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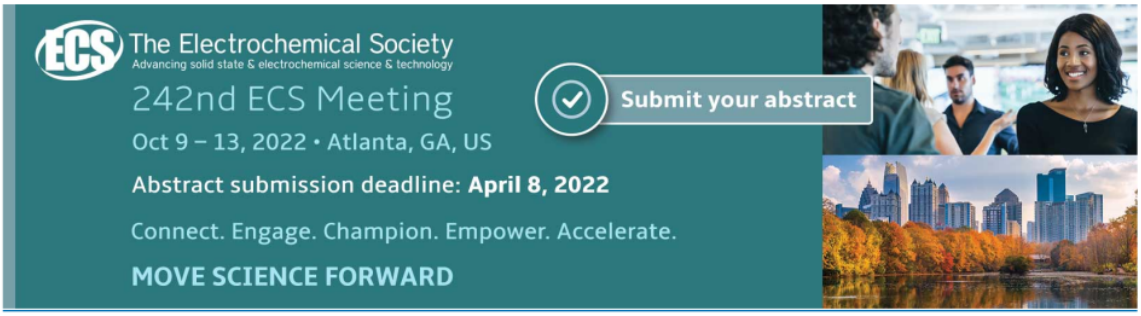

Variation of eurycomanone content within and among populations of *E. apiculata* A.W. Benn.

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Abstract. Information on the eurycomanone content of *E. apiculata* A.W. Benn from natural populations in Indonesia is unknown. This study aimed to assess the variation of eurycomanone content within and among populations of *E. apiculata*, determine the correlation of eurycomanone content with environmental factors, and determine the collection sites of genetic material for the establishment of the breeding base population of *E. apiculata*. The analysis of eurycomanone content was carried out using High-Performance Liquid Chromatography. This study found that the highest eurycomanone content was observed in the Rumbio population (9.86 mg/g) and the lower value was observed in the Pokomo population (4.44 mg/g). The average variation of eurycomanone content within the population was 25.72%. The coefficient of eurycomanone differentiation among populations was 84.33%, indicating that the variation of eurycomanone content among populations of *E. apiculata* was higher than the variation of eurycomanone content within-population (15.67%). Pearson correlation of eurycomanone content with population environmental factors showed no significant correlation. Based on the eurycomanone content and clustering, the Rumbio population can be selected as a source of the genetic material of *E. apiculata* for eurycomanone production via the breeding program in the future.

1. Introduction

Eurycoma apiculata A.W. Benn is one of the members of the Simaroubaceae family. This species grows in Sumatra island, Indonesia, and Malaysia Peninsular. In Indonesia, *E. apiculata* is known as 'pasak bumi daun runcing', and the local name (Rumbio, Riau) is 'pasak bumi betina' [1]. *E. apiculata* is a small tree with a height less than 5 m [2]. This species is found in primary and secondary forests with altitudes less than 1,200 m above sea level. The research on *E. apiculata* was still limited to study on the leaf morphometric analysis [3,4], and chromosomes and karyotype analysis [5], while the phytochemical content of this species was not yet reported.

Eurycomanone compound is one of the secondary metabolic compounds from the Simaroubaceae family and *Eurycoma* genus [6–8]. Eurycomanone is a type of quassinoid that is widely extracted from the 'pasak bumi' plant, especially the roots, and is used as a drug to increase testosterone hormone [9–11], and anti-cancer [12,13]. This eurycomanone compound has also been used as a biomarker of pasak bumi to detect counterfeiting of raw materials for pasak bumi products [8,14,15].

As a medicinal plant, the breeding activities of *E. apiculata* are directed to enhance secondary metabolic compounds, namely the eurycomanone. In the development of breeding populations, high-quality plant materials, especially characters (eurycomanone compound) that are closely related to the purpose of breeding, must be available and collected from various natural populations. The genetic gain can be optimally obtained if selection activities are conducted in the traits desired.

Information on the eurycomanone content of each population that will be the target of the collection of genetic resources of the *E.apiculata* should be known. Until now, there has been no report on the eurycomanone content of *E. apiculata* in various regions in Indonesia, so research needs to be done. The screening study of eurycomanone compounds for the selection of *E. longifolia* genetic material for the development of its breeding population has been carried out in Malaysia [16]. The authors found the variation of eurycomanone content among populations studied. Many reports state that the same species from different populations will have different secondary metabolic contents [17–20]. This study purposes were to assess the variation of eurycomanone content within and among populations of *E. apiculata*, to determine the correlation of eurycomanone content with environmental factors, and to determine the collections site of genetic material for the establishment of the breeding base population of *E. apiculata*.

