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Antioxidant Activity of Red Betel Leaves Extract (*Piper crocatum* Ruiz & Pav.) By Difference Concentration of Solvents.

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ABSTRACT

Analysis of antioxidant activity (IC_{50}), total phenol, total flavonoids and compounds performed on dry powder extracts of red betel leaves (*Piper crocatum* Ruiz & Pav.). Extraction using maceration method by ethanol concentration of 50 %, 70 % and 90 %. Extract with the highest antioxidant activity, analyzed by GCMS. The results showed levels of total flavonoid extract of ethanol (50 %, 70 % and 90 %), respectively for 159.32 ± 4.71 (mg QE/g), 172.50 ± 4.37 (mg QE/g) and 197.95 ± 12.51 (mg QE/g). Levels of total phenol, respectively for 148.70 ± 4.21 (mg GAE/g), 165.77 ± 4.55 (mg GAE/g) and 182.68 ± 4.39 (mg GAE/g) and antioxidants activity (IC_{50}), respectively for 136.84±3.16 ppm, 127.35±2.25 ppm and 82.71±2.35 ppm. Phytochemical screening of ethanol extract positive for flavonoids, polyphenols, tannins, alkaloids, and terpenoids but negative for steroids. There are 58 compounds were detected by GCMS, but only eight dominant compounds based on the chromatogram peak and 13 compounds with ≥ 90 % was observed similarity of m/z.

Keywords: red betel, total phenol, total flavonoids, antioxidant activity/IC_{50}, phytochemical.

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INTRODUCTION

Red betel (*Piper crocatum* Ruiz & Pav.) is plant in Indonesia which is widely used as a tradisional drug, now [2]. The chemical contents of red betel leaves 96 % methanol extract are flavonoids, saponins, triterpenoids, and tannins. Flavonoids in red betel leaves form flavonon, isoflavones, auron, catechins, anthocyanidins and chalchones [16].

The compounds in red betel obtained by extracting it leaves. Extraction is the separation between the two phases of the immisible analyte distribution [22]. Maceration extraction most commonly used, it is the separation of vegetable material with a solvent followed by shaking at room temperature. Components of the 70% ethanol extract of red betel leaves are fatty acids, terpenoids, flavonoids, steroids, alkaloids, pyrimidine, essential oils, polyphenols and vitamin E [1]. Methanol extract fractionation of red betel leaves by column chromatography with eluent hexane:ethyl acetate as SGP (Step Gradient Polarity) and followed with ethyl acetate:methanol. The identification results are Lignan and β–sitosterol compounds. Phytochemical screening fraction of ethyl acetate and n-hexane in red betel leaves contain terpenoids and steroids. While butanol fraction containing phenolic compounds, flavonoids, terpenoids and steroid [9].

Antioxidants are compounds that can inhibit the oxidation process under the influence of atmospheric oxygen or oxygen species reactive [20] or inhibit the initiation or propagation of oxidative chain reaction [13]. The main function of antioxidants are to reduce the occurrence of oxidation process [25], as a compound for the body health and prevent damage from the free radical [7] and prevent/repair the damage to the body cells [11].

The measurement of antioxidant activity using DPPH method. This method is simple, easy to do, requires little sample and need short time in the measurement of antioxidant activity [21].

The antioxidant activity (IC_{50}) assay the extract of red betel leaves (*Piper crocatum* Ruiz & Pav.) with DPPH (2,2-diphenyl - 1 - pikryl - hidraziy) are on the reactivity of samples tested with a stable radical. Maceration extraction using 50% ethanol, 70% ethanol and 90 % ethanol is expected to provide the high level antioxidants activity of red betel leaves extract.

MATERIAL AND METHODS

Chemicals:

Dry powder of red betel leaves, ethanol (50 % , 70 % , 90 %), distilled water, quercetin, DPPH (2,2 - diphenyl - 1 - Pikryl - hidrazyl) 0.2 M, gallic acid, Folinciocalteau reagent, Na_2CO_3, NaNO2 5 %, $AlCl_3$ 10 %, NaOH1 M, Mg powder, $FeCl_3$ 1 %, concentrated HCl 2 N, meyer reagents, dragendrof reagents, acetic anhydride, chloroform, H_2SO_4.

Tools:

Digitial scales, paper filter, spatula, measuring pipette 5 ml and 10 ml, vortex, erlenmeyer flask, beaker glass, measuring cups, rotary shacker, test tubes, vacuum filtration, vacuum rotary evaporator, visible spectrophotometer and GCMS machines.

