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TLC-based fingerprinting for *Phyllanthus niruri* from diverse geographical origins in East and Central Java Indonesia

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TLC-based fingerprinting for *Phyllanthus niruri* from diverse geographical origins in East and Central Java Indonesia

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Abstract. *Phyllanthus niruri* L. (meniran), the member of *Euphorbiaceae*, is a medicinal plant that is commonly found in tropical and sub-tropical areas such as Asia, America, and China. Various factors such as environment, geographical location, harvest time, and post-harvest process can affect the quality of crude drugs produced from *P. niruri*. The objective of this study was to evaluate the quality of meniran herbs obtained from 15 geographical origins in East and Central Java, Indonesia using Thin Layer Chromatography (TLC) profiles analyzed by chemometrics. TLC was carried out using TLC plate Si Gel 60 GF₂₅₄ as stationary phase; toluene, ethyl acetate, methanol, 85% formic acid (75:25:25:6) as mobile phase; and visualized using NP/PEG Reagent. The results showed TLC-fingerprinting combined with chemometric (PCA and CA) analyses were able to discriminate the origin of *P. niruri* from different geographical origins. *P. niruri* from 15 locations of East and Central Java Indonesia were classified into 5 groups based on their chemical similarity. The samples that are grouped in one cluster have the similar quality of chemical compounds, while the samples in different clusters also have different qualities.

Keywords: chemometric, geographical origin, herbal medicine, principal component analysis, TLC

1. Introduction

Phyllanthus niruri L. (meniran) belongs to *Euphorbiaceae* family and grows in tropical and sub-tropical environment such as Asia including Indonesia [1]. This plant has various pharmacological activities such as anti-inflammatory, antioxidant, antihyperuricemic, antimalarial, anticancer, antihyperlipidemic, hepatoprotector, and immunomodulatory [2, 3]. The chemical compounds contained in meniran herbs include flavonoids (rutin, quercetin, quercitrin, astragaline, catechin), alkaloids (norsecurinine, nirurine, phyllochrysin), terpenoids (limonene, p-cymene, lupeol), lignans (phyllanthin, hypophyllanthin, niranthin, nirtetralin, phyltetralin, lintetralin), tannins (repandusinic acid, geraniin, corilagin), coumarins (ellagic acid, methyl brevifolincarboxylate), and saponins (diosgenin) [4].

In general, quality of herbal medicine raw materials is determined by a number of variables, i.e., environment, geographical location, harvest time, and post-harvest process [5-7]. Considering that *P. niruri* can be grown in various habitat, it is possible that the quality of the crude drugs and its products will also vary. Therefore, a specific method is needed to evaluate the quality of meniran herbs obtained from various growing environment.



Quality evaluation of the crude drugs can be done by using specific parameters and non-specific parameters. Specific parameters can be determined utilizing a chemical marker and fingerprint profile approaches. The marker approach involves one or several specific compounds in plants, while fingerprinting profile involves the use of information from all or almost all chemical compounds in plants obtained through spectroscopic or chromatographic methods [8-10]. Thin Layer Chromatography (TLC) is a simple, fast, and inexpensive chromatographic method. The results are visually more attractive and informative than other chromatographic methods [11]. Multivariate data produced from TLC profiles need a special technique analysis called as chemometrics. It uses statistics and mathematics for chemical data processing [12]. Various statistical methods can be used in chemometrics, including Principal Component Analysis (PCA) and Cluster Analysis (CA). The objective of the study was to evaluate the quality of meniran herbs obtained from 15 geographical origins in East and Central Java, Indonesia based on TLC profiles analysed by chemometrics.

2. Material and methods

2.1. Plant materials

Phyllanthus niruri samples were collected from 15 locations in East and Central Java, Indonesia (table 1) and were authenticated by the Center for Information and Traditional Medicine Development (PIPOT), Faculty of Pharmacy, University of Surabaya with a letter of determination no. 1432/D.T/I/2021.

