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Reconstruction of *Curcuma aeruginosa* Secondary Metabolite Biosynthetic Pathway using Omics Data

(Pembinaan Semula Tapak Jalan Biosintetik Metabolit Sekunder *Curcuma aeruginosa* Menggunakan Data Omiks)

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ABSTRACT

Curcuma aeruginosa *or* temu hitam *is herbaceous plant with high therapeutic values in its rhizome that is widely used in traditional medicine. However, molecular studies on the secondary metabolite biosynthetic pathway of* C. aeruginosa *is still limited. Hence, the aim of this study was to explore and reconstruct the secondary metabolite biosynthetic pathway of* C. aeruginosa *rhizome by integrating the metabolite profiling and transcriptomic data. A total of 81 metabolites were identified in the rhizome of* C. aeruginosa*; amongst others are curzerene and β-Cubebene which are potent antioxidants. A total of 28,225 unigene were obtained from the transcriptomic sequencing of* C. aeruginosa *rhizome and analysed to identify potential genes associated with the biosynthesis of its metabolites. Of these, 43 unigenes were identified and mapped onto five sub-pathways; i.e. carotenoid biosynthetic pathway, diterpenoid biosynthetic pathway, monoterpenoid biosynthetic pathway, terpenoid and steroid biosynthetic pathway, and sesquiterpenoid and triterpenoid biosynthetic pathway. This study demonstrated a systematic bioinformatic approach to reconstruct a metabolic pathway in the rhizome of* C. aeruginosa *using bioinformatic approach.*

Keywords: Data integration; metabolic pathway; metabolomics; pathway reconstruction; transcriptomic

ABSTRAK

Curcuma aeruginosa *atau temu hitam merupakan sejenis tumbuhan herba yang mempunyai nilai terapeutik tinggi pada bahagian rizomnya dan telah digunakan secara meluas dalam perubatan tradisi. Namun begitu, masih banyak yang belum diketahui tentang penghasilan metabolit sekunder di dalam* C. aeruginosa. *Kajian ini dijalankan untuk membina semula tapak jalan biosintesis* C. aeruginosa *dengan menggunakan data pemprofilan metabolit sekunder dan transkriptomik. Sebanyak 81 metabolit telah dikenal pasti di dalam rizom seperti* curzerene *dan* β-Cubebene *yang berfungsi sebagai anti-oksidan. Sejumlah 28,225 unigen yang terhasil daripada penjujukan transkriptomik rizom* C. aeruginosa *telah dianalisis untuk mencari dan mengenal pasti sebarang gen yang terlibat di dalam penghasilan metabolit di dalam rizom* C. aeruginosa. *Terdapat 43 unigen telah dikenal pasti terlibat di dalam lima tapak jalan biosintetik utama iaitu biosintesis karotenoid, biosintesis diterpenoid, biosintesis monoterpenoid, biosintesis terpenoid dan steroid serta biosintesis sesquiterpenoid dan triterpenoid. Kajian ini juga memfokuskan kepada strategi pembinaan semula tapak jalan biosintetik yang terlibat dalam rizom* C. aeruginosa *dengan menggunakan pendekatan bioinformatik.*

Kata kunci: Integrasi data; metabolomik; pembinaan semula tapak jalan; tapak jalan metabolik; transkriptomik

INTRODUCTION

Curcuma aeruginosa Roxb also known as *temu hitam* is an interesting aromatic blue rhizome belongs to Zingiberaceae family. This herb is popular in Asian countries and commonly found in the forests and river banks. It has been widely used in ethnomedicine as its rhizome has multiple therapeutic properties to cure various gastrointestinal disorders (Rajkumari & Sanatombi et al. 2018) and rheumatic disease (Liu et al. 2013).

