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Short communication

Characterisation of agarwood type resin of *Gyrinops walla* Gaertn growing in selected populations in Sri Lanka

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ABSTRACT

Gyrinops walla is the only agarwood producing tree growing in Sri Lanka which is believed to be endemic. Agarwood is valuable resinous heartwood of Thymalaeaceae family. Present study is aimed to identify the characteristics of naturally formed agarwood type resins in *G. walla* stems. Samples were isolated from trees growing three different location in the wet zone of Sri Lanka. Naturally formed resinous part of wood was solvent extracted and analysed by GCMS. Further, the current study has developed an effective GCMS method to analyse agarwood type resin from *G. walla*. Tree diameters and the heights varied in the trees samples, which had no effect on resin formation. Resin contents were not significantly different between three populations although the chemical variations were considerably high. Among the 19 constituents identified by GCMS in the agarwood resins, free fatty acids and isopropyl naphthalene, 2-phenylethyl chromone compounds found to be common for most of the *G. walla* trees tested. Comonly found sesquiterpene compounds from the *G. walla* resin were Jinkhol, γ-eudesmol, valerenol and valerinal. Similar compounds have been reported in resin from *Aquilaria* spp. which is the more established source of agarwood. A future study would experiment on artificial resin induction methods and establishing plantations of *G. walla* to sustain its supply.

1. Introduction

Agarwood is a highly valuable non-timber product found as a dark fragrant resin in the stem, branches and roots of certain species of genera *Aquilaria*, *Gyrinops* (Borris et al., 1988; Eurlings et al., 2010; Ito and Honda, 2005), *Aetoxylon* and *Gonystylus* (Blanchette, 2003; Shaw, 1954; Compton and Zich, 2002) of family Thymalaeaceae. This resin impregnated woody tissues produced in the heartwood area are widely used in traditional medicines, incense and perfume in the Middle East and South and Far East Asia (van Beek and Phillips, 1999; Persoon, 2007; Persoon and Van Beek, 2008). However, agarwood is not naturally produced in the trees and it is hypothesised to be a product of a plant defence response to external damages (Zhang et al., 2012; Xu et al., 2013; Okudera and Ito, 2009; Yagura et al., 2005) and specifically fungal infections stimulating natural agarwood formation has been documented in the past (Tamuli et al., 2005; Mohamed et al., 2014). When certain species of the above mentioned genera are wounded, damage signals are induced and transmitted activating a defence response, after which the chemical substances such as sesquiterpenes and phenylethyl chromones are produced. Then agarwood is formed when these products combine with the wood tissue to avoid damage expansion. The aroma of agarwood comes from a complex mixture of many volatile sesquiterpene compounds, mainly classified as agarofurans, cadinanes, eudesmanes, valencanes, eremophilanes, guaianes, prezizanes and vetispiranes. In addition, the resin also contains number of 2-phenylethyl chromone derivates (Chen et al., 2012; Naef, 2011). It is also reported that agarwood varies in aroma properties and resin character based on the species and geographical distribution (Espinoza et al., 2014; Nor Azah et al., 2008; Chang et al., 1997).

Gyrinops walla locally known as "Walla Patta" is endemic to Sri Lanka, despite of the rare records of its presence in extreme south west India (Dassanayake and Fosberg, 1981; Gunatilleke et al., 2014). It is the only species found in Sri Lanka which produces agarwood type resins. The illegal harvesting of *G. walla* was frequently reported in Sri Lanka during the past years, though the authorities have taken necessary steps to restrain this situation. The ability of *G. walla* in producing agarwood was explored for the first time

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through our previous research (Subasinghe et al., 2012; Subasinghe and Hettiarachchi, 2013). These findings confirmed that *G. walla* is producing agarwood type resins which are chemically similar to that of *Aquilaria* species.

Despite the commercial and biodiversity significance, only limited research has been conducted agarwood type resins of *G. walla* (Subasinghe and Hettiarachchi, 2013; Subasinghe et al., 2012). Constituents of *G. walla* leaves and stem have been studied and reported previously (Schun et al., 1986).

Our previous studies on *G. walla* were conducted only on single specimens to confirm the presence of agarwood type resin. The present study is focused on studying the variations of naturally formed agarwood resin and its composition in *G. walla* stems sampled from different locations in Sri Lanka. Therefore the populations were selected for the present study from three districts of the western province of Sri Lanka where the maximum poaching of *G. walla* has been reported. It is further expected that the findings of this study would help to establish the key characteristics agarwood resins of *G. walla*., If a notable variation is found, then the selected tree locations and the tree size data could be used in identifying factors favourable for natural resin formation. Understanding of these characteristics would lead to develop an agarwood industry using *G. walla*, thus the local communities are benefited from a new non-timber crop while preserving the wild populations.

