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Research Article

Pharmacognostic Profile of Simplicia and Ethanolic Leaves Extract from Indonesian *Piper betle var. nigra*

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ABSTRACT

Piper betle var. *nigra* has potency as an herbal medication. Thus it can be used as a source of medicinal raw materials. *Piper betle* var. *nigra* simplicia and extracts must be controlled to obtain unswerving quality and ensure their pharmacological effects by standardizing them through several specific and non-specific parameters. Specific parameters were analyzed organoleptically in the ethanol extract, namely having a thick consistency, brownish-green or blackish-green color, a mild peculiar odor, a bitter, cherish taste, and a slightly spicy flavor. The microscopic simplicia powder profile is as follows: the lower epidermis with idioblasts in oil cells contour and upper epidermis, sclerenchyma, covering hairs, and transport bundles ladder-type thickening, and idioblasts in oil cells contour. Extract content analysis of simplicia and black betle leaves ethanol extract showed water-soluble extract content of 2.77% and 12.45% and ethanol-soluble extract content of 1.38% and 19.1%. Secondary metabolites in the ethanol extract are flavonoids, polyphenols, tannins, saponins, alkaloids, and steroids. The non-specific parameters of the simplicia and extract are as follows; total ash content of 12.1% and 7.43%; acid insoluble ash content of 4.45% and 1.57%; drying shrinkage of 14.5% and 15.85%; total lead (Pb) contamination 47.5 ppm and 1.2 ppm; and the total cadmium (Cd) contamination <0.2 ppm. The total bacterial contamination in the extract was 4.3x105 colonies/g. The total yeast contamination was 8.3x105 colonies/g and volumetric mass density of the water-soluble extract of 0.96 g/mL and the volumetric mass density of the ethanol-soluble extract of 1.01 g/mL.

Key words: *Piper betle* var. *nigra,* Non-specific parameters, Specific parameters, Black betle, Standardization.

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INTRODUCTION

The goods of Black betle (*Piper betle* var. *nigra*) leaves extract had been reported, giving rise to chances for black betle to be marketed. Several black betle leaves extract bioactivities are included antimicrobial,[1,2] anti-inflammatories,[3] cytotoxic[4] and antioxidant[5] properties. Black betle's economic gains make it engage the demands to become a commodity crop. Consequently, it will provide financial growth sustainability for the grassroots community. One of the main provisions in herbal medicines commercialization or industrialization is raw materials of affluence and good quality. Raw materials must also have common characteristics ranging from the most detailed, such as plant morphology, to specific characteristics such as representing dominant compounds and types of biologically active compounds. The characteristics of the black betle plant are vines with leaves that resemble heart shape and have a petiole. The leaves grow alternately on the stem and have dark green-blackish color. Morphology of black betle leaves has a round leaves stem, purplish-green color, and no flowers. The Single leaves have a shape like a heart, and the tip of the leaves is tapered. Leaves that thrive on an average of 10 cm and 5 cm leave feel thick and stiff when held. The classification of black betle based on identification results at the Lembaga Ilmu Pengetahuan Indonesia (LIPI) Bogor with specimen number 749 / IPH.1.01 / If.07 / VII / 2020. The classification results are kingdom Plantae, subkingdom tracheobionta, super division Spermatophyta, division Magnoliophyta, class Magnoliopsida, subclass Magnoliidae, order Piperales, family Piperaceae, genus piper, species of piper sp. Based on this classification, it is known that this plant's sub-species are still missing, so it can be said that the black betle has not undergone much assessment. Black betle's morphology can be seen more clearly in the image below.[2]

Numerous types of plants have been used as herbal medications by Indonesians from generation to generation because plants have secondary metabolites that can cause specific biological effects when utilized. The use of herbal medicines is also more desirable because they have few side effects and low toxicity. Therefore, it encourages more extensive herbal medicine development.