2. Materials and methods

2.1. Samples collection

The root samples of the *E. apiculata* were taken from six natural populations (four populations in Riau province and two populations from Riau island province). Each population was represented by three samples, so that the total was 18 samples. The roots harvested were washed with water to clean off adhering soil, then dried at room temperature. The dried root was chopped manually, then made into powder with a powder size of 250 μm using a grinder. The root powder of *E. apiculata* was placed into a plastic bag and stored at room temperature until eurycomanone extraction was performed.

2.2. Extraction

The extraction of eurycomanone compounds followed the method of [21]. Extraction was carried out using methanol as a solvent because methanol is considered as a universal solvent that has a polar OH group and a nonpolar CH_3 group so that it can extract polar and nonpolar compounds [22]. 20 g of *E. apiculata* root powder was taken and then soaked in 180 ml of methanol solvent with a ratio of powder to solvent of 1:9 (g/ml).

The extraction process was carried out by heating a mixture of powder and solvent for 8 hours at a temperature of 60°C in a water bath. The extract solution was then filtered through filter paper. The residue from the first stage of extraction was re-extracted with methanol following the previous process. Extraction was performed only twice in this study. This was based on the results of the study of [23] which obtained high eurycomanone content only in the first and second extractions, while in the third to fifth extraction the eurycomanone content obtained was very low.

The filtered solutions from the first and second extractions were combined and then evaporated using a vacuum rotary evaporator for 45 minutes at 50°C. The crude extract obtained was then dried at room temperature, after that it was weighed to calculate the yield obtained.

2.3. Determination of eurycomanone concentration with HPLC

A standard eurycomanone curve was made to determine the eurycomanone content of each sample. Standard eurycomanone powder from ChemFaces manufacturer, Wuhan, China was weighed 0.5 mg

and dissolved in 1 ml of HPLC grade methanol to obtain a concentration of 500 ppm, then filtered with a PTFE filter of 0.45 μm. Furthermore, serial dilutions were carried out with the same solvent to obtain a standard concentration of 250 ppm, 125 ppm, 62.5 ppm, 31.25 ppm, and 15.625 ppm.

Determination of eurycomanone content was performed using HPLC (High-Performance Liquid Chromatography) Agilent Varian ProStar following the method of [21]. As much as 20 μl of the standard solution sample was injected into the HPLC column using a Hamilton syringe. The column used was column C18 (Microsorb-MV 100-5: size 150 mm x 4.6 mm x 5 m), the detector wavelength was 254 nm, and the run time was 17.5 minutes. The stationary phase was silica gel and the mobile phase was water and acetonitrile with the ratio of water and acetonitrile of 85:15 (v/v). The flow rate of the mobile phase mixture was 0.8 ml/min. The resulting chromatograms were recorded and analyzed using Galaxie software.

After the standard curve of eurycomanone was obtained, the determination of the eurycomanone concentration of each sample was carried out. The crude extract was dissolved with 1 ml of HPLC grade methanol, vortexed and then filtered with 0.45 m PTFE. 20 μl of sample solution was injected into the HPLC column using a Hamilton syringe. The determination of the peak of the eurycomanone chromatogram for each sample was determined by comparing the retention time of the sample (analyte) with the retention time of the standard eurycomanone. The total injection was three replicates per each sample.

2.4. Data analysis

The parameters observed in this study were as follows:Extraction yield, extraction yield calculation is carried out by the formula:$R\ (\%) = \frac{(A}{B)}\ x\ 100$ where R is the extraction yield (%), A is the extracted weight (g), and B is the initial powder weight (g). The eurycomanone content in the sample was determined based on the peak area of the eurycomanone chromatogram generated from HPLC, then calculated using a linear regression equation (Y = bx + a) from the standard curve.

The data of extraction yield and eurycomanone content obtained were calculated the average value (X), standard deviation (SD), and coefficient of variation (CV). The coefficient of phenotypic differentiation (V_{ST}) between populations was calculated by following the formula: $V_{ST} = \frac{\sigma^2_{t/s}}{(\sigma^2_{t/s} + \sigma^2_s)}$ [24], where $\sigma^2_{t/s}$ was component variance value of among populations, and σ^2_s was the component variance value within the population. Principal component analysis (PCA) and cluster analysis using the UPGMA method were performed to group the population. All of the above analyzes were performed using SAS software version 9.00 [25], and cluster analysis was performed using NTSYS ver.2.01 software [26].