2. Materials and Methods

2.1. Plant material

G. walla is commonly found in the low country wet zone of Sri Lanka where the elevation is below 1000 m and the average annual rainfall is above 2000 mm and average temperature varies from 25 °C to 27.5 °C. Three separate populations of all three districts present in the western province were selected for the present study (Fig. 1). Two samples (I1 and I2) were collected from Ingiriya (Colombo district) bordering to a natural forest at elevation of 100–200 m. Four samples (M1–M4) from Mirigama (Gampaha district) were collected from home gardens at elevation of 60–65 m. Total of ten samples (Y1–Y10) from Yagirala (Kalutara district) were collected bordering to a natural forest at elevation of 75–95 m. Diameter at breast height (dbh) and total height were measured for the sampled trees and the geographic locations were marked for each sampled tree using a GPS (Garmin Montana 650©). All samples were extracted from the main stems of the trees which produced resins as a result of natural injuries at 0.3 m–1.5 m from ground level. Then the dark colour agarwood resinous areas were separated from light coloured stem tissues by the use of a carving chisel.

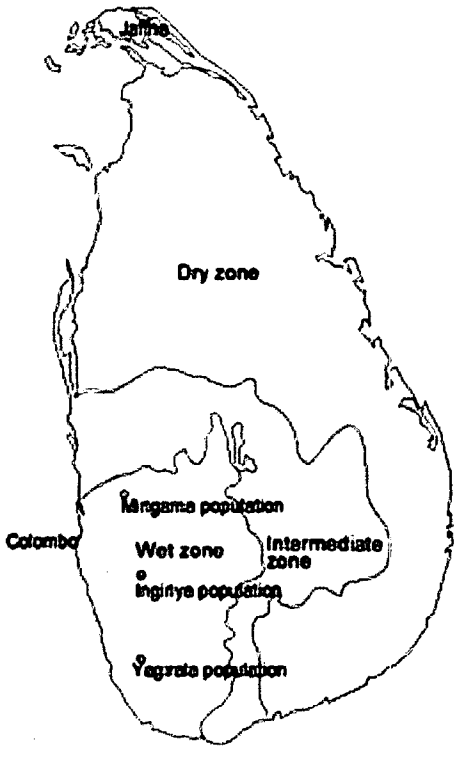

Fig. 1. Locations where the samples collected in Sri Lanka with reference to the climatic zone.

2.2. Resin extraction

Collected tissues with resins were size reduced using a low speed cutter mill (internal temperature <40 °C). Sample of 2 g equivalent was placed in a scintillation glass vial and 20 ml of dichloromethane was added. Extract was collected at every 12 h for 36 h by renewing the solvent. Combined extract was then evaporated under a low pressure stream of nitrogen at room temperature and stored away from light until further analysis.

2.3. Gas chromatography mass spectroscopy analysis

A known weight of each extract was dissolved in dehydrated acetone to make a 100 ng ml^{-1} solution. Internal standards used were l-menthol 100 ng ml^{-1} in dehydrated acetone. Instrument used was HP 6790 GC and HP 5971 (Agilent Technologies, USA). The capillary column used was a 30 m 95% phenylsiloxane with a inner diameter of 0.25 mm and a film thickness of 0.25 μm (AT-5MS, Alltech, USA). The injector was kept at 220 °C and the oven had a 10 °C min^{-1} gradient temperature from 100 °C to 200 °C. Data were processed by Chemstation© software (Agilent Technologies, CA, USA). Compounds were identified by comparing the mass fragmentation patterns with published data and online database library (NIST 5, NIST, USA; Wiley 275, John Wiley and Sons Inc. USA). Retention indices were calculated for the compounds by calibrating the GC column with C8 to C40 alkanes series (Sigma–Aldrich, USA).

3. Results and discussion

3.1. Tree size distribution

Variations of the average dbh and height of the three *G. walla* populations are given in Fig. 1. The dbh of trees sampled in Ingiriya population are of 12.5 cm (I1) and 21.0 cm (I2) while their heights varied from 8.0 m (I1) to 12.5 m (I2). The dbh of Mirigama trees varied from 15.5 cm to 18.0 cm while height varied from 10.0 m to 13.0 m. Variation of dbh of the trees sampled in Yagirala population varied from 15.5 cm to 24.5 cm and their variation of height was 12.0 m to 16.5 m. Although dbh values were not significantly different between the three populations ($F = 2.61$ and $p = 0.107$), heights of the trees in Yagirala were significantly different from that of the other two populations ($F = 8.43$, $p = 0.004$) at 95% probability level.