One of the plants that have potential as herbal medicine is black betle. Empirically, the people of East Kalimantan use *Piper acre* Blume (black betle) as a medication for itching, cysts, prostate, pain (abdominal pain), and jaundice.[6]

Medicinal Raw materials for simplicia and ethanol extract of black betle leaves need to be standardized by referring to official procedures from the Indonesian Ministry of Health (2000) regarding General Standard Parameters of Medicinal Plant Extracts and Indonesian Herbal Pharmacopoeia (2017). Standardization intends to promote the product state and warrant its pharmacological effects to be more attainable and safe for the broader citizenry as standardized herbal medicine.

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Besides, this process concedes black betle leaves to be manifested into legitimately standardized herbal medicinal raw materials so that people can employ it with the becoming identity and proved quality.

EXPERIMENTAL

Material and methods

This research used experimental laboratory methods on the standardization of simplicia, and black betle leaves extract by looking at specific and non-specific parameters.

Plant material

Black betle leaves were collected from Samarinda, East Kalimantan, Indonesia, in June 2020. Pla,nt identified By Dr. Atik Retnowati, a staff from Herbarium Bogoriense, Bogor, West Java, Indonesia, with specimen number 749 / IPH.1.01 / If.07 / VII / 2020. The part of the plant used is the leaves. The leaves are cleaned thoroughly and then wilted in an oven at 40 ° C. The dried leaves are then pulverized into a powder.

Chemical

The materials used are ammonia (Merck), anhydrous acetic acid (Merck), hydrochloric acid (Merck), sulfuric acid (Merck), distilled water (Merck), ethanol 96% (Merck), Ferric (III) chloride (Merck), gelatin, filter paper, chloroform (Merck), N.A. (Nutrient Agar) medium (Sigma), medium PDA (Potato Dextrose Agar) (Sigma), sodium chloride (Merck), dragendroff reagent, and magnesium powder (Merck).

Equipment

The laboratory apparatus used includes stirring rods, reagent bottles (Schott-Duran), weigh bottles, bunsen, Krush dishes, Petri dishes, porcelain dishes, funnels, desiccators, beakers, hotplates, incubators, watch glasses, slide glasses, cuvettes, Erlenmeyer flasks, measuring flasks, bowl, microscope, oven, pycnometer, dropper pipette, measuring pipette, propipette, rotary evaporator, horn spoon, shaker, metal spatial, atomic absorption spectrophotometer, test tube, furnace, thermometer, scale, and glass jar.

Extraction and extract preparation

Simplicia powder is macerated using 70% ethanol in a glass container. The maceration process is allotted to last for three days, stirring infrequently using a stirring rod. The filtrate is refined using filter paper and concentrated using a rotary evaporator to produce a condensed extract.

Characterization of specific parameters

Organoleptic examination

The organoleptic analysis entails observation of the shape, taste, color, and scent of the extract.[7]

Microscopic test

Leaves powder is placed on a slide that has been plopped with chlorohydrate and then heated. The object was examined using a microscope to identify fragments in cells, cell contents, or plant tissue of black betle leaves simplicia powder.[1,8]

Determination of water-soluble extract content

5 g of crude powder is weighed carefully. Put the powder in a sealed flask, add 100 mL of chloroform saturated water, shake for the first 6 hours, and omit it for 18 hours. The mixture was filtered, and 20 mL of the filtrate was dissipated to aridity in a shoal flat bottom dish which had been heated at 105 ° C and leveled, heated at 105 ° C until steady weight. Calculate the content as a percentage of water-soluble extract.[9]

Determination of ethanol-soluble extract content

5 g of powder is weighed carefully. Put the powder in a sealed flask, add 100 mL of ethanol, regularly shake for the first 6 hours, and leave for 18 hours. The mixture is filtered, and 20 mL of the filtrate is dissipated to aridity in a shoal, flat-bottomed dish that has been heated at 105 °C and leveled, and the remaining filtrate heated at 105 °C to fixed weight. Levels were calculated in the percentage of the ethanol-soluble extract.[9]

Identification of secondary metabolites

Flavonoid: The extract was dissolved in ethanol, then added magnesium powder and condensed HCl. If a yellowish-orange to red color has risen, then it is definite for containing flavonoids[9].

