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ANTİ-CANDIDIASIS BIOMARKER DETERMINATION AND CHROMATOGRAPHIC FINGERPRINT CHARACTERIZATION OF ESSENTIAL OILS EXTRACTED FROM BALI ISLAND CULTIVAR *Piper betle L.* FOLIUM

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Ключевые слова: *Piper betle L. folium essentialoil; TLC-bioautography; biomarker; eugenol derivates*

Introduction

Balinese have been using PBLas an alternative herbal medicine for generations in their daily life to eliminate body odour, and to cure diarrhoea, sore throat, and skin allergies. Searching ethnopharmacognosy of the PBL use in Bali was based on words of "*usada* and *sirih*". *Sirih* is a Balinese word for PBL and *Usadha* word derived from Sanskrithas a meaning of herbal medicine. However Balinese people understand the meaning of *usada* as guidance for diagnosis of traditional healer, curing and treatment of diseases, preventing and recovering of diseases. The PBL has been used for generations to cure infection-related diseases, such asabscess, wounds, sores, rashes accompanied by fever, and thrush [1]. Decocted leaves have also been used for gargle in the treatment of mouth hygiene. Thrush is a type of infection in mouth cavity and throat caused by fungi. Candidiasis is a type of thrush that has been extensively reported [2]. In the treatment of infection-related diseases, PBLis normally mixed with other herbal ingredients.

Anti-candidiasis activity of PBL has been extensively studied and as a result large varieties of compounds have been successfully isolated from this plant. Some chemical constituents found in PBL essential oils were mono-terpenes, sesqui-terpenes, alcohols, aldehydes, oxides, phenols, phenolic ester, and ester [3]. Eugenol derivates of PBL, such as eugenol, isoeugenol, chavicol, methyl chavicol, and hydroxyl chavicol were reported to have anti-candidiasis [2, 4]. The chemical compounds contained in the essential oil of PBL varied with their varieties, cultivars, geographical locations, and harvesting seasons.

TLC bioautography has been used in the identification of antifungal bioactive compounds. Data set of TLC numeric parametersis necessary for further identification of biomarkers. GC-MS results provide us with information of compound identities contained in the PBL essential oils. By combining the TLC bioautography, TLC-densitometer, and GC-MS results, it will enable us to identify precisely the antifungal bio-active compounds of PBL essential oils. The relationship between the minimum inhibition concentration (MIC) of PBL essential oil and its biomarker concentration can be analysed with the help of statistical multivariate analysis. The main objectives of this study were to determine anti-candidiasis biomarkers of PBL essential oils extracted from leaves of this plant which were collected from ten sites within Bali Island and to determine their chromatographic fingerprints.

Materials and methods

The chemicals with analytical grades, such as NaCl, $NaSO_4$ anhydrate, methanol, toluene, ethyl acetate, and TLC SiGF 254 from Merck-Germany, ultra-high purity helium from a local supplier and soburaud dextrose agar (SDA) from Sigma-Aldrich were used in this project. *Piper betle* L. leaves were harvested from Petang-Badung (S1), Penebel-Tabanan (S2), Kintamani-Bangli (S3), Baturiti-Tabanan (S4), Tejakula-Singaraja (S5), Blahbatuh-Gianyar (S6), Banjarangkan-Klungkung (S7), Susut-Bangli (S8), Selat-Karangasem (S9), and Pekutatan-Jembrana (S10). The *Candida albicans* ATCC ATCC 10231 was obtained from microbiology laboratory of Faculty Medicine-Udayana University.

The equipment used in this study included TLC instruments from Camag-Switzerland (TLC Scanner 4, twin trough chamber 20x10 cm, and automatic TLC sampler), GC-MS with HP-5ms column (6890N GC – 5973 MS-Agilent Technologies), glassware (IWAKI-Pyrex-Indonesian),

analytical balance (AND-Japan), and moisture analyzer balance (Shimadzu-Japan).

Sample preparation.

The taxonomy classification of the PBL sample was identified by botanist expert in the Technical implementation unit for plant conservation – Bali botanic garden of Indonesian Institute of Sciences. Some1 kg of freshly chopped PBL was distilled with boiling water, saturated with NaCl, and transferred to a separating funnel. The separated volatile oil was collected with a centrifuge tube, added with $NaSO_4$, and centrifuged at 4000 rpm for 10 minutes. The essential oil was collected in a dark born vial [5].