3.2. Resin content

Percentage yield of dichloromethane extract was considered as the resin content of the resinous wood in this study. Although the slight variation of the colours were observed, significant differences were not identified between the resin contents extracted from the trees growing in three populations ($F = 0.28$, $p = 0.763$). Both the lowest and highest resin contents, i.e., 0.66% (w/w) and 5.80% (w/w)

Table 1
Resin content and composition of identified chemical compounds of *G. walla* samples collected from 16 samples.

Sample number		I1	I2	M1	M2	M3	M4	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	Y10
Resin content (% w/w)		0.87	2.78	0.41	7.68	0.25	0.96	0.66	0.65	0.15	0.66	1.17	9.80	7.09	0.85	1.48	3.12
Compound	RI	Percentage composition (% w/w)															
3-Phenyl butanone	1371	1.79	5.03	*T*	*T*	*T*	*T*	2.55	4.45	*T*	7.85	*T*	3.49	*T*	*T*	*T*	*T*
Azulenone	1681	*T*	*T*	3.2	1.54	6.65	1.47	*T*	*T*	1.06	*T*	1.28	1.09	*T*	1.19	*T*	*T*
Beta-seline	1721	*T*	2.52	*T*	1.14	*T*	*T*	*T*	2.73	*T*	1.23	1.12	*T*	1.95	1.92	*T*	*T*
Isopropyl naphthalene derivative	1736	2.46	4.98	4.05	13.92	4.09	16.39	*T*	22.95	2.78	*T*	2.68	6.81	7.79	24.86	3.47	13.5
Jinkohol	1739	*T*	*T*	*T*	2.4	*T*	2.39	*T*	3.13	*T*	*T*	*T*	1.47	1.25	2.35	*T*	2.37
Agarospirol	1743	*T*	1.94	2.17	4.39	4.04	2.71	*T*	3.97	*T*	*T*	2.38	3.15	3.03	5.07	2.1	4.05
γ-Eudesmol	1758	*T*	*T*	*T*	6.18	*T*	7.77	*T*	10.33	1.15	*T*	*T*	*T*	*T*	11.86	*T*	5.02
Valerenol	1773	*T*	1.47	*T*	*T*	*T*	*T*	*T*	*T*	*T*	*T*	*T*	*T*	3.33	1.02	1.68	1.31
Gamma elemene	1781	*T*	*T*	*T*	1.45	*T*	4.39	*T*	1.68	3.11	*T*	*T*	*T*	1.22	2.06	*T*	1.17
2,2,6,8-Tetramethyl bicyclo undece-7-en-3-ol	1790	*T*	*T*	*T*	*T*	*T*	*T*	*T*	*T*	*T*	*T*	*T*	*T*	9.56	*T*	*T*	*T*
Aromadendrine oxide	1825	1.13	*T*	5.48	1.24	11.42	1.05	*T*	*T*	*T*	1.16	1.92	*T*	*T*	*T*	*T*	*T*
Hexadecanoic acid	1863	2.87	3.27	16.78	7.12	25.21	10.31	2.03	6.25	8.1	12.09	9.5	2.82	4.92	10.08	9.38	7.88
Camphene	1867	*T*	*T*	*T*	*T*	*T*	*T*	*T*	*T*	*T*	1.01	3.2	9.29	1.17	*T*	1.91	2.19
6-Acetyl-7-hydroxy-2,2-dimethyl benzopyran	1882	*T*	*T*	2.04	*T*	*T*	*T*	*T*	*T*	1.06	*T*	4.46	1.97	4.53	*T*	7.92	4.02
Valerenal	1894	*T*	1.11	*T*	*T*	*T*	*T*	*T*	*T*	*T*	*T*	2.44	*T*	3.99	*T*	4.44	2.04
9-Ocatdecanoic acid	1927	2.98	*T*	4.69	16.31	6.13	3.61	*T*	3.81	*T*	16.75	1.71	*T*	*T*	3.97	7.1	1.73
Dodecanoic acid	1960	5.94	6.24	17.19	7.3	22.19	8.17	*T*	6.61	1.28	3.22	12.69	12.22	2.38	4.52	5.25	5.4
2(2-Phenyl)- chromone derivative	2011	*T*	4.01	31.74	11.76	*T*	*T*	*T*	*T*	15.98	*T*	10.57	*T*	18.48	*T*	8.048	1.71
2(2-Phenyl)- chromone derivative	2019	19.38	17.94	*T*	17.26	11.72	0.95	10.13	13.82	0.87	18.01	1.39	1.78	*T*	4.31	*T*	0.92

T – less than 0.1% w/w.
Retention indeces relative to C8–C40 on a 95% phenylsiloxane column (30m × 0.25 mm × 0.25 μm).

were observed in the trees sampled in Yagirala location. Resin content varied from 1.35 to 2.78% (w/w) in Ingiriya population and 0.96–3.68% (w/w) in Mirigama population. However, the factors influenced for the resin formation in the trees used for the current study are not known.

