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Genetic diversity and differentiation of *Aquilaria malaccensis* Lam. Using RAPD markers

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Aquilaria malaccensis Lam. (family Thymelaeaceae) commonly known as agarwood or gaharu producing tree in Malaysia. The tree is being heavily exploited due to its highly valuable agar oil used in the production of high grade perfumes and traditional medicines. Consequently, their population in nature is threatened greatly. Conservation of this tree species is of the main concern, however, identification of *A. malaccensis* from other *Aquilaria* sp. based on morphology is very difficult and time consuming. This study aimed to determine the genetic diversity among three selected *Aquilaria* sp. namely *A. malaccensis*, *A. sinensis* (Lour.) Spreng. And *A. subintegra* Ding Hou using random amplified polymorphic DNA (RAPD) markers and to differentiate *A. malaccensis* from *A. sinensis* and *A. subintegra*. Out of ten RAPD primers, four primers (G12, R15, U13 and OPA 05) produced the most clear and reproducible bands. A total of 24 bands were scored from the four primers. Construction of dendrogram resulted in two major clusters; cluster I consisted of only *A. malaccensis* accessions, and cluster II consisted of *A. subintegra* and *A. sinensis* accessions. This indicates that *A. subintegra* is more closely related to *A. sinensis* while *A. malaccensis* is genetically distant from both. Species-specific bands for *A. malaccensis* were produced at 875, 1000 and 2500 bp by G12 primer, and at 2500 bp by OPA 05 primer. This study laid the foundation for a creation of rapid and cost effective molecular identification of *A. malaccensis.*

Keywords: *Aquilaria malaccensis*, agarwood, gaharu, genetic diversity, RAPD, molecular identification.

INTRODUCTION

The genus *Aquilaria* (family Thymelaeaceae) consisted of 21 species of which 13 species are reported to form agarwood or gaharu (Lee and Mohamed, 2016). The term agarwood or gaharu refers to the resins produced from the trees which turn to aromatic and highly valuable agar oil used not only in the production of incense and perfumes, but also in traditional medicines. For example, *Aquilaria* extract has shown to have strong antioxidant, antimicrobial and antidiabetic activities (Mahmod et al. 2017; Wan Ahmad et al., 2019). In Malaysia, native *Aquilaria* species such as, *A. malaccensis*, *A. hirta* Ridl., *A. beccariana* Tiegh., *A. rostrata* Ridl., and *A. microcarpa* Baill, and introduced species such as, *A. crassna* Pierre ex Lecomte (from Vietnam), *A. sinensis* (from China) and *A. subintegra* (from Thailand). Different *Aquilaria* species produce resins with different smells and quality.

Aquilaria malaccensis is known to be the main producer of high grade agarwood oil in Malaysia (Nor Azah et al., 2008; Chua, 2008).

Presently, Malaysia has been reported as the primary exporter of agarwood along with Indonesia (Soehartono and Newton, 2000). The agarwood oil is obtained by distilling selected parts of the infected wood of *Aquilaria* trees. However, the presence of agarwood in the trees is not easily noticeable; hence many uninfected or less infected trees are often cut down and harvested in search of agarwood. Zhang et al. (2010) reported that agarwood resources in nature forests are declining drastically due to uncontrolled harvesting and forest clearance. As a result, *A. malaccensis* has been categorized as critically endangered species globally by IUCN (http://www.iucnredlist.org). To avoid further destruction towards its gene pool, various conservation efforts have been done. This includes introducing *A. malaccensis* along with foreign species for plantation as well as cultivation in the nurseries or home gardens. In addition, the application of biotechnology such as plant tissue culture was adopted for propagation of *A. malaccensis* seedlings as an alternative for conventional propagation method (Saikia et al. 2012).

Conventional identification of agarwood producing species is difficult as the morphological structures among the species are almost similar and keep changing according to environmental conditions (Lee et al., 2011). Misleading identification could happen due to lack of knowledge, training or intentionally for business advantages. Experienced taxonomist or botanist, in most cases, is required to identify and differentiate all the agarwood producing species. According to Pern et al. (2018), identification of *Aquilaria* sp. relies heavily on the flower and fruit morphology, not on the vegetative parts of the trees, which made identification of *Aquilaria* sp. at seedling stage not possible.