Extraction:

This study use maceration method with ethanol (50%, 70 % and 90 %). The powder of red betel leaves macerated with ratio of 1: 8 (w/v) for 72 hours with 4 hours of agitation over the shacker every day and allowed stand for 24 hours. After it, filtered and replaced with the same new solvent and re-macerated. The entire filtrate has been obtained, mixed and vaporized by rotary evaporator. The pressure used is -700 hPa, with a speed of 40-45 rpm at temperature of 40 ^{0}C.

Phytochemical screening:

Flavonoid identification [10]:

5 gram sample is weighed and added to 50 ml of water and then heated for 5 minutes. After 5 minutes add a few drops of concentrated HCl and added a Mg powder. A positive result showed a deep red color, pink.

Polyphenols Identification [12]:

The sample is weighed 5 grams and added 50 ml water and then heated for 5 minutes. After 5 minutes, added $FeCl_3$ 1%. A positive result shows green, blue, purple, dark blue, dark green.

Tanin identification [8]:

1 ml sample was added FeCl 3 1 %. Then observed it changes, if positive shows green, blue, purple, dark blue or dark green.

Alkaloid identification [10]:

1 ml samples in each 3 test tube are added few drops of meyer reagents and or dragendrof. A positive result on meyer reagents precipitate formed white meyer. While the precipitation dragendrof reagent orange or red- brown Gragendrof.

Saponin identification [10]:

1 ml samples was added 2 ml of hot water and shaken strongly. A positive result indicates the formation of permanent froth for no less than 10 minutes as high as 1-10 cm. Then add 1 drop of concentrated HCl. A positive result indicates the foam will not be lost permanently.

Triterpenoid identification [4]:

1 ml sample was added 0.25 ml of chloroform and added 3 drops of acetic acid anhydride then added 1 drop of concentrated H_2SO_4.Then observed the changes, positive results of steroid compounds showed a bluish green color, if positive triterpenoids show red or orange brown.

Determination of Total phenol [14]:

0.4 ml of the extract samples were inserted into the flask 10 ml. Prepared form of distilled water, then 0.4 ml of Folin - Ciocalteu reagent and shaken. After five minutes, 4 ml of $Na_2CO_3$7 % mixed. Defined with distilled water until reaching volume of 10 ml and incubated for 90 min at 23 °C. Sample absorbance is read by a spectrophotometer with a λ 760 nm.

Determination of Total Flavonoid [3]:

Levels of total flavonoids were analyzed using spectrophotometry (modification) with standard $AlCl_3$ reagent. Using quercetin concentration of 20, 40, 60, 80 and 100 ppm. The procedure used is 1 ml sample of extract was added to 4 ml of distilled water and 0.3 ml of $NaNO_2$ 5 % into a test tube and homogenized. Then incubated for 5 minutes After 5 minutes, add 0.3 ml of $AlCl_3$ and incubated for 6 minutes and add 2 ml of NaOH1 M and distilled water until reaching a volume of 10 ml and homogenized. Sample absorbance value measured with a λ of 510 nm.

Antioxidant Activity Assay [24]:

The antioxidant activity assay (IC50) using DPPH (2,2- diphenyl -1- pikryl-hidrazyl) with visible spectrophotometer. Standard concentrations used 50, 100, 200, 250, 300 ppm. The procedure, enter 2 ml of

sample extract into a test tube and add 1 ml of 200 µM DPPH solution then homogenized and incubated for 30 minutes at temperature of 30 ^{0}C. Sample absorbance value measured at a λ of 517 nm.

Extract Analysis by GCMS:

The ethanol extract with the highest antioxidant activity (lower IC_{50}) was analyzed by GCMS to determine the compounds in the extract. GCMS used is HP 6890 Gas chromatogram, equipped with a capillary column Aglient models 1909 1 S-433HP-5 MS (5 % Phenyl Methyl siloxane), diameter of 250 um, 0.25 um thickness and capillary length of 30 m with a flow rate of 1 ml/min. Oven temperature program 80 ^{0}C/min - 325 ^{0}C/15 min. The carrier gas such as helium at a flow rate of 19.9 ml/min with a pressure of 9.32 psi. Injector temperature is 300 ^{0}C. Samples were injected with a split ratio of 20:1. The injection volume is 1 microliter. Gas chromatography-mass spectrophotometer data were obtained on a - HP 6890 Capillary Column Aglient 1909 1 S-433 HP-5 MS (5% Phenyl Methyl Siloxane) under the same temperature with a gas chromatograph. That compounds reads adjusted to the library in GCMS machines with data base Wiley 275.L

Statistics analysis:

The data obtained were analyzed by statistical tests using Anova followed by HSD test (Tukey) 5% (Minitab, version 16).

