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Metabolite *Fingerprintiing* of *Eleutherine palmifolia* (L.) Merr. by HPTLC-Densitometry and Its Correlation with Anticancer Activities and *In Vitro* Toxicity

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ABSTRACT

Eleutherine palmifolia (L.) Merr. (E. *palmifolia* or Dayak Onion) is an anticancer plant used for traditional medicines. The difference of cultivation sites may affect metabolites content, pharmacological activity and toxicity profiles. This study aimed to determine the metabolite fingerprinting, anticancer and toxicity profiles of E. *palmifolia* from several regions in Indonesia for authentication, efficacy, safety and quality control. Samples were obtained from six different locations in Indonesia which included West Java (WJ), Central Java (CJ), East Java (EJ), East Borneo (EB), Central Borneo (CB), and South Borneo (SB). Metabolite fingerprinting was determined by HPTLC-densitometry method and profile of anticancer and toxicity were analyzed by MTT-ELISA method. The difference among metabolite fingerprinting, anticancer, and toxicity profile was analyzed by Principal Component Analysis (PCA) and Hierarchical Component Analysis (HCA), whereas the association among them was analyzed by Partial Least Square (PLS). The PCA results showed a difference in E. *palmifolia* metabolite fingerprints and the HCA results showed that six different regions were the same cluster. The PLS-DA analysis showed four significant metabolites proposed as anticancer markers with Rf 0.34, 0.59, 0.76, 0.93 and three significant metabolites proposed as negative markers with Rf 0.02, 0.44 and 0.59. E. *palmifolia* from East Java had the lowest IC_{50} (86.98±4.62µg/mL) and higher SI value (5.5).

Keywords: E*. palmifolia*, metabolite fingerprinting, anticancer profiles, toxicity profiles

INTRODUCTION

E. *palmifolia* (Dayak Onion) has been used empirically by Indonesian people to treat several diseases such as cancer. E. *palmifolia* bulb contains glycosides, flavonoids, steroids, tannins, and phenolics compounds. The flavonoids contained are considered responsible for the anticancer activity through inhibition of the colon cancer cell cycle in the G1/S and G2/M phases (Ren *et al.,* 2003; Chahar *et al.,* 2011). Eluetherine and elecanacin compounds in this plant are also known to inhibit TCF/β-catenin transcription in SW480 colon cancer cells (Li *et al*., 2008) and to show selective activity against colorectal cancer (Fitri *et al.,* 2014).

Differences in cultivation sites may affect metabolite content, activity, and toxicity (Verma and Shukla, 2015) including internal and external factors (Kim *et al*., 2011). Internal factors include genetic factors and physiological variations (Verma and Shukla, 2015), whereas external factors (environment) include climate change, soil type, nutrient content, fertilizer use, damage caused by microorganisms, stress induced by UV radiation, heavy metals, and pesticides (Canas *et al*., 2015).

Table I. Origin of samples

Sample	Sites of Cultivation	Altitude (m)	Average Temp (°C)	Average Rainfall (mm)	Climate Type	Voucher Number
EB	East Borneo	29	26.4	2376	Af	074/348/102.7/2017
SB	South Borneo	10	26.8	2765	Af	074/348/102.7/2017
CB	Central Borneo	31	27.2	2627	Af	074/348/102.7/2017
EJ	East Java	127	25.0	1819	Aw	074/348/102.7/2017
WJ	West Java	668	24.0	3454	Af	074/348/102.7/2017
CJ	Central Java	1221	19.1	3299	Am	074/348/102.7/2017

Metabolite fingerprinting of medicinal plants is very important to ensure authenticity, safety, and efficacy because of varying metabolite content (Patel *et al*., 2015). Metabolite fingerprinting is an analytical technique based on the chromatogram pattern of compounds that provide special characteristics (Wolfender *et al*., 2015, Fiehn, 2002). HPTLC can be an alternative for analyzing plant extracts because it is more efficient in evaluating herbal medicines (Dhalwal *et al*., 2008). To develop E. *palmifolia* into phytomedicine, several requirements must be met. Phytomedicine is a preparation of natural ingredients in which efficacy and safety has been proven scientifically with clinical trials and pre-clinical trials along with in which raw materials and end-products has been standardized (Indonesia National Agency of Drug and Food Control, 2005). Until now, there is still no report on metabolite fingerprinting of E. *palmifolia* from Indonesia. In this work, HPTLC and ELISA were used to determine metabolite profiles, anticancer activity, and toxicity profiles of E. *palmifolia* to obtain marker compounds, efficacy, and safety profiles of this plant.

