Heliyon 10 (2024) e35861

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Metabolite comparative variation related lipid metabolisms among fruit, leaf, and stem of *Jatropha curcas*

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ARTICLE INFO

Keywords:
GC-MS
Jatropha curcas
Lipid biosynthesis
Metabolomics
Renewable energy

ABSTRACT

The issue of non-renewable energy scarcity has persisted over an extended period, primarily due to the depletion of fossil fuel reserves and the adverse effects of their utilization. This scarcity stems from the finite nature of fossil energy resources. The development of oil energy or biofuels aims to utilize oil-producing plants such as *Jatropha curcas* to develop alternative energy resources. However, metabolomic studies in *Jatropha curcas* are limited and need more investigations. Therefore, this research was essential to find biomarkers of metabolites among the fruit, leaf, and stem of *Jatropha curcas* using the GC-MS technique. We tested the metabolite profile with the R program, especially the metaboanalystR package, to determine fold change metabolite and pathway analysis. We found that 54 metabolites were detected in both fruit, leaf, and stem tissues of *Jatropha curcas* L, of which 19 metabolites were upregulated in the fruit, 20 metabolites in the leaf, and 15 up-regulated metabolites in the stem. The metabolites found formed three clusters based on correlation and networking metabolites analysis. The three clusters showed a relationship with the lipid biosynthesis pathway. In this study, provisional information was obtained that there was a different pattern of expression of metabolites between fruit, leaf, and stem tissues in *Jatropha curcas*, which was thought to be related to the critical metabolites of oleic acid and methylcyclohexane carboxylate in the biosynthetic pathway of fatty acids and unsaturated fatty acids. This information is essential as an initial reference for genetic engineering *Jatropha curcas* so that it can be used to transform plants, especially lipid-producing plants, as a source of oil.

1. Introduction

The crisis of non-renewable energy has occurred for a long time and can be in the form of limited raw materials and the impact of their use. The limitation of raw materials is a limitation of fossil energy resources. Using fossil energy increases environmental

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https://doi.org/10.1016/j.heliyon.2024.e35861
Received 1 May 2024; Received in revised form 23 July 2024; Accepted 5 August 2024
Available online 6 August 2024

pollution and changes in climatic conditions. Both things stimulate efforts to develop oils as a renewable energy source supporting ecological conservation efforts. Conversely, new and renewable energy technology is an alternative potential resource; these energy sources are commonly in biomass and biofuel, especially the biomass of plant products, one of which is *Jatropha curcas* [1]. Biofuel is environmentally friendly because it produces cleaner exhaust emissions and contributes less to global warming than fossil fuels. Exhaust emissions resulting from using biofuels in the form of carbon monoxide, unburned hydrocarbons, particulates, and toxic air are lower than using petroleum fuels. Biofuels can be produced from various renewable natural resources such as palm oil, non-edible oil (*Jatropha curcas*), and lignocellulosic biomass (from woody plants) [1].

The development of oil energy or biofuels aims to utilize oil-producing plants such as *Jatropha curcas* to develop alternative energy resources, pay attention to a sustainable environment, and make area energy independent, especially liquid fuels [2]. This goal is expected to be achieved without displacing other crops in land use. *Jatropha curcas* is one of the plants that can be selected as the leading non-edible oil producer [1]. As a potential source of Biofuel, *Jatropha curcas* has many advantages over other edible crop sources. This plant has good adaptability to various environments [3,4], is drought tolerant, resistant to pests and diseases, grows fast, and can still produce on land with low fertility such as marginal land so that it can support land conversion efforts [5,6]. Therefore, *Jatropha curcas* is very likely to be developed in Indonesia because there are 49.53 million ha suitable for *Jatropha curcas*, consisting of 14.28 million ha, very suitable, 5.53 million ha, quite suitable, and 29.72 million ha marginally suitable which have not been utilized and managed correctly [5].

Jatropha curcas belongs to the Euphorbiaceae family and is a tropical plant native to Mexico and Central America [5,6]. The *Jatropha* is widely cultivated in Central America, South America, Southeast Asia, India, and Africa [5]. This plant was widely distributed by Portuguese sailors through the Cape Verde Islands and Guinea Bissau to other countries in Africa and Asia [6]. In Indonesia, this plant is also known as jarak budeg, jarak gundul, or jarak cina. *Jatropha* is a woody plant with round stems and contains much sap. The leaf apex is acuminate, and the margin is wavy [5,6]. *Jatropha curcas* is a non-edible crop, so it has the potential to be mainly used as a source of plant material for producing biofuels. In corn and other crops, there is competition or dualism of use, i.e., as a fuel and food crop. In *Jatropha curcas*, utilization as a source of biofuel can be utilized optimally [1]. The potential use of *Jatropha curcas* as a renewable alternative bioenergy resource is crucial in overcoming the national and international energy crisis. However, research on metabolomics, genomics, and transcriptomics related to oil production and potential in *Jatropha curcas* still needs to be completed [7,8]. Several metabolites related to oil production in *Jatropha curcas*, especially in fruits and seeds, including tri-acylglycerols, curcin, and phorbol esters, are still limited [8]. Therefore, further studies on pathways in various organs of *Jatropha curcas* are still needed. This study aimed to examine and characterize the potential of *Jatropha curcas* as a renewable bioenergy source through metabolomics and molecular genetics approaches.

2. Materials and methods

2.1. Growth of Jatropha curcas L

The *Jatropha curcas*, which is two years old, is then transplanted into polybags using the cutting technique. We have collected *Jatropha curcas* around Bogor City (The latitude: 6.595038; The longitude: 106.816635; 290 m above sea) and propagated by cuttings in the experimental garden of Pakuan (West Bogor). We have harvested and analyzed fruit (Ft1, Ft2, Ft 3), leaf (Lf1, Lf2, Lf3), and stem (St1, St2, St3) of *Jatropha curcas* L after the plants were 12 months old with three biological repeats and three technical replications, respectively. The growth and development of *Jatropha curcas* potential were carried out by the following (Wang & Ding 2012). The tissues, mainly fruit, leaf, and stem, were harvested with characteristics, size, and development phase in each replication. For fruit, we harvest when the fruit is dark brown. For leaves, we harvested 11–17 cm in size, freshly textured and green in color, respectively. For the stem, we have harvested 30 cm for leaf primordia/or crown tips, respectively. Each sample was put into liquid N_2 and stored in the freezer (−80 °C) before maceration.