RESEARCH RESULTS

Maceration extraction:

Maceration extraction with 50%, 70 % and 90 % ethanol produce different yields. But the results of Anovatest followed by Tukey test provides no significant effect. Yield results in table 1.

Table 1: Yield Average in dry powder extracts of red betel leaves

Ethanol concentration	Yield average (%)*
50%	17,30 ± 0,85^a
70%	16,41± 0,62^a
90%	16,14± 0,59^a

*(Values are Expressed as mean ± Standard Deviation, n=5)

The above table shows the results of ethanol yield extract. Ethanol concentration not significant effect on the end yield. Anova statistical test with advanced test HSD (Tukey) level of 5 % shows the value of P > 0.05. The lower concentration of ethanol produces higher yield. The reason is the ability of ethanol to evaporate at differences concentration is different. The higher concentration of ethanol, accelerating the evaporation rate, and the lower concentration of ethanol has steam power is also getting slower. But at HSD test (Tukey 5 %) showed the ethanol concentration does not significantly influence in the end yield.

Analysis of Total Phenol, Total Flavonoids and Antioxidant Activity (IC_{50}):

Total phenol is calculated based on a standard curve of gallic acid expressed as mg GAE/g, while the total flavonoids calculated based on standard curve of quercetin expressed as mg QE/g and antioxidant activity (IC_{50}) expressed as ppm. The results are shown in Table 2.

Table 2: Total Phenol, Flavonoids and IC50 Average in Dry Powder Extract of Red Betel leaves

Ethanol Concentration	Total Phenol (mg GAE/g)*	Total Flavonoid (mg QE/g)*	IC_{50} (ppm)*
50%	148.70 ± 4.21^a	159.32± 4.71^b	136.84 ± 3.16^a
70%	165.77 ± 4.55^b	172.50± 4.37^b	127.35 ± 2.25^b
90%	182.68 ± 4.39^c	197.96± 12.51^a	82.71 ± 2.35^c

*(Values are Expressed as mean ± Standard Deviation, n=5)

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The above table shows the total phenol content of ethanol extract respectively was 148.70 ± 4.21, 165.77 ± 4.55 and 182.68 ± 4.39 mg GAE/g, while total flavonoid content of ethanol extract respectively was 159.32 ± 4.71, 172.50 ± 4.37 and 197.96 ± 12.51 mg QE/g. for antioxidant activity / IC_{50} respectively was 136.84 ± 3.16, 127.35 ± 2.25 and 82.71 ± 2.35 ppm.

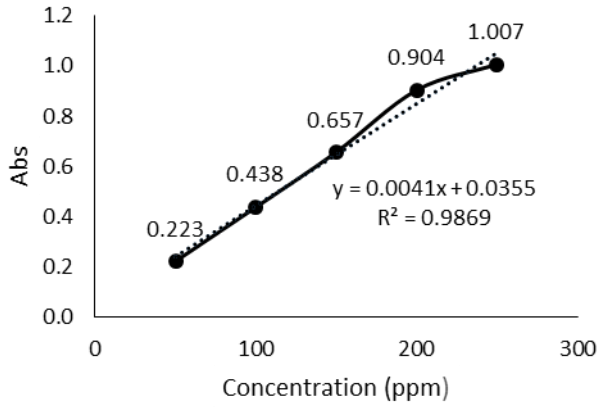

Fig .1: Calibration curve of gallic acid standard

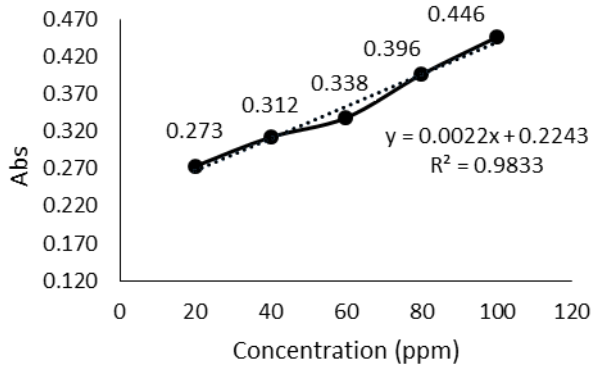

Fig .2: Calibration curve of quercetin standard

Phytochemicals Screening:

Phytochemical screening performed on extracts of ethanol. This is a qualitative analysis and the resulting data is qualitative data. Phytochemical screening done on flavonoids, polyphenols, saponins, tannins, alkaloids and terpenoids (triterpenoids and steroids). The results are shown in Table 3.

