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PHYTOCHEMICAL ANALYSIS OF *Gyrinops walla* AND COMPARISON WITH *Aquilaria malaccensis*

SUMMARY

Agarwood is a fragrant dark resinous wood formed in the heartwood of *Aquilaria* sp. (*Thymelaeaceae*), especially in *A. malaccensis*. The aromatic resin, agarwood, is used for the world's most expensive perfumes. *Gyrinops walla* (*Thymelaeaceae*) is an evergreen tree which grows in wet zone regions in Sri Lanka. Recently, it has been identified that *G. walla* possesses agarwood producing ability, which is similar to other species in family *Thymelaeaceae*. The objective of the present study was to preliminary identification of phytochemicals present in *G. walla* and to compare them with *A. malaccensis* which was the true agarwood resin forming species. Air dried coarse powders of different stem samples and leaf sample of *G. walla* and a stem sample of *A. malaccensis* were used and phytochemicals were extracted with each sample using soxhlet extractor with dichloromethane at 70 °C. After extraction, solvent was evaporated under reduced pressure and crude was re-dissolved in ethyl acetate for the phytochemical screening, using GC-MS. An external standard method was used to identify peaks and analyte concentrations in the chromatogram. From the stems, sixteen phytochemicals were identified in *A. malaccensis* and thirteen were found in *G. walla* all presented in agarwood resin. Out of those, nine compounds were found to be common in both species. Also, few important phytochemicals were identified from *G. walla* leaves. Therefore, *G. walla* could be confirmed as a species with significant influence on social, economic and natural environment in Sri Lanka and globally as an alternative of expensive agarwood resin for perfumery industry.

Keywords: Agarwood, GC-MS, Phytochemicals, *Thymelaeaceae*.

INTRODUCTION

Gyrinops walla Gaertn. (*Thymelaeaceae*) tree grows up to 15 m height with a straight, slender trunk and small, rounded crown (Dassanayake *et al*., 1981). The "Sri Lankan agarwood", which is the common name used for *G. walla* is also called "walla patta" and "Sri Lanka agaru". *G. walla* is a vulnerable species found in the wet zone of Sri Lanka, where the elevation is below 1000 m and the average annual rainfall is above 2000 mm with an average temperature of 25 - 28 °C. Outside Sri Lanka, *G. walla* occurs only in the extreme Southwest of

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India, where it appears to be very rare (Subasinghe and Hettiarachchi, 2015). *Aquilaria malaccensis* Lamk. (*Thymelaeaceae*) tree, up to 50 m tall, with a bole up to 60 cm in diameter, usually straight, sometimes fluted or with thick buttresses. Commonly found in primary and secondary forest, mainly in plains but also on hillsides and ridges up to 1500 m altitude. *A. malaccensis* species scattered, in Peninsular Malaysia and north-eastern India and many South East Asian countries. It grows best with an annual rainfall of 1500-6500 mm, a mean annual maximum temperature of 22 - 28 °C (Mohamed, *et al*., 2010).

Aquilaria species and also *Gyrinops* species in the family *Thymelaeaceae*, mainly produced agarwood which is the resinous, fragrant and highly valuable heartwood. The high demand occurred regarding *A. malaccensis* and *G. walla* in the recent past is due to cosmetics and medicines produced by the agarwood (Liyanage, 2014). There is a high demand from European countries, Middle East countries, Japan, Korea, China, etc. for agarwood products. Agarwood essential oil is a highly valued perfumery product in modem cosmetics and traditional Attar. *G. walla* is closely related to genus *Aquilaria* and in the past *G. walla* was considered to belong in genus *Aquilaria*. *G. walla*, together with genus *Aquilaria*, which is best known as the principal producer of the resin agarwood has high demand as economical plants (Barden, 2000).

Plants and its products are more reliable for its renewability and considered as a catalyst for human welfare. Therefore, in the past few decades, there was a growing research interest in plants as a source of phytochemical agents (Mahesh and Satish, 2008). *A. malaccensis* and *G. walla* have been investigated for the presence of phytochemically important substances which have biological, pharmacological and economic importance. This factor forced the scientists to develop methods of bioprocesses for the production and extraction of compounds from natural, renewable sources for their potential application in cosmetics and pharmaceutical industries (Velioglu *et al*., 1998). Considering the importance of the bioactive compounds it has become a necessity to build up an integrated approach to extract, purify and characterize the active compounds. From the plant materials, the extraction of bioactive compounds is the first step in the utilization of phytochemicals. Bioactive compounds can be extracted from fresh, frozen or dried plant samples. Usually before extraction plant samples are size reduced by milling, grinding or homogenization, which may be preceded by air drying or freeze drying (Abascal *et al*., 2005).