2.2. Identification of morphology and physiology of Jatropha curcas

The morphological identification of potential *Jatropha curcas* was carried out by direct observation following [4]. Morphological and physiological identification of *Jatropha curcas* is necessary to ensure the harvest time and the maximum number of metabolites that can be detected metabolically by the gas chromatography-mass spectrometry (GCMS) technique. Precisely, the process of harvesting *Jatropha curcas* fruit is adjusted to the physiological stage of the plant at the ripening stage according to the procedure performed by [9].

2.3. Extraction of metabolites and gas chromatography-mass spectrometry (GC-MS) assay in Jatropha curcas

Maceration of *Jatropha curcas* was performed according to that done by Refs. [9,10] with a slight modification, especially in the weight of the tissue used and solvent type. A total of 10 g of *Jatropha curcas* tissues were extracted using ethyl acetate (50 ml solvent) at 25 °C for 72 h, shaken with a 50 rpm shaker (proportion: 1 g sample:10 ml solvent), modified by Park et al. (2019). *Jatropha curcas* extract was evaporated using an evaporator (Caliper-Life-Science, USA) for 60 min at 45 °C. GC-MS was performed based on [9]. The extract (5 ml) in split is then injected into the GC-MS Instrument with the following equipment specifications: main instrument (7890, Agilent Tech-Palo Alto-USA), auto-sampler (7693, Agilent Tech-Palo Alto-USA), Mass Selective Detector, and a Chemstation Dataset System (5975 inert MSD Detector, Agilent Tech-Palo Alto-USA). Instruments covering injection temperature and temperature of

various instruments, mass spectrum detection, and metabolite identification were done following [10]. We set the instrument temperature (T), particularly ion source temperature (230 °C), injection temperature (250 °C), quadrupole temperature (140 °C), and interface temperature (280 °C). The metabolites were detected based on the Wiley W8N08.L and HMDB metabolites database [11].

2.4. Biomarker and statistical analysis of Jatropha curcas metabolites

We performed biomarker and statistical analysis based on [10]. We used Metaboanalyst R version 4.0 [12]. We set all metabolites as unpaired and threshold expressions into 2. All Metabolites were investigated using univariate and multivariate analysis [9,13,14] using R version 3.5.1 [15,16] and MetaboAnalystR version 4.0 [12,13,17–19].

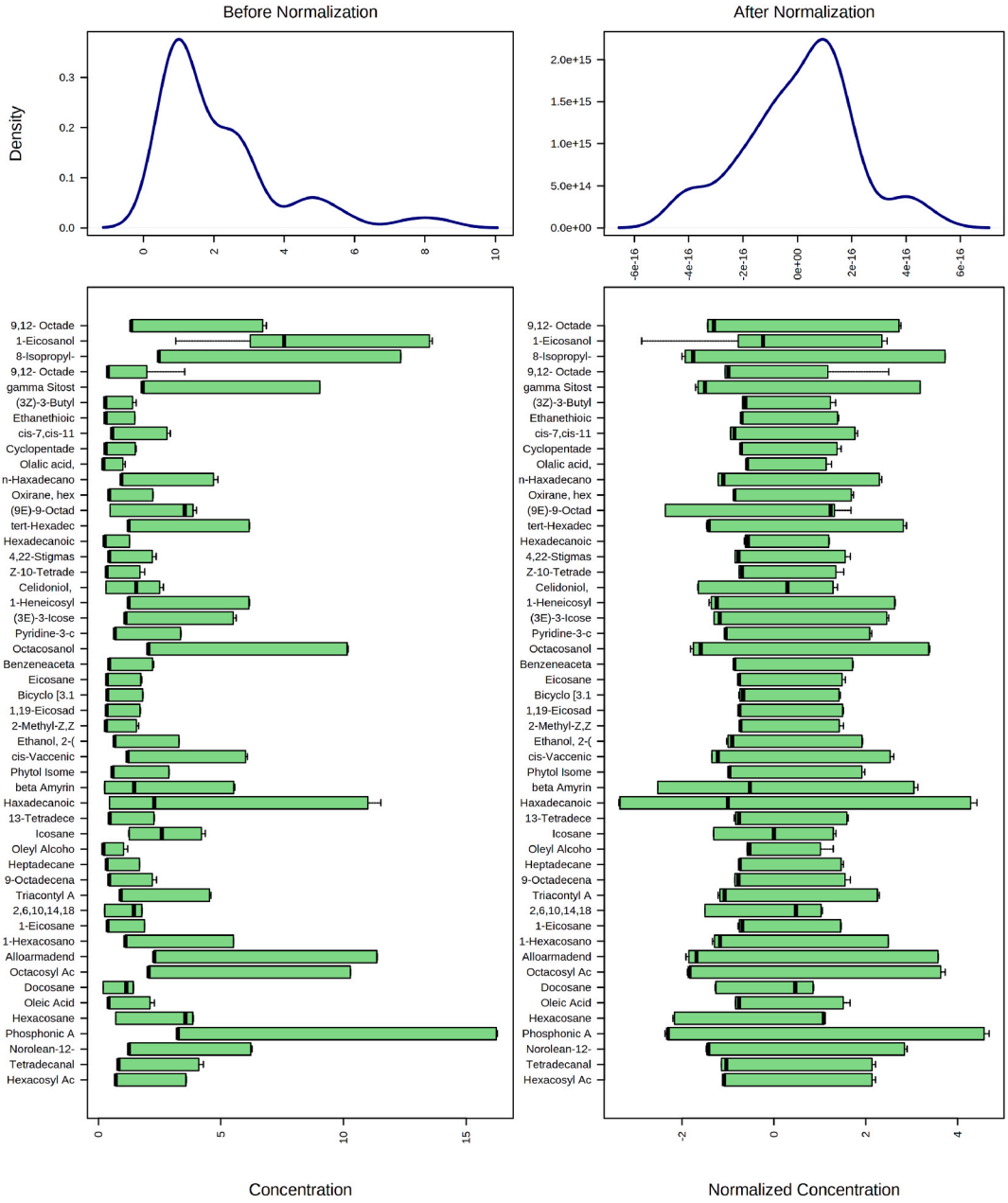


Fig. 1. Data processing of *Jatropha curcas* metabolites. All metabolite data was normalized using Metaboanalyst. R using main centered default.

2.5. Classification and clustering analysis

Classification and clustering analyses were carried out with several studies, including K-means clustering, Partial Least Square (PLS), heatmap-dendrogram expression, and mean decrease accuracy. The four analyses were performed by default on the program and MetaboAnalystR version 4.0 [10,12].