Table 1. Geographical location of *Phyllanthus niruri* from East and Central Java, Indonesia.

No.	District	Height (m a.s.l.)	Latitude, Longitude	Time of collection	Moisture content (%) ^a
1	Surabaya	2	7°15' S; 112°45' E	June 2019	6.64 ± 0.11
2	Gresik	3	7°09' S; 112°39' E	July 2020	5.52 ± 0.19
3	Sidoarjo	3	7°28' S; 112°40' E	September 2020	5.23 ± 0.44
4	Pasuruan	5	7°38' S; 112°53' E	August 2020	6.73 ± 0.08
5	Banyuwangi	25	8°13' S; 114°22' E	July 2020	5.25 ± 0.18
6	Mojokerto	30	7°28' S; 112°26' E	June 2019	6.69 ± 0.33
7	Bangkalan	47	7°01' S; 112°45' E	June 2019	6.39 ± 0.32
8	Lumajang	51	8°50' S; 113°14' E	August 2020	5.84 ± 0.55
9	Nganjuk	56	7°36' S; 111°53' E	June 2019	6.90 ± 0.13
10	Kediri	60	7°50' S; 112°01' E	June 2019	6.93 ± 0.53
11	Jember	83	8°11' S; 113°40' E	September 2020	5.71 ± 0.18
12	Tulungagung	85	8°03' S; 111°54' E	August 2020	5.02 ± 0.43
13	Blitar	167	8°05' S; 112°09' E	June 2019	6.74 ± 0.60
14	Batu	831	7°52' S; 112°31' E	August 2020	5.72 ± 0.35
15	Tawangmangu	1200	7°42' S; 111°08' E	August 2020	6.72 ± 0.38

^amean ± SD (n = 3)

2.2. Preparation of extracts

The *P. niruri* parts used in this study were all plant parts above the ground (shoot). The shoot was washed with tap water, air dried, ground and sieved. The water content was then determined using moisture content balance (Moisture Analyzer HB43 Mettler-Toledo GmbH, Laboratory & Weighing Technologies, Switzerland). One gram of powdered material was then extracted with 10 ml methanol (Merck KGaA, Germany) using an ultrasonic bath (As One, Japan) at 42 kHz for 15 min. The extract was filtered into a 10 ml volumetric flask and then stored in a closed vial. Extraction was conducted in triplicate for each plant sample.

2.3. TLC condition and selection of mobile phase

Phyllanthus niruri extract and phyllanthin (Sigma Aldrich Co., USA) were applied on a silica gel 60 GF₂₅₄ TLC plate (Merck KGaA, Germany). The plate was inserted into a chamber that has been pre-saturated with a mobile phase. The plate was eluted and then derivatized using NP/PEG reagent (Merck KGaA, Germany). It was then observed and documented using TLC-Visualizer (Camag, Switzerland) under white light, 254 nm UV light, and 366 nm UV light. Each single solvent, i.e., chloroform, tetrahydrofuran, ethanol, dioxane, n-hexane, toluene, ethyl acetate, acetic acid, 2-propanol, diethyl ether, and dichloromethane (Merck KGaA, Germany) were used for optimization of the initial mobile phase. The selected mobile phase for the next stage was the mobile phase which shows the highest number of spots with the best separation. The three mobile phases that show these characteristics were then combined with the mixture design method for optimization of the mixed mobile phase.

2.4. TLC-fingerprint analysis

Phyllanthus niruri extracts from 15 different locations were spotted on a TLC plate and eluted using optimized mobile phase. The chromatogram was then transferred into videoscanner to obtain a videodensitogram, R_f value, area, and peak height. The data matrix was then analyzed chemometrically using Minitab v.16 software (Minitab Inc., State College, PA). Chemometrics were performed using Principal Component Analysis (PCA) and Clustering Analysis (CA) methods.