MATERIALS AND METHODS

Plant material and extraction

E. *palmifolia* was obtained from West Java, Central Java, East Java, East Borneo, Central Borneo, and South Borneo (Table I). The specimens were determined at the UPTD Materia Medika Malang with collection number 074/348/102.7/2017 and were stored in the Pharmacognosy Laboratory of Pharmacy Department of State Islamic University of Maulana Malik Ibrahim Malang. Simplicial powder was extracted by UAE method using 96% ethanol with a ratio of 1:20. The ethanol extract was stored in an oven at 40°C and was ready to use for further testing.

Mobile phase optimization

Ten mg of E. *palmifolia* 96% ethanol extract was dissolved in 1mL of 96% ethanol. The 2µL of extract was applied on the 20x10cm F_{254} Silica Gel HPTLC plate and was eluted using 2 types of mobile phases i.e. chloroform: methanol (8:2 v/v) and n-hexane:ethyl acetate (6:4 v/v). HPTLC plate results were scanned by TLC Scanner (CAMAG, Germany) at 254nm and 366nm, were sprayed with 10% H_2SO_4 as its stainer and were heated on the hot plate (CAMAG, Germany) at 105°C for 5min.

Method validation

The validation method for metabolite fingerprinting was carried out using two parameters including precision and stability test (on a Merck HPTLC plate). Precision and stability tests were carried out by applying 5µL of samples on a plate (concentrations of 20.000 and 10.000ppm) at 0, 15, 30, 45, 60, 75 and 90min by 3 times replication. Precision test (interday) and stability test (on the plate) were analyzed using PCA (Indah, 2015).

Metabolite fingerprinting test

Each ten mg of 96% ethanol extract of E. *palmifolia* from six different locations including West Java (WJ), Central Java (CJ), East Java (EJ), East Borneo (EB), Central Borneo (CB) and South Borneo (SB) was dissolved in 96% ethanol to obtain concentration of 20.000ppm and 10.000ppm, was filtered using a membrane filter of 0.20µm. Five µL of samples was applied (10.000 ppm) on a 20x10 cm F_{254} Silica Gel HPTLC plate and was eluted using selected mobile phase (chloroform:methanol 80:20 v/v) and 3 times replication, was sprayed using 10% sulfuric acid solution, and HPTLC plate was scanned using TLC Scanner (CAMAG, Germany) at 254nm. All spots on all tracks were scanned at 200-700nm and were

visualized using TLC Visualizer (CAMAG, Germany) (Taufik, 2017).

Anticancer activity and toxicity test using the MTT method

Cell culture

Cells were taken from a liquid nitrogen tank at -80°C and were thawed at 37°C. Subsequently, the cells were transferred to a conical tube containing 1640 serum RPMI media for WiDr cancer cells and M199 for normal cells. Cells were centrifuged to separate the pellets from the supernatant. The supernatant was discarded, and the procedure was repeated twice. One mL of 10% FBS solvent was added and was resuspended until homogeneous. Cells were transferred to a culture flask and were incubated in an incubator at 37°C 5% CO_2 for 2x24 hours. The media was replaced continuously, and the number of live cells was checked regularly (Mutiah, 2017).

Cytotoxicity test using MTT assay method

Cells were calculated in a hemocytometer and were diluted to a certain density i.e. 174.25x104 cells for WiDr cells and 74.75x104 cells for Vero cells. Each cell was transferred into a-96 well plate by 100µL/well and was incubated in a 5% CO_2 incubator at 37°C for 24h. After 24h, the media on the plate was discarded. A number of 96% ethanol extract of E. *palmifolia* L with a certain weight was diluted in a 1% v/v DMSO to make concentration (µg/mL) of 500; 250; 125; 62.5; 31.25; 15.625 and 7.625 for WiDr cells and concentration (µg/mL) of 1000; 500; 250; 125; 62.5; 31.25; 15,625 for vero cells, was transferred to well three times replication, and was incubated for 24h at 37°C and 5% CO_2. After incubation, the media was discarded and was replaced with 100µL/well of MTT reagent, was incubated for 3h. If formazan had been formed, it was added a 100µL/well of 10% SDS in 0.1N HCl, was incubated in a dark room or was covered with aluminum foil for 24h at room temperature, and had its absorbance measured by ELISA reader at 590nm (Mutiah, 2017).