2.6. Correlation analysis

Specifically, we analyzed the correlation and set using the syntax: dimension 'feature,' distance measure 'Pearson rank correlation,' view mode 'overview,' and clustering 'true.' We set the settings in the R Program with MetaboAnalystR Packages based on the previous setting described above [9,12].

2.7. Debiased Sparse Partial Correlation (DSPC)

We use the Debiased Sparse Partial Correlation (DSPC) network Based on Basu et al. (2017). The nodes were input metabolites in the Debiased Sparse Partial Correlation (DSPC) network, while the edges represented the association measures.

2.8. Pathway analysis

Metabolites from all tissues were incorporated into a single data entry and were analyzed using the KEGG database, followed by

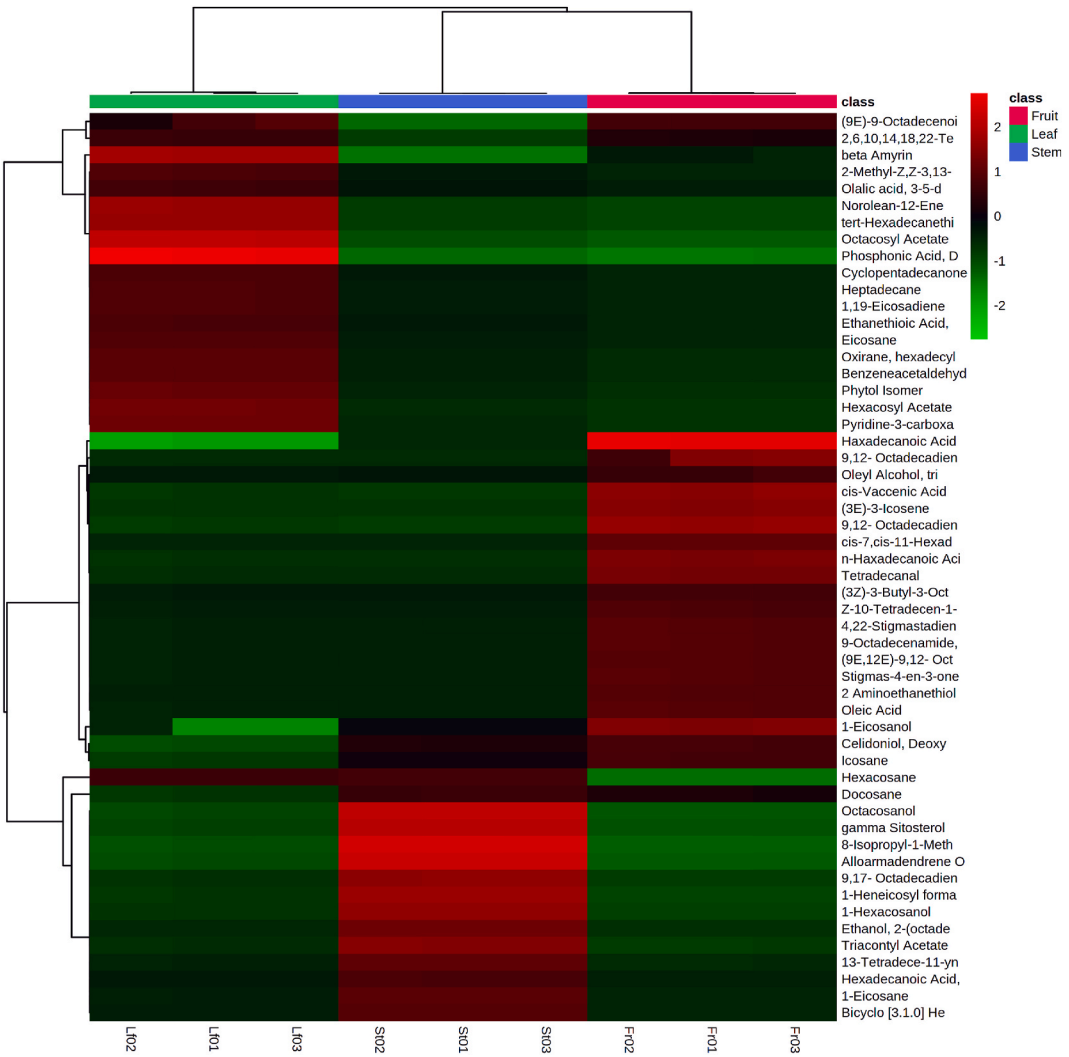


Fig. 2. Metabolites variation among fruit (Fr01, Fr02, Fr03), leaf (Lf01, Lf02, Lf03), and stems (St01, St02, St03) of *Jatropha curcas*.

Ref. [9]. All significant metabolites were investigated narrowly based on significant pathway scores.

3. Results

3.1. Metabolite profiling, biomarkers, and fold change of Jatropha curcas metabolites

Fifty-four metabolites were detected in the fruit, leaf, and stem tissues of *Jatropha curcas*. All detected metabolites have been normalized using Metaboanalyst standardization by default (Fig. 1). A total of 19 metabolites were upregulated, and 35 metabolites were downregulated in the fruit section. In the Leaf, 20 metabolites are upregulated, and 32 are downregulated based on fruit comparison. Specifically, we found 15 upregulated metabolites in stem tissues and 30 downregulated metabolites compared to fruit. Uniquely, we found one metabolite that was relatively expressed in all tissues, namely 2,6,10,14,18,22-Tetracosahexaene. In addition, the metabolite expression of Celidoniol was relatively upregulated in the fruit and stem parts of *Jatropha curcas* but was down-regulated in the leaf organs. Hexacosane was also upregulated in the leaf and stem tissues but was downregulated in the fruit. (9E)-9-Octadecenoic Acid was shown to be upregulated in leaves and fruit while stems were down-regulated (Fig. 2). Differences in the expression of metabolites may be caused by genetic expression patterns [20–22].

