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Standardization of leaf extract of red betel(*Piper crocatum*) leaves using ethanol

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ABSTRACT

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Background: Red betel vine (*Piper crocatum Ruiz & Pav*) is known empirically to have properties to cure various kinds of diseases. It contains flavonoids, alkaloids, polifenolat compounds, tannins and essential oil compounds. Standardization in the pharmacy is required to ensure the level of quality, fulfill the standard requirement of chemical, biological, and pharmaceutical, including the guarantee of stability as pharmaceutical products generally.
Objective: To investigate the standard specific and non-specific parameters set by Food and Drug Supervisory Agency (FDSA) of ethanol leaf extract of red betel leaves.
Methods: The study began with sample preparation, then extracted by maceration method to get the active compounds in the lumpy extract. Extract was analyzed with standard specific and non-specific parameters set by FDSA. Results were analyzed with descriptive analysis method.
Results: The features of leaf extract of red betel vine leaves using are organolepticly viscous, dark green,and has a distinctive odor with a bitter spicy taste. The yield, water content, ash content, and specific gravity of extract were 14.8%, 0.353%, 0.16%, 0.729 respectively. Total mold contamination of extract satisfied the standard criteria that was below the limit of a maximum of 10 colonies/gram. Metal contamination of lead (Pb) was 1. 404 mg/kg and Cadmium (Cd) was 0. 223 mg/kg. Chromatographic profile of the Gas Chromatography-Mass Spectrometry (GC-MS) red betel vine leaves ethanol extract contains compounds caryophyllene, germacrene-D and some other compounds with low similiaritas index, and has a marker compound which is suspected possibly trimethoxyallyl benzen.
Conclusion : The ethanol extract of red betel vine (*Piper crocatum Ruiz & Pav*) leaves is an extract of the organolepticly viscous, dark green color, distinctive smell, bitter taste, and spicy. The ethanol extract of red betel vine leaves satisfies the standard level set by FDSA and has the marker compound which is expected likely Trimethoxyallyl benzen.

Latar Belakang: *Sirih merah (Piper crocatum Ruiz & Pav) berpotensi menyembuhkan berbagai jenis penyakit. Telah diketahui sirih merah mengandung flavonoid, alkaloid, senyawa polifenolat, tanin dan senyawa minyak atsiri. Standarisasi dalam kefarmasian dibutuhkan untuk menjamin kualitas mutu,*

memenuhi syarat standart kimia, biologi, dan farmasi, termasuk jaminan stabilitas sebagai produk kefarmasian umumnya.

Tujuan penelitian: *Untuk mengetahui nilai parameter standar dan mendapatkan ekstrak etanol daun sirih merah yang terstandar sesuai standar BPOM.*

Metode Penelitian: *Penelitian dilakukan dengan cara melakukan preparasi sampel, mengekstraksi dengan metode maserasi untuk mendapatkan senyawa aktif dalam ekstrak kental. Menganalisis ekstrak dengan nilai-nilai parameter spesifik dan parameter non spesifik standar, yang telah ditetapkan BPOM. Hasil yang diperoleh dianalisis dengan metode analisis diskriptif.*

Hasil: *Ekstrak etanol daun sirih merah secara organoleptik adalah ekstrak kental, berwarna hijau tua, bau khas daun, dengan rasa pahit pedas. Rendemen ekstrak 14,8%, kadar air ekstrak didapat sebesar 0,353%, kadar abu ekstrak 0,16% dan, bobot jenis 0,729. Cemaran mikroba maupun bakteri patogen negatif. Total cemaran kapang dari ekstrak memenuhi syarat standar yaitu berada di bawah batas maksimum 10 koloni/gram. Cemaran logam timbal (Pb) adalah 1,404 mg/kg dan Kadmium (Cd) adalah 0,223 mg/kg. Profil kromatografi dari GC-MS ekstrak etanol daun sirih merah menunjukan senyawa yang dikandungnya adalah, caryophyllene, germacrene-d dan beberapa senyawa lain dengan indeks similiaritas yang rendah, serta memiliki senyawa penanda yang diduga adalah trimethoxyallylbenzen.*

Kesimpulan: *Ekstrak etanol daun sirih merah (Piper crocatum Ruiz & Pav) secara organoleptik adalah ekstrak kental, berwarna hijau tua, bau khas daun, dan rasa pahit dan pedas. Ekstrak etanol daun sirih merah memenuhi syarat standar dan memiliki senyawa penanda yang diduga adalah Trimethoxyallylbenzen.*

INTRODUCTION

Indonesia is a country enriched with medicinal plants (herbal medicine). The existence slogan is back to nature to minimize side effects of chemical drugs, and increase the use of medicinal plants. Regarding this aspect, one of the potential medicinal plants is red betel (Piper crocatum)

A medicinal plant is considered to have efficacy as a drug based on scientific evidence (Evidence Based Medicine). The widely reported medicinal properties of red betel are the antibacterial activity against gram positive and gram negative[1], antioxidant activity[2], anti-hyperglycemic[3], anti-proliferative on cancer cells[4] and others. However, there is no data regarding the standardization of the ethanol extract. As a herbal medicine standardization of red betel leaf is highly required to ensure the product quality.