Selection of the type of solvent used in the extraction procedure, greatly depend on the successful determination of biologically active compounds from plant material and it will also depend on the targeted compounds to be extracted (Das *et al*., 2010). The polarity of the targeted compound is the most important factor for solvent choice. Along with that molecular affinity between solvent and solute, mass transfer, low toxicity, ability to evaporate at low heat, rapid physiological absorption of the extract, inability to cause the extract to complex or dissociate are the main phenomena that should concern prior to the usage of

any solvent (Eloff, 1998). Solvent extraction techniques are the most commonly used methods to prepare agarwood extracts from plant materials due to their ease of use, wide applicability and efficiency. Usually, for extraction, solvents are used from nonpolar to polar. Solvents such as methanol, ethanol, acetone, ethyl acetate, acetic acid, petroleum ether, chloroform (Xu and Chang, 2007) and their combinations are widely used for solvent extractions. Gas chromatography-mass spectrometry (GC-MS) is one of the key techniques used for screening, identification and quantification of many groups of non-polar and polar chemical compounds or their derivatives in the agarwood. The highest feasible separation of GC in combination with different types of MS detectors engaging various detection principles to which it can be coupled makes GC-MS an important, recurrently irreplaceable tool in the analysis of trace levels of chemical compounds (Hajšlová and Cajka, 2007).

MATERIAL AND METHODS

Two different stem samples and a leaf sample (L1) of *G. walla* and mature stem sample of *A. malaccensis* were selected for this experiment. In *G. walla*, one stem sample was dark in colour (G1) (assumed to be an agarwood bearing stem) and other sample was light straw colour (G2). Mean girth at breast height (GBH) of the trees from which the stem samples were collected was 30.0 - 40.0 cm. Samples were stored in a refrigerator at 4 °C prior to the experiment.

Samples were size reduced manually and air dried at room temperature. Then the dried samples were ground into a coarse powder. Phytochemicals were extracted using soxhlet extractor. About 10.0 g of each sample was extracted at a temperature of 70 °C for 20 - 30 extraction cycles over a period of 3 hours in dichloromethane (250.0 cm^3).

After extraction, solvent was evaporated, using a rotary evaporator, yielding the crude extract under reduced pressure at 40 °C. Then the crude extracts were dissolved in ethyl acetate solvent. Following soxhlet extraction, 2.0 µL aliquots were screened for the presence of phytochemicals, using GC-MS. Agilent 7890A GC (5% Phenyl Methyl Siloxane) capillary column was used for the separation and 5975C inert XL EI/C1 MS detector identified the compounds present (Table 1). An external standard method was used by the GC-MS machine to identify peaks and to find out the relationship between peak areas and analyte concentration in the chromatogram. Each chromatogram obtained were compared.

Table 1: Column conditions for GC-MS analysis

	Rate (°C/min)	Value (°C)	Hold time (min)	Run time (min)
Initial		70	4	4
Ramp	10	280	4	30

RESULTS AND DISCUSSION

It was observed that dry weight of crude obtained from dark colour stem sample (G1) of *G. walla* was the highest (48.27 mg/g), followed by dried leaf sample (L1) (10.92 mg/g) and straw colour stem sample (G2) (8.46 mg/g). That might indicate the presence of extra amount of phytochemicals in the G1, which believed agarwood bearing stem. L1 showed higher weight compared to G2. The crude obtained from *A. malaccensis* stem (A1) was 62.60 mg/g.

About twenty one phytochemicals were identified in dark colour stem samples (G1) of *G. walla* and some of them are constituents in commercially available agarwood oil. Some phytochemicals which are common in many aromatic plant species have been observed in G2. Meanwhile, eleven important phytochemical constituents were observed in mature leaf samples (L1). This indicates that mature leaves of *G. walla* also have the potential to be used as a source of agarwood oil (Table 02).

Different types of chemical constituents identified from the dark colour stem sample (G1) of *G. walla*, which believed that agarwood bearing stem are 4,7-diisopropenyldecan-3,8-diol; is an agarofuran, spiro[4.5]dec-6-en-8-one derivatives; a sesquiterpene, few naphthalene derivatives; eudesmane sesquiterpene which is a common structure found in plant derived flavours and fragrances, 2-cyclohexen-1-one derivative; a nootkatane sesquiterpene, 6-hydroxy-2-methyl-5-nitro-chromone; volatile chromone constituent responsible for the long lasting pleasant odour, aromatic compounds i.e. D-limonene, 2-fluorobenzoic acid ester derivatives and benzoic acid trimethylsilyl ester derivatives identified in commercially available agarwood oil.

Other compounds such as ledol; a sesquiterpene, myrtenyl acetate; naturally occurred fruit flavour, 2-octenoic acid methyl ester derivative; food and flavour ingredient, longifolene derivatives; abundant aroma constituent with good scent, 4-α-isopropenyl-2-carene; colourless liquid with sweet and pungent odour, andrographolide; a potential cancer therapeutic agent, quinazolinone derivative; shows wide spectrum of biological properties like antibacterial and antifungal, hexadecanoic acid ester derivatives, 2-butenylbenzene, 4,8-methanoazule-9-ol, undecanol derivative, cycloicosane and squalene present in the dark colour stem samples (G1).