3.2. Hierarchical and partitional clustering among fruit, leaf, and stem

Hierarchical Clustering showed that 54 metabolites had relatively high fold changed values ranging from −2 to +2. The results of the clustering test showed three classes of metabolites, namely fruit, leaf, and stem classes. Regarding the research, the dendrogram

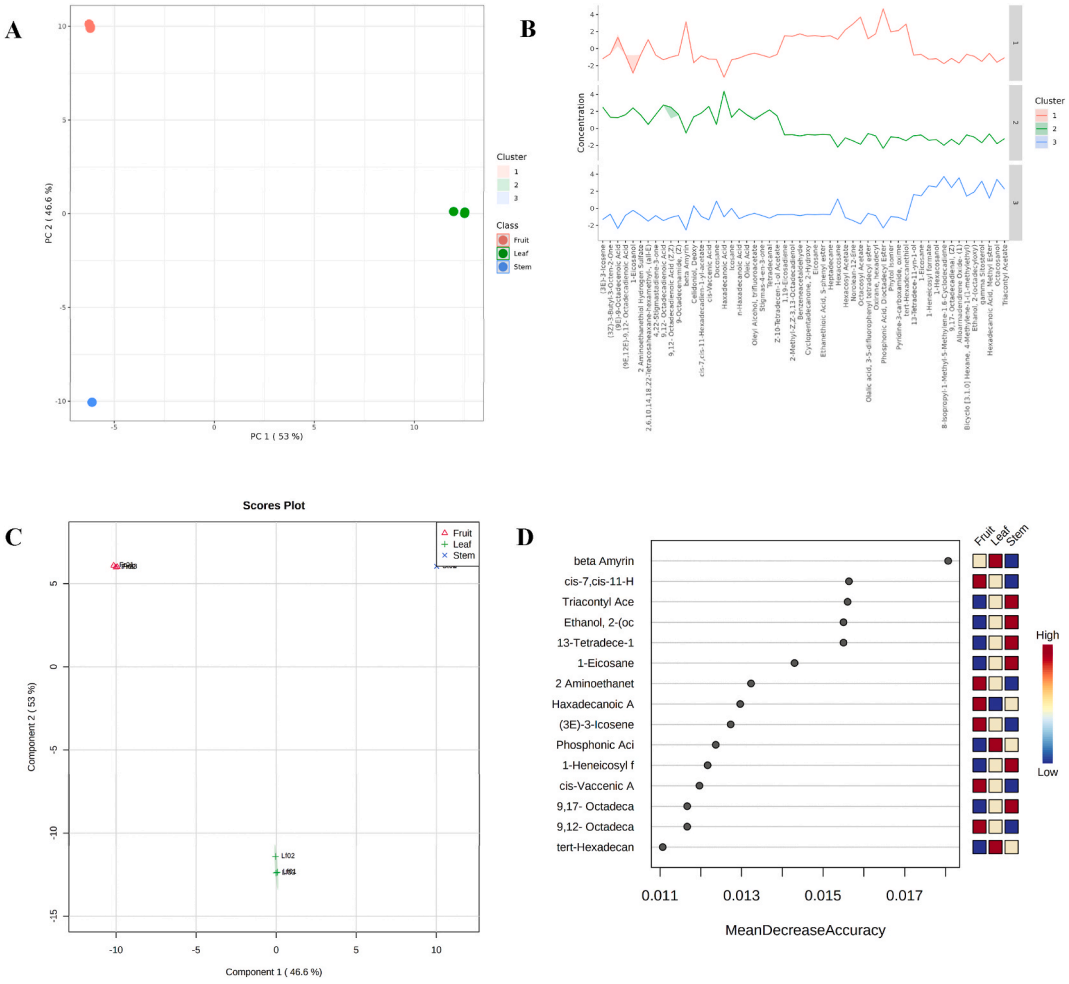


Fig. 3. Metabolites classification in several tissues (fruit, leaf, and stem) of *Jatropha curcas* Clustering K-means analysis (A) and overview metabolites plot among the tissues (B). Based on metabolite profiling, The cluster was divided into three types: 1, 2, and 3. Partial Least Square (PLS) of *Jatropha curcas* based on metabolites profiling (C). Mean Decrease Accuracy (MDA) analysis among metabolites in *Jatropha curcas* (D). Positively High MDA showed red color, and negatively high MDA showed blue color.

shows that the metabolites in the stem and fruit are relatively located in one group, while metabolites in the leaf class tend to separate (Fig. 2).

Partitional clustering was performed with K-means analysis and an overview of metabolite plots. The K-means test results showed that the fruit tissue metabolite profile tends to separate from the leaf and stem metabolites. The three tissues have relatively different metabolite profiles with a variation value of 99.6 %, with principal component 1 (PC1) values of 53 % and PC2 at 46.6 % (Fig. 3A). The same thing was also obtained in the overview metabolites plot analysis; there were three clusters with different patterns: the metabolites cluster from fruit, leaf, and stem tissue. The concentration values of the three showed fluctuating upregulation and downregulation of metabolites ranging from −2 to 4 with relatively different patterns (Fig. 3B).

3.3. Chemometrics Analysis, classification, feature selection of Jatropha curcas metabolites

Chemometrics Analysis was tested by Partial Least Squares - Discriminant Analysis (PLS-DA). The results of the PLS-DA test showed that the metabolites tend to separate based on the origin of the tissue, which has three main groups, i.e., the fruit, leaf, and stem metabolites group. The PLS-DA results showed a diversity of 99.6 %, with values ranging from −10 to 10 for component 1 and -15 to 5 for component 2 (Fig. 3C). Classification and Feature selection is done by investigating the Mean Decrease Accuracy (MDA) value. Features are ranked by their contributions to classification accuracy based on MDA scores. Based on the mean decrease accuracy (MAC) test, beta Amyrin, *cis*-7,*cis*-11-Hexadecadien-1-yl-acetate, and Triacontyl Acetate (Fig. 3D) showed the highest MAC scores.

