10' 41.830'' |
| | | | Y6 | 8.5 | 8.0 | 6° 21' 40.259'' | 80° 10' 42.708'' |

dbh = diameter at breast height, that is, 1.3 m above the ground.



Figure 1. Photomicrograph of the agarwood (white colour areas) produced tree stem. Source: <http://forestpathology.cfans.umn.edu/agarwoodadd.htm>.

Combined extract was then evaporated using a rotary vacuum evaporator at room temperature and stored away from light until further analysis.

Gas chromatography analysis

A known weight of each extract was dissolved in ethyl acetate to make a 10 mg ml^{-1} solution. $1 \mu\text{l}$ of this solution was then injected to the gas chromatography instrument (GC2010, Shimadzu Scientific, Japan) using an auto

sampler (AOC20i, Shimadzu Scientific, Japan). A 5% phenyl-polysiloxane coated $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$ column (AT-5, Alltech, USA) was used for the separation. Injector chamber was kept at 250°C with helium as carrier gas maintained at a linear velocity of 30 cm sec^{-1} . Oven was programmed to increase from 120 to 250°C at the rate of 5°C min^{-1} and held for 5 min at 250°C . Flame ionisation detector was held at 300°C . Standard alkane series of C8 to C40 (Sigma-Aldrich, USA) was used for the determination of retention indices. Chromatograms and indices obtained from authentic agarwood samples

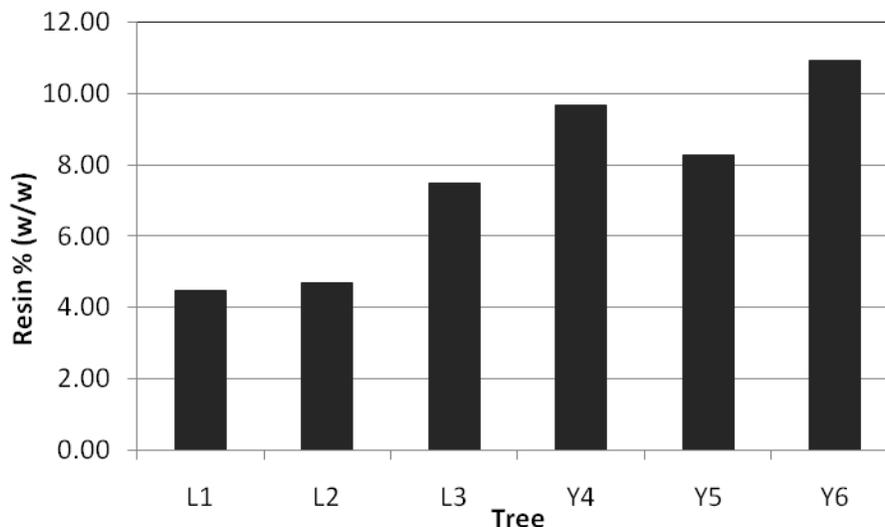


Figure 2. The percentage values (w/w) of resin contents of the selected trees.

Table 2. Comparison of retention indices of *G. walla* and some *Aquilaria* species.

| Compound | <i>G. walla</i> * | <i>A. crassna</i> ¹ | <i>A. crassna</i> ² | <i>A. sinens</i> ³ | <i>A. agallocha</i> ⁴ |
|---------------------------|-------------------|--------------------------------|--------------------------------|-------------------------------|----------------------------------|
| jinkho-eremol | 1641 ^a | 1643 ^a | 1643 | -- | 1650 |
| selina-3,11-diene-9-one | 1689 ^a | 1670 ^b | 1687 | -- | -- |
| selina-3,11-diene-14-al | 1733 ^a | 1737 ^a | 1735 | 1733 | -- |
| 9,11-eremophiladien-8-one | 1741 ^a | 1743 ^a | 1740 | -- | -- |
| guaia-(10),11-dien-15-ol | 1766 ^a | 1791 ^b | 1770 | -- | -- |
| oxo-agarospirol | 1818 ^a | 1828 ^a | 1822 | -- | -- |

1 = Authentic samples of Wescorp Agarwood Limited.; 2 = Wetwitayaklung et al. (2009); 3 = Chen et al. (2011); 4 = Nor Azah et al. (2008); * Values are reported as mean (n=3); Means with different superscript letters are significantly different (p<0.05).

of different *Aquilaria* species (Nor Azah et al., 2008; Wetwitayaklung et al., 2009; Chen et al., 2011) were used for comparison of the data of the present study.