3. Results and discussion

3.1. Extraction yield

The average value of extraction yield of *E. apiculata* root powder in this study was 2.99%. It was higher than the yield of *E. longifolia* root extraction with methanol solvent as reported by [27] 2.67%, [28] 1.00%, [29] 2.20%, but it was lower than the extraction yield reported by [30] and [31], 8.00% and 4.1%, respectively. The extraction yield of this study is also higher than the extraction yield with water solvent i.e. 2.30–2.60% [32] and ethanol solvents i.e. 0.47% [33], but it was almost the same as those reported by [34] and [35].

The high extraction yield of this study is presumably because the extraction was carried out by heating at a temperature of 60°C for eight hours, and the small size of the sample powder (250 μm) so that the secondary metabolites present in the sample were extracted effectively. The heating in extraction aims to increase solvent penetration into the materials (powder) so that secondary metabolic compounds will be more extracted, in addition, the small powder size will also enhance the contact area to be larger. Several factors affect the extraction yield, including the solvent used, the ratio of the weight of the material to the volume of the solvent, temperature, extraction time, and sample size [36].

The average value of the extraction yield of each population of *E. apiculata* can be observed in Figure 1. The average value of the extraction yield of *E. apiculata* populations ranged from 2.20% – 3.91%. The lowest extraction yield was obtained in the Lingga-1 population while the highest extraction yield was obtained in the Lingga-2 population. The results of analysis of variance (ANOVA) showed that the extraction yield of *E. apiculata* between populations was not significantly different ($P > 0.05$).

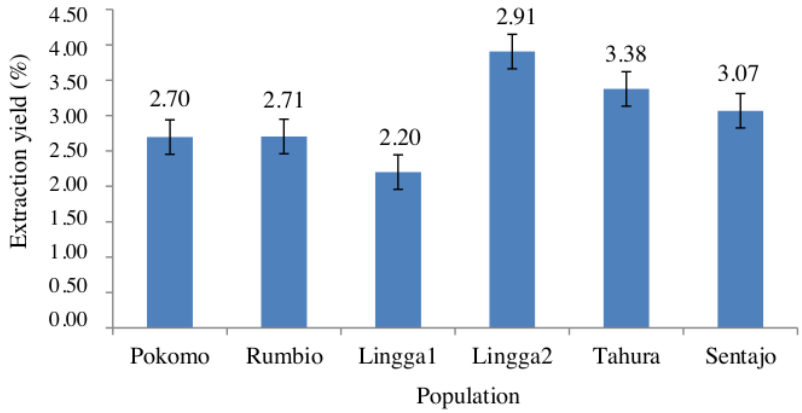

Figure 1. The average value of the extraction yield of *E. apiculata* with methanol solvent

3.2. Eurycomanone standard curve

The eurycomanone content in the sample was calculated based on the regression equation of the eurycomanone standard curve, i.e. Y = 0.0344x – 0.2526, in which the value of the correlation coefficient (r) is 0.999. The average retention time (RT) of standard eurycomanone was obtained at 2.76 minutes. The eurycomanone retention time of the tested samples of *E. apiculata* ranged from 2.70– 2.79 minutes with an average retention time of 2.75 minutes.

The retention time in this study is lower than the results of the study by Nhan and Loc [21] which found that the retention time of eurycomanone compounds was 4.1 minutes, the difference in retention time is caused by the different lengths of the C18 column used, Nhan and Loc [21] using the column C18 measures 250 mm in length while this study uses a C18 column with a length of 150 mm. This is in accordance with the results of the study of Sun et al. [39] who found that shorter column lengths resulted in faster retention times compared to longer column sizes.

3.3. Eurycomanone content and variation within population

The eurycomanone content of *E. apiculata* roots in this study was 7.13 mg/g or (0.731%). This eurycomanone content of root extracts of *E. apiculata* was higher than the eurycomanone content of *E. longifolia* roots from natural forests in Johor-Malaysia of 1.40 mg/g [40], eurycomanone content of *E. longifolia* roots from eight populations in Malaysia, i.e. 5.80 mg/g [16], eurycomanone content of *E. longifolia* roots from several populations in Malaysian Peninsular of 0.353 mg/g [18], and eurycomanone content of 2.10 mg/g from plant propagation of *E. longifolia* using tissue culture techniques [21].

