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GC-MS composition of leaf extract of *Piper cf. arcuatum* blume and their antioxidant activity and toxicity studies

Candra Irawan, Foliatini and Hanafi

Abstract
In order to study the chemical characteristic and bioactivity of red betel (*Piper cf. arcuatum* Blume) leaf extracts, a simple and reliable GC-MS method was developed to identify the chemical components from the extract, followed by antioxidant activity assay using DPPH (1,1-Diphenyl-2-picrylhydrazyl) method and toxicity studiy using Brine Shrimp Lethality Test (BSLT) study. Purification step was carried by column chromatography, containing silica gel as stationary phase, and mixture of *n*-hexane, ethyl acetate and methanol as mobile phase. The DPPH assay showed that methanolic extract has the highest antioxidant activity, compared to *n*-hexane and ethyl acetate extract, and fraction B has the highest antioxidant activity, compared to fraction A and C. GC-MS analysis of fraction B revealed that the fraction was mainly composed of 6,7-dimethoxy-2,2-dimethyl-1-benzopyran. Fragmentation and the most probable biosynthetic route were also proposed in this study. Brine Shrimp Lethality Test (BSLT) study showed that methanolic extract has the highest toxicity with LC_{50} value of 16.15 ppm. The main compound in this extract was a derivative of benzopyran, which has high antioxidant capacity and self-defence capability against predator. The results demonstrated that GC-MS method was proven to be an excellent tool to identify chemical constituens in the red betel (*Piper cf. arcuatum* Blume) leaf extract.

Keywords: leaf extract of Piper cf. arcuatum Blume, fractional extraction, GC– MS, antioxidant activity, BSLT.

Introduction
Red betel (*Piper cf. arcuatum* Blume) is one of original plants from Indonesia which is widely used by people as a traditional medicine. Red betel leaf is a part of the plant that is empirically used by communities to cope with various diseases, including cancer [1-2]. Several studies reported that extract of red betel leaf has antioxidant activity [3], hepatoprotective activity [4], and antibacterial activity against *Streptococcus mutans* [5], methicillin-resistant Staphylococcus aureus, vancomycin-resistant Enterococcus [6], *Salmonella species, Escherichia coli, Klebsiella* and *Bacillus subtilis* [7]. The extract of red betel leaf was also proved as antiinflammatory [8], antidiabetic [9], antifertility [10], antidermatophytic [11], and anticariogenic agent [12]. The antibacterial activity of raw extract of red betel leaf provides the potential application of this extract as an antiseptic for handwashing [13]. Hartini *et al.* [14] showed immunomodulatory effect of the extract of red betel leaf (*Piper crocatum* Ruiz & Pav) on mouse liver and kidney. Though the bioactivity of the extract of red betel leaf has been reported in several studies, however scientific studies on the identification of chemical compounds contained in the red betel leaf (*Piper cf. arcuatum* Blume) have not yet been found.
The extraction process is a separation technique commonly performed on part of plants [15-16]. Extraction can be performed using *n*-hexane, ethyl acetate, and methanol and the selection of these solvents are based on the fact that different secondary metabolites has different solubility. Thus the usage of these various solvents in one step can be advantageous based on assumption that all of secondary metabolites in the sample can be completely extracted. In this study, we conducted extraction using various solvents and followed by identification of chemical compounds in the red betel leaves (*Piper cf. arcuatum* Blume) using TLC and GC-MS. Fragmentation route and possible mechanism of biosynthesis pathway of the metabolites in the extract were also proposed in this paper. The antioxidant activity and Brine Shrimp Lethality Test (BSLT) study were also performed in this research to draw a correlation between these parameters and the main constituens of the extracts.

Experimental
General experimental procedures
Red betel plants (*Piper cf. arcuatum* Blume) were harvested from Ciapus, Bogor. The plant

was identified by Herbarium Bogoriense, Biological Research Centre, LIPI, Indonesia. The leaves were washed with running tap water to remove the dirt, prior to the drying process. The leaves were cut into small pieces, dried in room temperature, and then were powdered.

All chemicals used were of analytical grade. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Dragendorff's reagent, Mayer's reagent, methanol, ethyl acetate, *n*-hexane, concentrated sulfuric acid, concentrated HCl, ferric chloride hexahydrate ($FeCl_3.6H_2O$), DMSO, acetic acid anhydride, acetic acid glacial, chloroform were purchased from Merck. *Artemia salina* Leach shrimp larvae was obtained from LIPI, Indonesia.