Cytotoxicity analysis

The absorbance data obtained from each well were converted to percentage of cell viability using equation:

$$\text{Percentage of cell viability (\%)} = \frac{(\text{abs. treatment} - \text{abs. media control})}{(\text{abs. cell control} - \text{abs. media control})}$$

The percentage of cell viability was calculated to get IC_{50} value. IC_{50} is a concentration in which 50% of population cell growth is inhibited reflecting the cytotoxic potential of an extract (Mutiah, 2017). IC_{50} values were determined by probit analysis using SPSS v25.

Analysis of Metabolite Fingerprinting Data

Metabolite fingerprinting data of E. *palmifolia* ethanol extract from each location (Rf value, AUC, maximum absorbance) were identified and were processed using PCA and HCA (Multibase v2015 adds in Excel v2013) to obtain the difference among them, whereas the anticancer profile of WiDr cells and Vero cells were processed using one-way ANOVA (SPSS v16).

RESULT AND DISCUSSION

Method validation

The validation method was carried out before the metabolite fingerprint of E. *palmifolia* extract were tested to determine whether the analysis method used in this study had met the requirements in the validation parameters i.e. stability test after it was applied on HPTLC plates and interday precision tests (Indah, 2015).

Precision testing

The precision test aimed to obtain the proximity of measurement results when the analysis method was repeated (Ravichandran *et al*. 2010). The results obtained showed that samples with a concentration of 10.000ppm were more precise than those with concentrations of 20.000ppm. The profile of PCA analysis for a precision test (Figure 1) showed that the repetition at the concentration of 10.000ppm was more precise than the concentration of 20.000ppm. This result was used to determine which concentration was best to be chosen for metabolite fingerprinting testing.

Stability test

The stability test is a stage of pre-validation to show sufficient stability of an analyte in a matrix or on an HPTLC plate in a certain time. This stage is the most important stage in conducting research (Yuwono and Indriyanto, 2005). Stability test of E. *palmifolia* extract on HPTLC plate was carried out in minutes 0, 15, 30, 45, 60, 75 and 90. The results of the stability test showed that analyte could be well separated and stable until 90min on the HPTLC plate (Figure 2).

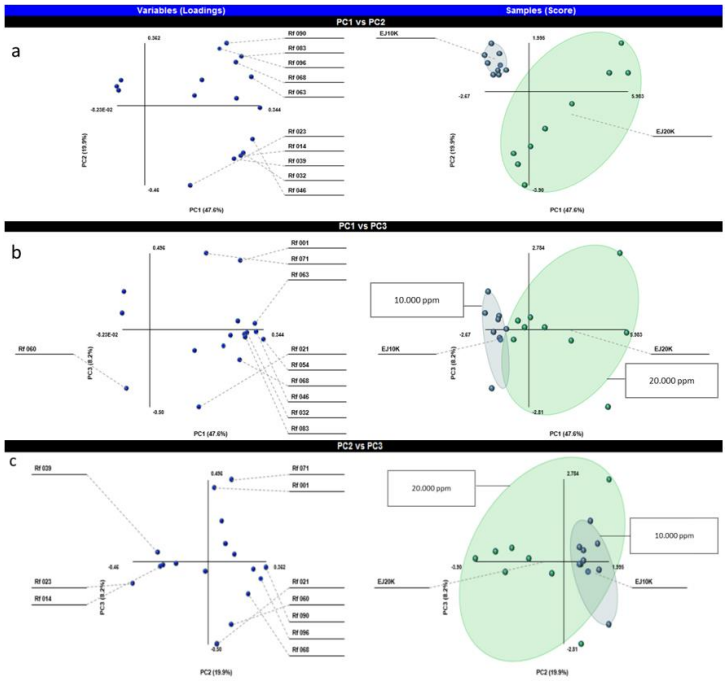

Figure 1. PCA profile (in principal component/PC) for precision test (on HPTLC plate) between concentration 10.000 ppm and 20.000 ppm in minutes 0, 15, 30, 45, 60, 75 and 90: (a) PC1 vs PC2; (b) PC1 vs PC3; (3) PC2 vs PC3