In pharmaceutical field, Standardization is generally a series of parameters or procedures and measurements of which results are quality-related elements, the paradigm of pharmaceutical quality (meet the standard requirements of chemistry, biology, and pharmaceuticals), including the guarantee of stability as pharmaceutical products. Standardization also means ensuring the end product (drug, extract, or extract product) that has a constant value of certain parameters and fixing of the end product beforehand.[5] Government regulation through FDA (Food and Drug Administration) regulation categorizes the type of natural medicine (plant) into three groups, namely herbs, standardized herbal medicine (SHM) and phytopharmaca. herb. They have beneficial or efficacy landmark based on traditional information and method of development which is executed by trial and error (empirical). SHM has the efficacy feature based on the results of pre-clinical studies of animal trials. Phytopharmaca, is a cornerstone of expediency or group with the highest efficacy because it is based on the pre-clinical tests and clinical trials (proven in humans). An effort to enhance the status of traditional medicine into the dosage form, phytopharmaca must be processed in the form of standardized extracts, as well as met up requirements of standard level set by the Food and Drug Supervisory Agency (FDSA).[6]

With the progress of time, currently medicinal plants are not being used much in the form of intact material (such as bulbs, roots), but they are processed as juice by adding solvent. The Guarantee of consistency of the chemical constituents in the extract is carried out through standardized method such as studies and arrangements to ensure the constancy of the content at corresponding set value (must be present in the product), so that the benefit or the expected healing properties will be obtained.[5]

Red betel (*Piper crocatum*) is one of the

potential medicinal plants which is empirically known to have properties to cure various diseases[7]. Based on previous chromatographic research findings, it can be reported that red betel contains flavonoids, alkaloids, poly-phenolic compounds, tannins and essential oils. This compound is known to have efficacy as antibacterial, anti-inflammatory, and antipyretic. Empirically red betel leaf is used singly or formulated with other medicinal plants to eradicate various diseases.[8]

Standardization is necessary to ensure the quality of products. The known value of constancy or correct standards and appropriate trilogy (quality, safety, and benefits) are extracted through this method specific and non-specific parameters were considered in the study. It was also intended for further development, whether it can be used as simpilisia, standardized extracts, standardized herbal medicine, or even as phytopharmaca. so it is necessary to study the standardization of the ethanol extract of red betel leaf. Results of this study were expected to expand and develop its use as a traditional medicine, as well as justification the usage of red betel leaf.

METHODS

1. Material

Red betel leaf was taken from the Prujakan village, Kaliurang road at 9.8 Km. Other materials were absolute ethanol (96%), nitric acid, hydrochloric acid, distilled water, 0.9% NaCl, Plate Count Agar (PCA), Brilliant Green Lactose Broth (BGLB), Czapex Dox Agar (CDA), ice cubes.

2. Equipment

Analytical balance, stirrer glass, knives, drying cabinets, glass jar, white cloth, rotary evaporator (Heidolph-L4000), Erlen-meyer, glass beaker, measuring cup, flask, funnel, filter paper, pycnometer, pans, thermometer, Krüss porcelain, oven (Vulcan A-550), heating, Karl Fischer instrument (Mettler toledo V30), calculator, incubator, petri dish, LAF, test tubes, Durham tube , lids, micropipette, pipette, pipette volume, AAS instrument (Perkin-Elmer 5100 PC), GC-MS instrument, centrifuges.