There was no significantly important phytochemicals in agarwood oil has been observed in the chromatograms of straw colour stem samples (G2) of *G. walla*. However, as a mature stem of an aromatic plant, it showed many secondary metabolites in chromatogram such as phenolic compounds, alkanes, alkenes, cyclo compounds, benzene derivatives, naphthalene derivatives, pyridine derivatives, alcohols, etc. which can be found commonly.

Economically important phytochemicals present in *G. walla* and *A. malaccensis* by means of percentage similarity in dichloromethane solvent was identified and compared from the chromatograms obtained. Sixteen (16) agarwood phytochemicals were identified in *A. malaccensis* and only thirteen (13) were found in (G1) stems of *G. walla*. Out of those, only nine (09)

compounds were found to be common in both species. Present study revealed that, dichloromethane as a better solvent for extracting agarwood phytochemicals present in *A. malaccensis* and *G. walla*. Chromatograms obtained from GC-MS analysis (Figure 1) revealed that higher number of economically important phytochemicals with higher abundance, found in *A. malaccensis* than *G. walla* with dichloromethane solvent.

Table 2: Some important phytochemicals present in stem sample (G1) and leaf sample (L1) of *G. walla* extracted with dichloromethane

No.	Compound	Percentage similarity	
		Stem (G1)	Leaf (L1)
1	4,7-diisopropenyldecan-3,8-diol	88	-
2	spiro[4.5]dec-6-en-8-one derivatives	90	-
3	naphthalene derivatives	87	--
4	2-cyclohexen-1-one derivative	87	-
5	6-hydroxy-2-methyl-5-nitro-chromone	84	-
6	D-limonene	96	94
7	2-fluorobenzoic acid ester derivatives	85	-
8	benzoic acid trimethylsilyl ester derivatives	93	-
9	ledol	96	-
10	myrtenyl acetate	95	-
11	2-octenoic acid methyl ester derivative	86	-
12	longifolene derivatives	92	-
13	4-α-isopropenyl-2-carene	90	-
14	andrographolide	94	-
15	quinazolinone derivative	85	-
16	hexadecanoic acid ester derivatives	87	-
17	2-butenylbenzene	89	-
18	4,8-methanoazule-9-ol,	90	-
19	undecanol derivative	86	-
20	cycloicosane	95	-
21	squalene	87	-
22	sigmatadinene-3-one	-	96
23	β-sitosterol	-	84
24	2-chloroethyl linoleate	-	85
25	phytol	-	90
26	α-tocopherol	-	89
27	pyrazine derivative	-	83
28	lup-20-en-3-one	-	95
29	pyrone,	-	83
30	azulene	-	85
31	olean-12-ene	-	90