At present there is a limited knowledge on the *C. aeruginosa* molecular information hence it is essential to investigate the metabolites and their associated bioactive compounds which have significant therapeutic properties. This investigation was achieved through metabolite profiling technique using gas chromatography-mass spectrometer (GC-MS) or liquid chromatography-mass spectrometer (LC-MS). Kamazeri et al. (2012) used a gas chromatography-mass spectrometry (GC-MS) technique to elucidate the bioactive compounds with anti-microbial and food preservative properties and to identify possible compounds to be used as essential oils. Meanwhile Simoh and Zainal (2015) have conducted a phytochemistry profiling of *C. aeruginosa* rhizome using different solvent extractions and 81 metabolites have been identified. Knowledge on the metabolite profiling is important to gain insight on the phytochemical composition in the rhizome of *C. aeruginosa* and as well as enabling it to be registered as one of the potential health products.

In addition to the metabolic profiles of *C. aeruginosa* rhizome, there were only 80 nucleotides and 30 protein sequences of *C. aeruginosa* were deposited in NCBI database (https://www.ncbi.nlm.nih.gov/gquery/?term=Curcuma+aeruginosa+) as of 20 March 2018. This limited genomic information has restricted the effort to the single-species metabolic pathway that can facilitate the understanding of biological mechanism involved in the rhizome of *C. aeruginosa*. Now the availability of metabolite profiles (Simoh & Zainal 2015) and transcriptomic data (Zaidan et al. 2016) of *C. aeruginosa* rhizome have provided an opportunity to reconstruct its metabolic pathway by integrating both omics data in combination with the functional characterization of genes and metabolites in search for the interesting bioactive compounds. Metabolic pathway reconstruction offers an essential step in deciphering the macromolecular relationship towards understanding the biological mechanisms involved in specific traits (Kitano 2002). Moreover, it serves as a platform to visualize the omic components involved in the biological and cellular processes (Francke et al. 2005). Therefore, findings from the reconstruction of metabolic pathway will serve as a valuable resource to facilitate the research on traditional herbs and development of natural products for therapeutic needs.

In this study, the unigenes and metabolites data were integrated and mapped onto the pathway template as part of reconstructing the species-specific pathways in *C. aeruginosa* rhizome. Five major biosynthetic pathways in rhizome of *C. aeruginosa* were elucidated; i.e., carotenoid, diterpenoid, monoterpenoid, terpenoids and steroids and sesquiterpenoids and triterpenoids biosynthetic pathways. The findings from this study will serve as a step forward in unravelling the major genes behind important biosynthetic pathway for better manipulation in the development of natural products for pharmaceutical applications.

Materials and Methods

Data Sources

Simoh and Zainal (2015) provided the *C. aeruginosa* rhizome metabolomics data set whilst unigenes data was obtained from the transcriptomic analysis performed by Zaidan et al. (2016). Metabolomics data set consisted of compound descriptions such as compound name, molecular formula, molecular weight, retention time and peak area. Unigene data set comprised of descriptions of unigenes name, identifier, annotation descriptions, annotation identifier and KEGG identifier in tab-delimited format.

Classification of Metabolites Profiling

Metabolites profiles were classified into primary and secondary metabolites by comparing the information from CheBI (Hastings et al. 2013), KEGG (Kanehisa et al. 2016) and PlantCyc (Schlapfer et al. 2017).

Mapping of Genes and Metabolites

The mapping of gene identifiers and metabolites was performed using data mined from CheBI, PlantCyc (Schlapfer et al. 2017), Medicinal Plant Metabolomics Resources (Wurtele et al. 2012), STRING (Szklarczyk et al. 2017), BioGRID database (Chatr-Aryamontri et al. 2017), UniProt database (Bateman et al. 2015) and TAIR (Berardini et al. 2015) databases.

Reconstruction of Metabolite Biosynthetic Pathway

The biosynthetic pathways of *C. aeruginosa* rhizome were constructed by mapping the relevant/related genes and metabolites data onto relevant skeleton pathways obtained from KEGG (Kanehisa et al. 2016) and were used as a reference model for the reconstruction of *C. aeruginosa* rhizome metabolite biosynthetic pathways.