The average value of eurycomanone content in the population of *E. apiculata* ranged from 4.44 to 9.86 mg/g (Table 1). The highest eurycomanone content was observed in the Rumbio population and the lowest eurycomanone content was observed in the Pokomo population. The results of the analysis of variance showed that the eurycomanone content of *E. apiculata* was significantly different between populations ($p < 0.05$), suggesting that individuals within the population were genetically different, and environmental conditions different between populations. The result of Duncan's multiple range tests demonstrated that the eurycomanone content of *E. apiculata* in the Rumbio population was significantly

different with Pokomo and Tahura populations, but Rumbio population was not significantly different with other populations.

There are several factors that may influence the differences in the eurycomanone content of *E. apiculata* between populations. First, the differences in the geographical location of each population because the sampling locations of plants were from different habitats. This is supported by a previous study on the Mentha spicata plant which shows that variations in plant phytochemical content are closely related to geographic location [41]. Furthermore, the author explained that differences in altitude, humidity, and location temperature contribute to changes in the content of secondary metabolic compounds. Second, differences in eurycomanone content between populations may be caused by different physical and chemical properties of the soil in which plants grow. Soil physical and chemical properties such as the amount and composition of available nutrients and soil pH may affect plant physiology and may result in differences in the secondary metabolic content of plants [42]. Biotic factors such as herbivorous communities can also induce differences and concentrations of plant secondary metabolites in both leaves and plant roots [43].

Table 1. The average ($\underline{x}$), deviation standard (SD), and coefficient of variation (CV) of eurycomanone content of six populations *E. apiculata*.

Population	$\underline{x}$ ± SD (mg/g)	CV (%)	CV category
Rumbio	9.86 a ± 1.70	17.26	Moderate
Sentajo	8.82 ab ± 2.71	30.70	Moderate
Lingga-1	6.98 abc ± 0.82	11.71	Moderate
Lingga-2	6.99 abc ± 1.42	20.26	Moderate
Tahura	5.70 bc ± 2.76	48.54	High
Pokomo	4.44 c ± 1.15	25.85	Moderate
average	7.13 ± 1.76	25.72	moderate

Note: numbers followed by different letters in the same column are significantly different ($p < 0.05$).

The value of the coefficient of variation (CV) is often considered the main indicator of population or character diversity [24]. The CV values of eurycomanone content for the entire population of *E. apiculata* are shown in Table 2. The CV value of eurycomanone content in six populations of *E. apiculata* varied from 11.71% to 48.54% with the average CV value of all populations was 25.72%. The highest CV value was observed in the Tahura population while the lowest CV value was observed in the Lingga-1 population. According to the classification of CV [44], the variation of eurycomanone in the Tahura population was high, while other populations were moderate. The diversity of eurycomanon *E. apiculata* in this study was 25.72% higher than that of ginkgolide B (CV = 18.46%), ginkgolide C (CV = 15.74%); total ginkgolide (CV = 19.41%) and total lactone (CV = 20.36%) in *Ginkgo biloba* plant [45].

The average value of variation of the eurycomanone content in the population of *E. apiculata* from the Riau Islands region (Lingga-1 and Lingga-2) was 15.99%, lower than the population of *E. apiculata* from Sumatra (30.59%). This showed that the population from the Sumatra mainland is more diverse than the population on the Riau Archipelago. The lower eurycomanone variation of the population from the Riau Islands is greater than the population in mainland Sumatra due to geographic isolation, consequences diminishing of gene flow among populations, and low genetic diversity of species [46]. In addition, variability in environmental factors in island population could reduce defense plants, reflecting in decline certain phytochemical content, such as reduction of tannin contents in *Periploca laevigata* [47], and terpene in *Thuja plicata* [48].

3.4. Coefficient of phenotypic differentiation (V_{ST}).

The coefficient of differentiation of eurycomanone among populations (V_{ST}) of *E. apiculata* was 84.33%, indicating that the variation of eurycomanone content among populations was higher than that of eurycomanone content within-population which was only 15.67%. The high variation of eurycomanone among populations can be caused by several factors, including i) geographical isolation

between the studied populations, gene flow will be inhibited, and resulting in loss of genetic variation and high differentiation among populations. This is also in line with that reported by Ha et al. [49], ii) different in geographical position among populations, iii) different in soil properties among populations study, and iv) different in herbivore community among populations.