3. Results

3.1. Selected mobile phase

Phyllanthus niruri powder from 15 locations are shown in figure 1. One of the *P. niruri* extract was then used for mobile phase optimization. In the initial stage, chloroform, dichloromethane, and ethanol were the three single solvents that revealed the best separation. In the next stage, using the mix design method, the results showed that chloroform, dichloromethane, ethanol (8:1:1) was the ratio resulting a good separation. However, the separation was not optimal yet. Therefore, other mobile phase mixtures were tried which produced better separation, i.e., toluene, ethyl acetate, methanol, 85% formic acid (75:25:25:6) (figure 2).



Figure 1. *Phyllanthus niruri* powder collected from Surabaya (1), Gresik (2), Sidoarjo (3), Pasuruan (4), Banyuwangi (5), Mojokerto (6), Bangkalan (7), Lumajang (8), Nganjuk (9), Kediri (10), Jember (11), Tulungagung (12), Blitar (13), Materia Medica Batu (14), and B2P2TOOT Tawangmangu (15).

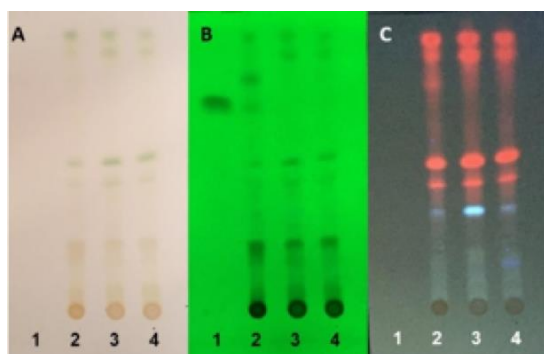


Figure 2. TLC profile of phyllanthin (1) and *P. niruri* extract (2, 3, 4) using optimized mobile phase. MP (mobile phase): toluene, ethyl acetate, methanol, 85% formic acid (75:25:25:6). D (detection): NP-PEG reagent, white light (A), 254 nm UV light (B), 366 nm UV light (C).

3.2. TLC-fingerprints of *P. niruri* collected from different origins

The TLC profile of *P. niruri* from 15 locations after derivatization can be seen in figure 3. The chromatogram was then transferred into videodensitogram to determine the R_f value, height, and area of each detected peak. As an example, a videodensitogram is shown in figure 3D.

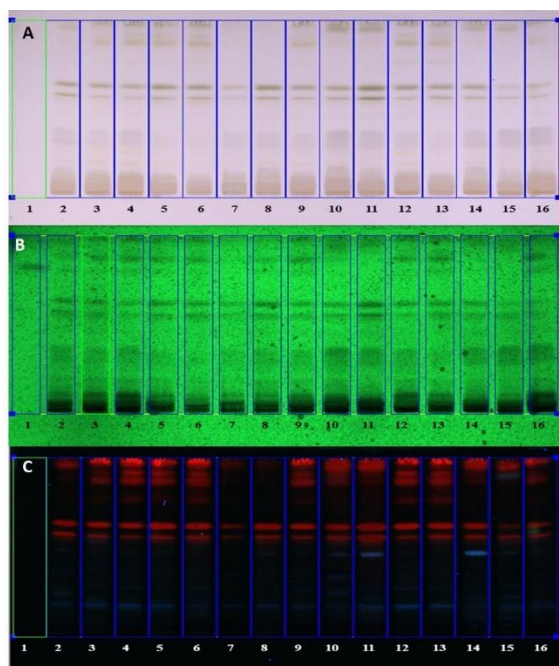


Figure 3. TLC profile of phyllanthin (1) and *P. niruri* collected from Surabaya (2), Gresik (3), Sidoarjo (4), Pasuruan (5), Banyuwangi (6), Mojokerto (7), Bangkalan (8), Lumajang (9), Nganjuk (10), Kediri (11), Jember (12), Tulungagung (13), Blitar (14), Batu (15), and Tawangmangu (16). MP: toluene, ethyl acetate, methanol, 85% formic acid (75:25:25:6). D: NP-PEG reagent, white light (A), 254 nm UV light (B), 366 nm UV light (C). The videodensitogram of *P. niruri* transferred from the track 2 of Figure 3B (D).