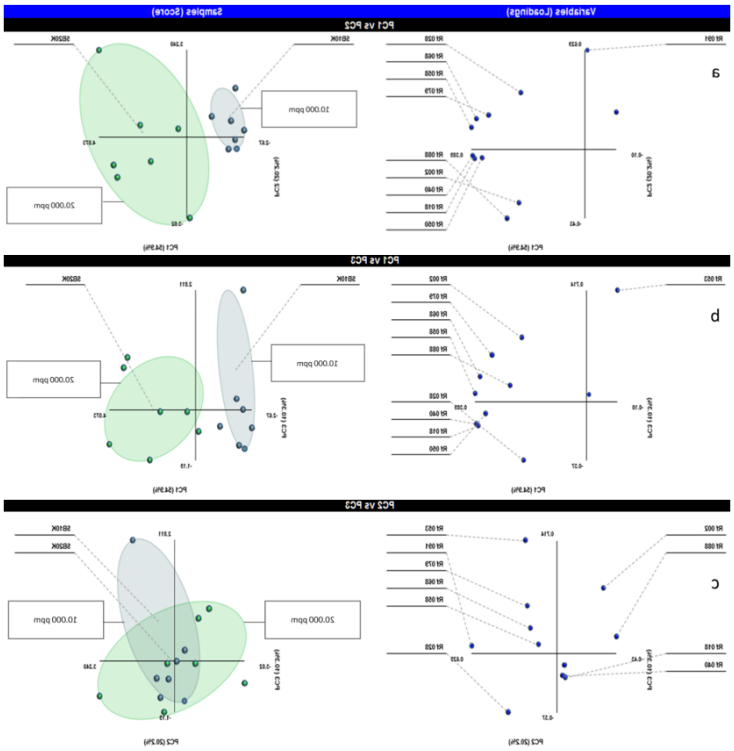

Figure 2. PCA profile (in principal component/PC) for stability test (on HPTLC plate) between concentration 10.000 ppm and 20.000 ppm in minutes 0, 15, 30, 45, 60, 75 and 90: (a) PC1 vs PC2; (b) PC1 vs PC3; (3) PC2 vs PC3

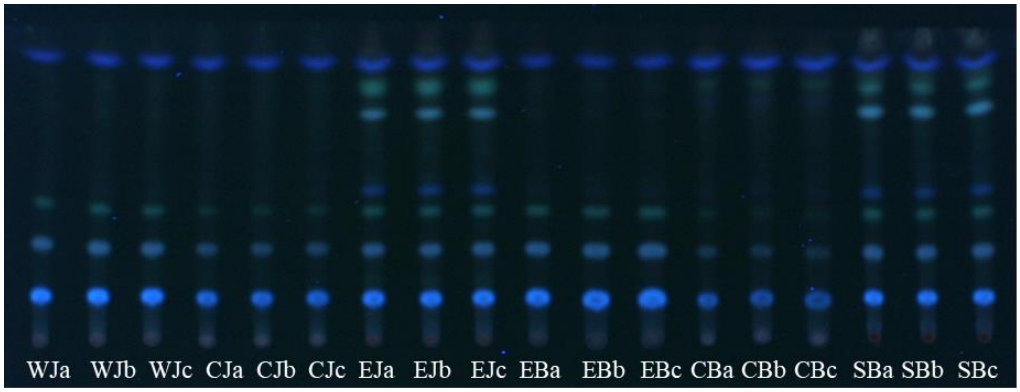

Figure 3. Metabolite fingerprinting of *E. palmifolia* ethanol extract, the WJa-WJc (West Java) sample, CJa-CJc (Central Java), EJa-EJc (East Java), EBa-EBc (East Borneo), CBa-CBc (Central Borneo), SBa-SBc (South Borneo) were eluted with the mobile phase chloroform: metanol (80:20 v/v) and derivatized using 10% H_2SO_4 on 366 nm UV with three times replication.