Moreover, authentic agarwood oil samples obtained from Wescorp Agarwood Ltd. were analysed using gas chromatography mass spectroscopy and compounds were identified and Kovat's retention indices were established for a similar column and instrumental conditions. The compound analysis was triplicate for *G. walla* samples collected for the present study and *A. crassna* authentic samples obtained from Wescorp Agarwood Limited for the purpose of analysing the significance of the compounds statistically.

RESULTS

As shown in Figure 2, the resin contents (w/w) of *G. walla* samples varied from 4.48 to 10.93% with the mean value of 7.59%. Among them, all three samples collected from Yagirala of Kalutara District, that is, sample numbers Y4,

Y5 and Y6 had comparatively higher resin percentages to that of Labugama sample site. However, agarwood resin development pattern in the *G. walla* plant tissues was not identified because artificial resin induction had not been done and only the naturally wounded areas of the selected trees were used for the present study.

Gas chromatography analysis revealed that the resin of *G. walla* contained aroma compounds commonly found in commercially available agarwood (Tables 2 and 3). Sesquiterpenes of guaiane and eudesmane skeleton were also present in *G. walla* resin. Many fatty acids were also found to be common between the authentic and test samples.

The comparison of retention indices of *G. walla* with the authentic agarwood oil samples was found to be corresponding (Table 2). Previously published data on *A. crassna* (Wetwitayaklung et al., 2009), *A. sinensis* (Chen et al., 2011) and *A. agallocha* (Nor Azah et al., 2008) are shown in Table 2. Apart from *A. crassna*, information for all tested compounds of *G. walla* was not found for other *Aquilaria* species in the literature. However, when the

Table 3. Percentage areas of identified compounds of the tested six trees.

| Tree | a | b | c | d | e | f |
|----------------|------|------|------|------|------|------|
| L1 | 0.38 | 0.59 | 7.82 | 1.64 | 2.65 | 1.21 |
| L2 | 0.49 | 0.51 | 7.22 | 1.57 | 1.79 | 0.87 |
| L3 | 0.78 | 4.87 | 5.14 | 0.92 | 1.61 | 0.78 |
| Y4 | 0.78 | 4.15 | 4.11 | 0.86 | 1.42 | 0.80 |
| Y5 | 0.46 | 1.02 | 4.99 | 0.86 | 1.49 | 0.59 |
| Y6 | 0.57 | 2.19 | 3.93 | 2.76 | 1.62 | 1.64 |
| Mean | 0.58 | 2.22 | 5.54 | 1.44 | 1.76 | 0.98 |
| Standard Error | 0.07 | 0.77 | 0.66 | 0.30 | 0.19 | 0.16 |

a = jinkho-eremol; b = selina-3,11-diene-9-one; c = selina-3,11-diene-14-al; d = 9,11-eremophadiene-8-one; e = guaia-(10),11-diene-15-ol; f = oxo-agarospirol. Values are reported as mean (n=3).

significance of the retention indices of *G. walla* resin compounds was statistically tested with authentic *A. crassna* oil samples obtained from Wescorp Agarwood Limited, it was found that all compounds other than selina-3,11-diene-9-one and guaia-(10),11-dien-15-al were not significant (Table 2). Apart from those two compounds, the retention indices of the tested compounds of *G. walla* were similar to that of the tested *Aquilaria* species. This proves a strong similarity of agarwood between *G. walla* and *Aquilaria* species.

