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RESEARCH ARTICLE

Inducement of Agarwood Resin Using Various Chemical Formulation of Stem Tissue (*Aquilaria malaccensis*)

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Abstract

Agarwood is described as fragrance wood that is usually derived from the trunk of genus *Aquilaria* which have been infected by a particular disease. The main focus of this research is to study the effectiveness of four chemical inoculants applied into 6 year old karas tree (*A. malaccensis*) plantations in Slim River, Perak, Malaysia using dripping techniques. The inoculants used were labelled as A, B, C and D. Commercial inoculant, labelled as K, was used for comparison purposes and control labelled as N. After eight months of inoculation, the karas tree were cut down and discoloured tissue samples were collected. Solvent extraction methods using dichloromethane (DCM) was carried out to obtain the resin crude extract from the plant tissue. Resin crude extract were then analysed using gas chromatography-mass spectrometry (GC-MS). The results showed that all chemicals successfully stimulated agarwood production. Inoculant A produced the highest crude extract recovery (8.63%) compared to inoculants B (7.45%), D (6.55%), C (5.95%), K (1.89%) and N (0.49%). GC-MS analysis revealed that aromatic compounds, sesquiterpenes, monoterpenes, sterols, alkanes and fatty acid methyl ester were identified from DCM extract. As an outcome, inoculant A is the best to be used as stimulant for agarwood due to product highest yield. This finding will hopefully contribute to new types of inoculants production in agarwood industries in Malaysia.

Keywords: *Agar wood, artificial inoculation, Chemical inducement, Aquilaria malaccensis, Malaysia*

Introduction

Agar wood is very popular around the world because it can be used with a variety of specific purposes such as luxury fragrance fluid in the Middle East, medicine, and beverages. It has been used to relieve kidney problems, abdominal pain, and diarrhoea and lung diseases by the Chinese community from the past [1, 2]. Malaysia is one of the 12 countries in the world which host more than 50000 species of plants that survive in a diversity of habitats.

They can be found from the lowland village to the deep interior of the forest. Malaysian rainforests is said to be the oldest in the world, hosting a complex ecosystem where both animals and plants mutually interact as they evolved more than 10 million years ago [3].

Thymelaecea is a family defined tree that generates a secondary metabolic compound that has fragrance smell[4]. It contains various genuses such as *Aquilaria*, *Gyrinops* and *Wikstroemia* that have long been known for its valuable resin. *Aquilaria malaccensis* is a largely planted species in addition to wildlife along with four more natural species in Malaysia's forests such as *A. microcarpa, A. hirta, A. beccariana and A. rostrata* [4].

There are 19 indigenous plant species in Malaysia that are capable of producing *gaharu*, 13 in Peninsula Malaysia, 11 in Sabah and 13 in Sarawak [5]. These recorded species come from 5 genera consist of 7 *Aquilaria* species; 6 *Gonystylus* species; 4 *Wikstroema* species; 1 *Aetoxylon* specie; and 1 *Enkleia* specie.

They also pointed out that *Aquilaria* spp which is *A. malaccensis* is the most popular source of *gaharu* among the karas trees [5]. Agarwood is sustained in high demand every year internationally for various uses including for treating cancer and tumours which resulted in its high price that depends on the grade obtained.

In 2009, The Convention on International Trades in Endangered Species of Wild Fauna and Flora (CITES), Malaysia's export permit of gaharu was worth RM 56.43 million [6, 7]. Whereas nowadays the highest grade of agarwood has reached up to USD 30 000 per kg depends on the demand and the relevant consumer country [8]. In addition, there is dramatic increase in the grade A gaharu price which reached approximately RM130, 000 per kg compared to the previous price of RM 20 000 to RM30, 000 per kg [9].

Gaharu is produced when the injuries that occur have allowed oxygen to enter the plant tissues and damage the living cells. As a result, plants will react to the process by producing resin extract containing aromatic compound such as agarwood [10]. The activation of the karas wood defense mechanism generally due to physical, biological and chemical injuries will result in the resin composting process. The persistent resin accumulation process will cause the wood tissue to grow denser and darker which eventually becomes known as agarwood.