TLC-Bioautography and anti-candidiasis activity.

Before being used, the Al-TLC SiGF 254 (20 cm × 10 cm) was prewashed with methanol and activated at 120°C for 30 minutes. Some 30 µL PBL essential oil previously diluted in methanol (4 µL PBL essential oil in 1 mL ethanol) was spotted (duplicate spots for each sample) onto a platein a band form by using an automatic sampler (ATS) applicator fitted with a 25 µL syringe and air sprayed band. The Band length was 3.5 mm. The first band was appliedon x = 13 mm, y = 10 mm, and with a 9.2 mm space between tracks. The spotted essential oils were developed to a distance of 9 cm with mobile phase (toluene: ethyl acetate, 93:7, v/v) in a twin-trough previously saturated with mobile phase vapour for 30 minutes. The developed plate was then dried at 60 °C for 5 min and scanned witha TLC-scanner 4 under wave length of 224 nm, with slit length of 80% of the bandwidth of the spots and the no factor of slit dimension under 2.6 [6], with a scanning speed of 20 mm s^{-1} and data resolution of 100 µm/step. The *in-situ* UV spectra of each peak in all assigned peak between 200 to 400 nm wavelengths were recorded. The parameter integration peak was set at the following filter factors: Savit-sky-Golay 19, lowest slope, peak thresholds (minimum slope: 5, minimum height: 10 AU, minimum area: 50 AU).

Each track was cut with a dimension of 1 x 9.2 cm, sterilized under UV light in a clean bench for 20 minutes, poured with 15 mL soburaud dextrose agar (SDA) containing 75 µL of *Candida albicans* ATCC 10231 suspension with a cell density of 1.5 x 10^8 CFU/mL, incubated (inverted position) at 37 °C for 24 h, and observed for inhibition zone around TLC spots.

Anti-candidiasis activity of PBL essential oil, extracted from samples collected from different sites within Bali Island, was performed in triplicate experiments on SDA. The 0.1% *Candida albicans* suspension was swabbed uniformly with a sterile cotton bud onthe sterilesolidified SDA and let dry for 15 mins. Sterilized paper disks with a diameter of 6 mm previously loaded with 15 µL of PBL essential oil with concentration of 25, 50, and 100 µL /mL, werethen placed at equidistance on the surface of this medium and then incubated at 37 °C for 24 h. The diameter IZ was measured with a verniercaliper. The minimum inhibition concentration (MIC) of PBL essential oil on the causative agent of candidiasis was determined on the basis of a zero intercept of a linear regression of the squared size of the IZ, plotted against natural logarithm of PBL essential oil concentrations [7].

GC-MS.

Some 1 µL PBL essential oil previously diluted in methanol (10 µL PBL essential oil in 240 µL methanol) was injected into a GC-MS with HP-5ms column (6890N GC – 5973 MS-Agilent Technologies) with a setting of one mL/min helium gas flow. The temperature oven was settled at 60 °C (held for 5 minutes), and then was gradually increased by 4 °C/min until it reached 220 °C (this washeld for 20 min). The running time of this programmed temperature was 65 minutes. Data were collected in full scan mode (m/z 50-600). The essential oil components were identified based on their retention indices and by comparing their mass spectra with those in the NIST 98 spectra library database.

Data processing

The *in-situ* UV spectra between TLC biomarker peaks were compared by using cross-correlation function. Peak areas of the detected substances, with the help of software, were arranged into a rectangular matrix (t observations x n variables) [5]. Multivariate statistical analyses of the matrix data set were conducted with help of Minitab 17 statistical software. The chromatographic fingerprints similarity was calculated with the help of HCA-ward linkage method.

Results

The results of taxonomy identification said that PBL sample for this study, was *Piper betle L.* The yield of PBL essential oils obtained from hydro-distillation of PBL samples collected from various sites within Bali Island was in the range of 0.17 – 0.32% (v/w). Based on the results of GC-MS analysis onthese essentialoils, 30 different substances were detected (see Table 1). The respective chromatographic fingerprints similarities among PBL essential oils for GC-MS and for TLC were 96.85% and 95.08%.

