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Active Compounds of Red betel (*Piper crocatum*) Extract for Safe Antioxidant as Cytotoxicity Test Revealed

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Abstract : The objective of this study was to explore the red betel active compounds promising as a safe antioxidant based on fibroblast cell toxicity test. **Methods**: Sample of red betel collected from Balai Materia Medica, Batu East Java. Red betel extracted with ethanol by percolation method. Phytochemical compounds were analyzed using Tin Layer Chromatography (CTLC), High Performance LiquidChromatography (HPLC) and Fourier Transform–Infra Red Spectroscopy (FT-IR). The scavenging activity of free radical was determined by 2,2-diphenyl 1-picrylhydrazyl (DPPH) method, and the cytotoxicity test was determined by exposed fibroblast cell to ethanol extract red betel for 72 hours. Viability test further assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay. **Results**: The main compounds of red betel extract are flavonoids and tannins. HPLC results showed epigenin is the main compound in red betel extract and the major functional group as follows C-O-C; C-NO2; C=O, CH; C=C; C-N; N-C-N, OH and NH. Red betel extract has 30,20 % antioxidant potency (IC_{50}). Red betel dose 400µg/ml is toxic to fibroblast cell. **Conclusion**: Red betel extract is safe source of antioxidant based on toxocity test.
Keywords: red betel extract, antioxidant, toxicity test.

Introduction

Development of natural antioxidant to replace commercially available synthetic antioxidant has been the subjects of many bio-pharmacology as well as medical researches due to the emerging common view about the possibility of side effects arise from the application of synthetic antioxidants[1,2]. Antioxidant can be described as chemical compounds that capable of prohibit metabolic oxidation process by reacting itself with reactively free radicals to form non-reactive and stable molecule[3]. The search of such natural resources antioxidant have been extended to the important of systematically identifying potential candidates for natural antioxidant by means of effectively reliable methods including cytotoxicity test[4]. The most feasible target for such immensely growing research area is any kind of medicinal plants family member. In addition, the promising candidates for natural antioxidant should be easily found and grown.

Many medicinal plants family member from Indonesia known as potential candidate for natural antioxidant are rambutan (*Nephhelium lappaceum* sp)[5], mengkudu (Moringa citrifolia)[6] and Piperbetel(*Piper betel*)[7]**.** Red betel is commonly found in tropical area and its leaves are conventionally used by the surrounding natives as medical treatment to overcome several diseases such as diabetes, inflammation[8], and wound healing[9]. Beside its unique smell results from its essensial content, red betel leaves also possess several functional active

compounds including flavonoid, alcaloid, saponin, and tannin[10]. Many considerable investigations have reported that active compounds exhibit hypoglycemic activity and antioxidant activity[2] whereas comparable to which shown by Butileted Hidroxi Tuluene (BHT). However, the potentials use of redbetel extract as natural source of antioxidant must be completed by the understanding of its side effects.

Motivated by those facts above, we have carried out antioxidant activity test of red betel specimens to shed light on the safety of its potential application as natural source of antioxidant without any further negative side effects. We have employed 1,1-diphenyl-2-picrylhidrazyl (DPPH) in so called cytotoxicity test for the investigation. The specimens' resistance on DPPH will provide the insight of their capability to overcome free-radicals without necessarily distinguish the radical's type.

Experimental Method

Red betel extract preparation

The leaves of Red betel have been provided by BalaiMateria Medica, Batu district, East Java. The leaves were then let dry at ambient air condition and were thoroughly ground by mortar and pestle to obtain fine powder of the leaves of Red betel. Afterwards, the water content of the fine powder was determined by using AOAC method. The one gram of powder was weighed precisely prior to annealing process in the furnace at 110^0C for 30 minutes, and then it was weighed. Important data are the mass of sample before and after annealing process. The subsequent annealing processes were done until the mass of the sample was constant/no significant different between before and after annealing process.

One hundred gram of powder were added into Ethanol 70% with gently stirred for 48 hours. The filtrate was obtained by filtration technique and to ensure the dryness, the filtrate was subjected to the rotary evaporator at 48^0C. 0.5 gram filtrate dissolved aqua bidest until 5 ml and then centrifuged 10000 rpm 10 menit. Supernatant was used stock solution.