The process of producing natural agarwood normally will take a very long time or decades. Nowadays various studies have been conducted by scientists and stakeholders in the agarwood industry to find inoculant that can cause karas trees to produce gaharu in a short time, higher quality resin which is comparable to the natural wild gaharu. High-grade agarwood usually contains aromatic compounds and terpene groups such as α-guaine, ß-selinene, aromadendrene, α-bulnesene and agarospirol [8].So far the best inoculants formulation of agarwood in karas tree is still understudy [11, 13].

Material and Methods

Study Site

The study was conducted at Al Hilmi Agrofarm plantation, Slim River, Malaysia. The karas tree age is about 6 years old and was identified and deposited at the herbarium of Universiti Pendidikan Sultan Idris for reference purposes with voucher number MFE001, MFE002 and MFE003.

Chemical Treatments

Inoculation formulations were produced using several selected chemicals by try and error method. It is done based on weight calculations per volume. A total of four different formulations were produced which were labelled as inoculants A, B, C and D. For comparison, one commercial inoculant that was bought from private company was used and labelled as K. In term for control purposes only a healthy karas tree without any treatment is used.

The control tree (N) was not cut down but only the plant stem tissue was scrapped out for analysis. About 80 ml of each inoculant that have been produced were kept in the reagent bottle before being taken to the study area. At the field, dripping technique is use during inoculation. The hole is punched on the tree using an electric drill with a diameter of 10mm. Only one hole is punched on each tree with as much as 80 mL of inoculants per hole. Each type of inoculate were injected to two karas tree for replication purpose that resulted 10 karas trees have been used in total. The trees were left for 8 months before it was cutting down.

Crude Extraction

Samples obtained will be cut into small size. Then it was dried in oven at 50°C for 3 days and weighed. Samples that have been dried will be crushed and blended using a dry mill. Extraction process is done by using solvent extraction (cold extraction techniques). A total of 50g plant samples are weighed for each sample. The plant samples of each inoculant were soaked in DCM for nine days. After 3 days, the solvent will be replaced with new solvent. All the filtration liquid were concentrated using rotary evaporator. The crude extract then weighed and stored in the freezer at -4°C until it is used.

GC-MS Analysis

The crude extract were analysed using GC-MS Perkin Elmer in order to identify the chemical composition. The operating temperature for Elite-5MS column as followed: initial temperature 30°C held for 1 min, then rose to 150°C at rate of 10°C/min and held for 1 min. After that the

temperature raised for the second time to 280°C at a rate of 5°C/min and held for 6 min. running time is 45 min with the mass range of 50-500m/z. For the identification of individual components was performed by matching their mass spectral data with those from the mass search program form NIST/EPA/NIH Mass Spectral Library version 5.0.

Figure 1 below showed the profile for agarwood formation of karas tree after 8 month of inoculation. Based on the observation, it showed that there are significant differences between each karas tree due to their response towards the chemical. There were slightly different according to their colour and the thickness of the agarwood.