3. Working procedure

a. Sample preparation :

At least one month aged Red betel leaves were taken from the at least 4 months aged old plants. The collection of samples time was morning after sunrise to noon 11.0 a.m. Identification of plants were executed in the Laboratory of Pharmaceutical Biology at the Islamic University of Indonesia. The fresh, good conditioned and proper shaped leaves were soaked for 30 minutes, then washed and cleaned. Chopping, slicing of every leaves into about 2-3 pieces, was executed by using a clean, sterile, and sharp cutter Chopped leaves were put in a box with a dryer and dispersed evenly without any pedestal in a drying cabinet for 1 week.

b. Sample Extraction

The extraction method was maceration using 96% ethanol. The stored dried leaves were powdered. In macerator 100g of powdered red betel leaf (*Piper crocatum*) was soaked into 1 liter of 96% ethanol for 6 hours. Then occasionally stirring was done and the maceration solution was let to stand for 24 hours. The macerated product was separated by filtration using a white cloth into a 5 L capacity glass jar. Then the residual pulp of macerate was incorporated in the initial glass jars , given 1 liter of fresh 96% , and allowed to stand for 24 hours. This process is called re-maceration. Re-maceration was done 2 times. All macerate was collected and evaporated using rotary evaporator in order to concentrate the extract as well as obtain a viscous extract.

c. Non specific parameters

1) Specific gravity

Specific gravity was measured using a pycnometer. The specific gravity of the liquid extract was obtained by dividing the density of the extract with the density of water in the pycnometer at 25°C temperature.

2) Water level

The level of water content was determined by Karl Fischer method. The extract was injected in the Karl Fischer. Then the result of the parameters in the device was observed. The same procedure was replicated 3 times. Results were analyzed and the value of rela-

tive standard deviation (RSD) was calculated.

3) Ash content
i. Determination of Ash content

Approximately 2-3g crushed extract was weighed carefully, then put into a porcelain crucible which had already been heated. The extract of porcelain crucible was weighed and then heated slowly with heating ± 800ºC for 8 hours until the exhaustion of charcoal. Subsequently, it was cooled and reweighed. If this way of charcoal had not been eliminated, then hot water was added, filtered with ash-free filter paper. The rest of the paper and filter paper were heated in Krüss at same rate. The filtrate was inserted into Krüss, then evaporated and heated to fixed weights, and then weighed. The ash content was calculated in the material that had been dried in the air.

ii. Determination of ash content insoluble in acid

The obtained ash during the determination of ash content was boiled with 25 ml of dilute sulfuric acid C for 5 minutes. The insoluble part was collected in acid, filtered through Krüss glass macerate or ash-free filter paper, then washed with hot water, heated to fix weight and then weighed. Finally insoluble ash content in acid was calculated in the material that had been dried in air.

4) Residues of Pesticide

Testing methods of pesticide residues in agricultural commodities was determined following the commission pesticide Department of Agriculture in 1997 with modifications in below:

i. If analysis of the inhibitor chemical constituents having non-polar properties relatively small as the extracts obtained by concentrated water or ethanol yield less than 20%, the analysis can be done semi quantitatively using thin layer chromatography directly without following through the stage of cleaning in advance or using gas chromatography if there is no chemistry with N elements such as chlorophyll, alkoloid and other non-polar amines.

ii. Extract is obtained by ethanol, and does not contain high levels of non-polar nitrogen compounds, it can be tested using thin layer chromatography or gas chromatography directly without cleaning. If the extracts contain the detrimental chemical content then it must be tested according to standard methods. Examination of pesticide residue in ethanol extract of red betel was analyzed and standardization parameters were checked by GC-MS method.

5) Heavy Metal Contamination

Approximately 3g extract was weighed carefully, put in a porcelain crucible and leveled. Then it was slowly heated at a temperature of 800°C for 8 hours. Nitric acid was added and heated in a fume hood until the perfect destruction. Subsequently the level of heavy metal contamination was determined by AAS (Atomic Absorption Spectroscopy).

6) Microbial Contamination
i. Test of microbial number determination

The medium used was the Plate Count Agar (PCA). Samples were cultured in medium with 5 various dilution. Furthermore, the number of growing colonies was observed and calculated.

ii. Estimated Coliform test

Brilliant Green Lactose Broth (BLGB) was used for this testing. Samples were analyzed by variation of three 10-1 sample and three 10-2 samples dilution as treatment. While, the other three tubes were used as negative controls. The result of breeding was observed and analyzed.

7) Contamination Fungus and Yeast

Czapek Dox Agar (CDA) media was used for the determination of fungus and yeast contamination. Duplo was made for every variation of dilution and blank test. The incubation was executed at 20-25°C temperature for 5-7 days. Agar plate was observed plates carrying 40-60 colonies of fungi / yeast

d. Specific Parameter

1). Description of Plants :

Determination red betel was carried out in the Laboratory of Pharmaceutical Biology of the Faculty of Mathematics and Natural Sciences at the Islamic University of Indonesia referring to the book , Flora of Java.[9]

2). Organoleptic Extract

Test used the five senses with the description of the shape, color, smell, taste of the extract.