Secondary Metabolic Characterization of Red betel extract

Color Test

Flavonoid: 1 mL of specimen + 1 gram of Mg powder were dissolved in HCl PA. The yellow color appearance is indicator that extract is containing flavonoid.Terpenoid: 1 mL of specimen + 1 mL of glassy-CH_3COOH were dissolved into H_2SO_4. The appearance of red color is indicator that the specimen is containing terpenoid whereas blue color is indicating that the specimen contains steroid.Tannin: 1 mL of extract + 10 drops of $FeCl_3$ 1% and the appearance of blue color indicates the existence of tannin and phenolic within the Red betel extract.Saponin: 1 mL of extract was added into test-tube and was then vertically shaked, gently drop HCl into this solution will generate bubbles if the extract contains saponin.

Alcaloid: 1 mL of extract was dissolved into Dragendorff solvent, precipitation happens if the extract contains alkaloid.

Thin Film Chromatography (TLC)

Identification of compounds within the Red betel extract were carried out by employing thin film chromatography technique. A small spot of Red betel extract was applied onto a 2x10 cm plate containing thin layer of silica gel. After a certain moment, it was then submitted into eluent (appropriate solvent system).

The appropriate solvent for alkaloid is made as follows. Etil acetat:methanol:aquadest = 100:16.5:13.5. The plate was then sprayed with Dragendorff reactant and will exhibits brown color at visible light range, yellow for UV 254 nm wavelength, and ligth green under UV 366 nm radiation.

The appropriate solvent for saponin is mixture of hexana:acetone (4:1). Sprayed using acetil-acetat $SbCl_3$ will indicating the existence of saponin for pink up to purple colors appearances under visible light and yellow under radiation of UV 366nm.

The appropriate solvent for flavonoid is mixture of butane: acetic acid: water (3:1:1). The existence of flavonoid that isthe light yellow color appearance under visible light and blue color under radiation of UV 366nm after sprayed amonia.

Fourier Transforme–Infra Red Spectroscopy (FT-IR)

The FT-IR characterization has been carried out in FT-IR Spectroscopy machine Shimadzu FT-IR8400.

DPPH test

Modified method from (Goldfarb, 1993) and (Brand-Williams et al., 1995) has been used to evaluate the DPPH activity of fruits peel on the free radicals[11,12]. The reactant for DPPH was prepared by dissolving DPPH into 40 µg/mL methanol (PA), keeping it fresh at low temperature and avoid the light exposure. The 1.5 mL solution test/standard was dissolved into 3 mL DPPH solution following by the measurement of its absorption under 516 nm wavelength radiations. The standard solution used was made of 1.5 mL methanol (PA) reacted with 3 mL DPPH. The percentage of free-radicals activity shown by DPPH (%) was calculated by using formula (A standard-A sample): (AstandartX100%). The percentage of activity (%) were analyzed and calculated by using the EC_{50} number value through probit analysis. The EC_{50} is concentration that capable of resisting 50% DPPH.

In-vitro toxicity

Cell culture medium used in this characterization is Dulbeco's Modified Eagle Medium (DMEM). The fibroblast cell was obtained from Mesenchyme Stem Cell, isolated and collected based on method by Helgason et al (2005)[13]. The tibia bone and femur of adult rat was washed by using PBS plus Fetal Calf Serum (FCS) 0.1% and gentamycin 50µg/ml. After washing step, it was subjected for centrifugal process using PBS and DMEM for 3 and 2 times repetition. The pellet was re-suspended in culture medium DMEM. The culture growth was done on petri dish with 35 mm diameter coated with gelatin 0.1%. Cell (10^5 cell/mL) were grown in 2 mL of DMEM inside CO_2 5% incubator at 37^0 for 24 hours. Afterwards, medium was replaced to make unattached cells. The attached cells were identified as fibroblast cells. The medium was then replaced with the new one containing red betel extract with variation of concentration is 6.25µg/mL, 12.5µg/mL, 25µg/mL, 50µg/mL, 100µg/mL, 200µg/mL and 400µg/mL, respectively and then incubation for 72 hours. Positive control containing cells in culture medium was regarded as 100% living cells and negative control containing culture medium only was regarded as 0% living cells. At the end of incubation process, medium and the extract were removed whilst the cells were washed by using PBS 5 mg/mL. The 100 µL culture medium and 10 µL MTT (Sigma) 5 mg/mL were added into every cell. The cells were then submitted for incubation process for 4 hours inside an incubator. The cell metabolic activities were read by using ELISA reader under 570 nm wavelength radiations following by viability calculation using formula below

$$\% \text{ Living Cell} = \left[\frac{\text{Treatment} + \text{Medium}}{\text{Cell} + \text{Medium}}\right] \times 100\%$$