Previous studies reported that several molecular approaches have been piloted for identification and characterization of *Aquilaria* sp. such as those using microsatellite markers (Tnah et al., 2012; Zhang et al., 2010), inter-simple sequence repeat (ISSR) markers (Azhar et al., 2013; Azhari et al., 2015; Zahari et al., 2020) and plastid *trnL-trnF* intergenic spacer sequences (Eurlings and Gravendeel, 2005). Eventually, none of these approaches could differentiate *A. malaccensis* from other *Aquilaria* species. Random amplified polymorphic DNA (RAPD) markers have been successfully used as an efficient tool for identification and analysis of genetic diversity in many plant species including *Cucumis* sp. (Manohar et al., 2013), *Piper* sp. (Sen et al., 2010), *Jatropha curcas* L. (Boora and Dhillon, 2010) and *Mangifera indica* L. (Britto et al., 2011). Thus, this study aimed to investigate the potential of RAPD markers in analysis of genetic diversity of three selected *Aquilaria* sp. (*A. malaccensis*, *A. sinensis* and *A. subintegra*) and for differentiation of *A. malaccensis*.

MATERIALS AND METHODS

Plant materials

In this study, five accessions of each *Aquilaria* species namely *A. malaccensis* (labelled as M), *A. subintegra* (labelled as S) and *A. sinensis* (labelled as SI) were used. Seedlings of *A. malaccensis* were collected from Forest Research Institute Malaysia (FRIM) Forest Reserve, Merchang, Terengganu (4°58'58.4"N 103°19'19.8"E); *A. subintegra* were collected from Alor Gajah, Melaka (2°23'03.3"N 102°13'10.2"E); and *A. sinensis* were collected from Sri Kembangan, Selangor (3°00'48.5"N 101°42'12.1"E). All the seedlings were sampled during June - August 2018 and the sampled seedlings were approximately two years old. The seedlings were then planted at Ladang Pasir Akar, Jertih, Terengganu, Malaysia (5°38'41.8"N 102°28'09.3"E).

DNA isolation and RAPD-PCR amplification

The DNA was isolated from fresh leaves using DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. Initially, the optimization of RAPD primers was performed using five different annealing temperatures (43 °C, 44 °C, 45 °C, 46 °C, 47 °C). The DNA samples were screened with ten RAPD primers (Table 1, Eurofins, Genomics) for polymerase chain reactions (PCR) amplification. Primers which generated most clear banding patterns were chosen for the final PCR analysis.

Table1: List of RAPD primers used in this study.

No.	Primer	Sequence (5' – 3')
1	D20	ACCCGGTCAC
2	A17	GACCGCTTGT
3	G12	CAGCTCACGA
4	R15	GGACAACGAG
5	U13	GGCTGGTTCC
6	OPA 20	GTTGCGATCC
7	OPB 12	CCTTGACGCA
8	OPA 18	AGGTGACCGT
9	OPA 02	TGCCGAGCTG
10	OPA 05	AGGGGTCTTG

The PCR was performed using New England Biolab's (NEB) kit in a final reaction volume 25 µl containing 2.0 µl of 30 ng/ml genomic DNA, 2.5 µl of 10X buffer, 0.5 µl of 10 µM dNTP, 0.1 µl of Taq polymerase, 1.0 µl of 10 µM primer and 18.9 µl of sterile distilled water. Amplifications were carried out on a Thermal Cycler (Applied Biosystems™) for 35 cycles programmed at initial denaturation of 94 °C for 3 minutes, followed by denaturation at 94 °C for 54 seconds, annealing at 44 °C for 43 seconds and extension at 72 °C for 2 minutes, and a final elongation step at 72 °C for 5 minutes. This method was referred to Lee et al. (2011) with some modification on the annealing temperature. Amplification products were run on 1 % (w/v) horizontal agarose gel prepared in 1 X TBE buffer and visualized under an UV transilluminator. 1 kb DNA ladder (Promega) was included in the gels as a size reference.