3.3. Principal Component Analysis (PCA) results

Videodensitogram data (i.e., peak height at a certain R_f) of all *P. niruri* samples from the chromatogram observed under 254 nm UV light were then tabulated into data matrix 15 x 10 (table 2). These data were then analysed with chemometric using PCA and CA. The scree plot of the PCA (figure 4) exhibits that total variance was shared among the ten Principle Components (PCs). figure 5 plots the scores of the 2 Principle Components (PC1 and PC2) for the fifteen *P. niruri* herbs in table 1, whereas figure 6 shows the loading plot on the first 2 PCs.

3.4. Clustering analysis

The next analysis was Cluster Analysis (CA) which divide 15 *P. niruri* herbs based on their similarity. The dendrogram resulted from CA can be seen in figure 7.

4. Discussion

In general, the powder showed a dark green color, except those from Surabaya (1), Bangkalan (7), Nganjuk (9), Blitar (13), Batu (14), and Tawangmangu (15) which are brownish green. This is an early indicator that meniran originating from these locations may have similar characteristics. The moisture content of all *P. niruri* powder was less than 10% (table 1) and this was in accordance with the Indonesian Herbal Pharmacopoeia [13]. It indicates that the drying process of plant materials was appropriate.

Thin Layer Chromatography (TLC) is an analytical technique that has been commonly used for both qualitative and quantitative analysis, even for testing biological activity when combined with bioautography. TLC is the choice of screening technique because it is fast, simple, and inexpensive. TLC is also a method with high flexibility because 20 samples can be analyzed simultaneously under the same conditions. With the advancement of technology, modern high performance thin layer chromatography (HPTLC) has been successfully developed, which is a reliable and powerful analytical technique, which can meet the requirements of today's GMP (cGMP) [14-16].

Table 2. Matrix data of *P. niruri* origin vs height of peaks detected from TLC chromatogram.

Origin	Height of peak at a certain Rf value									
	0.0-0.1	0.1-0.2	0.2-0.3	0.3-0.4	0.4-0.5	0.5-0.6	0.6-0.7	0.7-0.8	0.8-0.9	0.9-1.0
Surabaya	2138.1	3271.3	3267.6	3329.9	3377.9	3064.3	3085.0	2790.2	2164.7	1068.7
Gresik	1894.9	3626.0	3638.1	3741.5	3738.5	3306.7	3281.1	2971.2	2277.0	1424.8
Sidoarjo	1959.8	3808.6	3726.4	3973.3	4100.7	3823.7	3736.1	3376.1	2369.0	1722.1
Pasuruan	2860.3	3808.6	3568.6	3797.4	3736.3	3370.4	3287.7	3223.9	2330.4	1441.5
Banyuwangi	2253.6	3627.4	3654.8	3675.9	3486.6	3378.9	3488.6	3015.7	2407.6	1563.2
Mojokerto	2779.5	3575.8	3253.9	3677.7	3638.9	3313.2	3339.3	3121.6	2484.4	1246.7
Bangkalan	2387.3	3493.8	3190.5	2733.2	2995.3	3204.2	3238.0	2803.8	1973.5	1003.3
Lumajang	1892.5	3899.3	3959.4	3318.1	3745.6	3900.3	3902.9	3389.7	2322.8	1569.0
Nganjuk	2623.8	3727.7	3593.7	4110.3	4250.4	3997.5	3895.5	3506.2	2817.5	942.9
Kediri	2536.0	3985.2	3359.3	3773.5	3750.2	3600.9	3640.0	3297.5	2722.4	1352.2
Jember	2450.5	3925.6	3726.9	4037.8	4179.4	3954.1	3861.2	3386.3	2608.6	1548.7
Tulungagung	2357.8	3483.0	3666.8	3598.6	3664.8	3394.3	3454.2	3049.6	2257.4	1303.0
Blitar	2060.3	3585.8	3413.0	3736.6	3888.9	3930.8	3829.1	3536.6	2836.3	1766.1
Batu	2331.5	3555.2	3100.4	3498.2	3677.2	3554.7	3427.8	3499.7	2879.7	1868.4
Tawangmangu	1709.2	3463.3	3362.8	3707.8	4051.0	3911.6	4070.3	3649.0	2740.0	2125.1