Results and Discussion

Classification of Metabolite Data

A total of 81 metabolites were identified from the metabolite profiles of *C. aeruginosa* rhizome (Simoh & Zainal 2015). Of these, four of them are primary metabolites whereas 77 are secondary metabolites (Table 1). The primary metabolites are 8,9 b- Dimethyl -4a, 9b-dihydrodibenzol [b,d] furan-3 (4H)-one, D-Fructose,1,3,4,5,6-pentakis-O- (TMS)-, O-methyloxime, D-Glucose,2,3,4,5,6-pentakis-O(TMS)-O-methyloxime and α-D Glucopyranoside,2,3,4,5,6 tetrakis-O-(TMS)-β-D-fructofuranosyl 2,3,4,6-tetrakis-O-(TMS). Primary metabolites such as carbohydrate, lipid and protein are involved in plant growth and metabolism process (Sanchez & Demain 2008) whereas secondary metabolites such as alkaloid, steroid, tannins, and phenolic are usually involved in plant defence mechanism and are known to have antioxidant, anti-cancer and anti-inflammatory properties (Filippis 2016); making them as potential materials in the development of functional food and pharmaceutical products (Cameron et al. 2005).

Identification of Secondary Metabolite Biosynthetic Pathway in the Rhizome of *C. aeruginosa*

Further investigation was performed on the 77 identified metabolites to elucidate the biosynthetic pathways in the rhizome of *C. aeruginosa* and 72 pathways were identified. However, only five major biosynthetic pathways were highlighted in this study with its potential in pharmaceutical application (Rajkumari & Sanatombi 2018); i.e. terpenoid and steroid biosynthetic pathway, carotenoid biosynthetic pathway, diterpenoid biosynthetic pathway, monoterpenoid and sesquiterpenoid biosynthetic pathway and triterpenoid biosynthetic pathway (Table 1).

Monoterpenoid and terpenoid are bioactive compounds involved in pharmacological activities with many pharmaceutical applications (Sawai & Saito 2011; Singh

TABLE 1. Summary of five major biosynthetic pathways in *C. aeuroginosa* rhizome

KEGG Identifier	Biosynthetic Pathway	List of metabolites
map01062	Terpenoid & steroid biosynthetic pathway	*3-Carene* *Camphene* *Borneol* α-*Terpineol* β-*Sitosterol* *Butanedioic acid, [(TMS) oxyl-, bis (TMS) ester* *Citric acid, ethyl ester, tri-TMS* *Isocitric acid (TMS)* *Borneol-TMS ether* *Germacra-1(10),4-diene-12-oic acid 6 alpha hydroxy gamma lactone*
map00906	Carotenoid biosynthetic pathway	*Tetracosane*
map00904	Diterpenoid biosynthetic pathway	*Anthiaergostan-5,7,9,22-tetraen-14-ol-15-one*
map00902	Monoterpenoid biosynthetic pathway	*Camphene* *2-Thujene* β-*Pinene* *Cineole (Eucalyptol)* *Borneol* α-*Terpineol* *8,9 b-Dimethyl-4a,9b-dihydrodibenzol[b,d] furan-3(4H)-one* *3-methyl cyclopentane-1-yl-TMS ether*
map00909	Sesquiterpenoid & triterpenoid	*Caryophyllene* *Germacrene B* *Z-α-farnesene* *Germacrone* *Cycloisolongifolene, 8,9-dehydro-9-formyl* *Germacra-1(10),4-diene-12-oic acid 6 alpha hydroxy gamma lactone*

& Sharma 2015). Sesquiterpenoid and triterpenoid are biosynthesized via MEP pathway whereas monoterpenoid and diterpenoid are biosynthesized via MEP pathway (Sawai & Saito 2011). Meanwhile, carotenoid biosynthetic pathway contributes to the biosynthesis of orange pigmentation and a good source for vitamin A (Matsuba et al. 2015; Othman et al. 2014; Satoru & Saito 2011).