Data analysis

In order to calculate RAPD polymorphism, a binary data matrix was made based on the RAPD band profiles. Only clear and distinct bands were scored visually, where '1' was attributed to the presence of the targeted band and '0' to the absence of the band. The data was then analyzed by PAUP* software (version 4.0). The genetic distances were assessed with Nei's unbiased genetic distance (Nei, 1987) measure and converted to a dendrogram by unweighted pair group method using arithmetic averages (UPGMA, Sneath and Sokal, 1973). The percentage of polymorphic bands for each primer was manually calculated based on the given formula (Tidke and Ranawade, 2017):

$$\text{Polymorphism} = \text{No. of polymorphic band} / \text{Total no. of band} \times 100$$

RESULTS

Optimization of annealing temperature and screening of primers

In this study, five annealing temperatures (43 °C, 44 °C, 45 °C, 46 °C, 47 °C) were tested. Using the primer U13 and DNA sample of *A. sinensis*, it was found that the clearer bands were observed at 44 °C (Figure 1). Out of ten random primers screened, only four primers (G12, R15, U13 and OPA 05) produced clearer bands and were further used in the RAPD analysis (Figure 2). No bands were produced by primer A17 and OPA 20 after the first trial of screening. No amplification products were observed with these primers, hence they were not chosen in the present study.

RAPD-PCR amplifications

The RAPD-PCR amplification by two primers, G12 and OPA 05 produced species-specific banding patterns for *A. malaccensis*. The primer G12 produced six bands ranging in sizes from 875 to 3500 bp (Figure 3a). The banding patterns for G12 showed variations in all *Aquilaria* species. Nonetheless, bands at 875, 1000 and 2500 bp were to be specific to *A. malaccensis*. For OPA 05, ten bands were produced, ranging in sizes from 625 to 3000 bp (Figure 3c). The potential diagnostic band was identified at 2500 bp, which was specific to *A. malaccensis*. This indicated that the potential bands from G12 and OPA 05 could be used to differentiate *A. malaccensis* from the other two species.

The primer R15 produced six bands with molecular sizes ranging from 750 to 3500 bp (Figure 3b), while U13 produced only two bands ranging in sizes from 1500 to 2000 bp (Figure 3d). However, no species-specific band was produced by R15 and U13 primers.

RAPD polymorphisms and genetic diversity

The four primers (G12, R15, U13 and OPA 05) used in this study were found to be polymorphic (Table 2). The primer OPA 05 was highly polymorphic (100%), while U13 was the least polymorphic (50%).

Table 2: Percentage of polymorphisms obtained from primers used in this study.

Primer	Total no. of bands	No. of polymorphic band	Polymorphisms Percentage (%)
G12	6	5	83
R15	6	5	83
OPA 05	10	10	100
U13	2	1	50

The dendrogram constructed using UPGMA based on Nei's genetic distance obtained from RAPD analysis (Figure 4, Table 3) formed two major clusters. Cluster I consisted of all accessions of *A. malaccensis* and cluster II consisted of all accessions *A. subintegra* and *A. sinensis*. The major cluster I formed two sub clusters, IA and IB. The larger sub cluster IA consisted *A. malaccensis* from accessions M1, M3 and M8, while M5 and M7 were clustered in sub cluster IB.

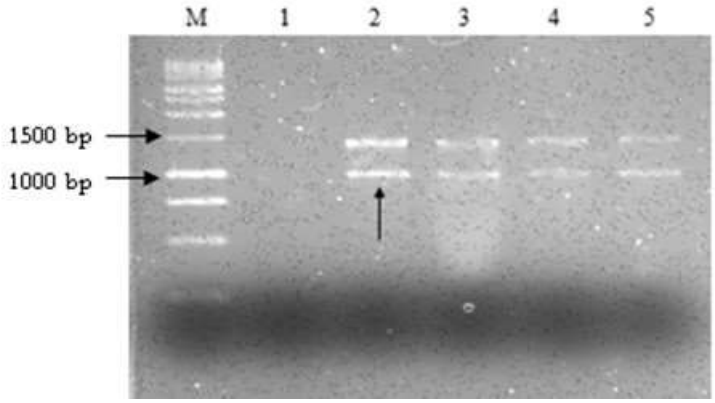

Figure 1: Banding patterns produced by five different annealing temperatures (using primer U13 and DNA sample of *A. sinensis*). M = 1 kb marker, 1 = 43 °C, 2 = 44 °C, 3 = 45 °C, 4 = 46 °C, 5 = 47 °C.