The percentage areas of the tested compounds of *G. walla* resulted after gas chromatography analyses are shown in Table 3. The descriptive statistics were also calculated and added to in the same table. Among these compounds, jinkho-eremol had the lowest mean value while selina-3,11-diene-14-al had the highest mean value. The largest standard error values were given by selina-3,11-diene-9-one and selina-3,11-diene-14-al respectively (Table 3). The standard error values of other compounds of the tested samples were comparatively smaller.

DISCUSSION AND CONCLUSION

For the first time, the present study identified the ability of *G. walla* for producing agarwood. In addition to that, the present study also confirmed the quality of agarwood produced by *G. walla* is similar to that of certain *Aquilaria* species available in the market.

In his study on *Aquilaria agallocha*, Gibson (1977) stated that every tree does not produce agarwood. Although not properly estimated, it was also rare to observe the formation of agarwood in all naturally grown *G. walla* trees in the present study. The reason could be that the lack of injuries occurred under the natural conditions.

The sample sizes that could be collected were small and therefore solvent extraction was used for the present study. However, various techniques have been used for

agarwood oil extraction in the past such as hydro-distillation, solvent extraction, and supercritical fluid extraction (Naef, 2011). Each technique has advantages and disadvantages. The classical method that is currently used in commerce for the agarwood oil extraction is hydro-distillation. This method consumes 7 to 10 days and high energy for extraction (Wetwitayaklung et al., 2009). The supercritical fluid carbon dioxide extraction is known as non-flammable, non-toxic, chemically stable and less energy consumption method. It provides some advantages over classical method, since super critical carbon dioxide has low viscosity, high diffusivity, good transport properties and gives faster extraction and high yields (Anklam et al., 1998). Nevertheless, agarwood oil is most frequently extracted by hydro-distillation methods because it is safer to operate and environmentally friendly (Liu et al., 2008).

All six samples tested in this study using gas chromatography analysis have shown similar compounds according the gas chromatographic trace. Majority of these compounds were unidentified due to the lack of references. In future, however, a gas chromatograph coupled with a mass spectrometer will be used to identify the remaining compounds.

According to Yoneda et al. (1984), oxo-agarospirol and jinkoh-eremol are found in all types of agarwood. Even though the presence of these compounds was lower in the analysed *G. walla* samples of the current study, they are important markers in identifying agarwood aroma. These compounds are known to produce characteristic camphor like aroma with woody and floral notes (Ishihara et al., 1991). However, these resins tested were found to lack agarofuran, vetivae sesquiterpenes and chromone derivatives, which are key components of the resin formed in *Aquilaria* species (Naf et al., 1993). More sample analysis is needed to achieve a proper conclusion, however, in this regard.

In order to explain the the reason of significant difference of selina-3,11-diene-9-one and guaia-(10),11-diene-15-ol from that of authentic *A. crassna* samples,

more *G. walla* samples should be examined. However, according to Yang et al. (1989), agarwood oils vary in their composition: some oils contain a large amount of sesquiterpene compounds and others contain principally benzylacetone. A study conducted on agarwood oil by Takemoto et al. (2008) also proved that the constituents of agarwood oil vary between trees. The results of the present study are similar to the aforementioned studies and the resin compounds and the oil contents vary from one tree to the other. According to Takemoto et al. (2008), it is the variation that occurs naturally due to the biological characteristics of trees. Nor Azah et al. (2008) stated that the colour of the oil may also vary depending on the oil extraction method. However, a colour difference was not visually observed for the agarwood resins extracted in the present study. The reason could be the use of single method for extracting oils from all samples.

Takemoto et al. (2008) did an analysis of volatile components of agarwood oils using SPME-GC-MS. Solid phase microextraction (SPME) is suitable for analysing volatile compounds, absorbing compounds in the headspace of the sample vial, and desorbing them directly into GC injection port. In addition to that, Bhuiyan et al. (2009) did the essential oil analysis successfully for *A. agallocha* using the same methodology.

According to the analysis conducted and the comparison of retention indices of the present study, it can be concluded that agarwood resins produced in *G. walla* are similar to that produced by commercially used *Aquilaria* species.

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