Results and Discussion

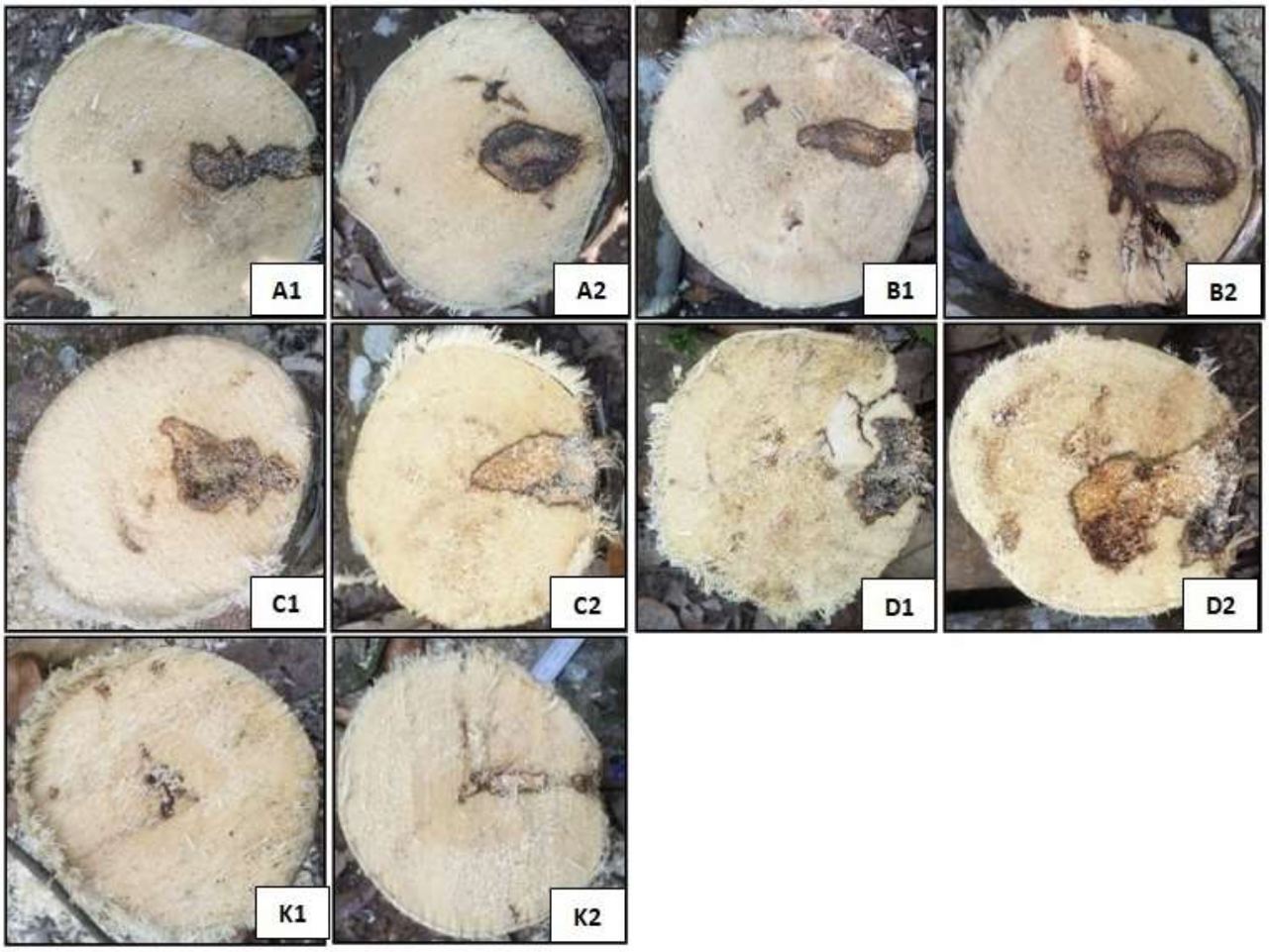

Figure 1: The trunk cross sections treated with 4 groups of agarwood inoculants A-D and a commercial inoculant K

In the natural environment, all healthy wood of karas tree appear as a white, soft and without scented resins. Usually it has a softer wood core and light in colour. When the karas trees are exposed to pathogen infection or parasites, it will produce special resin which will caused the infected tissue to turn darker or dark red in colour [14, 15].

The oleoresin that formed also is the result of the karas trees when its respond to another stimulation such as fungus infection, lightning strikes, fire and insect attacks Its takes several year or decades for karas tree which still alive after wounding to form agarwood.

There is no agarwood obtain from a healthy tree [16]. The cross section of tree treated with inoculants show in Figure 1.

The entire trees were cut down after 8 months and exhibited the developed resinous and dark area. Pictures of A1 and A2 referred to the same inoculants but at two different trees and so on. However K1 and K2 did not showed any significant changed into a dark area.

According to the vendor's description, they normally used up to 2 to 3 litres of liquid for a single hole. In our study, we only used as much as 80ml of liquid. It is likely indicated

that inoculants K is less effective when it is used in small quantities. Based on the result, it can be concluded that all the karas trees responded to the inoculants stimuli to produce agarwood resin. The tree with A2 labelled showed the darkest colour and followed by B1, B2, A1, D1, C2, D2, K1 and K2, respectively. Meanwhile, Figure 2 showed the measurement of agarwood length spreads inside the stem. There are slightly a significant different between all the karas stem. Picture A showed the cutting stem and it is measured using a measuring tape. Picture B indicated a portion of the stick that contained a resinous column, whereas C indicated the boundary edge of the distribution that has been reached and D showed a normal boundary without any dark tissue.