3.5. Correlation of eurycomanone content with environmental factors

Correlation analysis is a valuable analytical parameter to know the relationship between one variable and another variable. The correlation coefficient value of the eurycomanone content of *E. apiculata* with environmental factors of each population was shown in Table 2. This study found that the eurycomanone content of *E. apiculata* was not significantly correlated with geographical factors (longitude position, latitude position, and altitude), and climatic factors (annual average temperature, and average annual rainfall).

These results are in line with the research of [50] who found that the content of secondary metabolic compounds in the roots of the *Tithonia diversifolia* plant was not influenced by environmental factors such as rainfall, humidity, temperature and solar radiation but was influenced by the availability of nutrients in the soil, especially macronutrients (Ca, Mg, P, and K) as well as micronutrients such as Cu. Furthermore, Sampaio et al. [50] stated that environmental factors such as rainfall, humidity, temperature and solar radiation only affect the profile of secondary metabolic compounds in the stems and leaves.

Table 2. Correlation coefficient value of eurycomanone content of *E. apiculata* with geographic and climate factors.

Eurycomanone content	Longitude	Latitude	Altitude	Average of temperature annually	Average of rainfall annually
r value	-0.01	-0.28	-0.31	-0.15	-0.39
P value	0.97	0.27	0.21	0.54	0.11

Many researchers have found that population geographic factors greatly affect the content of secondary metabolic compounds in plants.The secondary metabolic content among populations is closely related to population geographic factors, such as latitude and altitude because geographic changes will be associated with variations in biotic and abiotic factors [51, 52].

The results of this study contradict the results of the previous study of who reported a significant correlation between the content of plant active ingredients (five types of anthraquinone compounds) with latitude position while longitude negatively correlated with accumulation of anthraquinone secondary metabolic compounds. Pratt et al. [53] conducted a common garden test on Artemisia californica and found that the concentrations of terpenes and monoterpenes were associated with the latitude of the original population and differences in rainfall.

The most secondary metabolites of *S. baicalensis* plants were negatively correlated with latitude and positively correlated with temperature [54]. The content of 21 active ingredients of *S. baicalensis* is higher at low latitudes than at high latitudes. Bont et al. [55] has analyzed the secondary metabolic content of latex in the roots of the *Taraxacum officinale* plant on 63 populations in Switzerland with different climatic conditions, soil abiotic factors, and soil herbivores. Authors concluded that the secondary metabolic concentration of root latex had the strongest correlation with climatic conditions, while soil abiotic factors and root herbivore stress did not show a strong relationship with the secondary metabolic concentration of latex in the *Taraxacum officinale* roots.

3.6. Relationships between populations

The UPGMA dendrogram of the pasak bumi *E. apiculata* based on the similarity coefficient of eurycomanone content between populations is shown in Figure 2. The UPGMA *E. apiculata* dendrogram grouped the study population into three groups. The first group is the Rumbio and Sentajo populations, the second group is the Tahura, Lingga-1 and Lingga-2 populations, the third group is the Pokomo population. Population grouping based on principal component analysis was also carried out

(Figure 3). The results of grouping of *E.apiculata* are the same as with the UPGMA dendrogram as seen in Figures 2. The PCA results grouped six populations of *E. apiculata* into three groups, namely the first group was the population of Rumbio and Sentajo, the second group was the population of Lingga-1, Lingga-2, and Tahura while the third group was the population of Pokomo (Figure 3). The grouping population in this study was different from the clustering population of *E. apiculata* based on leaf morphometric [3], the authors grouped the six similar populations into two clusters.

3.7. The implications for the breeding of pasak bumi plants

The collection of genetic material is the main key in the plant breeding process. One of the considerations in choosing a location for genetic material collection is the presence of characters that are the target of breeding objectives, in this case, the content of eurycomanone compounds in the *E. apiculata*. Plant populations that contain high eurycomanone compounds can be used as candidate sites for the selection of genetic material of *E. apiculata*. The origin population of the seed source will determine the composition and content of secondary metabolic compounds of plants. Based on eurycomanone content data and limited funding for the collection of genetic material, the minimum collection of *E. apiculata* genetic material can be carried out in the Rumbio population.