% Living cell = percentage of the amount of living cell after measurement
Treatment = optical density of formazan for every sample
Medium = optical density of formazan for control medium
Cell = optical density of formazan for control cell

The cell viability were obtained by analyzing the percentage of living cell using one-way Anova statistic method with significant standard 5% followed by Tukey High Significant Different analysis method.

Result and Discussion

1. Scunder Metabolic Chararterization of Red betel

Phytochemical test

Phytochemical test carried out for red betel extract shows that the extract contained flavonoid and tannin, indicated by red and black color appearances(Table 1).

Table 1. Qualitative phytochemical test results

No	Group	Extract	
		wáter	Ethanol 70%
1	Flavonoid	+	+
2	Tannin	-	+
3	Terpenoid	-	-
4	Saponin	+	-
5	Alcaloid	+	-

Thin Layer Chromatography (TLC) Test

The results from TLC test show that red betel extract possess flavonoid indicated by the appearance of yellow color under visible light wavelength radiation and blue color under UV 366 nm wavelength radiation at Rf scale 0.87 and 0.52, respectively. The tannin existency in the ethanolic red betel extract was found from the indication shown by black color from TLC test with Rf scale 0.92. The water based red betel extract also shows the existence of saponin and alkaloid.

Fourier Transform –Infra Red Spectroscopy (FT-IR)

FT-IR has been used to analyze the existence of functional groups in any tested compound based on the unique adsorption of those functional groups respect to certain wavelength. The FTIR pattern obtained from red betel extract is shown on figure 1.

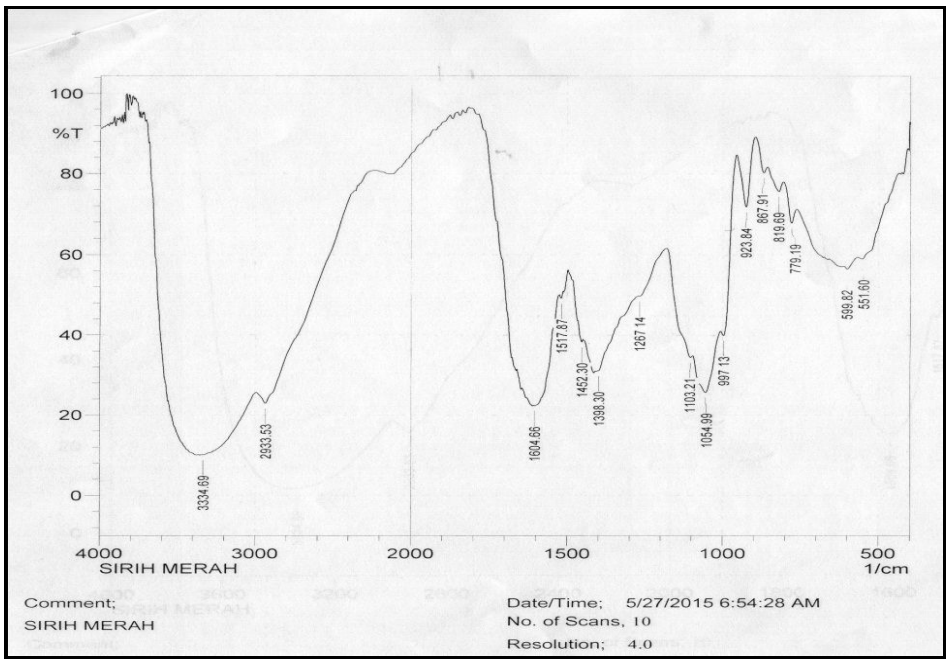

Figure 1. Peaks of functional groups in red betel extract shown by FT-IR spectroscopy results

We can conceive from Figure 1 that 9 out of 16 peaks shown by FTIR results belongs to the similar functional groups CH2 (551.6-1103.21cm-1).The peaks 1267.14-1517.87 belong to C-O-C, C-NO2, and C=O, while the peaks 1604.66 – 2933.53 belong to C=O, CH, C=C, C-N, N-C-N,and CO2. We can see functional groups OH and NH also appear at the vicinity of 3334.69. In details, we tabulated those functional groups respect to their peak position on Table 2.