Figure 2: The lengths of agar wood inoculation column

Table 1 and Figure 3 presented the agarwood column length after harvested. It showed that tree which was treated with formulation B showed the highest length and followed by A, C, D and K, respectively (note; N is controlled without cutting down the tree).Length of the agarwood (inoculation column) distribution has been determined by the observation of dark colored tissue. Cutting process is stop until dark tissue is not visible inside the stem.

Table 1: The length of the inoculation column

Inoculants	Length (Mean± SEM) (cm)
B	101.0±9.00
A	94.0 ± 4.00
C	87.5 ± 6.50
D	69.0±4.00
K	6.5 ±3.50
N (control)	-

Note,* the mean difference is significant at p<0.05

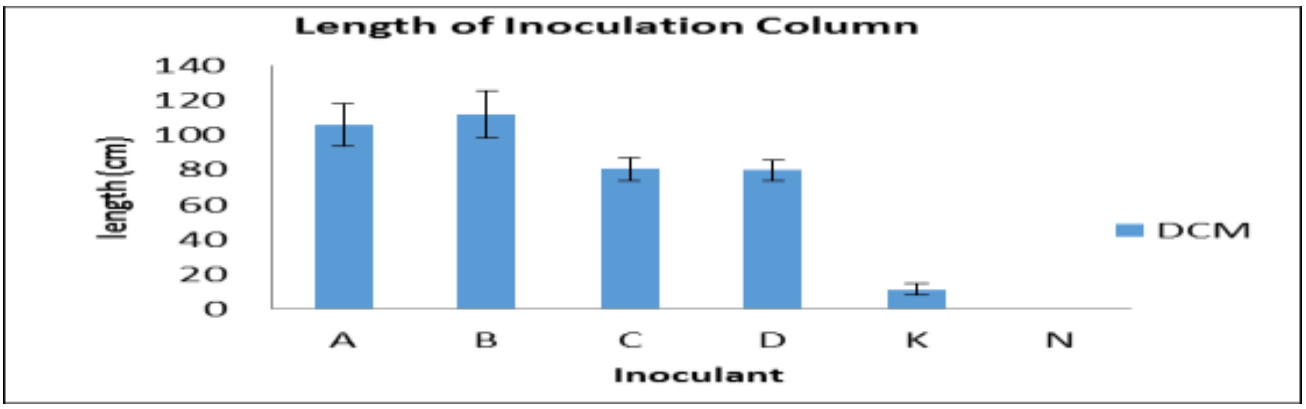

Figure 3: Agar wood inoculation column per tree treated with different inoculation formulation. Results as mean ± SEM (n=2)

The length along the stem to form agarwood was about 6.5cm to 101cm from the transfusion hole. This is lower compared to [12] that they get about 1.5m to 2m length of agarwood forming when using chemical based inducer. From this study, the length of agarwood induced by inoculant B was significant highest than those by inoculants A, C, D and K. The result obtained also showed no significant relationship between the diameter of the stems and the length of the agarwood formation because the diameter (DBH) of each tree used is almost the same. Table 2 and Figure 4 showed the weight of DCM crude extract obtained. Based on Table2, it was found that inoculant a produced more resin and followed by inoculants B, D, C and K.

Table 2: Crude extracts (DCM)

Inoculants	Crude extract mean (g) (Mean± SEM)	Percentage (%)
A	4.313 ± 0.022	8.63
B	3.674 ± 0.001	7.45
D	3.275 ± 0.127	6.55
C	2.976 ± 0.013	5.95
K	0.949 ± 0.006	1.89
N	0.245 ± 0.072	0.49