IDENTIFICATION

At present, the whole genome of *C. aeruginosa* is still unavailable hence the transcriptomic data of *C. aeruginosa* rhizome (Zaidan et al. 2016) was used to identify the genes involved. 28, 225 unigenes were successfully assembled and annotated. The annotation of these unigenes provided valuable information in search for genes related to the production of metabolites with high therapeutic values. Each unigene consists of gene name, gene identifier, gene description as well as KEGG pathway identifier. Functional annotation of unigenes has allowed the classification of unigenes on their respective biosynthetic pathways by using the identifiers-mapping approach. It has been demonstrated that the mapping between identifiers is known as pathway-based integration and it has been employed to integrate the transcriptomic and metabolomics data in elucidating the metabolic biosynthetic pathways involved (Cavill et al. 2015).

Since the primary focus of this study is on the five major biosynthetic pathways, further analyses were conducted to investigate the gene encoding enzymes that involved in these biosynthetic pathways resulting to the identification of monoterpenoid, carotenoid, diterpenoid, and terpenoid and steroid pathways that were assigned to 128, 67, 43 and six total number of unigenes, respectively and only one unigene was found in relation to the sesquiterpenoid and triterpenoid biosynthetic pathway (Figure 1).

As details on the genes and metabolites in the rhizome of *Curcuma aeuroginosa* are still unavailable in all biological databases, it has limit our effort to reconstruct the biosynthetic pathway. To overcome this issue, *Arabidopsis thaliana* was used as a reference and model to map the genes and metabolites of *Curcuma aeuroginosa* rhizome. The availability of the genomics and genetics information of *A. thaliana* has made it possible for the gene mapping identifier approach. By mapping the gene names from *C. aeruginosa* rhizome against TAIR database, 43 genes were successfully mapped to the reference gene and protein identifiers. A total of 32 genes were found to reside in the monoterpenoid biosynthetic pathway (Table

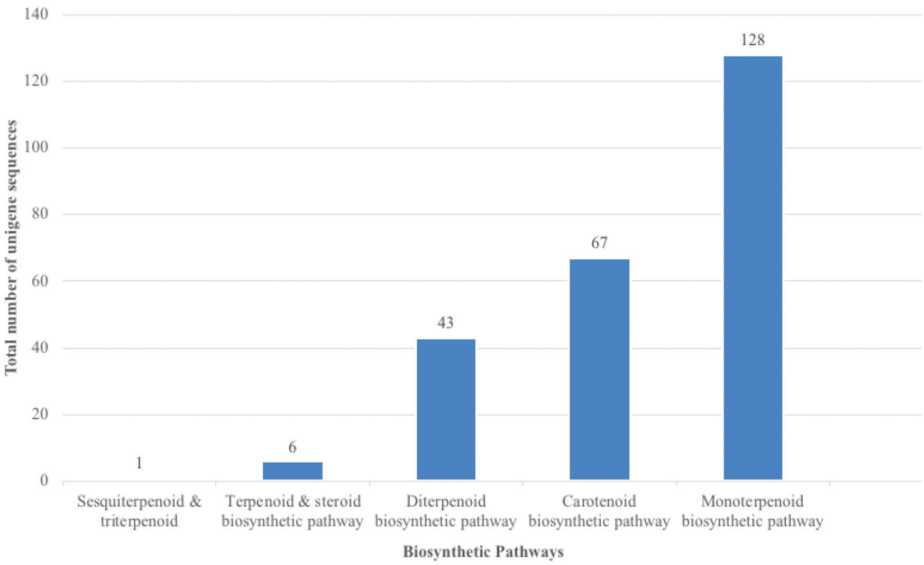

FIGURE 1. Distribution of unigenes in five major biosynthetic pathways of *Curcuma aeuroginosa* rhizome

2). Whilst, carotenoid, terpenoid and steroid, diterpenoid, sesquiterpenoid, and triterpenoid were associated with 5, 4, 5 and 2 genes of *A. thaliana*, respectively. This gene mapping analysis also showed that one gene can be involved in more than one biosynthetic pathway. This finding demonstrated that the 43 biosynthetic genes were mostly expressed in rhizome of *C. aeruginosa*. The interaction between molecules depends on the type of cell, condition, cell circulation phase, cell development, protein modification and binding cofactors (de Las Rivas & Fontanillo 2010).