3). Compounds Identity

The compounds contained in the ethanol extract of red betel leaf were identified by GC-MS. The dominant compounds that frequently appeared in the analysis were analyzed by showing with high peaks in the GC chromatogram and a large index similar to the data of MS.

4). Chemical Ingredients of Extract

GC-MS was used to determine the chemical constituent of red betel leaf extract. The obtained extract was centrifuged to obtain a clear supernatant. Then it was analyzed in GC instruments following by MS instrumentation. Results in chromatogram were read, analyzed, and described the existing compound.

4. Analysis of Result

The filtrate obtained from the maceration of the ethanol extract of red betel leaf was tested. Besides, the specific parameters and non-specific parameters were also analyzed with three replication. Data were analyzed with descriptive analysis method.

RESULTS

In this study 96% ethanol was used for maceration of red betel leaf sample (*Piper crocatum Ruiz & Pav*) because it is capable of dissolving almost all the substances. Extract of ethanol was concentrated by using rotary evaporator to obtain the thick extract at constant weight. The yield percentage from maceration was 14.8%. The result of lab test for identification indicates that the used sample plants are red betel plant (*Piper crocatum Ruiz & Pav*) where key determinant factors were as follows :

1b-2b-3b-4b-6b-7b-9a . . . .(class 4)
41b-42b-43b-54b-59b-61b-62b-63b-64a . . . .(*Piperaceae*)
37.1 Piper 1a-2b-3b . . . .(*Piper crocatum Ruiz&Pav.*)

The chemical compound in ethanol extract of red betel leaves were caryophyllene, germacrene-d and some other compounds with low similarity index indicated on the chromatogram of Gas Chromatography (GC) and the library analysis of (Mass Spectrometry) MS.

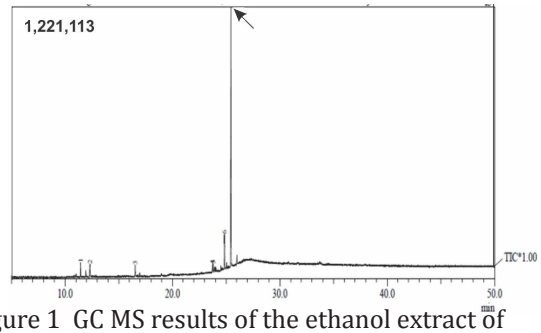

Figure 1 GC MS results of the ethanol extract of red betel leaf with peak of chromatogram height (arrows) in the GC and large similar index for MS data

The expected marker compound of ethanol extract of red betel leaf in this study was Trimethoxyallylbenzene. The result of calculated specific gravity of extract I , extract II, and Extract III was 0.728, 0.730, and 0.730 respectively. The purpose of the experiment was to provide the limitation of the amount of mass volume unity that represents the specific parameters of liquid extracts to concentrated (condensed) extract which is still pourable. The percentage of measured water content from three times replication of the ethanol extract of red betel leaves samples was 0.25%, 0.26% and 0.55% respectively. The information regarding the moisture content of extracts is necessary to determine the minimum value of the magnitude of the water content within the material, as it can affect the purity and contamination. The ash content was calculated by w/w basis before and after heating of extract .The ash content of samples was 0.16%, 0.17%, 0.17%, 0.16% respectively. The specified levels of FDSA is no more than 5µg/kg, however the analysis it was not detected at all. The result showed that the positive contaminated heavy metals were Pb and Cd contaminated, with the highest levels of 1.404 mg/kg and 0.244 mg/kg respectively, levels of tolerance from FDSA for Pb contaminant was not more than 10mg/kg and for Cd contaminant was not more than 0.3 mg/kg.

The test results of the calculated microbe numbers from the 11 cup showed that there is no microbial growth (negative), thereby extract does not contain microbes. Likewise all the coliform test tubes showed no gas

bubbles in the Durham tube, which means that extracts and negative control does not contain coliform.

The determined numbers of fungi and yeasts showed that the growing media CDA had overgrown with fungi and yeasts, but met the standard requirement that is under the maximum limit of 10 colonies/gram.