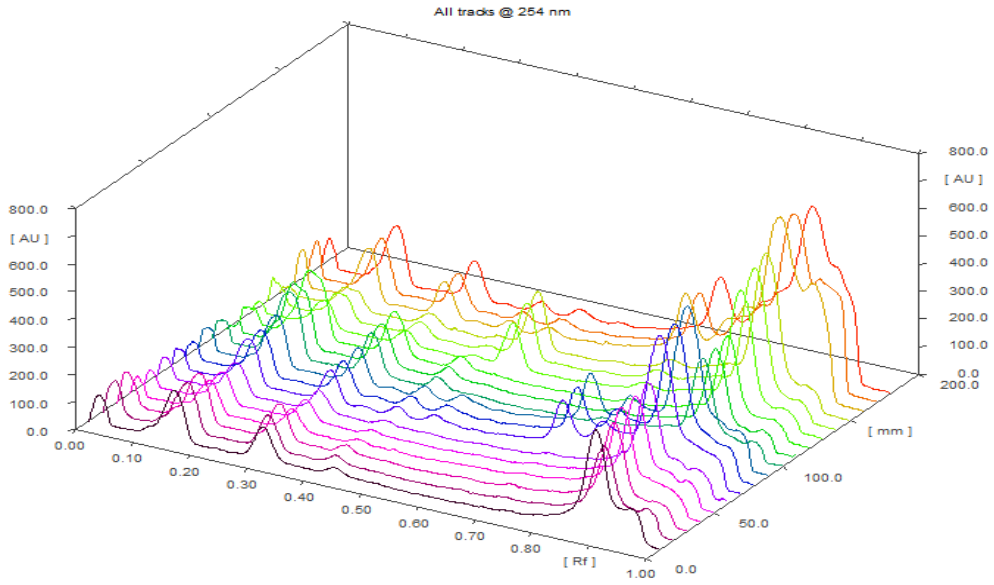

Figure 4. Densitogram of HPTLC using TLC Scanner-4 (Camag) on 254nm UV, with a scan speed 20mm/s, the data resolution 100µm/step, the first track position X 10.0mm, distance between the track 10.0mm, the position started from scan Y 15.0mm and the last scan Y 90.0mm

Metabolite fingerprinting of E. *palmifolia* extract

Metabolite fingerprinting from eighteen samples of E. *palmifolia* extracts obtained from six different locations i.e. West Java (WJ1a-WJ1c), Central Java (CJ1a -CJ1c), East Java (EJ1a-EJ1c), East Borneo (EB1a-EB1c), Central Borneo (CB1a-CB1c) and South Borneo (SB1a-SB1c) were analyzed with a normal phase HPTLC-densitometry. The stationary phase used was an 20x10 cm F_{254} silica gel 60 HPTLC plate and the selected mobile phase was chloroform: methanol (80:20 v/v) with extract concentration of 10.000ppm in ethanol.

The metabolites profiles of E. *palmifolia* extract from six different locations were similar although they grew from different environments (Figure 3). There were 145 metabolites detected using HPTLC-densitometry (Figure 4) and each region averagely contained eight metabolites.