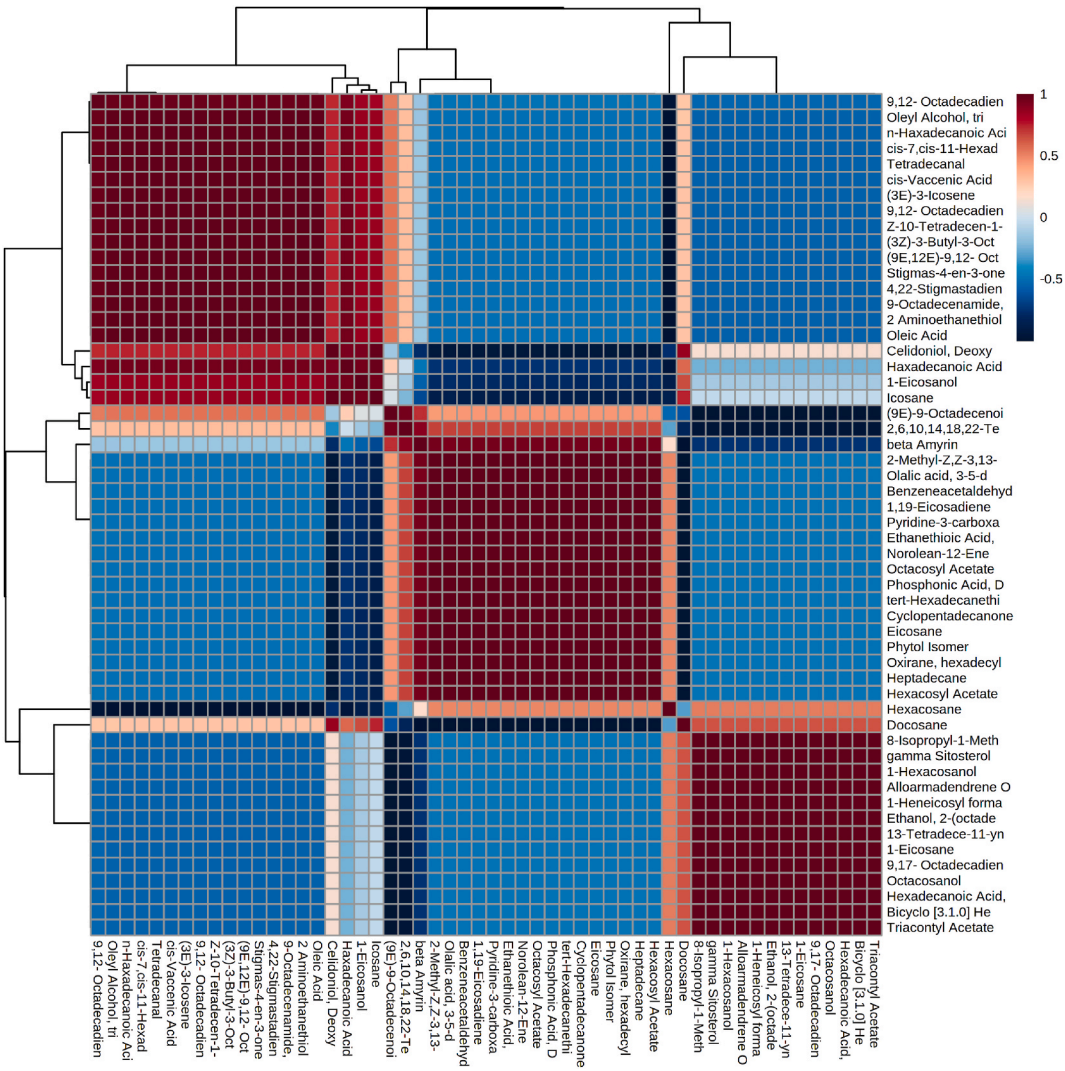


Fig. 4. Intercorrelation analysis of all metabolites in fruit, leaf, and stem of *Jatropha curcas* Significant correlation was determined from −1 to +1. The names, whether vertical or horizontal, are names of compounds.

3.4. Correlation heatmaps of Jatropha curcas metabolites

Metabolites detected in fruit, leaf, and stem tissue were grouped into three clusters, namely correlation clusters 1, 2, and 3. The distribution of the three clusters was based on the level of a strong positive correlation between one metabolite and another metabolite. Specifically for cluster 1, the metabolite 9,12- Octadecadienoic Acid was positively correlated with 15 other metabolites, i.e., with Oleyl Alcohol trifluoroacetate, n-Haxadecanoic Acid, *cis*-7 *cis*-11-Hexadecadien-1-acetate, and so on. In cluster 2, beta Amyrin compounds have a high degree of correlation with several vital compounds, i.e., 2-Methyl-Z, Z-3,13-Octadecadienol, and Olalic acid, 3-5-di fluorophenyl tetradecyl ester. The degree of correlation in cluster 3 is shown by 8-isopropyl-1-methyl-5-methylene-1.6-cyclodecadiene with gamma Sitosterol (Fig. 4). It is suspected that the level of correlation in this cluster has a strong relationship with the lipid metabolism pathway, so it is essential to examine the pathway analysis in this study, especially on the fruit, stem, and leaf tissues of *Jatropha curcas*.

3.5. Debiased Sparse Partial Correlation (DSPC)

DSPC analysis is carried out to see the relationship of one compound to another compound, which, in this case, is divided by the number of networks, nodes, and edges. Tools that must be considered in DSPC include reset network, degree filter, betweenness filter, minimum network, and correlation filter. The network generated in the DSPC test of metabolites of all networks showed three types, i.e., subnetwork1 (Fig. 5A), subnetwork2 (Fig. 5B), and subnetwork3 (Fig. 5C). In subnetwork1, the compound Olalic acid, 3-5-di fluorophenyl tetradecyl ester, is the main node closely related to 2-Methyl-Z, Z-3,13-Octadecadienol. 2-Methyl-Z, Z-3,13-Octadecadienol then form a spherical network of 1,19-Eicosadiene and other major compounds (Fig. 5A).

3.6. Pathway analysis

Based on the pathway test results, the metabolites detected in the three organs of *Jatropha curcas* showed that there were the highest influencing pathways, i.e., the fatty acid biosynthesis and unsaturated fatty acid biosynthesis pathways (Fig. 6A and B). The essential compounds detected in the specific pathway test were oleic acid and methylcyclohexane carboxylate (Fig. 6C and D). Further studies on the fatty acid biosynthesis pathway show this pathway involves essential compounds such as acetyl CoA, Malonyl-CoA, Octadecanoic acid, Decanoic acid, Hexadecanoic acid, and Tetradecanoic acid (Fig. 7). Specifically to the significant pathway in the biosynthesis pathway of unsaturated fatty acids, the fatty acid pathway in the n-3, n-6, n-7, n-9, and n-10 families shows significance with the key metabolite detected in the form of e-linolenic acid (ELA), Icosapentanoic acid (EPA), and Docosahexaenoic acid (DHA) (Fig. 8).