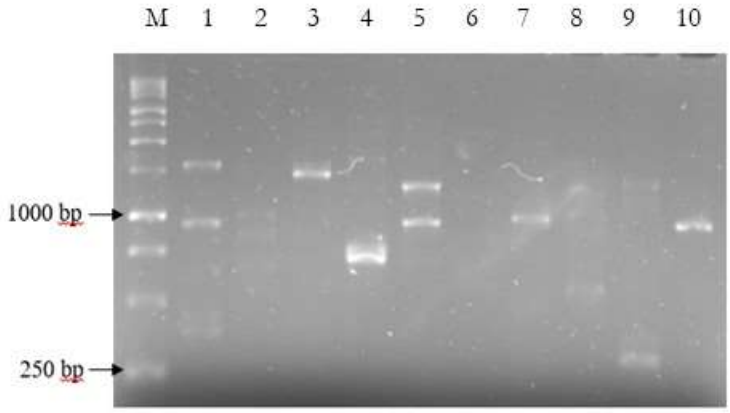

Figure 2: Banding patterns produced by ten primers using DNA sample of *A. sinensis*. M = 1 kb marker, 1 = D20, 2 = A17, 3 = G12, 4 = R15, 5 = U13, 6 = OPA 20, 7 = OPA 12, 8 = OPA 18, 9 = OPA 02, 10 = OPA 05.

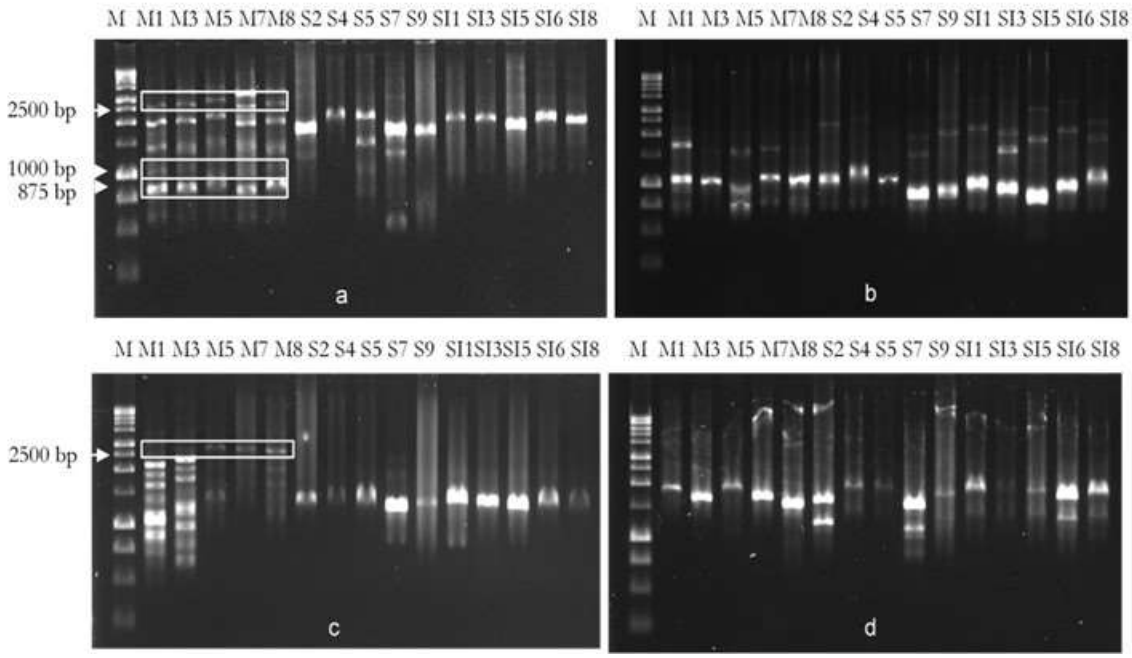

Figure 3: Banding patterns obtained with primers, (a) G12, (b) R15, (c) OPA 05 and (d) U13. M = 1 kb marker, M1-M8 = *A. malaccensis* accessions, S2-S9 = *A. subintegra* accessions, SI1-SI8 = *A. sinensis* accessions.