A higher resin yield would require a longer time for resin formation and it has been reported that the factors that stimulate agarwood formation in *Aquilaria* can result different qualities in the resin (Bhuiyan et al., 2008). Therefore it is expected in future to conduct a distillation analysis to make a reasonable comparison of agarwood resins formed in *G. walla* with commercially available agarwood oils extracted from other species.

Research conducted in West Kalimantan, Indonesia, demonstrates that the yield of *Aquilaria* resin does not correspond with tree diameter or timber volume, even when trees have similar indications of infection (Paoli et al., 2001; Soehartono and Mardiastuti, 1997; Soehartono and Newton, 2002). Lafrankie, (1994) suggested that only one-tenth of mature *Aquilaria* trees above 20 cm dbh produce agarwood naturally. Chakrabarti et al. (1994) reported that infected trees produce resin from the age of 20 years onwards; Soehartono and Mardiastuti (1997) suggesting that best yields are obtained from trees aged 50 years and over. Recent studies undertaken by Persoon and Van Beek (2008) have shown that agarwood formation can occur in cultivated trees as young as three years of age in Vietnam.

3.3. Chemical composition

Gas chromatography mass spectroscopy (GCMS) analysis separated 19 key compounds with significant abundance in the 16 trees analysed in this study based on the co-occurrence between samples (Table 1). Following Chen et al. (2012) and Naef (2011), compounds were identified based on the mass fragmentation patterns and the retention indices given in literature. Absolute identification of the two 2(2-phenylethyl) chromone derivatives and one isopropyl naphtalen derivatives, however, could not be obtained. Not all the 19 compounds could be quantified as they were present in trace levels. Moreover, variations were observed between the samples for the presence and the abundance of these compounds. Other compounds found in the resinous wood showed high variation between the samples, thus not discussed within the scope of this study (Table 1). Free fatty acid hexadecanoic acid was the only compound seen in all trees sampled. Another free fatty acid which is dodecanoic acid was found in all the trees except one. Free fatty acid are reported to be present in the resinous wood of *Aquilaria* (Liu et al., 2013)

2(2-Phenylethyl) chromone derivatives were found to be resinous compounds found commonly all the trees; however their semi-volatile nature would hinder the identification by using gas chromatography. Unidentified isopropyl naphthalene derivative was also found in all the trees except for two trees sampled from Yagirala.

Commonly seen sesquiterpene compounds in the collected samples were jinkhol, γ-eudesmol, valerenol and valerinal. The least common constituent was camphene, which is recorded as miscellaneous compound in agarwood resins. The trees of the Mirigama population showed the presence of aromadendrine oxide compared to the trees of the other two populations studied. However, phenyl-butanone, which is common agarwood resin compound, was not found in any of the samples from Mirigama population. It was important to observe that two trees sampled from Ingiriya have very few sesquiterpene compounds.

Chen et al. (2012) reported total of 61 essential compounds from three samples of *Aquilaria sinesis* with major constituents being sesquiterpenes, such as guaia-1(10), 11-dien-9-one, guaiol, benzylacetone, hinesol and baimuxinal. Current study has followed classifications and mass spectroscopic data to identify possible classes based on chemical structures and available literature (Chen et al., 2012; Espinoza et al., 2014; Lancaster and Espinoza, 2012; Naef, 2011; Xu et al., 2013; Zhang et al., 2012). Majority of the key compounds identified in the current study belong to eudesmane type and ermophilane, similar to the observations made on artificially inoculated *Aquilaria crassna* trees by Hettiarachchi et al. (2013) where the major sesquiterpenes were also eudesmane type.

All the samples analysed in this study were naturally formed agarwood type resins developed without any human intervention. The age of the trees or the timeline of resin development are not known to the authors. Therefore the variations depicted in this study cannot establish a relationship with factors affecting resin formation. However, the minor variations shown between samples could be used in a future study on artificial resin inducement methods on *G. walla*.

4. Conclusion

Current study has developed an effective gas chromatographic method to analyse agarwood type resin from *G. walla*. It was further established that the resin from *G. walla* has similar characteristics to more commercially used *Aquilaria* species. *G. walla* trees studied from three different populations in Sri Lanka did not show a significant variation in tree dbh, resinous wood content and the extractable resin content. However, the tree heights and the chemical constituents changed significantly between the sample trees. The key components of the studied resins were sesquiterpenes which could be compared with agarwood type resins previously reported from *Aquilaria* species. It was understood that the chemical variation of resin depends upon the geographical as well as physical and biological damage caused to the tree.

A future study would emphasise on genetic variation and artificial inducement of resin development in *G. walla*. Findings of the current and future studies would support a future agarwood industry from *G. walla* in Sri Lanka.

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