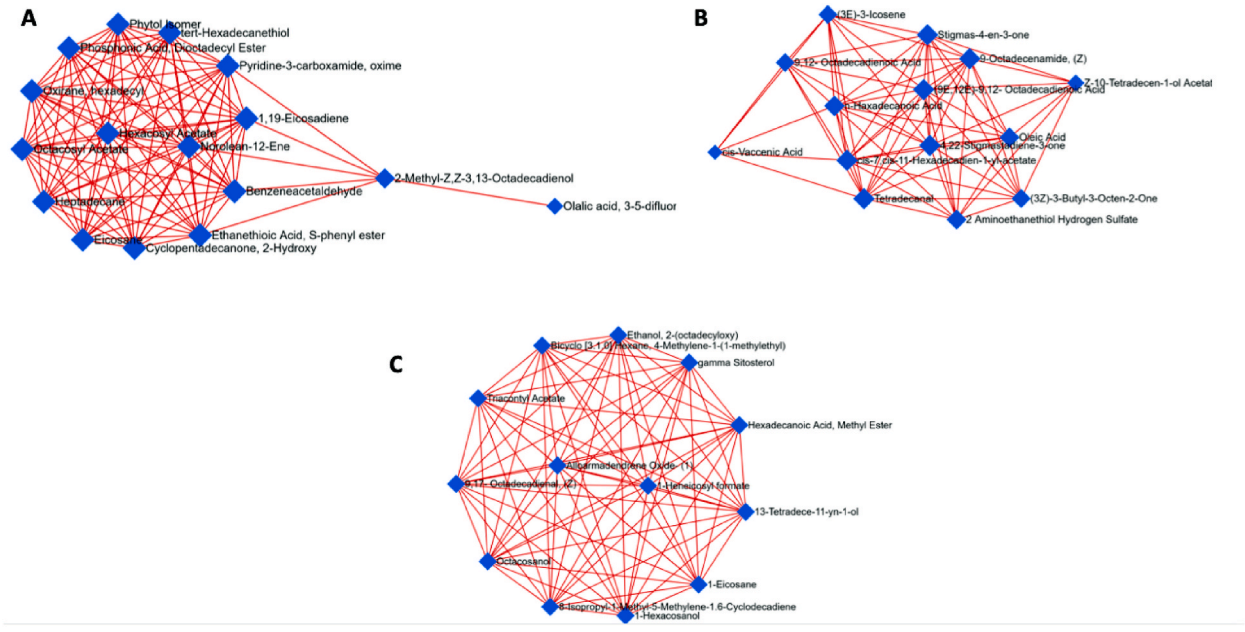
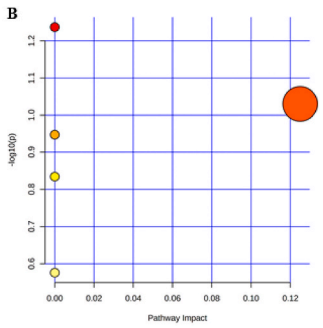
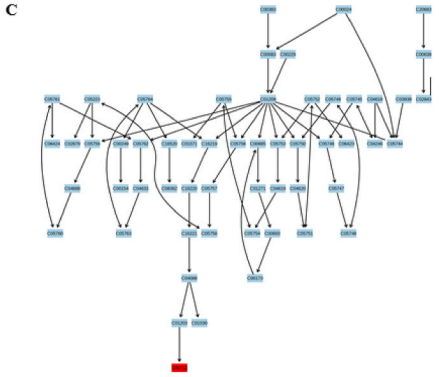


Fig. 5. Metabolites networking in *Jatropha curcas* using Debiased Sparse Partial Correlation (DSPC). Analysis of DSPC was performed in this study. Blue nodes input all metabolites in *Jatropha curcas*, while red edges represent the association measures among the metabolites.

A

Pathway Name	p	-log(p)	Holm p	FDR
Fatty acid biosynthesis	0.266	0.576	1.0	1.0
Biosynthesis of unsaturated fatty acids	0.113	0.947	1.0	1.0
Phenylalanine metabolism	0.058	1.236	1.0	1.0
Cyanoamino acid metabolism	0.146	0.834	1.0	1.0
Cutin, suberine and wax biosynthesis	0.093	1.030	1.0	1.0





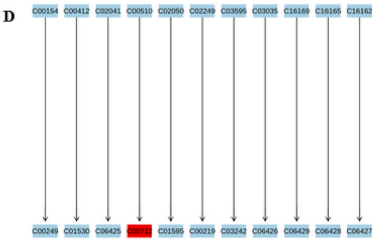


Fig. 6. Pathway analysis of all metabolites in *Jatropha curcas* Pathway is significant based on metabolites –log(p), holm p, and FDR scores (A). Five top hits of the metabolites pathway impact all Jatropha curcas tissues (B). Fatty acid biosynthesis (C) and Biosynthesis of unsaturated fatty acids (D) pathways in fruit, leaf, and stem of *Jatropha curcas* Red color represents highly significant metabolites hit, and blue color represents low to moderate metabolites hit.

4. Discussion

Metabolomics is one of the main areas of research in Omic technology that focuses on the expression of both upregulated and downregulated metabolites. The target of analysis in metabolomics studies is compounded with low molecular weights that are synthesized or broken down by an organism at certain times and under certain conditions. The primary purpose of metabolomics studies in plants is to measure the presence of metabolomes or all compounds present in an organism. Metabolic analysis is usually done to look for specific biomarkers in a tissue, for example, to see particular compounds in specific tissues not found in other tissues. This study has been carried out on snake fruit plants to distinguish metabolite markers in red and white arillus [10]. Many metabolomics studies have also been carried out on other plants, such as Cucumis sativus [23], Mukia javanica [24], etc. Metabolomics in plants combines a compound identification strategy with a statistical approach to measure the profile of metabolites present in cells and tissues. Metabolic studies consist of metabolic profiling, which is a quantitative estimation of a particular group of metabolites [9], metabolic fingerprinting, namely quantifying the complete profile of metabolites [10], and isotope-based analysis, which is intended to analyze specific compounds from intermediate metabolites of a biochemical pathway [9]. Although many studies have been carried out on metabolite profiles and pathway analysis in plants, research on *Jatropha curcas*, which examines biomarker analysis between fruit, leaf, and stem tissues, has never been carried out. However, this research is essential to know the metabolic pathways associated with producing oil in the fruit. In addition, by understanding the metabolite profile in the leaves and stems, we can ascertain the specific metabolites in the fruit and whether there is a potential for accumulation of metabolites deposited in the fruit.