Figure 3 shows the different TLC profiles of each sample visually. For example, tracks 2 (Surabaya), 7 (Mojokerto), 8 (Bangkalan), 14 (Blitar), and 15 (Batu) in figure 3C produce less red bands than the other samples. This red band under 366 nm UV light is predicted as chlorophyll. Therefore, samples from Surabaya, Mojokerto, Bangkalan, Blitar, and Batu are predicted to contain different types and

levels of chlorophyll from other samples. This finding is consistent with the color of meniran powder, where samples from Surabaya, Bangkalan, Blitar, and Batu show a paler color than other samples (figure 1). To identify whether there were other differences, chemometrics analysis using PCA and CA were then applied.

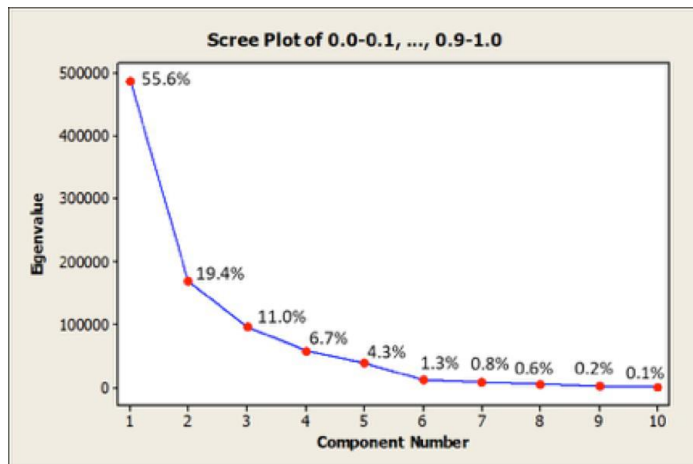


Figure 4. Result of eigen analysis and scree plot of PCs.

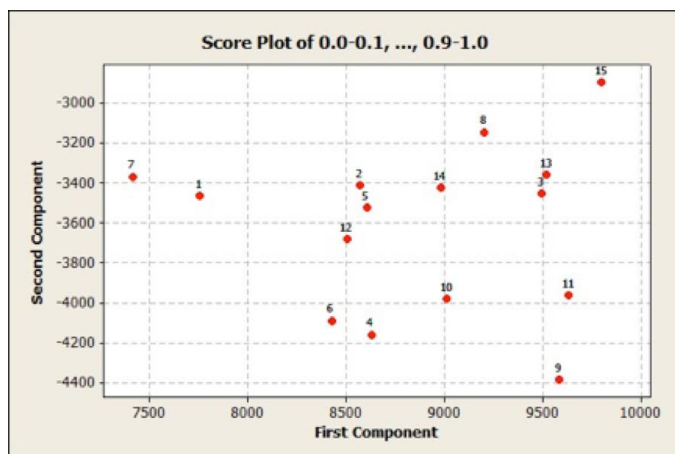


Figure 5. The PCA score plots of *P. niruri* from different locations on the first two PCs. 1-15 were *P. niruri* collected from Surabaya (1), Gresik (2), Sidoarjo (3), Pasuruan (4), Banyuwangi (5), Mojokerto (6), Bangkalan (7), Lumajang (8), Nganjuk (9), Kediri (10), Jember (11), Tulungagung (12), Blitar (13), Materia Medica Batu (14), and B2P2TOOT Tawangmangu (15).