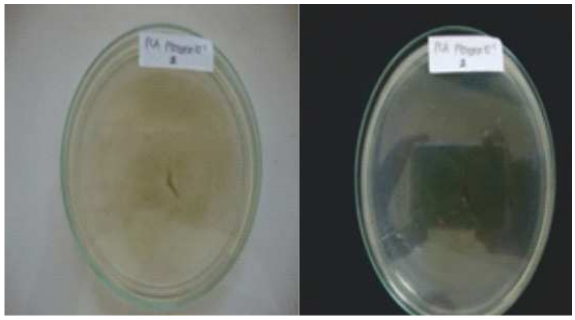

Figure 2 Results of the determined microbial numbers at 10-2 dilution (negative)

Figure 3 The test results of coliform estimation 10-2 dilution (negative)

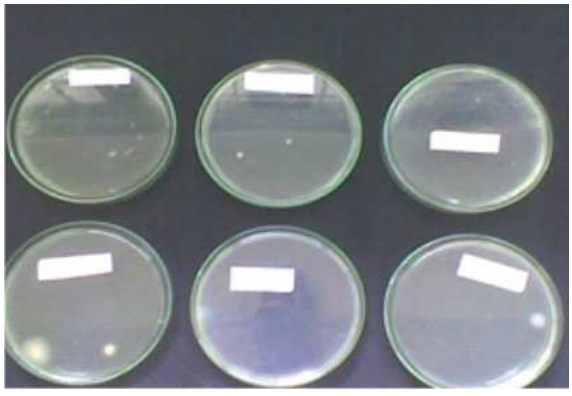

Figure 4 The result of contaminated molds and yeasts at 10-1, 10-2, 10-3 dilution

Table 1 Summary of the results of standardized ethanol extract of red betel leaves

No	Parameter	Result
1.	Yield	14.8%
2.	Organoleptic	Smell: Specific Taste: Bitter, spicy Color: Dark green Form: Viscous
3.	Water level	0.353%
4.	Ash content	0.16%
5.	Specific gravity	0.729
6.	Total Microbial Contamination	Microbes figures: Negative Forecasting of Coliform test: Negative
7.	Total contamination of Fungus & Yeast	10 colonies
8.	Total heavy metal contamination	Pb : 1,404 mg/kg Cd : 0, 223 mg/kg
9.	Residue of pestiside	Organochloro dan Organophosphate Negative
10.	Chemical constituent in Extract	Alkaloid, Flavonoid, and Terpenoid
11.	Identification of compound	Trimethoxyallylbenzene

DISCUSSION

Figure 2, 3, and 4 are the result of observation on the non-specific parameters to test the determination of the numbers of microbes and coliforms determination test and also test the determination of the numbers of fungi and yeasts. The purpose of determining the number of microbes and coliforms was to provide assurance that the extract does not contain microbial pathogens and non-pathogenic bacteria above the set limit. The purpose of determining the test parameters of molds and yeasts test was to confirm that the extract does not contain fungal contamination above the set limit, as this may affect the stability of extracts and dangerous to health.

From this study it can be reported that in general the ethanol extract of red betel meets the standard level which is set by the Food and Drug Supervisory Agency (FDSA). As the identified compound was Trimethoxyallyl-

benzene. Some Trimethoxyallylbenzene was reported to have been isolated from natural materials. Such as Elemicin (3,4,5-trimethoxyallylbenzene), Calamus (2,3,5-trimethoxyallylbenzene or 2,3,6-trimethoxyallylbenzene), and calamol successfully was obtained from the rhizomes of Acorus. Then the successfully obtained isomer was 2,4,6-trimethoxyallylbenzene and 2,4,5-trimethoxyallylbenzene (beta-asarone). Beta-asarone as other forms of family asarone has the potential to be developed into a therapeutic agent to address the cognitive impairment associated with conditions such as caused by Alzheimer's disease.[10]

CONCLUSION

From the results of this study it can be concluded that the features of Leaf extract of red betel using ethanol (*Piper crocatum Ruiz & Pav*) were organolepticly viscous, dark green color , distinctive smell, bitter taste, and spicy. The obtained water content, ash content, and specific gravity of the extract were 0.353%, 0.16%, 0.729 respectively. Microbial contamination and bacterial pathogens in ethanol extract of red betel leaves were negative. Total fungus and yeast contamination of extracts qualified as standard level that is under the maximum limit of 10 colonies/gram. Test of metal contamination revealed that the amount of lead contamination (Pb) was 1.404 mg/kg and Cadmium (Cd) contamination was 0.223 mg/kg. Profile of GC-MS chromatography showed that the chemical constitutions of ethanol extract of red betel leaves are caryophyllene, germacrene-D, and some compounds with low similarity index, where Trimethoxyallylbenzene is the suspected marker compound. Based on the analysis of parameters standardization it can be said that ethanol extract of red betel leaf meets the standard level set by FDSA, and is qualified as a standardized extract.

SUGGESTION

In future study on quantitative standardization of ethanol extract of red betel leaf should be executed. To promote a research on integration of preclinical testing and clinical trials with parameter of standardized ethanol extract of red betel leaves.

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