Polyphenol: The extract was dissolved in ethanol, then 1% FeCl3 was added. If green, red, purple, dark blue, blackish-blue, or greenish-black are set, it betokens the presence of phenolic compounds.[10]

Tannin: The extract was dissolved in ethanol, then 10% NaCl and 1% gelatin were added. If a white precipitate is formed, it indicates the presence of tannins.[11]

Saponin: The extract was dissolved in ethanol, then added with distilled water, and next heated. If foam forms, it indicates the presence of saponins.[8]

Steroid: The extract was dissolved in ethanol, then added with ten drops of anhydrous acetic acid and three drops of condensed sulfuric acid. If it forms green color, this indicates steroids; if it includes brownish color, it shows triterpenoids.[8]

Alkaloid: The extract was dissolved in ethanol, then added with chloroform, ammonia, and condensed sulfuric acid, then homogenized. If it shows the presence of alkaloid compounds, the top layer is taken. It is indicated by the formation of white deposits when added with Mayer reagent, and a red-orange precipitate is formed when dragendroff reagent is added.[8]

Standardization of non-specific parameters

Determination of specific gravity

pycnometer scaled and dried. The pycnometer is calibrated by arranging the weight of the pycnometer and the weight of water that has just been boiled at 25°C. The temperature of the liquid extract is set to roughly 20°C, and next put into a pycnometer. The filled pycnometer is adjusted to 25°C, sheds excess liquid extract, and weighs it. The weight of the pycnometer that has been loaded is subtracted from the weight of the empty pycnometer. The specific gravity of the liquid extract is the result attained by dividing the weight of the extract by the weight of water in a pycnometer at 25 ° C.[7]

Determination of total ash content

2-3 g of the fine material powder is precisely weighed and put into a silicate pot, which has been incandescent and leveled, gradually incandescent until the charcoal runs out, cooled, and weighed. The total ash content is calculated against the weight of the test material, denoted as a percentage.[7,9]

Determination of acid-insoluble ash content

ash (collected from the Determination of total ash content) is boiled with 25 mL HCl for 5 minutes. The part that is not dissolved in the acid is gathered, sifted through an ash-free filter paper, doused with hot water, and incandescent in a pot until the weight remains. The ash content which is not dissolved in acid is calculated against the weight of the test material, denoted as a percentage.

Determination of drying shrinkage

1-2 g of simplicia or extract is weighed carefully in a shallow weigh bottle with a lid that has been previously heated to a planned temperature and leveled. The material is flattened in the weigh bottle by shaking the bottle, making a layer about 5 to 10 mm thick. Bottles are put in the drying chamber, opened the lid, and dried at a planned temperature to a fixed weight. Before each drying, the vial remains closed and cooled in a desiccator at room temperature.[9,12]

Heavy metal contamination

Heavy metal contamination was estimated using an atomic absorption spectrophotometer at a wavelength of 217 nm for estimating lead (Pb) levels and a wavelength of 288.8 nm for assessing cadmium (Cd) levels.

Microbiological contamination

Testing for bacterial contamination (ALT) and mold (AKK) is one of the tests for the pureness of an extract. The extract is weighed as much as 1 gram, then dissolved in 10 mL of sterile water in a sterile container to obtain a sample suspension with a 10-1 dilution. Next, 9 mL of sterile water was arranged into three sterile containers for further dilution. 1 mL of the 10-1 dilution is put into a sterile container containing 9 mL of sterile water so that it shifts a suspension with a 10-2 dilution. The dilution was extended until 10-3 and 10-4 suspense were obtained. Then, 1 mL of each dilution was put into 6 Petri dishes in each microbial contamination test, and made two replications.[13]

Total plate numbers: 10-15 mL of Nutrient Agar (N.A.) medium are poured into a petri dish that has been loaded with suspension from each dilution. The petri dish was swayed and revolved in such a way that the suspension was homogeneous. Blanks were made bearing 1mL of diluent and Nutrient Agar (N.A.) medium. The solidified medium was incubated for 24-48 hours at 35-37 ° C in the incubator. The number of colonies that grew was then examined and calculated.[13]