Genes in the rhizome of *C. aeruginosa* with significant medicinal value include squalene synthase (SQS), a key enzyme in catalysing the sesquiterpenoid and triterpenoid biosynthetic pathway (Abe et al. 1993). The importance of SQS in the sesquiterpenoid and triterpenoid biosynthetic pathway has been reported in the herbal medicine such as *Bacopa monniera* and *Panax ginseng* (Lee et al. 2004). The molecular cloning and characterization of SQS in *B. monniera* has allowed the researcher to investigate and understand the molecular mechanism involved as the level of triterpene accumulates in low concentrations (Vishwakarma et al. 2015). Hence, by understanding the molecular mechanism of SQS will help in genetic engineering the sesquiterpenoid and triterpenoid biosynthetic pathway for increasing the production of triterpene content towards pharmaceutical application.

RECONSTRUCTION OF SECONDARY METABOLITE BIOSYNTHETIC PATHWAY OF *C. AERUGINOSA*

A putative biosynthetic pathway of *C. aeruginosa* rhizome was reconstructed using gene-metabolite mapping approach (Figure 2) and consisted of five major secondary metabolite biosynthetic pathways. The reconstructed pathway comprised of five biosynthetic pathways, 31 metabolites and 43 genes encoding enzyme. However, more experimental work is needed to complete and validate this reconstructed biosynthetic pathway. For instance, the identification of enzyme commission (EC) number, reactions as well as gaps.

Currently, the biosynthetic pathway reconstruction in herbs are very limited thus much effort is needed to reconstruct the secondary metabolite biosynthetic pathways using omics data. This strategy has been used in the reconstruction of starch biosynthetic pathway in cassava (Saithong et al. 2013) and in the reconstruction of metabolic network of *Setaria italic*a (de Oliveira Dal'Molin et al. 2016). Similar study has also indicated that it is essential to integrate the information from various metabolite pathway databases such as KEGG and BioCyc (Seaver et al. 2012) where this step has been successfully implemented in this study.

This study highlights major involvement of unigenes and metabolites in the terpenoid backbone biosynthetic pathway in the rhizome of *C. aeruginosa*. Five biosynthetic pathways; i.e., carotenoid, diterpenoid, monoterpenoid, terpenoids and steroids and sesquiterpenoids and triterpenoids biosynthetic pathways were derived from the terpenoid backbone biosynthetic pathway. Further study can be carried out on the establishment of these pathways for detailed understanding on their biological mechanisms towards the manipulation of high-value compounds for the industrial application (Li et al. 2018) using synthetic biology approach. In addition, the reconstruction of biosynthetic pathway can also be established as a platform to assist the cloning and characterization of potential enzymes with various industrial, pharmaceutical and nutraceutical benefits (Saithong et al. 2013).

CONCLUSION

Integration of transcriptomic and metabolomics data has led to the identification of five major secondary metabolite