Table 1. *The MIC - anti-candidiasis activity of PBL essential oils*

PBL essential oils		The IZ diameter (mm)			MIC (uL/mL)	R^2
ID	Yield (% v/b)	C_{25} (uL/mL)	C_{50} (uL/mL)	C_{100} (uL/mL)		
S1	0.295	8.3	12.6	22.0	11.738	0.955
S2	0.245	10.3	15.4	21.6	7.238	0.996
S3	0.290	0	12.0	17.4	22.873	0.953
S4	0.320	0	12.6	23.3	22.873	0.953
S5	0.300	11.3	20.4	25.5	7.773	0.974
S6	0.190	13.7	20.3	29.5	7.744	0.991
S7	0.170	10.3	15.2	21.0	6.727	0.996
S8	0.205	17.7	26.9	31.5	3.917	0.9647
S9	0.205	7.5	13.7	18.3	9.324	0.993
S10	0.205	8.3	14.0	24.7	13.251	0.969

The TLC bioautography images presented two inhibition zones (IZ). The first and the second IZ lied on Rf-values around 0.46 and 0.62, respectively. The diameter of the second IZ was found to be wider than that of the first one. The mean peak areas of the first and the second zone were 21407 AU and 38894 AU, respectively. The MIC-anti-candidiasis activity of PBL essential oils was arranged between 3.917 - 22.873 µL/mL (see table 2). Figure 1 presents the relationship between the altitude. The HCA calculation recommended a one cluster group with the similarity of 96.85% for GC/MS fingerprint and 95.06% for TLC fingerprint (see Fig. 2.a-b).

Table 2. *The identified substances on P. betle L. essential oil and the relationship between semi-logarithmic of MIC with the peak area of contained substances*

No	Retention time (min)	Identified Substance	Semi-logarthmic equation [log (MIC) = a (AUC) + b]
1	5.99	Sabinene hydrate	y = 0.136x + 0.916, R^2 = 0.125
2	6.22	α-pinene	y = 0.064x + 0.694, R^2 = 0.362
3	6.72	Camphene	y = 0.037x + 0.816, R^2 = 0.197
4	7.63	αphellandrene	y = 0.002x + 0.988, R^2 = 0.003
5	8.32	βPinene	y = 0.149x + 0.798, R^2 = 0.152
6	9.25	Bicycle 4,1, 0 heptane 3,7,7 trimethyl	y = 0.101x + 0.695, R^2 = 0.345
7	***9.72***	***β-phellandrene***	***y = -0.006x + 1.022, R^2 = 0.020***
8	9.79	Eucalyptol	y = 0.259x + 0.751, R^2 = 0.384
9	10.9	Terpinene	y = 0.071x + 0.686, R^2 = 0.322
10	12.04	4-Carene	y = 0.260x + 0.817, R^2 = 0.419
11	15.47	Terpineol	y = 0.046x + 0.559, R^2 = 0.261
12	***18.58***	***Chavicol***	***y = -0.026x + 1.534, R^2 = 0.393***
13	***21.45***	***Isoeugenol***	***y = -0.009x + 1.059, R^2 = 0.098***
14	***22.48***	***Eugenol***	***y = -0.010x + 1.978, R^2 = 0.308***
15	22.63	Phenol, 2-methoxy-3-(2-propenyl)-	y = 0.007x + 0.983, R^2 = 0.002
16	22.91	βElemene	y = 0.130x + 0.637, R^2 = 0.279
17	23.77	Caryophyllene	y = 0.092x + 0.218, R^2 = 0.592
18	24.84	Humulene	y = 0.109x + 0.271, R^2 = 0.509
19	25.59	α Amorphene	y = 0.108x + 0.176, R^2 = 0.581
20	25.72	Germacrene	y = 0.043x + 0.654, R^2 = 0.272
21	25.88	γ Selinene	y = 0.086x + 0.271, R^2 = 0.564
22	26.15	α Selinene	y = 0.068x + 0.268, R^2 = 0.574
23	26.28	βCadinene	y = 0.448x + 0.938, R^2 = 0.239
24	***26.54***	***β-bisabolene***	***y = -0.034x + 0.997, R^2 = 0.001***
25	26.79	α Panasinsen	y = 0.367x + 0.259, R^2 = 0.552
26	26.98	δAmorphene	y = 0.252x + 0.399, R^2 = 0.274
27	***27.23***	***α-patchoulene***	***y = -0.029x + 1.078, R^2 = 0.217***
28	27.95	g Elemene	y = 0.334x + 0.915, R^2 = 0.283
29	30.44	Cadinol	y = 0.160x + 0.969, R^2 = 0.048
30	***30.65***	***Allylpyrocatechol 3,4-diacetate***	***y = -0.535x + 1.039, R^2=0.120***