Yeast mold number: 10-15 mL of Potato Dextrose Agar (PDA) medium are poured into Petri dishes loaded with suspension from each dilution. Then the petri dish was swayed and revolved in such a way that the suspension was homogeneous. Blanks were made bearing 1 mL of diluent and Potato Dextrose Agar (PDA) medium. The solidified medium was incubated for 48-72 hours at 25-27 °C in the incubator. The number of colonies that grew was then examined and calculated on the condition that if the number of colonies was in the range 25-250, then it was summed and divided by 2, multiplied by the highest dilution. In conditions, the provision number of contaminants does not surpass the maximum limit.[13]

RESULTS AND DISCUSSION

Standardization

Black betle leaves can be used as a medication because it comprises compounds that can treat several diseases. Because of these deductions, it is imperative to regulate the raw materials from simplicia, and black betel leaves extract. The scope of standardization is to assure the quality and safety standards of a medicinal plant.

The quality standard is defined by analyzing the specific and non-specific parameters of the black betle leaves. Specific parameters look at the compound's qualitative and quantitative chemical characteristics that are subject to pharmacological activity. Meanwhile, non-specific parameters designate the safety and stability of raw materials.

Analysis of specific parameters involves organoleptic examination, microscopic test, Determination of water-soluble and ethanol-soluble extract, and unds inquiry. Erstwhile, non-specific parameter analysis entails the Determination of Specified gravity, total ash content, acid insoluble ash content, Drying shrinkage, heavy metal contamination, and microbiological contamination.

Moreover, the standardization grade is ascertained from the references that indicate that the simplicia and black betel leaves extract suffice the predetermined specifications. Nevertheless, the approved standardization reference for the black betle leaves has not been recorded in the Indonesian Herbal Pharmacopoeia issued by the Ministry of Health. Hence, the reference for black betle yet uses the comprehensive specifications regarding sirih leaves.

Samples were simplicia and ethanol extract of black betle leaves. Simplicia originated from fresh leaves, which are wilted at 40°C. Parched simplicia is coerced into powder, and extracted by the maceration method using 70% ethanol for three days while stirring occasionally.

CHARACTERIZATION OF SPECIFIC PARAMETERS

Organoleptic examination

Organoleptic analysis results of black betel leave simplicia (Figure 1) explain that single leaves blades have a round to oval frame. Leaves base displaying a heart shape or slightly arched, slightly notched. The leaves edge is flat and slightly rolled. Leaves pointed is a tip to taper. The upper and lower leaves surfaces are rough and look dull. The lower leaves surface is paler than the upper. The leaves bones are bowed, on the upper surface, slightly engulfed while the lower surface protrudes. Translucent spots are apparent when exposed to sunlight. Round petiole. Blackish green leaves color to black. The leaves have a distinctive smell and an imperceptibly spicy taste. While organoleptic examination results on black betel leave ethanol extract showed that the extract had a thick density; blackish green; It has a mild distinctive smell and a bitter, cherish taste, and spicy flavor (Table 1).