GC-MS apparatus and chromatographic conditions

Separation process was conducted by Thin Layer Chromatography, and further structure elucidation was performed by GC-MS, solvent removal was done by rotary evaporator, and Brine Shrimp Lethality Test was done using container with perforated septum.

Sample extraction

Sample preparation was conducted by maceration using several organic solvents. A 100 g of powdered red betel leaves were immersed in 5 L of n-hexane for 3 days, and then filtered. Filtrate was evaporated until dry sample was obtained, and this step resulted in raw extract of n-hexane. The residue from first immersion was entirely immersed back in 5 L ethyl acetate for 3 days to obtain raw extract of ethyl acetate. The solution was then filtered and evaporated, and the residue from this step was immersed in methanol for 3 days, resulted in raw methanolic extract. The maceration process was repeated several times to obtain clear extract containing all of expected chemical species.

Separation by Column Chromatography

Beaker glass containing silica gel (50 g) was added by *n*-hexane, stirred homogeneously until the gel appears like porridge. This porridge was introduced into a- 50 cm length, 2 cm diameter-column, which has cotton lid at the bottom of column. The tap of the column was opened thus the n-hexane solvent can drip and be collected in a flask. After the surface of remaining n-hexane was about 3 cm on silica gel surface, the tap was closed. This silica gel was acted as stationary phase.

Raw extract of n-hexane (0,2 g) was dissolved in small amount of n-hexane, added by silica gel, and homogenized. The *n*-hexane was evaporated using waterbath at temperature of 40^{o}C until residue was obtained, and then introduced to the column. Eluen which acts a mobile phase carefully poured into the column. The ratio of *n*-hexane to ethyl acetate was listed in the Table 1.

Table 1: The ratio of *n*-hexane to ethyl acetate as eluen mixture

n-Hexane (mL)	Ethyl acetate (mL)
100	0
90	10
80	20
70	30
30	70
0	100

The solution obtained from purification was collected in a 10 mL- vial, and analyzed by TLC. Identification of this solution was aided by UV lamp, at λ = 254 nm and 366 nm. The solution which showed spots in TLC sheet with the same Rf was collected in the same vial. This step was also done for raw extract of ethyl acetate (0.5 g) and that of methanol (0.5 g). The eluen mixtures which were used for each extract was listed in the Table 2.

Table 2: The eluen mixture for purification of extract of ethyl acetate and methanol

The eluen mixture for purification of extract of ethyl acetate	The eluen mixture for purification of methanolic extract
n-hexane : ethyl acetate (90 : 10)	n-hexane : ethyl acetate (90 : 10)
n-hexane : ethyl acetate (80 : 20)	n-hexane : ethyl acetate (70 : 30)
n-hexane : ethyl acetate (70 : 30)	n-hexane : ethyl acetate (60 : 40)
n-hexane : ethyl acetate (60 : 40)	n-hexane : ethyl acetate (40 : 60)
ethyl acetate (100)	ethyl acetate : methanol (90 : 10)
ethyl acetate : methanol (70 : 30)	ethyl acetate : methanol (70 : 30)
ethyl acetate : methanol (60 : 40)	ethyl acetate : methanol (40 : 60)
	ethyl acetate : methanol (20 : 80)
	methanol (100)

Phytochemical assay

Phytochemical assay of raw extract of *n*-hexane, ethyl acetate, and methanol was performed using *Ciulei* method [17]. The assay included several test for alkaloid, tannin, saponin, reducing sugar, flavonoid, glucoside, phenolic, glycoside-steroid, and sterol - triterpenoid.

Antioxidant activity

Antioxidant activity of red betel leaf extract was tested by monitoring radical scavenging activity using DPPH method [18]. Briefly, 1 mL of 0.5 mM DPPH (1,1-diphenyl-2-picryl-hydrazil) solution in methanol was pipetted and transferred to vial. The samples were prepared separately to obtain 1 mg/L (ppm), 3 ppm, and 5 ppm solution in methanol, and transferred to the vials which contain 0.5 mM DPPH. Each vial was diluted by adding methanol until the total volume of 5 mL. The absorbance of DPPH solution was measured by UV-Vis spectrophotometer at λ = 515 nm, every 5 minutes for total of 30 minutes. Antioxidant activity was calculated as a function of absorbance decrease of DPPH solution as a consequence of sample addition.