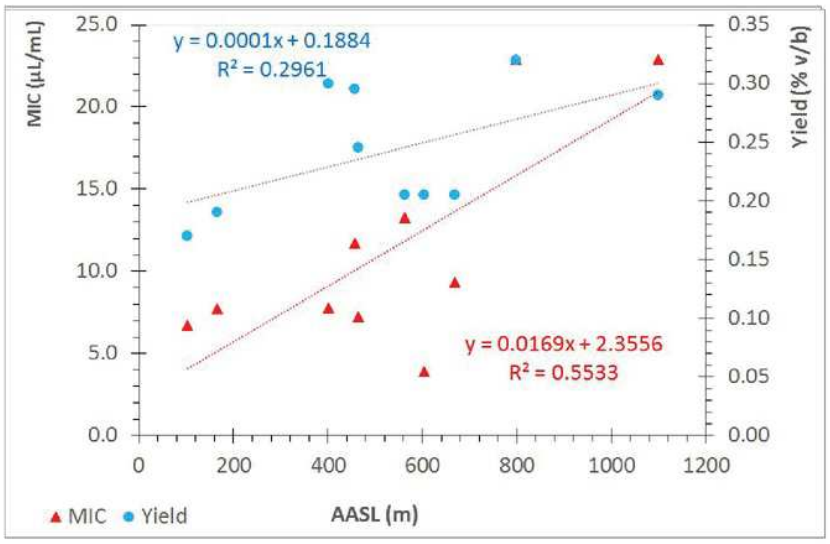

Figure 1. *The relationship between altitude, where the PBL grow (altitude above sea level "AASL") with MIC as well as yield of PBL essential oil*

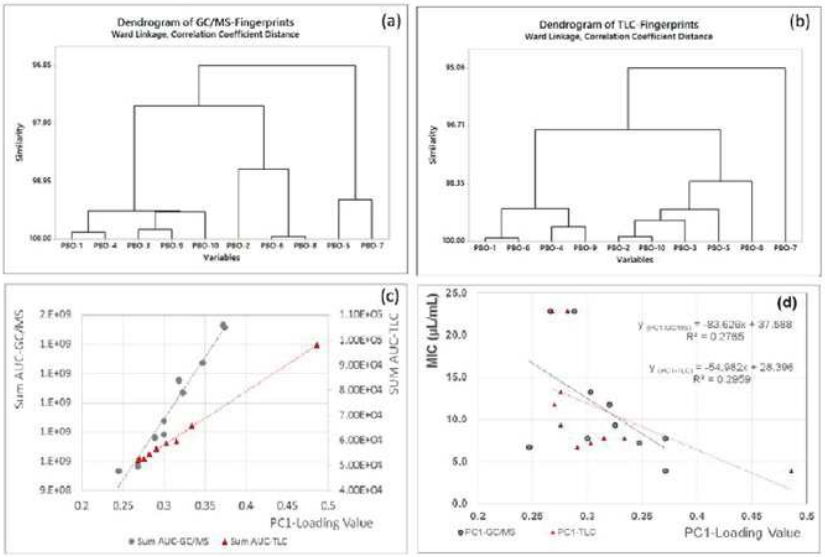

Figure 2. *The HCA-Clustering (a), the relationship altitude-% yield-% biomarker (b) and the relationship PC1 loading values-sum bio-active peak areas (c).*

Discussion

The respective chromatographic fingerprints similarities among PBL essential oils for GC-MS and for TLC were 96.85% and 95.08%. These high values of fingerprint similarity indicated no significant variations of chemical constituents contained in the PBL essential oils.

The TLC densitograms and the *in-situ* UV spectrum of both peak 1 of PBL essential oils (S1) are shown in Fig. 3. Comparison between *in-situ* spectra of the second IZ with that of eugenol library resulted similarity-values of between 94 – 97%. Due to the closeness of both hRf-values and their *in-situ* UV spectrum to eugenol library [8] the second IZ was confirmed to be belong to eugenol. In the meanwhile, the similarity value between the *in-situ* UV-spectrum of first IZ with

that of eugenol was 93 – 94%. Higher spectrum similarity could be obtained if the substance had a close chromophore function to eugenol. The two anti-candidiasis biomarkers of these PBL essential oils possessed a close chromophore functional to eugenol and the active substances have been presumed to be eugenol derivates. The five substances delivered by the GC/MS had chemical structures which were relatively close to eugenol (see Fig. 4). These five substances were chavicol, eugenol, isoeugenol phenol, 2-methoxy-3-(2-propenyl)-, and allyl pyrocatechol 3,4-diacetate. These substances possess relatively close chromophore structures and they could be suspected to be responsible for bioactivity of PBL essential oil against *C. albicans*.