TABLE 2. List of genes involved in selected metabolic biosynthetic pathways

Metabolic biosynthetic pathway	Gene identifier *C. aeruginosa*	Gene identifier *A. thaliana*	Gene name
Monoterpenoid	XP_009416430	AT1G60140	*alpha, alpha-trehalose-phosphate synthase [UDP-forming] 7*
	E3W9C4	AT1G01180	*Alpha-humulene 10-hydroxylase; P450 mono-oxygenase*
	XP_009414014	AT1G04920	*alpha, alpha-trehalose-phosphate synthase [UDP-forming] 9*
	XP_010930288	AT5G16970	*2-alkenal reductase (NADP(+)-dependent)-like*
	XP_008791841	AT1G26320	*2-alkenal reductase (NADP(+)-dependent)-like*
	EAZ26117	AT1G09610	*hypothetical protein OsJ_09979*
	XP_009409100	AT1G49530	*alpha, alpha-trehalose-phosphate synthase [UDP-forming] 6-like*
	XP_009401137	AT1G65560	*2-alkenal reductase (NADP(+)-dependent)-like*
	XP_009391422	AT3G14530	*alpha, alpha-trehalose-phosphate synthase [UDP-forming] 6-like*
	AJD09824	AT1G01290	*NADPH-dependent double-bond reductase 3 variant 2*
	EYU33434	AT1G11806	*hypothetical protein MIMGU_mgv1a001209mg*
	CDP13064	AT1G28620	*unnamed protein product*
	AGY49283	AT1G31950	*chloroplast terpene synthase*
	XP_009390520	AT1G06410	*probable alpha, alpha-trehalose-phosphate synthase [UDP-forming] 9*
	XP_009388531	AT1G01190	*cytochrome P450 734A6-like*
	BAJ39894	AT1G03410	*P450 mono-oxygenase*
	AAZ03640	AT1G13150	*putative cytochrome P450*
	AJD09822	AT1G09400	*NADPH-dependent double-bond reductase 2*
	AJD09823	AT1G12550	*NADPH-dependent double-bond reductase 3 variant 1*
	XP_008238638	AT1G11680	*7-ethoxycoumarin O-deethylase-like*
	XP_009415178	AT1G22410	*probable alpha, alpha-trehalose-phosphate synthase [UDP-forming] 7*
	XP_009413956	AT1G01280	*cytochrome P450 98A2*
	XP_009401244	AT2G23800	alpha, alpha-trehalose-phosphate synthase [UDP-forming] 5
	Q9SLN8	AT1G77120	*2-alkenal reductase (NADP(+)-dependent); Alkenal double bound reductase; Allyl-alcohol dehydrogenase; Allylic alcohol dehydrogenase 1; allyl-ADH1; Flavin-free double bond reductase; NtDBR; Pulegone reductase; NtRed-1*
	O48956	AT1G12740	*Cytochrome P450 98A1*
	O23617	AT1G63970	*Alpha, alpha-trehalose-phosphate synthase [UDP-forming] 5; Trehalose-6-phosphate synthase 5; AtTPS5*
	XP_009392100	AT1G11600	cytochrome P450 71A1-like
	XP_009380769	AT1G25083	*probable alpha, alpha-trehalose-phosphate synthase [UDP-forming] 9*
	XP_009409655	AT1G11610	*cytochrome P450 71A1-like*
	XP_009391250	AT1G24807	*probable alpha, alpha-trehalose-phosphate synthase [UDP-forming] 9*
	XP_009409766	AT1G24909	probable alpha,alpha-trehalose-phosphate synthase [UDP-forming] 9
	Q94AH8	AT3G14530	*Alpha, alpha-trehalose-phosphate synthase [UDP-forming] 6; Trehalose-6-phosphate synthase 6; AtTPS6*

continue

Continued TABLE 2.