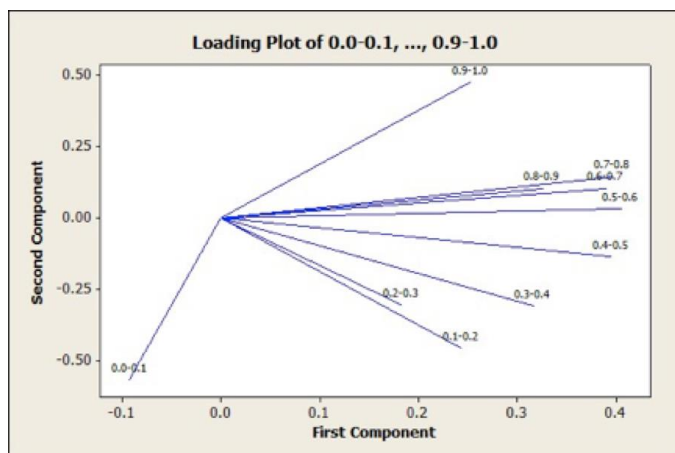


Figure 6. The PCA loading plots of *P. niruri* from different locations on the first two principal components.

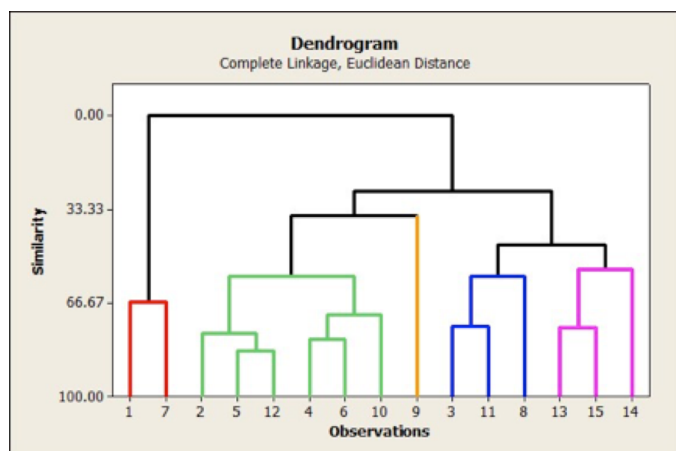


Figure 7. The dendrogram resulted from a complete linkage in the CA. 1-15 were *P. niruri* powder collected from Surabaya (1), Gresik (2), Sidoarjo (3), Pasuruan (4), Banyuwangi (5), Mojokerto (6), Bangkalan (7), Lumajang (8), Nganjuk (9), Kediri (10), Jember (11), Tulungagung (12), Blitar (13), Materia Medica Batu (14), and B2P2TOOT Tawangmangu (15).

From the PCA (figure 4), it is known that the total variance is shared among the 10 Principle Components (PCs). PC1 has a variance of 55.6% of the total variance. This value gives the largest proportion compared to the original variable. PC2 gives 19.4% of the total variance. Therefore, it can be concluded that PC1 and PC2 provide a variation of 75.0%. Thus, PCA was able to reduce the data that originally had 10 variables (peak height at 10 Rf values) can be explained by 2 new variables (up to PC2), because until PC2 was able to extract 75.0% information. figure 5 plots the scores of the 2 Principle Components (PC1 and PC2) for the fifteen *P. niruri* herbs in table 1. The loading plot (figure 6) illustrates how strong each character affects the PC. Two adjacent PCs with a narrow angle indicate a positive correlation, while two PCs that form an angle close to 90° show no correlation. On the other hand, two PCs that are scattered at an angle close to 180° indicate a tendency for negative correlation.

The dendrogram resulted from CA (figure 7) showed that the *P. niruri* from 15 locations could be divided into 5 groups. The first group consisted of samples from Batu, Tawangmangu, and Blitar. The second group is from Lumajang, Jember, and Sidoarjo. The third group is Nganjuk, the fourth group consists of Kediri, Mojokerto, Pasuruan, Tulungagung, Banyuwangi, and Gresik, while Surabaya and Bangkalan are included in group 5.