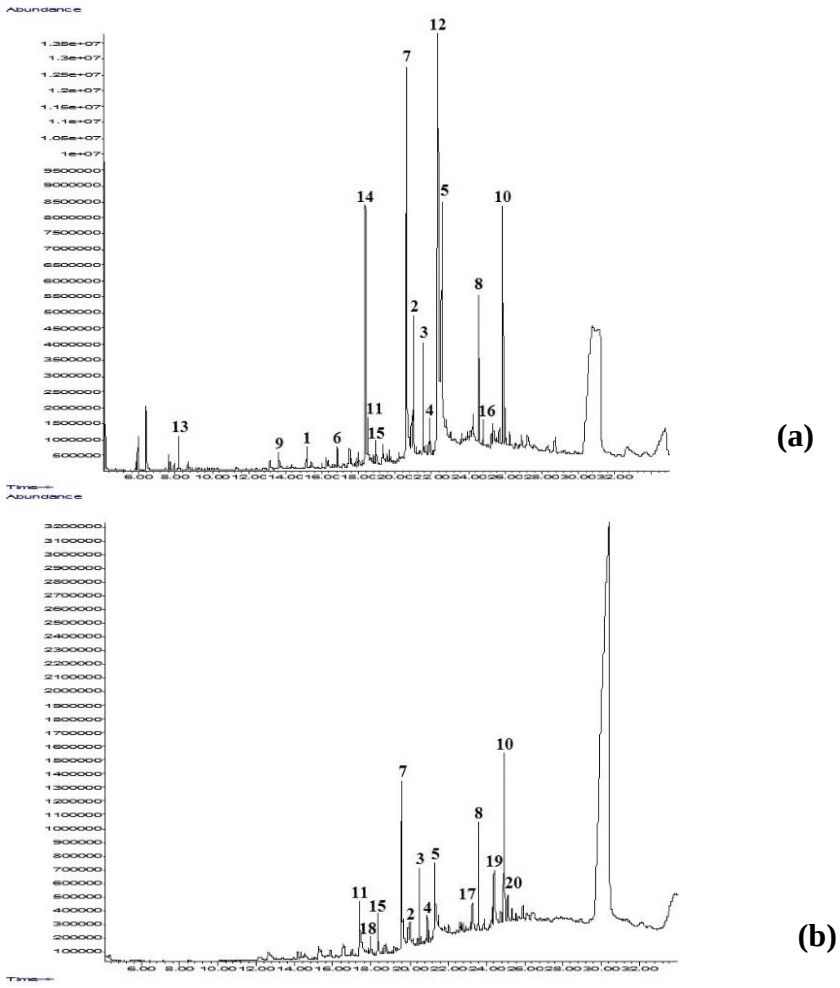

Figure 01: Chromatograms of stem samples of (a) *A. malaccensis* and (b) *G. walla* (G1) by GC-MS (Dichloromethane solvent)

Khalil *et al*. (2013) reported that, GC-MS analysis of the plant extracts led to the identification of 14 components from leaf extracts of two *Aquilaria* sp. Hexadecanoic acid was one of the major compounds in methanolic extracts. Other compounds were 1,4,7,10,13-pentaoxacyclopentadecane, acetic acid, 1,4,7,10,13,16-hexaoxacyclo octadecane, hexaethylene glycol monododecyl ether, 1,4,7,10,13-pentaoxacyclopenta decane, 2,6,10,14,18,22-tetracosahexaen and 2,6,10,15,19,23-hexamethyl. In the present study, wood samples of *A. malaccensis* contained pentadecanoic acid and octadecanoic acid. However other compounds were not observed in tested samples of *G. walla*. The presence of some volatile aromatic compounds that had been identified by Khalil *et al*.

(2013) were also found in tested *A. malaccensis* and *G. walla* samples of dichloromethane extracts.

Gas chromatography analysis revealed that the resin of *G. walla* contained aroma compounds such as sesquiterpenes of guaine and eudesmane which are commonly found in commercially available agarwood (Subasinghe *et al*., 2012). Some phytochemicals identified from the present study which used for different industries at the present, such as 2-methoxy-4-vinylphenol, an aromatic substance used as a flavouring agent and also the natural aroma of buckwheat, 2,6-dimethoxy phenol (Syringol), a naturally occurring dimethyl ether of pyrogallol, dodecanoic acid (lauric acid) which gives a faint odour of bay oil and ethyl hexadecanoate, a volatile ethyl ester which gives the characteristic fragrance for vine was observed in both *A. malaccensis* and *G. walla*. However, hexadecanoic acid ethyl ester (ethyl palmitate), benzylacetone with a sweet, flowery smell that is considered to be the most prominent compound in flowers, which responsible for attractiveness and one of volatile components of cocoa was only found in *G. walla*.

Some fatty acids (tetradecanoic acid, pentadecanoic acid, etc.) and alkanes (heneicosane, heptacosane, etc.) that are reported to be present in commercially available agarwood (Naef, 2011) were also found in both *A. malaccensis* and *G. walla* samples analysed in the present study. Benzaldehyde and vanillin were common in samples collected from unwounded *A. malaccensis* and *G. walla* plants, revealing that those compounds are not only present in resin produced, but also in the natural heartwood too. Presence of ϒ- sitosterol, which is a phytochemical present in commercially available agarwood oil (Wetwitayaklung *et al*., 2009) was observed in tested *G. walla* wood samples, but not detected in *A. malaccensis* samples. The present study revealed that although there are some common fragrance compounds present in *A. malaccensis* and *G. walla*, they do not share all fragrant compounds present in commercial agarwood samples. Therefore, felling *G. walla* trees to extract agarwood without knowing the exact mechanism behind resin induction is a waste of natural resources.

CONCLUSIONS

Sixteen important agarwood phytochemicals from *A. malaccensis* and thirteen from *G. walla* identified from stems and using dichloromethane solvent with soxhelt extraction procedure. Also, eleven important phytochemical constituents observed in matured leaves indicates that *G. walla* leaves have the potential to be used as a source for agarwood. To optimize a screening procedure of agarwood chemical constituents, primarily, phytochemical extraction procedure shall optimize with different solvent systems and conditions that ensure higher fraction of agarwood oil to be extract. Then it should identify few important agarwood constitutes i.e. sesquiterpenes, agarofurans, eudesmanes, nootkatanes and chromones, that is essential for grading agarwood and should use the standards of those chemicals to optimize GC-MS analysis. These optimization further could be implement as an internationally recognized test for identification of agarwood bearing stems of *A. malaccensis* and *G. walla* without felling them unnecessarily.

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