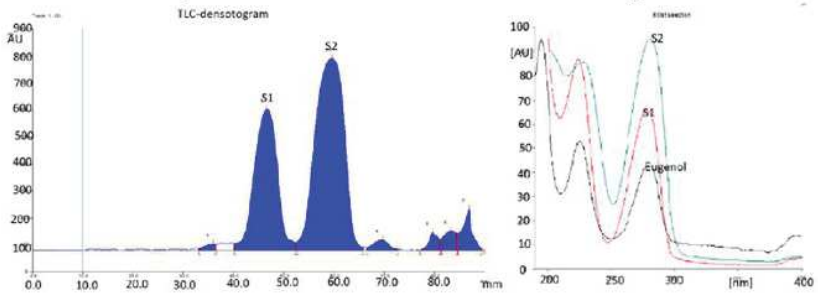

Figure 3. *The densitograms of P. betle L. essentialoil and the in-situ spectra of peak s1, s2 and eugenol library.*

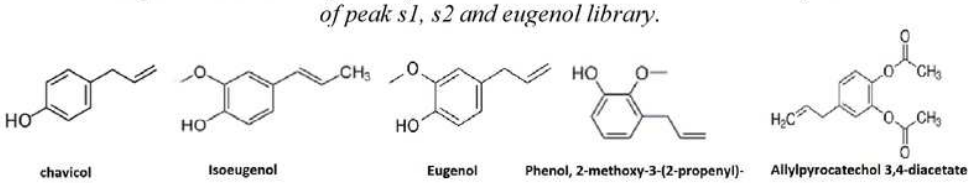

Figure 4. *The Eugenol derivate molecular structure of GC/MS detected substances in P. betle L essentialoils*

The MIC-anti-candidiasis activity of PBL essential oils was arranged between 3.917 - 22.873 µL/mL (see table 2). Although all PBL essential oil samples possessed close chromatographic fingerprints, their anti-candidiasis activity was highly varied. The more biomarkers contained in samples of PBL essential oil, the smaller their MIC-values. This relationship governed a negative slope on a linear regression equation between logarithmic values of MIC with peak areas of substances contained in PBL essentialoils (see table 1). The substances, such as β-phellandrene (Rt: 9.72min), chavicol (Rt:18.58 min), iso eugenol (Rt: 21.45 min), eugenol (Rt: 22.48 min), β-bisabolene (Rt: 26.54 min), α-pathoulene (Rt:27.32 min), andallyl pyrocatechol 3,4-diacetate (Rt: 30.65 min), provided negative slopes and were suspected to be highly responsible for the anti-candidiasis activities of the PBL essential oils.

The Coefficient determination (R-squared) of simple linear regression between logarithmic (MIC) of β-phellandrene, chavicol, iso eugenol, eugenol, β-bisabolene, α-pathoulene, andallyl pyrocatechol 3,4-diacetate, and their peak areas were 0.020, 0.393, 0.098, 0.308, 0.001, 0.217, and 0.120, respectively. The R-squared of correlation between the log (MIC) and sum of biomarker peak areas was 0.558. The closer the data to the fitted regression line, the higher its R-squared value. Each biomarker substance possessed lower R-squared value when compared to the total peak areas of all biomarker substances. This explained that anti-candidiasis activity of PBL-essential oil was a resultant effect of all biomarkers.

Figure 1 presents the relationship between the altitude, where the PBL grows, and their essential oil yield or their MIC-values. PBL harvested from lower altitude areas tends to produce fewer essential oil with higher biomarker contents, and therefore possess lower MIC-values. In other words, the altitude, where the PBL grows, affected anti-candidiasis activity of the essential oil yielded from extractionof this plant. To obtain higher anti-candidiasis activity of PBL, the plant should be harvested from low altitude areas.