Metabolic biosynthetic pathway	Gene identifier *C. aeruginosa*	Gene identifier *A. thaliana*	Gene name
Sesquiterpenoid & triterpenoid	P53800	AT2G44520	*Squalene synthase; SQS; SS; Full=FPP:FPP farnesyltransferase; Farnesyl-diphosphate farnesyltransferase*
	XP_009396917	AT4G34640	*squalene synthase-like*
Terpenoid & steroid	P49293	AT5G06130	*Phytoene synthase, chloroplastic; MEL5; Flags: Precursor*
	P37271	AT1G01046	*phytoene synthase 2, chloroplastic*
	XP_009386425	AT5G61670	*phytoene synthase 2, chloroplastic*
	P53797	AT1G01183	Phytoene synthase, chloroplastic; Flags:
Carotenoid	XP_009399529	AT5G05690	*cytochrome P450 724B1-like*
	P49293	AT5G06130	*Phytoene synthase, chloroplastic; MEL5; Flags: Precursor*
	P53797	AT1G01183	*Phytoene synthase, chloroplastic; Flags:*
	XP_009386425	AT5G61670	phytoene synthase 2, chloroplastic
	P37271	AT1G01046	*Phytoene synthase, chloroplastic; Flags:*
Diterpenoid	XP_009388282	AT2G19070	*benzyl alcohol O-benzoyltransferase*
	AFP33589	AT1G62730	*phytoene synthase 2a*
	XP_009399529	AT5G05690	*cytochrome P450 724B1-like*
	Q6F4F5	AT1G11680	*Cytochrome P450 724B1; Dwarf protein 11; OsDWARF11*
	Q6F4F5	AT1G11680	*Cytochrome P450 724B1; Dwarf protein 11; OsDWARF11*

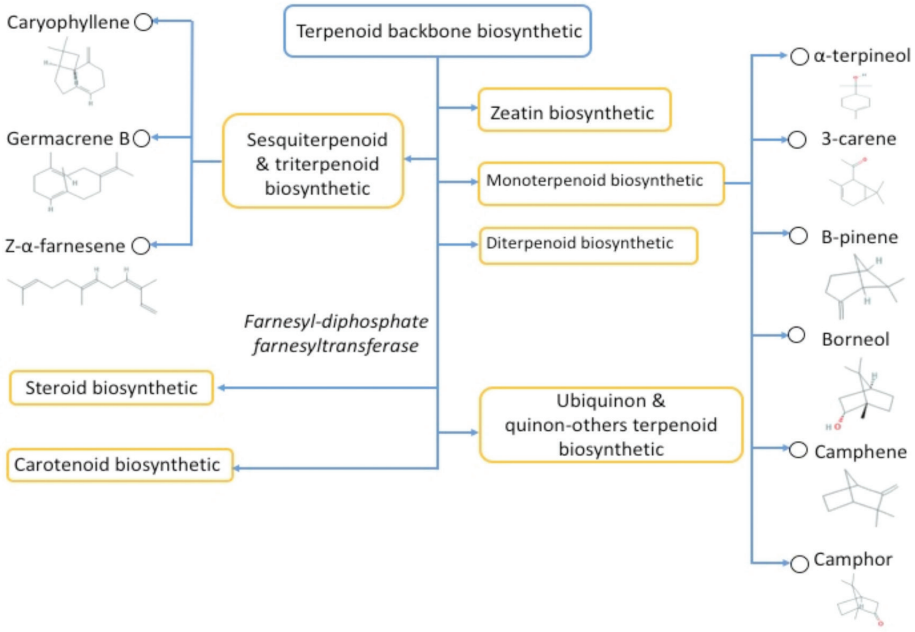

FIGURE 2. A reconstructed secondary metabolite biosynthetic pathway in the rhizome of *C. aeruginosa*. Yellow box refers to the biosynthetic pathway derived from the terpenoid backbone biosynthetic pathway. Nine potential metabolites and its structural chemistry were obtained from the metabolites profiles of *C. aeruginosa* rhizome

biosynthetic pathways in the production of potential metabolites with interesting pharmacological activities such as anti-tumor, anti-inflammatory, antimicrobial and antioxidant activity. This study has showed a putative pathway of *C. aeruginosa* rhizome constructed based on the interaction between genes and metabolites. This finding can be used to facilitate a better understanding on the biological mechanism of the secondary metabolite

production in *C. aeruginosa* rhizome. This will provide a valuable knowledge in search for potential materials with therapeutic values towards the development of health product based on local medicinal herbs.

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