Table 3: Phytochemicals Screening in dry powder extracts of red betel leaves

Compounds Test	The Sample Extract on Ethanol (Concentration)		
	50 %	70 %	90 %
Flavonoids	++	++	++
Polyphenols	++	++	++
Saponin	++	++	++
Tanin	++	++	++
Alkaloids			
Meyer*Reagent*	++	++	++
Dragendrof*Reagent*	+	+	+
Terpenoids			
Triterpenoids	++	++	++
Steroids	-	-	-

Description: sign + (Slightly), ++ (Many) and - (none)

Compounds Analysis By GCMS:

The results of extract analysis with the highest antioxidant activity, analyzed by GCMS. The highest antioxidant activity in 90% ethanol extract with antioxidant activity / IC_{50} of 82.71 ppm (a powerful antioxidant). There are 58 compounds were identified from the extract 90% of ethanol, but only eight dominant compound (on the chromatogram Figure 3) with the high area and % relative. The compound can be seen in Table 4. In addition to the dominant compounds also contained 13 compounds of the similarity ≥ 90 % (m/z) of the compound can be seen in Table 5.

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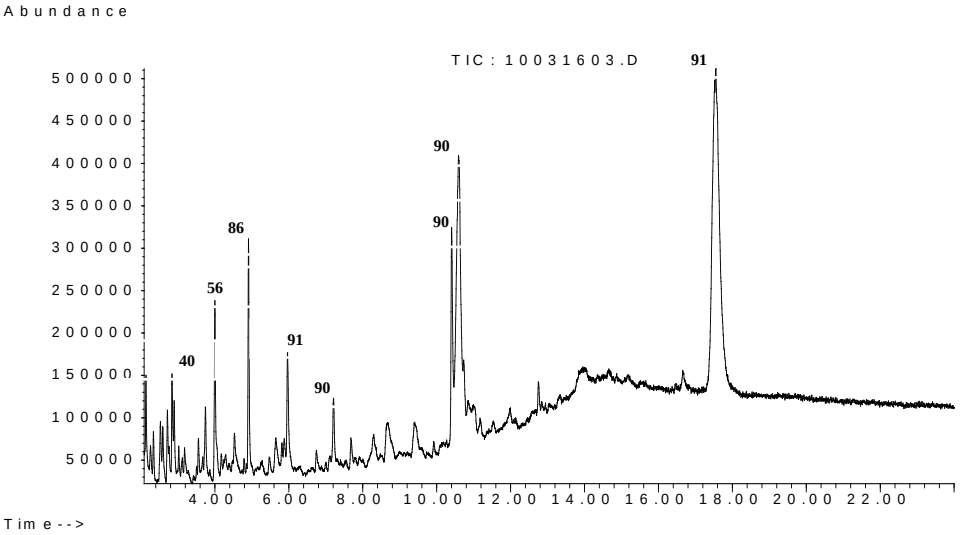

Fig 3. Chromatogram 8 Dominant compounds

Table 4: The Dominant Compound in 90 % dry powder extracts of red betel leaves

Retention Time	Name of chemical compound	chemical formula	Area	% Relative
2,85	1,2,3-Propanetriol	$C_3H_8O_3$	4508	1.21
4.00	2,4(1H,3H)-Pyrimidinedione	$C_4H_4N_2O_2$	17426	2.71
4,91	4H-Pyran-4-one	$C_5H_4O_2$	30617	3.14
5,97	2-Furancarboxaldehyde	$C_5H_4O_2$	10771	2.40
7,21	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	23329	1.59
10,41	2,6-Dimethyl-3-(methoxymethyl)-p-benzoquinone	$C_{10}H_{12}O_3$	64343	3.84
10,60	alpha.-D-Galactopyranoside	$C_6H_{12}O_6$	52266	14.60
17,55	1,2-Benzenedicarboxylic acid	$C_8H_6O_4$	105069	24.77

Table 5: The Compounds With ≥ 90 %Similarity.