The samples that are in one cluster indicate that the samples have similar chemical content, indicating the quality of *P. niruri* is also similar. Conversely, samples grouped in different clusters indicated different quality. The formation of 5 clusters of *P. niruri* in this study was estimated to be caused by differences in the geographical conditions of the sample origin. All samples used in this study were from the lowlands (<100 m asl), except for three samples from Blitar, Batu, and Tawangmangu. These three samples were from areas with an altitude of >100 m asl and based on CA, they formed one cluster. It is predicted that the altitude of the geographical origin has an effect on the grouping of the *P. niruri*. The grouping of samples due to the influence of the altitude has also been investigated on celery leaves [7]. However, whether this grouping is also influenced by other factors needs further research.

5. Conclusion

TLC-fingerprinting combined with chemometric (PCA and CA) were able to discriminate *P. niruri* originated from various origins. *P. niruri* from 15 locations in East and Central Java, Indonesia used in this study can be classified into 5 clusters. The samples that are joined in one group showed the similarity of chemical content both qualitatively and quantitatively. On the other hand, samples in different groups indicated differences in the quality of the chemical content.

Acknowledgments

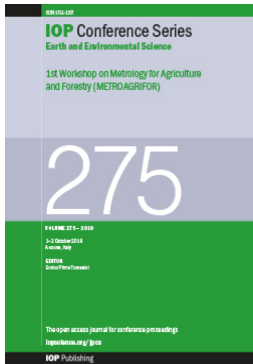
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Genetic construction of HBsAg gene subgenotype B3 in *Lactococcus lactis* as hepatitis B vaccine candidateA Z Mustopa, H H Putri, Kusdianawati, B R Budiarto, A Kusumawati, M Nurfatwa, N Ekawati, A Prastyowati, L Triratna, A Hertati *et al*
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Antagonistic capacity of dark septate endophytes (DSE) against *Candida* biofilms from oil palm (*Elaeis guineensis* Jacq.)