Table 2. Functional groups consisted in red betel extract from FTIR characterization

No	Peak	Group	PredictorCompones
1	551.6-1103.21	CH2	Othercompones
10	1267.14	C-O-C	Eter, nitroaromatik,
11	1398.30	C-NO2	oksin, keton
12	1452.30	C=O	
13	1517.87		
14	1604.66	C=O; CH; C=C;	alkena, diimida, amina
15	2933.53	C-N; N-C-N; >CO2	
16	3334.69	OH; NH	Amina primer, skunder, amida,

DPPH test

Antioxidant activity of ethanolic extract red betel to capture free radicals determined by IC_{50} using DPPH method (Table.3). Ethanolic extract red betel have a great ability to capture free radicals compared another sovent. That were showed on 40.2 µg/ml red betel ethanolic extract has ability capture free radicals from DPPH as 50%

Tabel 3. Antioxidant activity using DPPH method

No	Sample fraction	IC_{50} (ppm)
1	Ascorcib Acid	5.34
2	Red betel ethanolic extract	40.20
3	Red betel water extract	78.35

High antioxidant capacity was due to flavonid compound which sucessfully extracted from red betel with ethanol[8]. We did not distinguished the type of flavonoids which caused activity of antioxidant in ethanolic axtract red betel. Flavonoids with free hydroxyl radicals have a function to capture radicals. The avaibility of free hydroxy would improve antioxidant capacity[14]. According to Okuda, T. (1992) natural antioxidant are available in plants and of these compunds are scattered in various parts of the plants[7]. Natural antioxidant serves as a reducing agent, supresor singlet oxygen, capture free radicals and as a metal chelating. Those antioxidants include phenol derivative compounds such as flavonoids, hydroxynat derivatives compounds, coumarin and tocopherols.

Toxicity Test

Mean optical density of formazan, cells' viability percentage after exposed to red betel ethanolic extractsuch as Table 2.

Table4. Mean Optical Density of Formazan, Deviation Standard, Cells' Viability Percentage after Exposed To red betel ethanolic extract

Treatment	sum	Mean of DO	SD	% viability
Control medium	6	0.001	0.00	0.00
Control cell	6	0.418	1.72	100.00
P1(6.25µg/mL)	6	0.410	1.95	97.43
P2(12.5µg/mL)	6	0.361	5.41	86.33
P3(25.0µg/mL)	6	0.334	10.32	82.86
P4 (50.0µg/mL)	6	0.361	26.52	86.33
P5 (100.0µg/mL)	6	0.327	2.03	78.19
P6 (200.0µg/mL)	6	0.327	2.03	64.57
P7 (400.0µg/mL)	6	0.212	6.92	50.60

The cell viability of positive control 100%, while the negative control 0%, following by samples group I are 97.43%, group II 86.33%, group III 82.86%, group IV 86.33%, and group V 78.19%, group VI 64.57%, and group VII 50.6%. There is significant effect of variation of red betel extract concentration variation used. We can notice that the percentage of the amount of living cells for samples group I and II is more than 90%, while it is 67.24% for the samples group III.

There is no significant different between positive standard control and the cells viability under 6.5 µg/mL and 12.5-200 µg/mL dosage level exposure, receptively. But, there is interesting result to note that at level dose 400 µg/mL, there is almost only 55% cells can still survive. The graph of this cells viability as follow on Figure 1.