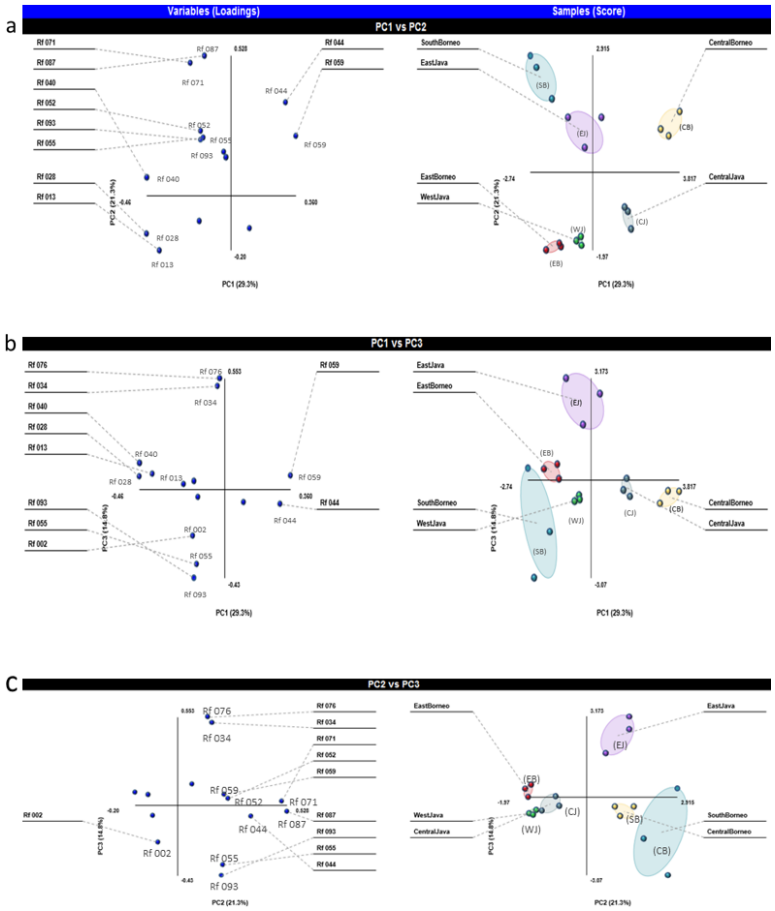

Figure 5. Metabolite fingerprinting of *E. palmifolia* using Principal *Component Analysis* (PCA) between *Loading Plot* (left) and *Score Plot* (right) explained variants PC1 (29.3%), PC2 (21.3%), PC3 (14.8%) and total variants explained by three principle components were 65.4%.

The retention factor (Rf) values found in each region showed similarities. The same Rf values found in all regions were 0.13, 0.28, 0.40, 0.71 and 0.87. The results of this metabolite fingerprinting of E. *palmifolia* extract could be proposed as a candidate of analytical marker compounds of this plant.

Principal component analysis (PCA)

The results of metabolite fingerprinting were analyzed using PCA. PCA showed the visualization of the score plot (right) and the loading plot (left). Score plots described the characteristics of the sample, whereas the loading plot described the relationship (correlation) between the variables in each component. PCA score plot and loading plot explained variants PC 1 (29.3%), PC 2 (21.3%), PC 3 (14.8%) and total variants explained by the three principal components were 65.4%. Visualization of PCA results showed that three significant metabolites influenced clusters formation because they appeared on each PC, both PC1, PC2, and PC3 (Figure 5). The significant metabolites were found on Rf values of 0.44, 0.55 and 0.59.

There were two clusters formed i.e. (I) EB, CJ, WJ and (II) EJ, SB, CB (Figure 5). The metabolites of E. *palmifolia* originated from EB, CJ and WJ showed similarities among them as well as those from EJ, SB, and CB. To confirm the clusters formed in PCA, HCA analysis was also carried out.

Hierarchical clustering analysis (HCA)

The results of HCA showed characteristics that were close among samples from West Java (green), East Borneo (red), Central Java (grey),

South Borneo (blue), Central Borneo (yellow) and East Java (purple). It could be concluded that all samples taken from six different locations were similar and located in one cluster (Figure 6).

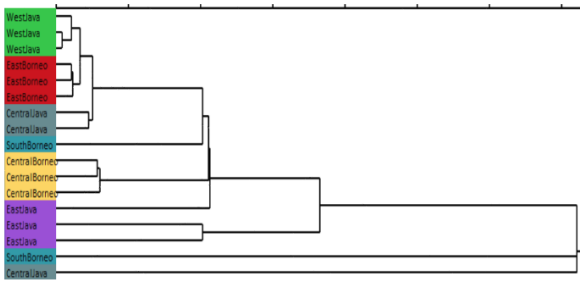

Figure 6. Dendrogram of Hierarchical Clustering Analysis (HCA) that showed the similarity of E. *palmifolia* from West Java (Green), East Borneo (Red), Central Java (Grey), Central Borneo (Yellow), East Java (Purple) and South Borneo (Blue)

The HCA results showed that the metabolite fingerprinting of East Borneo was similar to West Java and Central Java. Central Borneo has similarities with South Borneo and East Java. The contents of these metabolites were influenced by two factors i.e. internal factors and external factors (Heuberger, *et al*. 2014). Internal factors that influenced the composition of the compound included genetic and physiological variations, whereas external factors are factors such as geographical conditions (altitude), climate, humidity, light intensity, temperature, nutrient intake and radiation (Verma and Shukla, 2015).

Cytotoxic test of E. *palmifolia* extract

The cytotoxic test aimed to determine the anticancer potency from six different regions in Indonesia. The cytotoxic test was carried out using the MTT assay method on WiDr cells. WiDr cells were treated using a 96% ethanol extract of E. *palmifolia* bulb with concentration series (µg/mL) of 125; 62.5; 31.25; 15,625 and 7.8125.