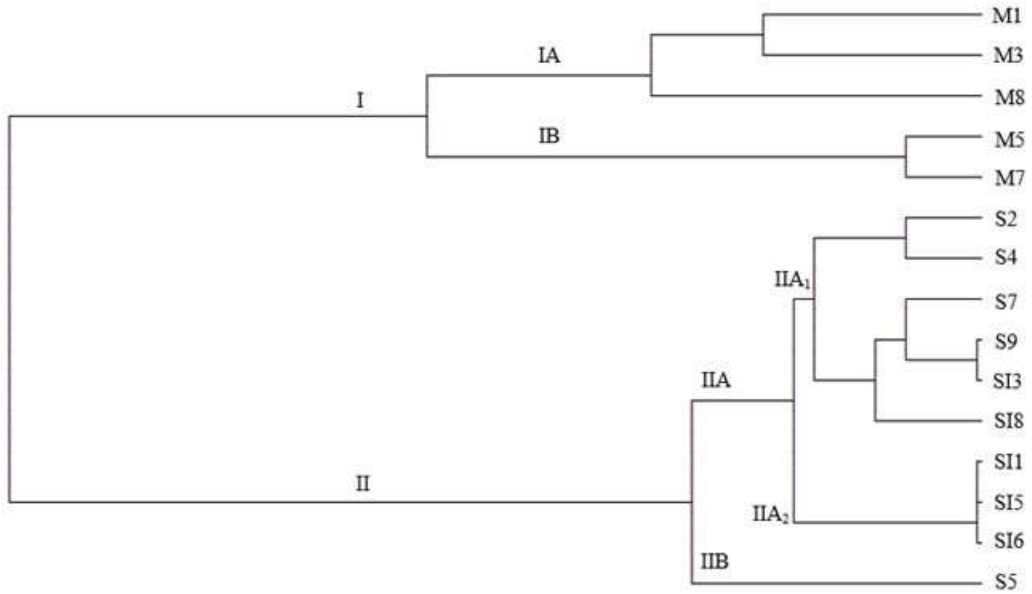

Figure 4: Dendrogram showing clustering of 15 accessions of *A. malaccensis* (labelled as M), *A. subintegra* (labelled as S) and *A. sinensis* (labelled as SI) constructed based on RAPD analysis.

Table 3 : Genetic distance matrix for 15 accessions of *A. malaccensis* M1, M3, M5, M7 and M8, *A. subintegra* S2, S4, S5, S7 and S9, *A. sinensis* SI1, SI3, SI5, SI6 and SI8.

	M1	M3	M5	M7	M8	S2	S4	S5	S7	S9	SI1	SI3	SI5	SI6	SI8
M1	-														
M3	0.1250	-													
M5	0.3750	0.3333	-												
M7	0.4167	0.3750	0.0417	-											
M8	0.2083	0.1667	0.1667	0.2083	-										
S2	0.7083	0.6667	0.4167	0.4583	0.5833	-									
S4	0.7500	0.7083	0.4583	0.5000	0.5250	0.0417	-								
S5	0.5833	0.5417	0.2917	0.3333	0.4583	0.1250	0.1667	-							
S7	0.6250	0.5833	0.3333	0.3750	0.5000	0.0833	0.1250	0.1250	-						
S9	0.6667	0.6250	0.3750	0.4167	0.5417	0.1250	0.0833	0.1667	0.0417	-					
SI1	0.7500	0.7083	0.4583	0.5000	0.6250	0.1250	0.0833	0.1667	0.1250	0.0833	-				
SI3	0.6667	0.6250	0.3750	0.4167	0.5417	0.1250	0.0833	0.1667	0.0417	0.0000	0.0833	-			
SI5	0.7500	0.7083	0.4583	0.5000	0.6250	0.1250	0.0833	0.1667	0.1250	0.0833	0.0000	0.0833	-		
SI6	0.7500	0.7083	0.4583	0.5000	0.6250	0.1250	0.0833	0.1667	0.1250	0.0833	0.0000	0.0833	0.000	-	
SI8	0.7083	0.6667	0.4167	0.4583	0.5833	0.0833	0.0417	0.2083	0.0833	0.0417	0.1250	0.0417	0.1250	0.1250	-

The major cluster II also composed of two sub clusters, IIA and IIB. The sub cluster IIA was further sub-clustered into two, IIA_1 and IIA_2 which contained combination of *A. subintegra* and *A. sinensis*. The accessions of *A. subintegra* namely S2, S4, S7 and S9, plus *A. sinensis* namely SI3 and SI8 were placed in sub cluster IIA_1, while sub cluster IIA_2 contained only *A. sinensis* accessions namely SI1, SI5 and SI6. The sub cluster IIB contained only *A. subintegra* accession S5. From the dendrogram obtained, *A. subintegra* was more closely related to *A. sinensis* while the *A. malaccensis* was genetically distant from both.