Note- The mean difference is significant at p<0.05

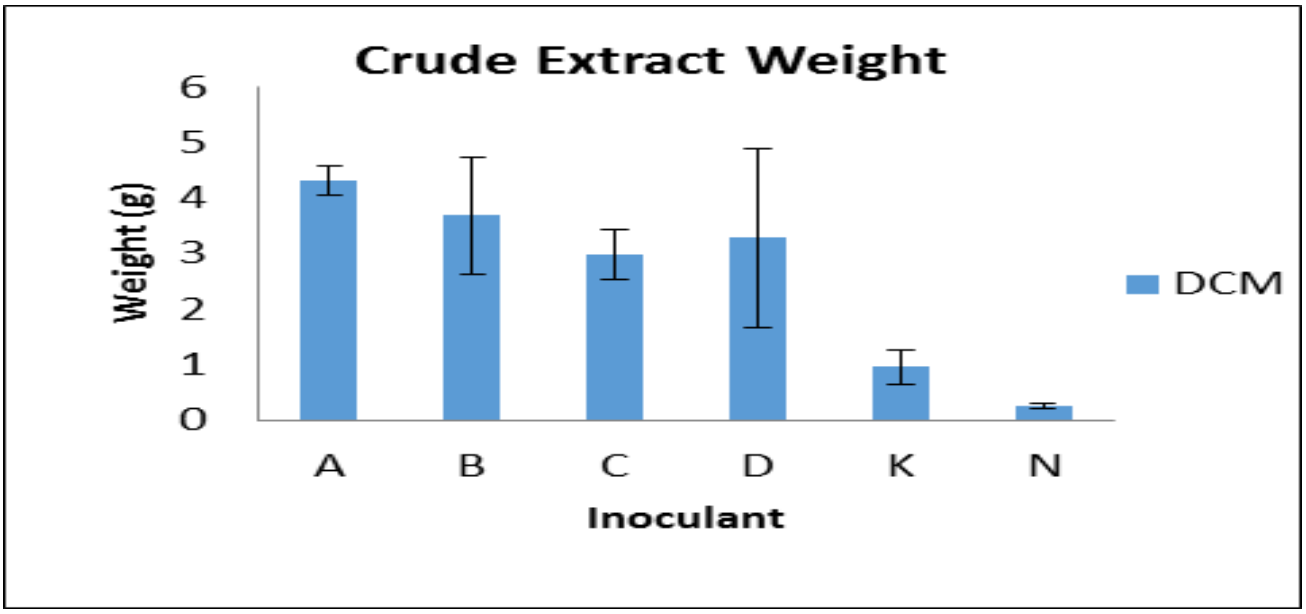

Figure 4: The crude extract of resin recovery after 8 month inoculation Results as mean ± SEM (n=2)

As showed in Table 2, the crude extract yield of agarwood obtained by inoculants A to N is range from 0.49% to 8.63%. There is significant different among them. These values were different to those of two control sample which is 0.245 ± 0.072 g. The higher weight oil recovery is inoculants A, B, D, C, K and N. The highest weights of resin are one of the indicators of inoculation effectiveness.

The other indicators are the colour tissue changes, weight of the resin and the chemical compounds present in the resin. The duration of 8 months is taken into account as it is expected to be appropriate because based on previous study [12, 14, 17] stated that the karas trees are able to produce resin as early as in the callus stage. They also stated that within a period of 3 months, the karas tree can produce resins when treated with a specific inoculants. Meanwhile, Table 3 and Figure 5showed the result of identified compound in all agarwood samples. A total of 130 compounds were identified from 12 samples.

While there are 50 major compounds were identified, the major constituents were derivatives of chromones, aromatic compound, sesquiterpenes, alkane group, sterol compound and fatty acid methyl ester.