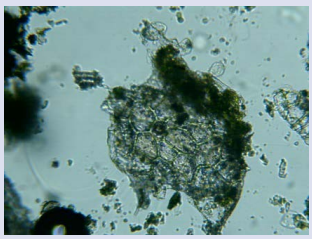
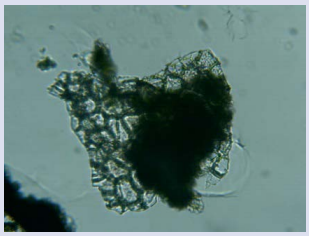
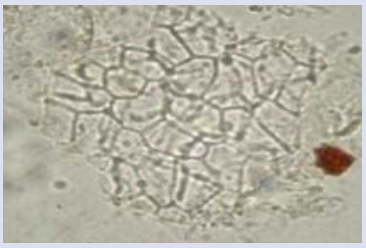
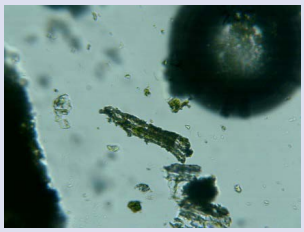
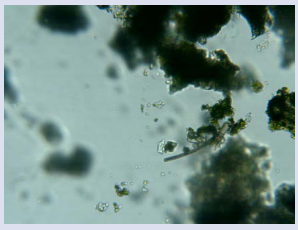
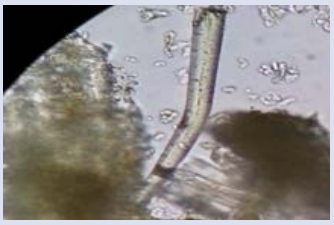
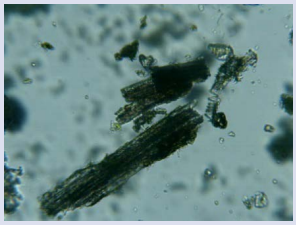
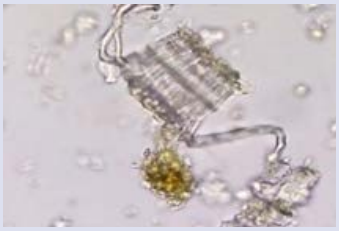
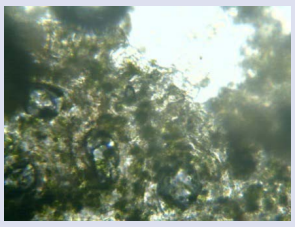
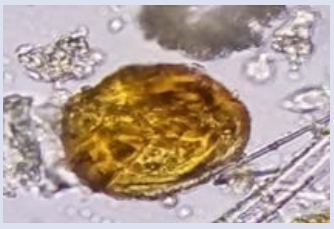
Microscopic test: Microscopic observation was conducted on black betel leaves simplicia powder. This test intends to determine the identifying fragments enclosed in black betle leaves to prevent simplicia falsification. The microscopic test results of identifying components on simplicia powders appeared, including the lower epidermis with idioblasts in the form of oil cells, upper epidermis, sclerenchyma, covering hairs, accent bundles with ladder-type thickening, and idioblasts in the form of oil cells (Figure 2).

Figure 1: *Piper betle var. nigra* (Simplicia).

Table 1: Organoleptic examination of Black betle extract.

Parameter	Result
Consistency	Thick
Colour	Brownish-green or blackish-green
Odor	A mild peculiar odor
Taste	Bitter, cherish taste, slightly spicy taste

Table 2: Microscopic comparison of black betel leaves simplicia with green betel leaves simplicial.

No.	Black betel leaves	Green betel leaves
1	 Lower epidermis with idioblasts in the form of oil cells	 Lower epidermis with idioblasts in the form of oil cells [4]
2	 Upper epidermis	 Upper epidermis [4]
3	 Sclerenchyma	 Sclerenchyma [4]
4	 Covering hair	 Covering hair [4]
5	 transport bundles with ladder-type thickening	 transport bundles with ladder-type thickening [4]
6	 Idioblasts in the form of black betel leave oil cells	 Idioblasts in the form of green betel leave oil cells [4]