DISCUSSION

RAPD has been widely used in genetic diversity study because it is relatively a simple, rapid, reliable and cost effective method (Esselman et al., 2000). In addition, RAPD primers are commercially available and no prior information on species genome required before RAPD screening (Sun and Wong, 2001; Josiah et al. 2008). An optimized protocol for RAPD-PCR is very important for reliability and reproducibility of the amplified DNA bands (Williams et al. 1993).

Determination of annealing temperature and screening of primers are the initial steps for genetic analysis using RAPD markers. Generally, the annealing temperatures for RAPD analysis in different *Aquilaria* species with different primers are ranging from 36 – 48 °C (Roslan et al. 2017). In this study, a gradient PCR was performed to test annealing temperatures varying from 43 – 47 °C. It was found that 44 °C was the optimal annealing temperature and further used for RAPD analysis. Four out of ten random primers showed distinct and reproducible bands. The remaining primers showed no or very faint amplification bands. The four primers (G12, R15, U13 and OPA 05) used in this study generated polymorphic bands indicating the usefulness of RAPD markers in analysis of genetic diversity in this taxon.

Recently, it was reported that ISSR markers were suitable for the assessment of polymorphism in *Aquilaria* species, however, no species-specific banding pattern for *A. malaccensis* could be obtained (Zahari et al., 2020). In addition, no species-specific bands were observed in *A. malaccensis* collected from Berau, East Kalimantan (Rimbawanto and Widyatmoko, 2011). In this study, two RAPD primers, G12 and OPA 05 produced species-specific banding patterns for *A. malaccensis* which will be useful for identification of this species. The finding of this study can lead to a creation of sequence characterized amplified regions (SCAR) markers by cloning and sequencing of polymorphic RAPD bands (Das et al. 2005; Lee et al. 2011; Roslan et al.2017).

Roslan et al. (2017) in previous study reported that RAPD analysis showed polymorphism in *Aquilaria* species and could be used to determine their genetic diversity. From the dendrogram obtained, all *A. malaccensis* were clustered into the same cluster while a combination of *A. subintegra* and *A. sinensis* were clustered into another cluster. According to Lee et al. (2011), *A. malaccensis* was closely related to *A. hirta*, another native species to Malaysia. This probably due to the different origin of these *Aquilaria* sp. where *A. malaccensis* is native to Malaysia while both *A. subintegra* (from Thailand) and *A. sinensis* (from China) were the introduced species to Malaysia. This finding was explained by Lasky et al. (2012), who stated that genomic variation in plant can be exhibited by the geographical distance and climate variation.

CONCLUSION

The RAPD markers showed polymorphisms in all three *Aquilaria* sp. tested. The optimal annealing temperature for RAPD-PCR in *Aquilaria* sp. was observed at 44 °C. Among ten RAPD primers screened, only four primers (G12, R15, U13 and OPA 05) produced the most clear and reproducible bands. The primers G12 and OPA 05 produced species-specific bands to differentiate *A. malaccensis* from *A. subintegra* and *A. sinensis*. OPA 05 showed highest percentage of polymorphisms which contributed 100% compared to the other primers. Hence, OPA 05 is suggested as the best primer for identification of *A. malaccensis*. Dendrogram constructed from RAPD analysis showed two major clusters. *A. subintegra* was found to be more closely related to *A. sinensis,* while *A. malaccensis* was genetically distant from both. Further, the potential RAPD markers can be developed into SCAR markers which will be helpful for a rapid identification of *A. malaccensis* regardless the age of the trees and could be used in breeding program.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

HNNF and NAJ designed and performed the experiments and also wrote the manuscript. NA and NHM contributed reagent and materials. KM and AMA reviewed the manuscript. All authors read and approved the final version.

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