Breeding of *E.apiculata* for the production of eurycomanone compounds can be done conventionally and in biotechnology. Conventionally, it is through progeny tests and stresses such as drought and shade. A progeny test is the testing method to predict the genetic composition of an individual by examining the characteristics of the offspring. In the progeny test, superior families will be selected and converted into seed orchards with high-quality classes. Biotechnology approaches for the production of eurycomanone compounds can be done through tissue culture techniques, chromosome manipulation with mutagens, and genetic engineering. Tissue culture techniques have been developed and intensively used for secondary metabolic production [56,57] such as callus culture and suspension culture. Modification of media and certain growth regulators in tissue culture can also be done to increase the main precursors in the biosynthesis of eurycomanone compounds. Collected plant material can be used as explants for tissue culture in order to increase the eurycomanone content

Chromosomal manipulation through mutations with colchicine may be an option to increase the eurycomanone content because many studies have reported that the higher the ploidy of a plant, the content of secondary metabolic compounds tends to increase [58–60]. Currently, several researchers are also developing hair root culture methods for the secondary metabolic production of pasak bumi plants [61–64]. Hair root culture is the induction of hair root formation by infecting Agrobacterium rhizogenes on explants to be cultured. Production of secondary metabolites by this method is higher than roots from normal plants. Finally, the selection of the method to be applied depends on the readiness of human resources, laboratory facilities and financial support.

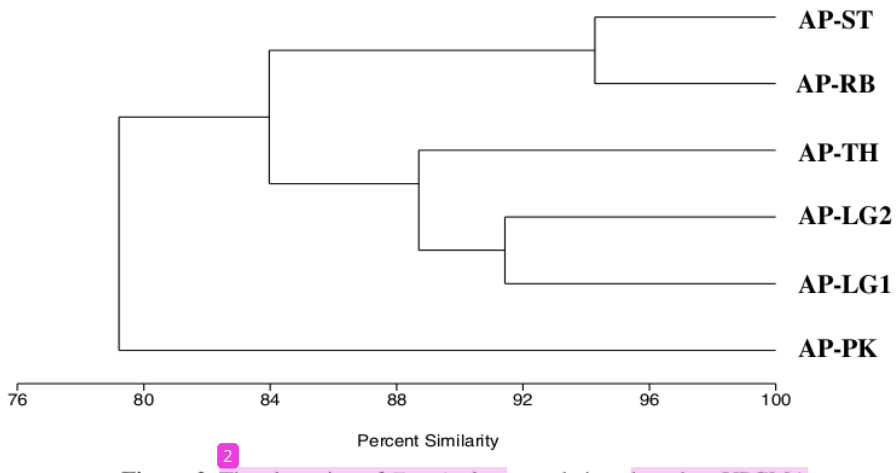

Figure 2. The clustering of *E. apiculata* populations based on UPGMA.

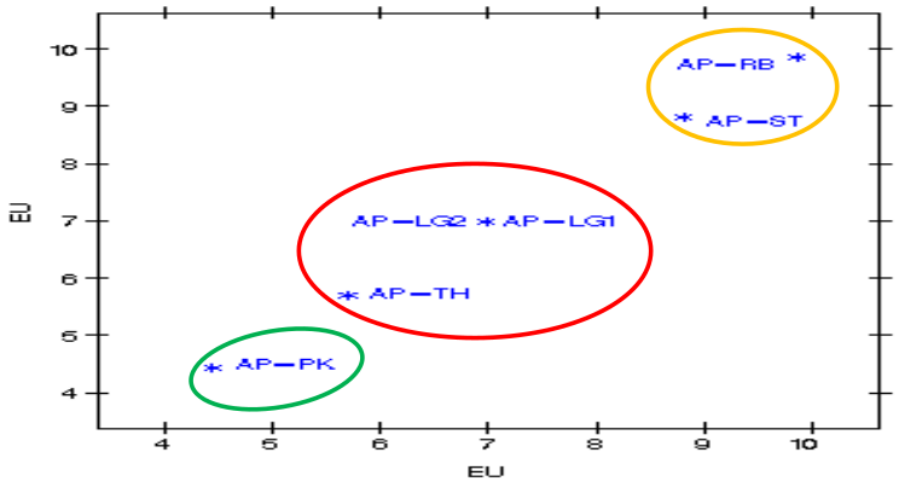

Figure 3. The clustering of *E. apiculata* populations based on PCA

4. Conclusion

The highest eurycomanone content was found in the Rumbio population. The average variation of eurycomanone content within the population of *E. apiculata* is 25.72%, while the coefficient of differentiation of eurycomanone between populations was 84.33%. The eurycomanone content in this study was not influenced by geographical factors (longitude position, latitude position, and altitude), and climatic factors (average of temperature annually, and average of rainfall annually). Collection of plant material for breeding activities can be carried out minimally in the Rumbio population.

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