The HCA calculation recommended a one cluster group with the similarity of 96.85% for GC/MS fingerprint and 95.06% for TLC fingerprint (see Fig. 2.a-b). It means there no signifi-

nt different of fingerprints among the PBL oils. The relationship between the PC1-loading values of both TLC- and GC/MS fingerprints and the sum peak areas of the biomarker described on Fig 2.c. The PC1-loading values correlated linearly to the sum peak areas of the biomarker with the regression coefficients of 0.982 for GC/MS fingerprints and of 0.998 for TLC fingerprints. The chromatographic fingerprint data consisted of the substances peak areas data series. These multi data series would be simplified through the PCA calculation into a single PC1-loading plot value. The PCA-loading plot was usually used to describe the relationship between observations (fingerprints) data among the rectangular matrix data set (t-observations x n-variables). A substance peak area of a chromatogram reflected its concentration level. This relationship could be assumed that the PC1-loading plot of this rectangular matrix data series presented the summary of each substance concentration, which composed the fingerprint data series. The Fig. 2 d showed the relationship between PC1-loading plot values of both prints and the discs diffusion diameter IZs of each PBL oils. The PC1-loading plot-values of GC/MS fingerprints obtained the regression coefficients of 0.613, but the higher coefficient linear regression presented by TLC-fingerprints (r = 0.925). It means, the IZ of PBL oil correlated nearly to their biomarker concentration level. This relationship could be used to control the quality of both biomarkers contained on PBL oil and their pharmacological effect.

For the quality control of herbal medicine should represent their efficacy control. The difficulty of this control was hard to find the biomarker as a reference standard. The result could be used as a simple method for herbal medicine quality control without the biomarker reference standard.

Conclusion

Based on the TLC-densitometry and TLC-autobiography results, the anti-candidiasis bio-actives possessed a close chromophore function to eugenol. The eugenol, chavicol, iso eugenol, allyl pyrocatechol 3,4-diacetate, β-bisabolene, α-pathoulene, and β-phellandrene were presumable to be the biomarkers of anti-candidiasis activity. The altitude, where the PBL grows, affected the essential oil yields, biomarker contents, and MIC-levelsof this plant. To obtain higher anti-candidiasis activity of PBL, the plant should be harvested from low altitude areas.

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Conflict of interest

We declared, that there was no financial/personal interest or belief that could affect their objectivity, or if there is, stating the source and nature of that potential conflict.

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BALİ ADASINDA BECƏRILƏN *Piper betle L.* YARPAQLARINDA ALINMIŞ EFIR YAĞLARININ ANTİKANDİDOZ BİOMARKERİN TƏDQİQİ VƏ XROMATOQRAFIK XARAKTERISTIKASI

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Acar sözlər: *Piper betle L. yarpağının efir yağı, NTX-bioavtoqrafiya, biomarker, evgenol törəmələri*

Nazik təbəqədə xromatoqrafiya-densitometriya üsulunun nəticələrinə əsaslanaraq antikandidoz bioloji fəallığı evgenola yaxın xromofor funksiya daşıyır. Zənn olunur ki, evgenol, xavikol, izoevgenol, allilpirokatexol-3,4-diasetat, β-bisabolen, α-patulin və β-fellandren antikandidoz fəallığın biomarkerləridir. *Piper betle L.* bitən ərazinin hündürlüyü bitkidə olan essensial yağların, biomarkerlərin miqdarına təsir edir. Yüksək antikandidiaz fəallığı əldə etmək üçün bitki, hündürlüyü daha az olan ərazilərdən tədarük edilməlidir.

ИССЛЕДОВАНИЕ АНТИКАНДИДОЗНОГО БИОМАРКЕРА И ХРОМАТОГРАФИЧЕСКАЯ ХАРАКТЕРИСТИКА ЭФИРНЫХ МАСЕЛ, ПОЛУЧЕННЫХ ИЗ ЛИСТЬЕВ *Piper betle L.* КУЛЬТИВИРУЕМЫХ НА ОСТРОВЕ БАЛИ

И Маде А.Г. Вирасута[1], Путу С. Юстиантара[1], Ингрид И.Ж. Ваге[1], Ни М.В. Астути[1], Ни Л.П.В. Парамита[1], Ян Рамона[2]

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Ключевые слова: *эфирные масла листьев Piper betle L., ТСХ-биоавтография, биомаркер, производные эвгенола*

Основываясь на методе ТСХ-денситометрии можно утверждать, что антикандидазная активность обусловлена хромофорной группой близкой к эвгенолу. Предполагается, что эвгенол, хавикол, изоэвгенол, аллилпирокатехола 3,4-диацетат, β-бисаболен, α-патулин и β-фелландрен являются биомаркерами антикандидозной активности. На содержание эфирных масел, биомаркеров в *Piper betle L.* влияет высота участков, где произрастает это растение. Для получения более высокой антикандидиазной активности необходимо собирать сырье в более низких районах.