In this experiment, the percentage of cell viability was lower with increasing extract dose and on the other hand it was higher with decreasing dose (Muti'ah *et al*., 2018) (Figure 7). This showed that there was an existence of a dose-dependent phenomenon between the concentration and viability of living cells. Then, the percentages of cell viability were converted using probit analysis to obtain IC_{50}. The results of probit analysis showed that there were differences in IC_{50} of E. *palmifolia* extracts from six different locations. The IC_{50} values (µg/mL) were 86.98 (East Jawa), 104.52 (Central Borneo), 133.23 (East Borneo), 159.07 (West Java), 269.80 (South Borneo) and 272.55 (Central Java). Dayak onion extract originating from the East Java region showed the highest cytotoxic effect on WiDr cells (the lowest IC_{50}) than other extracts and it also showed potent activity as an anticancer with an IC_{50} value <100µg/mL (Prayong *et al*., 2008).

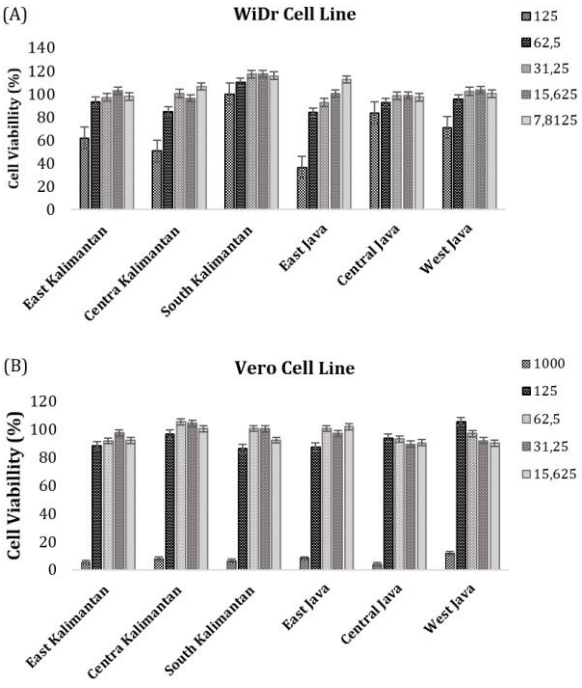

Figure 7. The percentage of viability cell (±SD) on colon cancer cell WiDr (A) and normal/vero cell (B) with a series dose of 96% E. *palmifolia* extract from six different regions.

In the development of an anticancer drug, selectivity index (SI) is very important to obtain a profile of anticancer activity of an extract on inhibiting cancer cell proliferation without damaging the normal cell. It is termed "selective" if the value of the selectivity index (SI) is >3 (Sutejo *et al*., 2016). The SI value was used to measure the safety of an anticancer drug (Dewi *et al*., 2015). The results showed that the SI value of E. *palmifolia* extracts were mostly selective except for exctract originated from South Borneo and Central Java. However, E. *palmifolia* extracts originating from East Java and Central Borneo showed a higher selectivity value compared to cisplatin as standard (Table II).

Table II. IC_{50} Analysis of Sample on WiDr Cells

Sample	IC_{50} WiDr Cells (µg/mL) ± SD*	IC_{50} *vero* Cells (µg/mL) ± SD*	SI	Interpretation
East Borneo	133.23±12.53	414.65±46.41	3.1	Selective
South Borneo	269.80±34.42	446.49±5.37	1.7	Non-Selective
Central Borneo	104.52±12.09	528.77±41.49	5.1	Selective
East Java	86.98±4.62	476.87±32.44	5.5	Selective
West Java	159.07±12.28	519.70±2,00	3.3	Selective
Central Java	272,55±42.47	393.16±24.66	1.4	Non-Selective
Cisplatin	47.16±2.22	232.35±43.00	4.9	Selective