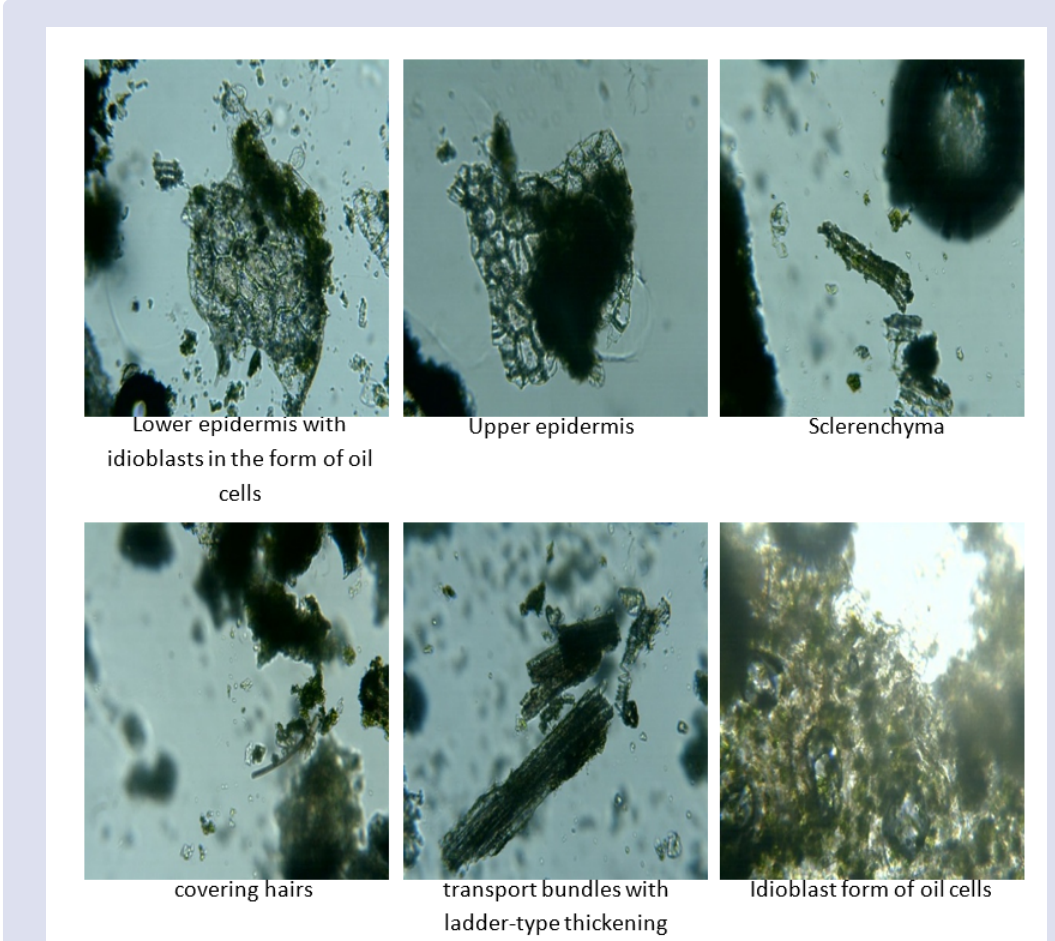


Figure 2: Microscopic assay on black betel leaves simplicial.

A comparison was made between the microscopic test results of black betle leaves, and the green betle leaves identification fragments from Indonesian Herbal Pharmacopoeia. After the results were collated, it was noticed that the identifier fragments found in green betle leaves have similarities with the test results fragments on black betle leaves (Table 2).

Content of dissolved compounds

Determination of compounds dissolved in water and ethanol predicts the portion of existing polar compounds (water-soluble) and semi-polar and non-polar (ethanol-soluble) in a sample.

Based on Table 3, the results of water-soluble content in simplicia were 2.77%, while the extract was 12.45%. The ethanol-soluble content in simplicia was 1.38%, while in the extract was 19.1%.

These results mean that the content in black betel leaves simplicia is more soluble in water than soluble in ethanol. Meanwhile, the extracted content in the ethanol extract exhibits a more significant percentage of substance that dissolves in ethanol than dissolves in water; this is because employing an ethanol extract so that the compounds comprised in the extract are semi-polar and non-polar.

Secondary metabolites analysis

Secondary metabolites analysis endeavors to render an initial picture of the compound composition in a sample. The black betel leaves ethanol extract was examined for its compound group composition. The analysis results manifested that there were secondary metabolites in the black betel leaf samples, including flavonoids, polyphenols, tannins, saponins, steroids, and alkaloids (Table 4).

Table 3: Level of dissolved compounds.

Solvent	Level (%) ± SD	
	Simplicia	Extract
Water	2, 77 % ± 0,04	12, 45% ± 0,05
Ethanol	1, 38 % ± 0,15	19, 1% ± 0,03

Table 4: Phytochemical compound assay.

Compound group	Result (Colour)
Flavonoid	+ (Yellow)
Polyphenol	+ (blackish blue)
Tanin	+ (colloid deposits)
Saponin	+ (formed foam)
Steroid	+ (bluish-green)
Alkaloid	+ (yellow precipitate)

Table 5: Non-specific parameter test results.