Name of Compound	Group of compound	m/z
2-Furancarboxaldehyde	Heterocyclic	91
k2-Methoxy-4-Vynil phenol	Heterocyclic	90
Phenol	Phenolate	90
2,6-Dimethyl-3-(methoxymethyl)-p-benzoquinone	Quinon	90
Alpha.D-Galactopyranoside	Heterocyclic	90
Cis-9-Hexadecanal	Chain unsaturated aldehydes	95
Oleic Acid	Ftty acid	90
Oleic Acid	Fatty acid	91
Thiosulfuric Acid	Acid	95
Cyclohexene	Alkena cyclic	90
Z-11-Tetradecen-1-ol-trifluoroacetat	Ester	95
1,2-Benzenedicarboxylic Acid	Aromatic carboxylic	91

DISSCUSSION

Levels of total flavonoid and total phenol dry powder ethanol extracts of red betel leaves 50% < 70 % < 90 %. This may be caused by the drying process during the making of red betel leaves powder. The content of flavonoid and phenol compounds can be influenced by the presence of temperature and radiation. The decrease in temperature and radiation, affect the increase in total flavonoids [23].

Differences concentration of ethanol affect the increase in total phenols and flavonoids. High phenol compounds cause in high antioxidant activity. Red betel leaves extract containing tannin compounds that have antioxidant properties and triterpenoids which have OH chain. The compounds with OH chain has a role in

providing the electron lone pairs on DPPH, causing instability of DPPH. Differences level of polar solvent determine the chemical structure of phenolic compounds extracted. Analysis of total phenol highly dependent on their chemical structure. Phenol compounds having a hydroxyl functional group (OH) a lot or in the free conditon (aglycone) produce high of total phenol [6].

Ethanol has hydroxyl groups that can bind with intramolecular hydrogen bonds on the phenolic hydroxyl group of compounds that cause an increase in the solubility of phenolic compounds in ethanol. Ethanol as an effective solvent to extract the phenolic compounds because of the low level of polarity [26]. Ethanol more attractive polar compounds present in red betel. Ethanol also dissolving compounds ranging from less polar to polar. Higher ethanol concentrations can attract more compounds. It causes the cell walls in plants that are less easily degraded and polar phenolic compounds easily get out of the plant cell. In addition semipolar organic solvents are more easily extract the phenolic compounds. Therefore the total phenolic content is highest at 90% ethanol extract.

The antioxidant activity/IC_{50} result showed extract 50% and 70% ethanol provide modest antioxidant properties, while the 90% ethanol extract provides strong antioxidant properties. Antioxidants are very strong if IC_{50} value of less than 50 ppm, is strong if IC_{50} values ranging from 50-100 ppm, is modest when IC_{50} ranging from 100-150 ppm, is weak when IC_{50} worth 150-200 ppm and very weak with IC_{50} over 200 ppm [18].

90% ethanol produce the highest antioxidant activity (low IC_{50}), followed by 70% ethanol and 50% ethanol. The lower of IC_{50}, also has higher antioxidant activity. The higher concentration of ethanol showed low IC_{50} values, because ethanol is more polar and more binding the compounds in red betel. Higher concentrations can attract more compounds, and therefore contributes to a role in antioxidant activity. Besides it, ethanol dissolves less polar compound up to polar. Antioxidant activity has decreased value is comparable to the decrease in the concentration of ethanol used.

The differences in the ability of antioxidants antioxidative compounds ethanol extract of red betel leaves against DPPH radical due to the differences in the ability to hidrogen atom transfer [19] to the radical DPPH forming yellow diphenylpikrylhidrasyn stable compound [5]. The activities capturing of free radicals DPPH influenced by polarity factors of the reaction medium, the chemical structure of radical catcher and the pH of the reaction mixture [24]. Molecules substituted hydroxyl group (OH) increasingly is more powerful in capturing free radicals of DPPH because of the ability to donate hydrogen atoms (H) are increasingly [17, 27]. Aglycone structure has higher antioxidant activity than the structure of glycosides [15].

CONCLUSION

The concentration of ethanol influence on the total phenols, total phenol content and antioxidant activity (**IC_{50}**). The higher concentration of ethanol, is better in dissolving polar and less polar compounds. Total flavonoids and total phenol affect the value of antioxidant activity. The higher value of total phenols and total flavonoids produce high antioxidant activity which is characterized by low **IC_{50}** values. Ethanol concentration of 90% provides optimum results in the extraction of red betel leaves. It extract showed there are eight dominant compounds with high area and % relative and 13 compounds with 90 % similarity.

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