There are several methods used in metabolomics studies, namely gas chromatography-mass spectrometry (GC-MS), liquid chromatography (LC)-MS, capillary electrophoresis (CE)-MS, and nuclear magnetic resonance spectroscopy (NMR) (Obata & Fernie 2012; Jorge et al., 2016). GC-MS is an advanced technology in metabolite analysis with high chromatographic resolution [25]. However, GC-MS cannot be used to detect polar compounds. Thus, HPLC-MS was used to cover this weakness, which has a wide range in detecting compounds even with a lower chromatographic resolution. Compared with NMR, metabolomic studies using GC-MS and LC-MS are considered relatively easy and inexpensive because they do not require isotopes in their implementation. Metabolomics studies have been used to study plant responses to stress, including stress in inundation conditions, drought, and other stresses [2]. In this study, we used GCMS to examine metabolites that would later be associated with lipid biosynthesis. GCMS is necessary for testing metabolites that tend to be volatile. We used ethyl acetate solvent, a polar-non-polar solvent, so it is expected to detect a broader range of volatile metabolites than water or alcohol solvents. Currently, biomarker studies are being studied very rapidly, usually in the form of morphological biomarkers [22–24], anatomy [26], physiology [27], molecular such as Snp DNA [27–29] or RNA transcriptomics [14,30] and metabolites [9,10]. In this study, we tried to develop biomarkers of metabolites based on fold change metabolites in fruit, leaf, and stem tissue in *Jatropha curcas*.

Omic technology includes metabolomics, transcriptomics, proteomics, and genomics [22,31]. Research on fold change analysis in metabolomics studies is widespread [32]. According to this research, Metabolites detected in fruit, leaf, and stem tissues showed a high category, namely 54 metabolites. The detected metabolites are relatively high and are similar to those of [2]. In general, the

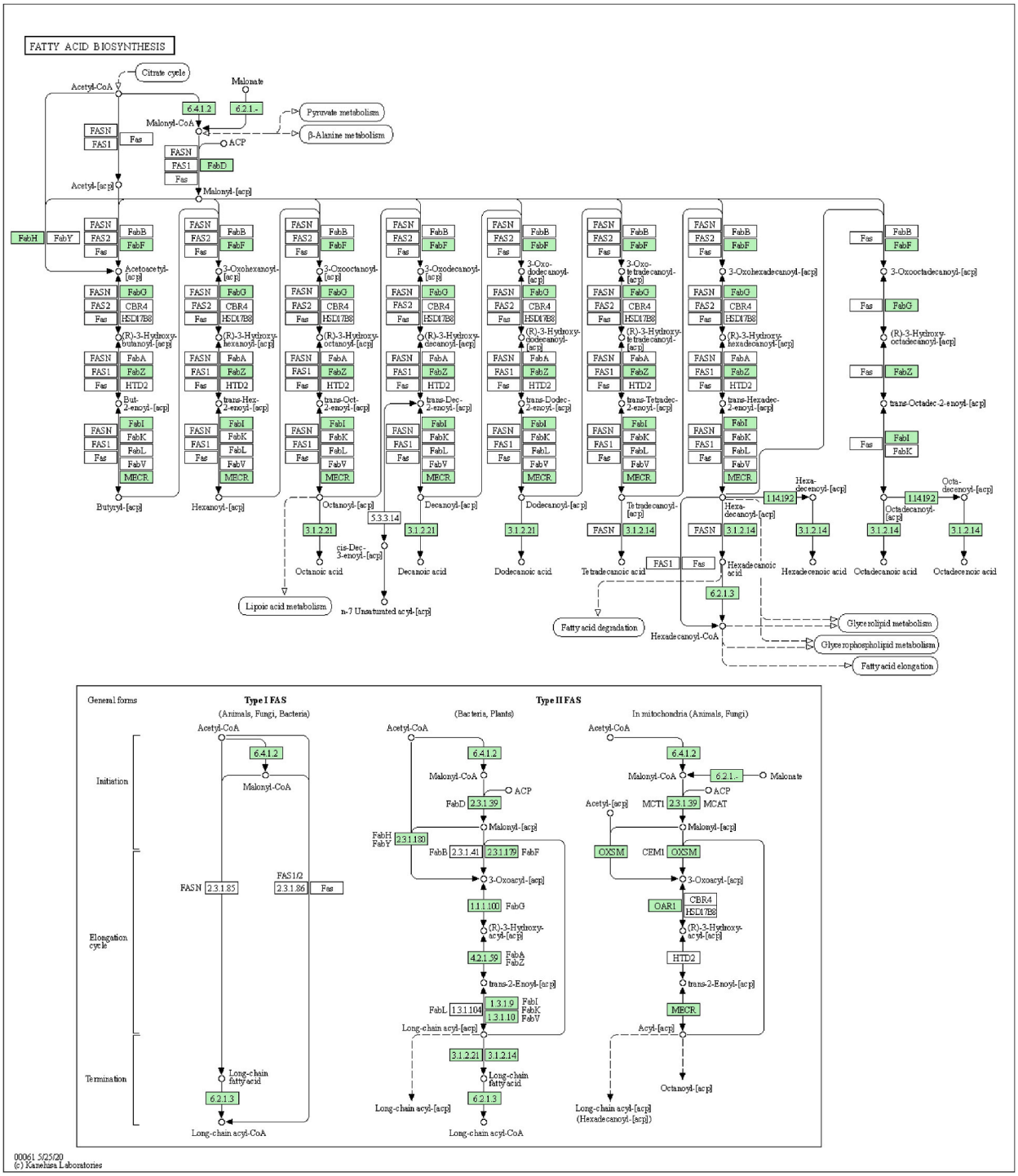


Fig. 7. KEGG pathway of fatty acid biosynthesis in *Jatropha curcas* Pathway was detected using Metaboanalyst R. Green border shows a significant impact of the metabolite in the pathway.

metabolites found in this study showed three clusters from the fold change expression analysis, K-means classification, Intercorrelation, and metabolites network analysis tests. This shows consistent results that the metabolites between fruit, leaf, and stem have statistically different profiles and expression patterns of metabolites. The metabolite profile in all three shows that these metabolites are significantly related, especially with the Fatty acid biosynthesis and Biosynthesis of unsaturated fatty acids pathways. This essential compound has the accession COO712. After investigation, this metabolic pathway was related to Oleic acid and Methylcyclohexane