Parameter	Result ± S.D.	
	Simplicia	Extract
Water-soluble specific gravity	-	0,96 ± 0,03 g/mL
Ethanol soluble specific gravity	-	1,01 ± 0,00 g/mL
Total ash content	12,1 ± 1,02%	7,43 ± 0,81%
Acid insoluble ash content	4,45 ± 0,5%	1,57 ± 0,15%
Shrink drying	14,5 ± 0,68%	15,65 ± 0,60%

Non-specific parameters

Specific gravity

Specific gravity is the ratio of substance density to water density, reckoned by mass per volume unit. Specific gravity represents a role in determining liquid matters, testing the identity and purity of medicinal compounds - especially in liquid form - and determining matter solubility. The results obtained were water-soluble of specific gravity of 0.96 and ethanol soluble of 1.01 (Table 5).

Total ash content

Total ash content is a parameter to estimate mineral and inorganic residues that linger after the material is incinerated to ashes. The leftover residue is physiological ash descended from plant tissue and non-physiological ash, a residue from extraneous matter such as sand or soil attached to the plant surface. The results showed that the total ash content in the simplicia was 12.1%, and the ethanol extract was 7.43% (Table 5). This indicates that the simplicia contains minerals and inorganic objects much higher than the ethanol extract.

Acid insoluble ash content

Acid insoluble ash content is a residual parameter obtained after boiling total ash with dilute HCl and incinerating the left insoluble material. The residue from combustion shows the number of impurities from the soil or sand, which adhere to plant parts. The results showed that the percentage of acid-insoluble ash content in simplicia was 4.45% and ethanol extract was 1.57% (Table 5). These results indicate that the simplicia carries a higher amount of impurities than that encountered in the ethanol extract.

Drying shrinkage

Drying shrinkage aims to implement an overview of the compounds that are lost through the drying process. Drying shrinkage is residual element measurement after the sample has been withered at 105°C to a steady weight, denoted as a percentage. From the test results, the Drying shrinkage percentage in simplicia was 14.5%, and ethanol extract was 15.65% (Table 5). These results indicate that other ingredients volatilize due to heating besides water content, ostensibly essential oils, and ethanol.

Heavy metal contamination

Heavy metal contamination is one of the non-specific parameters in raw materials standardization for simplicia and extracts. The contaminants marked for black betle leaves were lead (Pb) and cadmium (Cd). Contamination levels were estimated utilizing the atomic absorption spectrophotometer analysis instrument.

The test results recorded that lead contamination levels in the simplicia, and black betle leaves extract were 47.5 ppm and 1.2 ppm, respectively. Meanwhile, the analysis results for cadmium contamination levels in simplicia and black betel leaves extract were less than 0.2 ppm (Table 6).

If the test results are correlated with the BPOM Regulations on Quality Requirements for Traditional Medicines,[14] contamination level limits for lead (Pb) is ≤ ten ppm, and cadmium (Cd) is ≤ 0.3 ppm. Hence, contamination levels have sufficed the specifications except for lead contamination levels in black betel leaves simplicia, which exceed the predetermined standard limit.

Microbiological contamination

Analysis of bacterial and mold-yeast contamination ensures that the extract does not contain microbial contaminants that beat the planned border. Research pointed out that bacterial and mold-yeast contamination in the black betle leaves ethanol extract were 4.3x105 colonies/g and 8.3x105 colonies/g, sequentially (Table 7). Those figures do not match the guidelines if it is contrasted to the maximum numbers of microbial contamination according to the BPOM regarding Quality Requirements for Traditional Medicines. The maximum limit of bacterial contamination (ALT) is ≤105 colonies/g and for mold (AKK) is ≤103 colonies/g. This contamination may befall throughout sample processing into extracts, and during the extract's storage period, the air near the storage containers may spoil.

Table 6: **Heavy metal contamination.**

Parameter	Levels	
	Simplicia (ppm)	Extract (ppm)
Pb	47,5	1,2
Cd	< 0,2	< 0,2

Table 7: **Microbiological contamination.**

Dilution	Total Plate Numbers		Yeast and Mold Numbers	
	Replication 1	Replication 2	Replication 1	Replication 2
10-2	68	88	59	78
10-3	50	56	83	64
10-4	50	35	84	82
Report	4,3x105 colony/g		8,3x105 colony/g	