*SD= Standard Deviation from 3 times replication

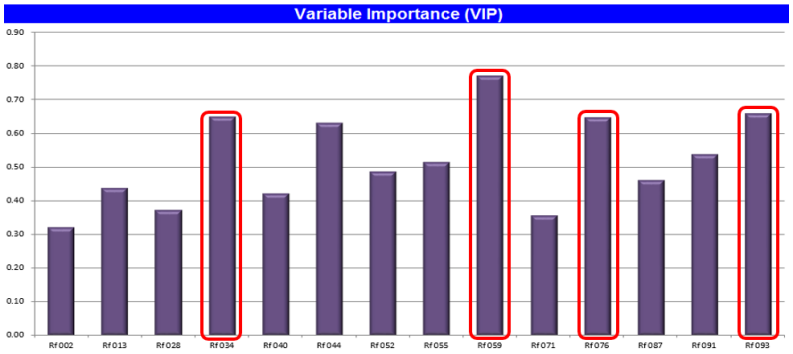

Figure 8. Partial Least Square (PLS) of metabolite fingerprinting and anticancer activity. Variable Importance (VIP) was the metabolite responsible for anticancer activity

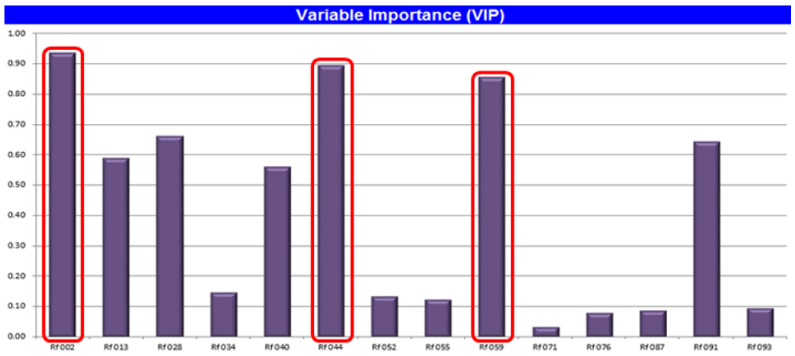

Figure 9. Partial Least Square (PLS) of metabolite fingerprinting and toxicity profiles. Variable Importance (VIP) was the metabolite responsible for toxicity activity.

Correlation between metabolite fingerprinting and *in vitro* anticancer activity

Anticancer activity of E. *palmifolia* from six different locations showed different activity eventhough they were originated from plants of the same type. Environmental factors might affect the content of secondary metabolites quantitatively and qualitatively so that their bioactivity might also vary. This could cause differences in the quality of phytomedicine (Kim *et al*., 2011). In this study, the Partial Least Square (PLS) analysis was conducted to determine the correlation of anticancer activity with metabolite fingerprints from six different regions.

The highest value of Variable Importance (VIP) was the metabolite responsible for the

anticancer activity (Figure 8). It could be seen that there were four highest Rf values: Rf 0.59 owned by East Java and Central Borneo, Rf 0.93 owned by all locations, Rf 0.34 owned only in East Java, and Rf 0.76 owned by Central and East Java.

Correlation between metabolite fingerprinting and *in vitro* toxicity

The results of PLS analysis of metabolite fingerprinting and *in vitro* toxicity of *E. palmifolia* extract were known according to the Variable Importance (VIP). The higher the VIP value, the greater the potency to cause toxicity. The metabolites responsible for the toxicity were metabolites with Rf 0.02 owned by extracts from all locations, Rf 0.44 owned by extracts from East Java, Central Borneo and South Borneo, and Rf 0.59 owned by extracts from East Java and Central Borneo (Figure 9). In the PLS analysis results of metabolite fingerprinting and toxicity, there were similarities to the results of PLS metabolite fingerprinting and anticancer activity i.e. Rf 0.44 and Rf 0.59 as metabolite responsible for anticancer activity and toxicity.

CONCLUSION

E. palmifolia from six different locations showed differences in metabolite fingerprint, activity profile, and toxicity profile. There were five metabolites that were used as candidates of analytical markers i.e. compound with Rf 0.13, Rf 0.28, Rf 0.40, Rf 0.71 and Rf 0.80 for this plant because they were found in all locations. Metabolites responsible for anticancer activity were found in metabolites with Rf 0.34, 0.59, Rf 0.76 and Rf 0.93 that could be proposed as positive markers compound for anticancer activity. Besides that, metabolites responsible for toxicity were metabolites with Rf 0.02, 0.44, and 0.59 that could be proposed as negative markers compound. *E. palmifolia* from East Java had the lowest IC_{50} (86.98±4.62µg/mL) and a higher SI value (5.5).

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