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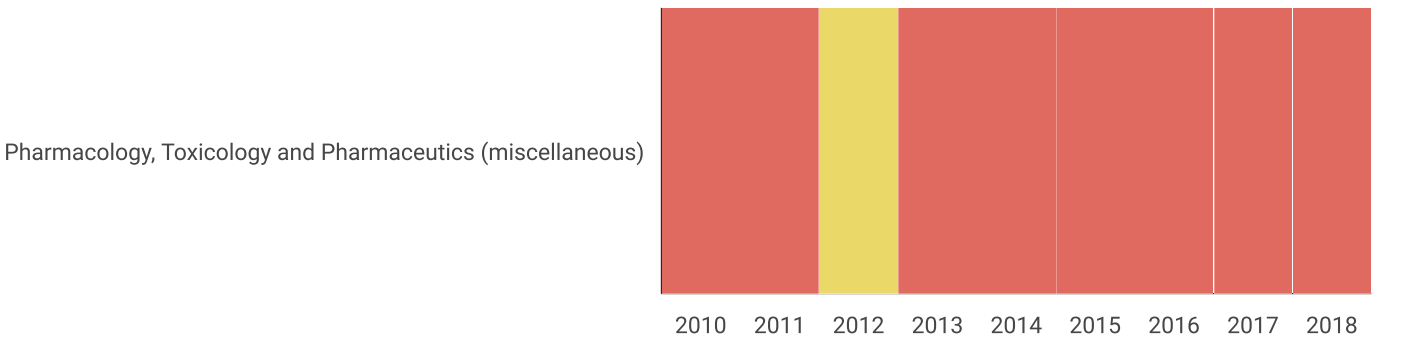

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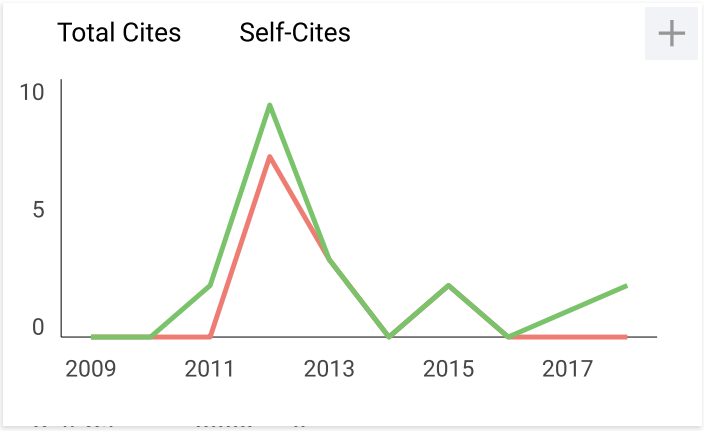