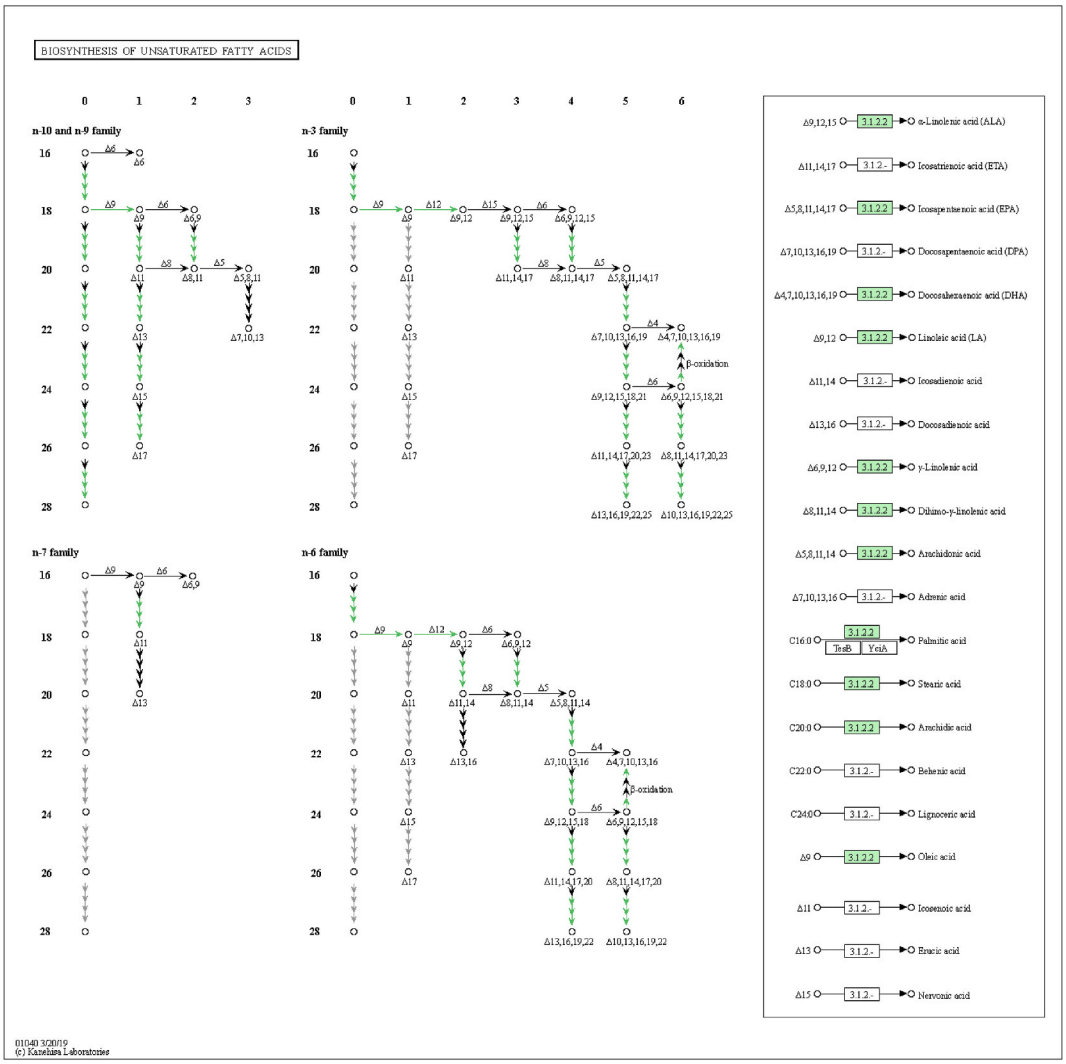


Fig. 8. The KEGG pathway of biosynthesis of unsaturated fatty acids of Jatropha curcas Pathway was detected using Metaboanalyst R. Green border shows a significant impact of the metabolite on the path.

carboxylate (Figs. 7 and 8).

Lipid biosynthesis in *Jatropha curcas* showed a strong relationship with lipid metabolism pathways, especially in the triacylglyceride (TAG) pathway. Lipid metabolism is the synthesis and degradation of lipids in cells, which involves the breakdown or storage of fat for energy [33]. These fats are obtained from consuming food and absorbing or synthesizing them by plants. Lipogenesis is the process of synthesizing these fats [34]. The types of lipids found in the body are fatty acids and membrane lipids [35,36]. Lipid metabolism often begins with hydrolysis, which occurs with the help of various essential enzymes [37]. Lipid metabolism also occurs in plants, although the process differs in animals [38]. In *Jatropha curcas*, in this study, it is suspected that this analytical pathway is related to lipid biosynthesis and involves the ACC and GPAT enzymes in the triacylglyceride (TAG) pathway starting from the acetyl-CoA precursor to oil bodies in plastids and reticulum. endoplasm [2]. In this study, provisional information was obtained that there was a different pattern of expression of metabolites between fruit, leaf, and stem tissues in *Jatropha curcas*, which was thought to be related to the key metabolites of Oleic acid and Methylcyclohexane carboxylate in the biosynthetic pathway of fatty acids and unsaturated fatty acids. This information is essential as an initial reference for the genetic engineering of Jatropha curcas so that it can be used to transform plants, especially lipid-producing plants, as a source of oil in the future.

5. Conclusion

In Summary, the identified metabolites formed three distinct clusters through correlation and metabolite network analysis. These clusters were found to be associated with the lipid biosynthesis pathway. This research has provided preliminary insights into variations in metabolite expression patterns among different tissues of *Jatropha curcas*, including fruit, leaf, and stem tissues. These

variations appear linked to key metabolites like oleic acid and methylcyclohexane carboxylate within the fatty acid and unsaturated fatty acid biosynthesis pathway. This information is an initial reference point for potential genetic engineering applications in *Jatropha curcas*. It can facilitate the development of genetically modified plants, especially those involved in lipid production, which will serve as future sources of oil.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Miftahul Huda Fendiyanto: Writing – original draft, Validation, Software, Investigation, Formal analysis, Conceptualization. **Muhammad Fuad Anshori:** Writing – original draft, Supervision, Resources, Methodology, Conceptualization. **Mentari Putri Pratami:** Writing – original draft, Data curation. **Daniel O. Wasonga:** Writing – review & editing, Validation, Conceptualization. **Mahmoud F. Seleiman:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Miftahul Huda Fendiyanto reports equipment, drugs, or supplies was provided by IPB University. Miftahul Huda Fendiyanto reports equipment, drugs, or supplies was provided by Laboratorium Kesehatan Daerah (LABKESDA).

Acknowledgments

This research was funded by Researchers Supporting Project number (RSPD2024R751), King Saud University, Riyadh, Saudi Arabia. In addition, the Ministry of Defense, Republic of Indonesia, supported the work, especially the Universitas Pertahanan Research Scheme, through Dr. Miftahul Huda Fendiyanto, S.Si., M.Si Funding year 2022. This work was also supported by Laboratory Plant Physiology and Molecular Biology, Department of Biology, IPB University. The authors acknowledged Laboratorium Kesehatan Daerah (LABKESDA), Jakarta, Indonesia, especially in supporting GC-MS analysis. Besides that, we also remembered to thank Hasanuddin University for the collaborative publication facility in developing this manuscript.

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