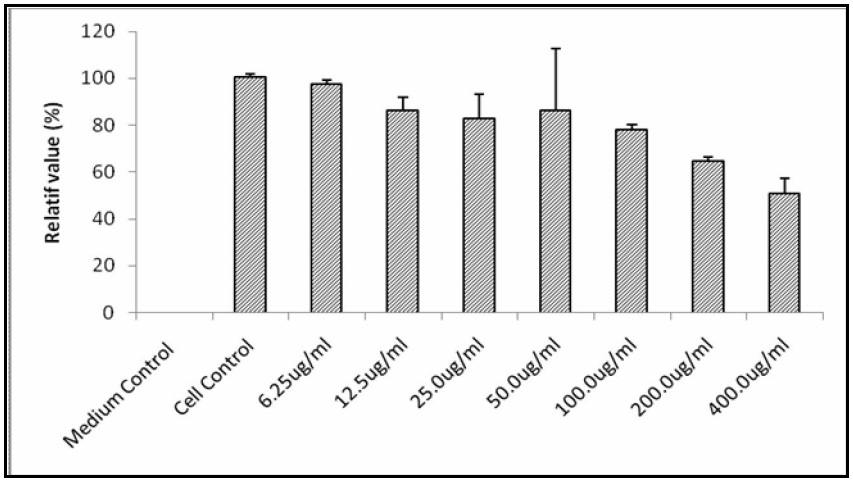

Figure 1.Fibroblast cells viability as the function of red betel extract dosage level

The average optical density of formazan (Table 2) decreasing as the concentration of red betel extract exposed to the fibroblast cells increase. The percentage of living cells, which is the percentage of optical density of mitochondrial dehydrogenase enzyme in fibroblast cells culture, also decrease.

The Red betel extract exposed to fibroblast cells is containing flavonoid which can act as antioxidant for any getting into the cells compounds. The free radicals compounds entering the cells have the capability of destroying cell membrane, as the consequence, the free radical taking oxygen from lipid cells membranes, the process goes on while the losing oxygen lipid cells membrane will capture oxygen from the nearest neighbour cells membrane increasing the rate of cells degradation result in cells death[7]. In addition, the free radicals can also deteriorate organel, particularly the mitochondria as the energy source inside the cells. If the energy source inside the cells dies, the cells itself will be death. Therefore, antioxidant playing important role to capture and neutralize the existence of free radicals inside the living cells[10].

Recall the conditions must be fullfilled for a substance to be used as antioxidant is that substance does not harmful morphologically as well as physiologically. The exposure of red betel extract on fibroblast cells is continuation of research to investigate whether the extract is safe or not to the cells physiology state condition. In this case, cells viability test using MTT assay can be done[3],[15]. The widely application of fibroblast cells culture for citotoxicity test in the medical as well as pharmaceutical area mainly because they provide advantages such as the durability of use in 50-70 times experiment, the high growth rate, cells integrity and its capability of multiplication in suspension. MTT assay is known as yellowish solute substance for assessing cell metabolic activity, based on the capability of living cells to reduce MTT salt including yellow tetrazolium salt[15]. The mechanism is a yellow tetrazolium salt to be reduced by activity of cells. Mitochondria metabolic activity in living cells plays an important role in producing the enzyme dehydrogenase. In the event of the death of the cell, the enzyme dehydrogenase will not be formed as a result formazan also not formed.

The level of toxicity in this work is based on CD_{50}, means that any substance can be acknowledge as toxic substance if the living cell percentage after exposure is less than 50%. In the case of red betel extract, it does not induce the death of fibroblast cells even at the level bigger than 50% indicate that is conveniently safe for use. This fact, will strengthen many of previous reported results that propose the potential use of red betel

extract for natural antioxidant in the term of safety.It is the existence of functional groups inside the red betel extract playing important role in capturing and neutralizing any dangeraous treath of free radicals in the living cells[3],[10]. Interesting result to note from this work is the increasing of red betel extract concentration exposure result in lowering the fibrobalst cells viability. It is probably contributed by other unidentified toxic cells coming into the cells as we can see from the flavonoid existence characterization, the identified flavonoid in the red betel extract still at the state of mixture, in other words, the present of flavonoid in red betel extract is also accompnied by other unidentified toxic substances. Therefore, it is strongly suggested to do further investigations toward better design and development of purification route to obtain the key substance responsible for the antioxidant activity of red betel extract. The results in this work can be useful for the base of obtaining the anti-inflammation agent from the red betel extract as well as en route the potential cure of the treath from dangerous harmful cancer cells. In general, this work suggest that red betel extract is promising candidate for safe natural antioxidant.

Conclusions

Red betelextract as a potential candidate and safe for natural antioxidant at the dosage level range between 6.25 – 200 mg/mL of normal cells (fibroblast cell). Important to note that at 400 mg/mL dosage level it caused almost 50% of those fibroblast cells dies.

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