Table 3: List of major volatile compounds found in 12 samples

Number	Retention Time	Compound	A1	A2	B1	B2	C1	C2	D1	D2	K1	K2	N1	N2	Total
1	00.21	2,2,3,4-tetramethylpentane	/	-	/	/	-	-	/	/	/	-	/	/	8
2	02.23	Heptane	-	-	-	-	-	-	-	/	-	/	-	/	3
3	04.20	Cyclopentaneacetic acid	/	/	/	/	/	/	/	-	/	/	/	/	11
4	04.93	3-methyl-5-propylnonane	/	/	/	-	/	-	-	-	-	-	-	-	4
5	05.59	1-(benzyloxy)-8-Naphthol	-	-	/	-	-	-	-	-	/	/	-	-	3
6	05.32	*o*-octylanisole	/	/	/	-	-	/	-	-	-	-	-	-	4
7	11.48	2,8-dimethylundecane	-	/	/	/	-	-	-	/	-	/	/	-	6
8	14.37	Octadec-9-enoic acid	-	/	-	/	/	-	/	/	-	/	-	/	7
9	14.72	6-ethyl-2-methyloctane	/	-	/	/	/	-	-	-	/	-	-	-	5
10	14.82	Isocitronellol	-	-	-	/	-	-	/	-	/	/	/	-	5
11	15.01	Dibenzofuranone	-	-	/	-	-	/	-	-	-	-	/	-	3
12	15.77	2,2,11,11-tetramethyldodecane	-	/	-	-	-	/	-	/	-	-	-	-	3
13	15.83	2,2-dimethyldecane	/	/	/	/	/	/	/	-	-	/	-	-	8
14	16.82	Lycopodine	-	-	/	-	-	-	/	/	-	-	-	/	4
15	16.86	Platambin	-	-	-	/	-	/	-	-	-	-	-	-	2
16	17.51	4-Hexanoylresorcinol	/	-	-	-	-	-	/	-	-	-	/	-	3
17	19.45	Anthracenedione	/	/	/	/	/	-	-	/	-	-	-	-	6
18	20.11	Azulene	-	/	-	-	/	-	-	-	-	-	-	-	2
19	20.19	3-methyltridecane	/	/	/	-	-	/	/	/	/	/	-	/	9
20	20.96	8,9-dehydro-9-formylcycloisolongifolene	-	/	-	-	-	/	/	/	-	-	/	/	6
21	22.76	1,4-dimethyl-7-(1-methylethyl)azulen-2-ol	/	/	-	/	/	-	-	-	/	-	-	-	5
22	23.02	*n*-Decanoic acid	/	/	/	/	/	/	/	/	/	-	-	/	10
23	23.78	ß-Panasinsene	-	-	-	/	-	-	/	-	-	-	-	-	2
24	24.37	Vanillin	-	-	-	-	-	-	-	-	-	-	/	/	2
25	24.61	Tetradecane	-	-	-	-	/	/	/	/	-	/	/	/	7
26	26.08	γ-Elemene	/	-	/	/	-	-	-	-	-	-	-	-	3
27	26.82	α-humulene	/	/	/	-	-	/	-	-	-	-	-	-	4
28	27.16	Aromadendrene	/	/	/	/	/	/	-	/	-	-	-	-	7
29	29.03	α-Farnesene	-	/	/	-	/	/	/	-	-	-	/	-	6
30	29.23	2-(4-methoxybenzylthio)-3-methylquinazolin-4(3H)-one	-	-	-	/	-	-	-	-	/	-	-	-	2
31	30.12	ß-Sitosterol	/	-	-	-	/	-	-	-	-	-	-	-	2
32	31.59	γ-Sitosterol	/	/	/	-	-	/	-	-	-	-	-	-	4
33	32.25	Methyl-9-octadecenoate	-	-	/	-	/	/	-	/	/	-	/	-	6
34	32.44	cis-9-Hexadecenal	/	/	-	/	/	/	-	-	/	-	-	/	7
35	32.89	Hexadecane	/	/	/	/	/	/	/	/	/	/	/	/	12
36	34.53	Styrene	-	/	-	-	-	-	-	-	-	-	-	-	1
37	34.69	Agarospirol	/	/	/	-	/	-	-	-	/	-	-	-	5
38	36.74	Heptadecane	-	/	-	/	-	/	-	/	-	-	-	-	4
39	36.85	Tridecanoic acid	/	/	/	/	/	/	/	/	-	/	/	/	11
40	37.29	Butorphanol	-	/	/	/	-	-	-	/	-	-	-	-	4
41	38.15	α-Santalol	/	/	-	/	/	-	-	/	/	-	/	-	7
42	40.22	6-(benzyloxy)-4,4-dimethyl-5-nitro chromanone	/	/	/	-	/	-	-	/	-	-	-	-	5
43	40.54	α-Naphtholphthalein	/	-	-	-	-	/	-	-	-	-	-	-	2
44	41.56	Elaidic acid	-	/	/	/	/	/	/	-	-	/	-	-	7

45	45.95	*n*-Hexadecanoic acid	/	/	/	/	/	/	/	/	/	/	/	/	12
46	48.15	Arteannuic acid	-	-	-	/	-	-	-	/	-	-	-	-	2
47	53.66	5-Methyldocosane	/	/	/	/	/	/	/	/	/	/	/	-	11
48	79.26	Stigmasta-3,5-dien-7-one	/	-	-	-	/	/	-	-	-	-	-	/	4
49	79.56	Stigmasterol	/	/	-	/	/	-	-	-	/	/	/	/	8
50	82.80	Tetratriacontane	/	/	/	/	-	/	/	/	-	-	/	-	8
		Total	28	31	28	27	25	25	19	23	17	15	18	16	272