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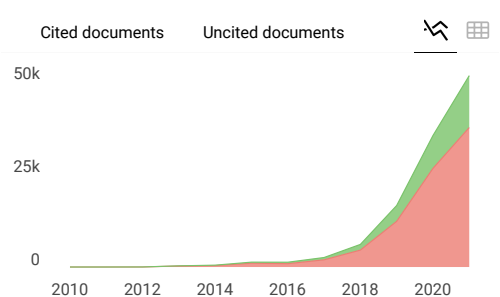
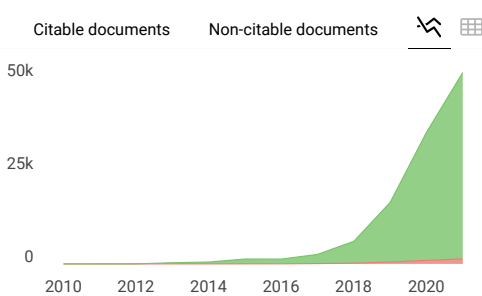
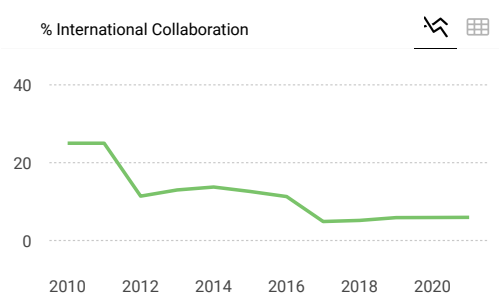
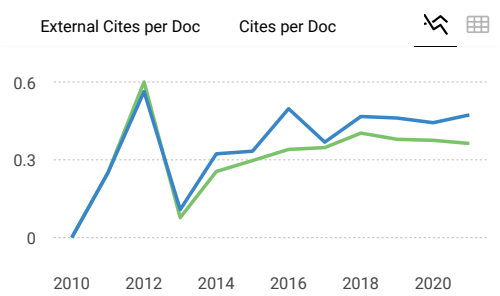
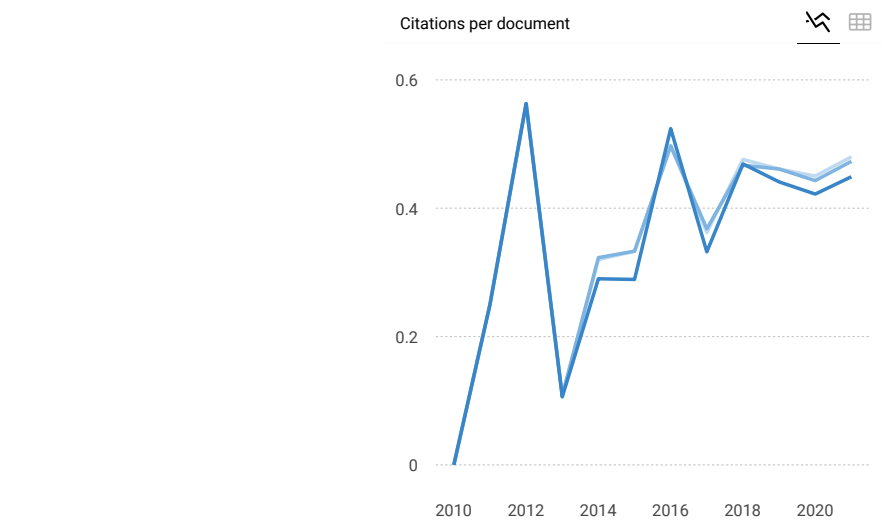
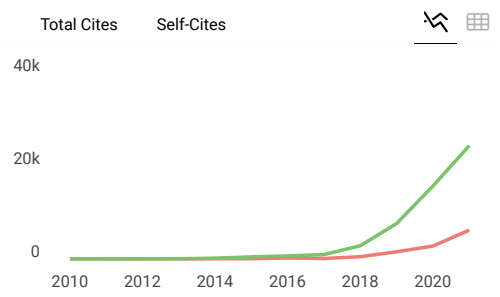
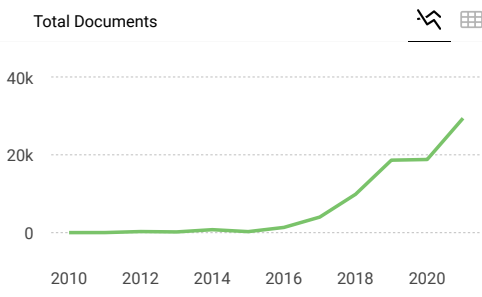
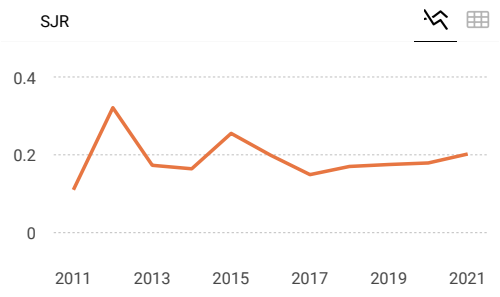
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ISSN: 1755-1307 E-ISSN: 1755-1315

- Subject area:
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 - Physics and Astronomy: General Physics and Astronomy

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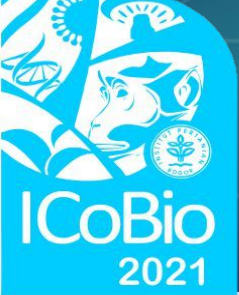
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in The 4th International Conference on Biosciences (ICoBio) and
The 17th National Congress of Indonesian Biological Society (PBI) 2021,
With theme “Bioscience Innovations for Sustainable Development Goals (SDGs)”
organized by the Department of Biology, IPB University, in collaboration with Indonesian Biological Society (PBI), and
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Bogor, 12 August 2021



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