CONCLUSION

Specific parameters: black betle leaves shape (*piper betle var. nigra* folium) is round to oval, round petiole, blackish-green to black color, has a distinctive smell, and has a spicy taste. The black betle leaves ethanol extract has a thick texture, is blackish-green, has a distinct mild scent, and has a bitter and spicy flavor. Black betle leaves simplicia powder microscopic observation showed fragments in the lower epidermis with idioblasts in oil cells appeared upper epidermis, sclerenchyma, covering hair, ladder-type thickening bundles, and idioblasts in the form of oil cells. The water-soluble extract content in simplicia was 2.77%, and the ethanol-soluble extract content was 1.38%. Meanwhile, the water-soluble compound in the extract was 12.45%, and the ethanol-soluble extract content was 19.1%. The ethanol extract contains flavonoids, polyphenols, tannins, saponins, steroids, and alkaloids.

Non-specific parameters: specific gravity of the water-soluble extract was 0.96 g/ml. The ethanol-soluble was 1.01 g/ml, The total ash content in the simplicia was 12.1%, and the extract was 7.43%. Acid insoluble ash content in simplicia 4.45% and extracted 1.57%. Drying shrinkage was f14.5% for simplicia and 15.65% for extract. The total Pb and cd contamination in simplicia was 47.5 ppm and <0.2 ppm, while in the extract was 1.2 ppm and <0.2 ppm. Total bacterial contamination was 4.3x105 colonies/g, and total mold-yeast contamination was 8.3x105 colonies/g.

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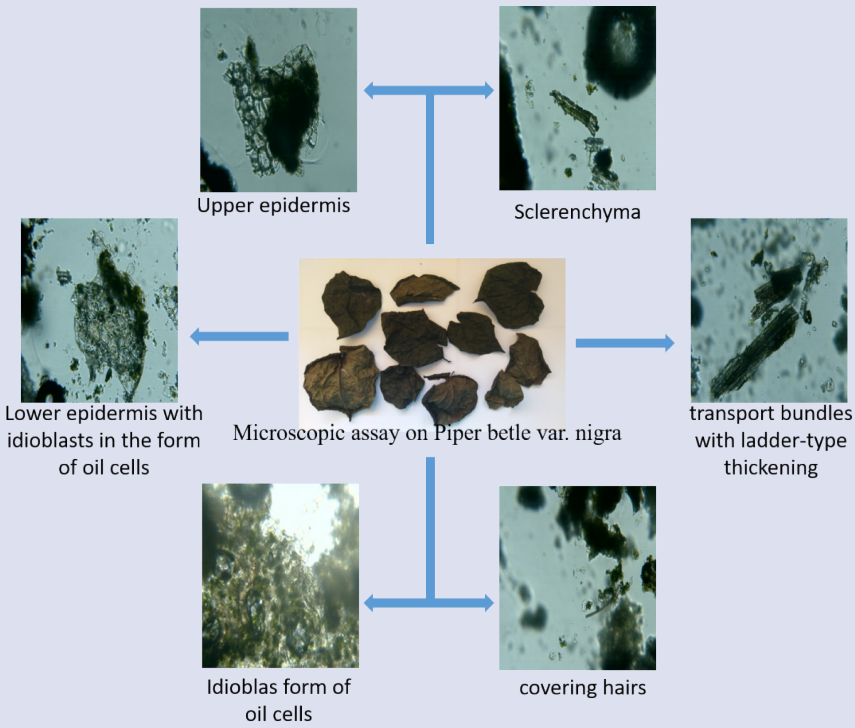
GRAPHICAL ABSTRACT



SUMMARY

The results show that the black betle leaves ethanol extract has a thick texture, is blackish-green, has a distinct mild scent, and has a bitter and spicy flavor.

Specific parameters; black betle leaves shape (piper betle var. nigra folium) is round to oval, round petiole, blackish-green to black color, has a distinctive smell, and has a spicy taste.

The ethanol extract contains flavonoids, polyphenols, tannins, saponins, steroids, and alkaloids.

The total Pb and cd contamination in simplicia was 47.5 ppm and <0.2 ppm, while in the extract was 1.2 ppm and <0.2 ppm.

Total bacterial contamination was 4.3x105 colonies/g, and total mold-yeast contamination was 8.3x105 colonies/g.

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