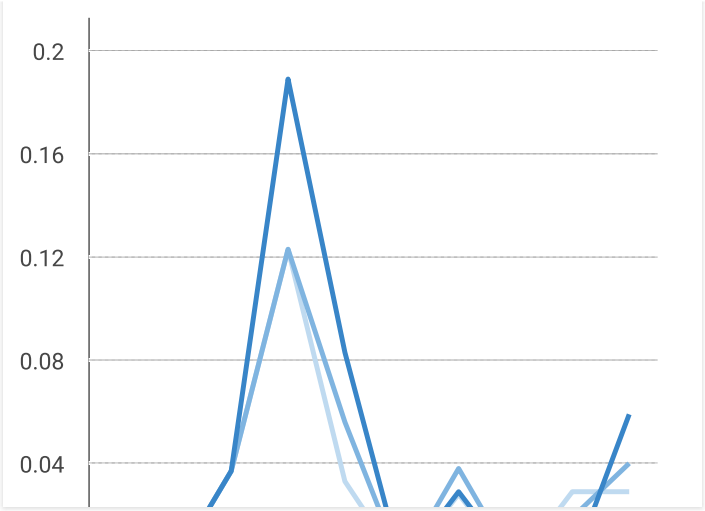

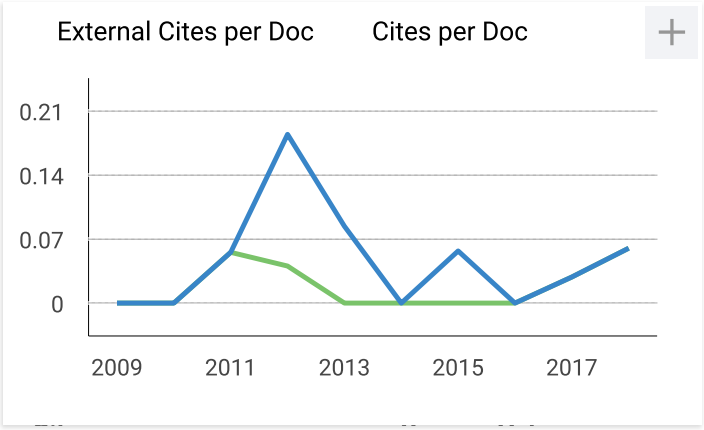

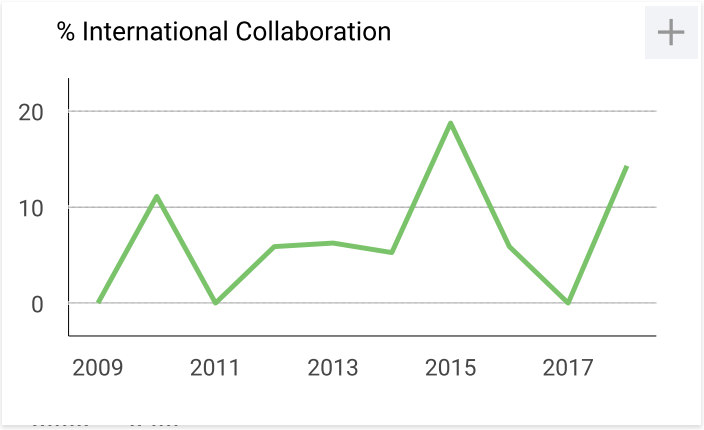

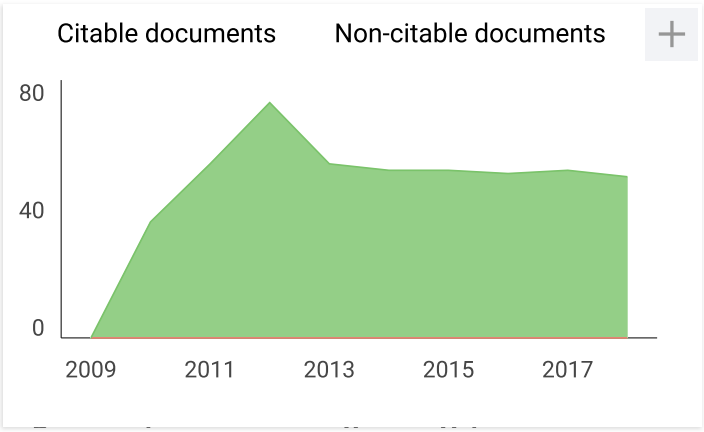

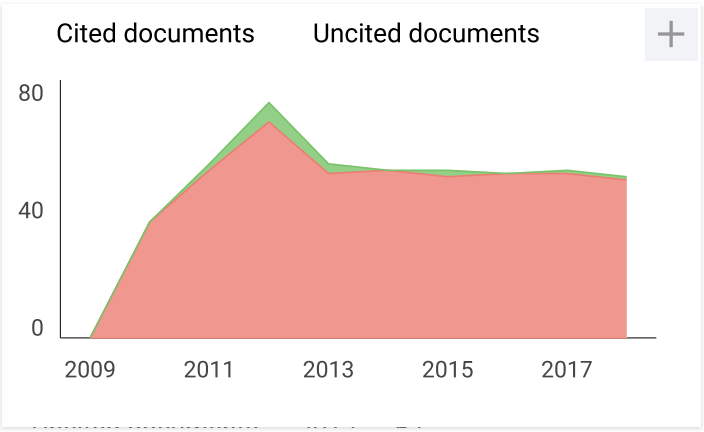

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