*Note,** A1-K2 represent the sample from each karas trees using specific inoculants after 8 months of inoculation. N1 and N2-healthy tree without inoculation "/" indicate detected "–" indicates not detected

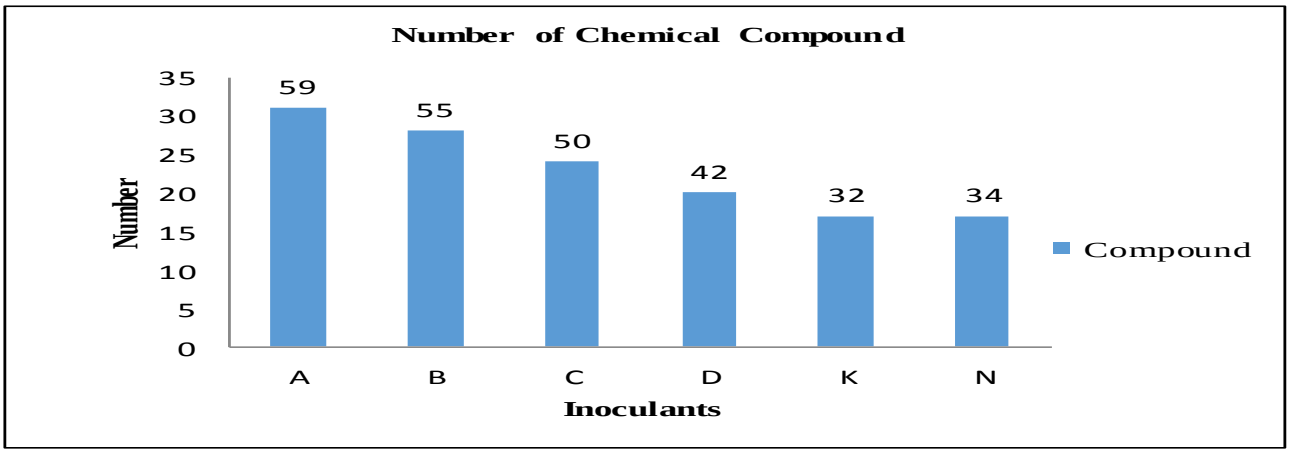

Figure 5: The number of major chemical compounds identified in agar wood samples * the results are combination of each replicates

Based on Figure 5, it showed the types and number of chemical compounds identified. It had a range of number 17-31 compounds. Inoculant A is recorded the highest number of 31 types of compound. This is followed by inoculants B (28), C (24), D (20), K (17) and N (17), respectively. The chemical compound profiling in DCM crude extract was evaluated to see the existence of chemical composition using GC-MS. Determination of chemical compounds is carried out by the selection of all terpene and chromone groups only because both groups are involved in the determination of the quality of the agarwood but not all the targeted compound can be identified.

The dominant compounds were alkane hydrocarbon group (20.5%) and terpenoid (15.9%). Chromones, although a major compound in agarwood, it has not reported from organic solvent extracted in *A.malaccensis* agarwood from Malaysia [19, 20]. Here, we reported the major compounds that identified in the agarwood sample. Other compound such as ß-agarafuran, α-agarafuran and 10-epi-γ-eusdemol were not detected in this study. Perhaps, because these were DCM extract and not used the water-distilled [21].

The findings from this study are very important as it can help researchers to compare the eight month result and the next twelve's month result. However, there is a difference in the number of compounds of terpene and chromone compounds produced in the resin. A total of 50 volatile compounds were found in the first eight month batch. The presence of volatile groups is an important indicator of the quality of the agarwood. This is due to the fact that agarwood is famous for its fragrant wood smell.

Conclusion

All inoculants can stimulate the karas tree to react and produce resin. The karas tree reacts when the pain response is received by the tree tissue. From the study inoculants formulation A and B should be given more attention because the formula produces more resin quantities compared to other formulations.

Similarly, GC-MS analysis results showed that the number and type of chemical compound produced are the same as reported by other researchers but have several different compounds and area percentage. All the formulation has been reproduced to carry

out a real-time study for a longer period of time before a tree was cut down for the determination of effectiveness and chemical profile for the purpose of determine the quality of the gaharu that was formed.

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