Structural elucidation of each fraction by GC-MS

White crystal of each fraction from raw *n*-hexane, ethyl acetate, and methanol extracts were further analyzed by GC-MS to determine the species contained in the samples.

Brine Shrimp Lethality Test (BSLT)

Raw extract of n-hexane, ethyl acetate, and methanol of red betel leaves were dissolved in seawater. At first step, the concentration of these extracts were 2000, 200, and 20 µg/mL, and after dilution were 20, 10, dan 2 µg/mL. The nonpolar samples which less soluble were added by DMSO for about 10 µL. For analysis with diluted samples, after 48 hours, 100 µL of seawater containing 10-15 shrimp larvae was put in sample vials. 100 mL of extract solution was poured in this vials, thus the extract concentration in each vials were 10; 5; and 1 µg/mL, respectively. These steps were done triplicate for each concentration. 100 µL of seawater containing 10-15 shrimp larvae was added by 100 µL of seawater, and was used as control. After 24 hours the surviving brine shrimp larvae were counted, and the BSLT analysis was performed using Sam method [19]. The percentage of mortality (%) was obtained by calculating the dead brine shrimp larvae divided by total number of larvae.

LC_{50} was assessed based on determination of probite number, by convert the percentage of mortality with probite table. Plotting of probite number as a function of log concentration of extract resulted in linear regression equation y = ax+b.

Result and Discussion

Sample Extraction

The results showed that different extracting agent resulted in different percentage of yield. From 100 g dry red betel leaf (*Piper cf. arcuatum* Blume), it yielded 1.70 g (1.67%) of green solution of raw n-hexane extract, 6.5 g (6.40%) of brownish green solution of raw ethyl acetate extract and 10.30 g (10.15%) of blackish green of raw methanolic extract. The results showed that the methanolic extract contains the largest yield compared to the other types of extracts.

The percentage of yield of extract indicated the extracting capacity of extracting agent. The highest yield of methanolic extract indicated that methanol has the highest extracting capacity for secondary metabolite in the red betel plant. On the other side, the lowest yield for n-hexane related to the fact that n-hexane has the lowest extracting capacity. Azmir *et al*. states that the efficiencies of extraction methods mostly depend on the understanding the nature of plant matrix and chemistry of bioactive compounds.[16] The possible explanation for this phenomenon was the fact that the secondary metabolites contained in the methanolic extract were polar or semipolar thus need the extracting agent which has the similar polarity. This explanation must be supported by the further phytochemical assay. The physical appearance of the extract solution also provided supporting information that different kinds of the secondary metabolites were extracted from different solvent.

Phytochemical assay

Phytochemical analysis of n-hexane, ethyl acetate, and methanol raw extract revealed that ethyl acetate and methanolic extracts contain alkaloid, reducing sugar and glucoside (Table 3). All of these metabolites were absent in *n*-hexane extract. Methanolic extract showed the most complete contents of secondary metabolite, compared to those of *n*-hexane and ethyl acetate extracts. Tannin, reducing sugar and glucoside were present in methanolic extract, thus it can be expected that the methanolic extract has high antioxidant activity, since the mechanism of antioxidation for polar antioxidant involves reaction with the hydroxyl group present in the metabolites. Indeed, phenolics such as tannins are composed of one or more aromatic rings bearing one or more hydroxyl groups and are therefore potentially able to quench free radicals by forming stabilized phenoxyl radicals [20].

Table 3: Phytochemical assay of red betel (*Piper cf. arcuatum* Blume) leaf

No	Parameter	n-hexane extract	ethyl acetate extract	methanolic extract
1	Alkaloid	-	++	+
2	Tannin	-	-	+
3	Saponin	-	-	++
4	Reducing sugar	-	+	+++
5	Flavonoid	-	-	-
6	Glucoside	-	+	++
7	Phenolic	-	-	-
8	Glycoside Steroid	-	-	+
9	Sterol Triterpenoid	-	-	-

TLC and Column Chromatography analysis

TLC analysis showed the difference of color and Rf value between each spots in TLC layer, with or without the aid of UV lamp. The chemical separation in the n-hexane extract was done using silica gel as stationary phase and the mixture of n-hexane and ethyl acetate as mobile phase, which has gradient composition (Table 1). When the ratio of eluen mixture was 7 : 3 (n-hexane : ethyl acetate), the separation yielded seven isolates, in which isolate 1 to 6 can be combined and called as fraction A, due to the same Rf value. This fraction has weight of 0.027 g.

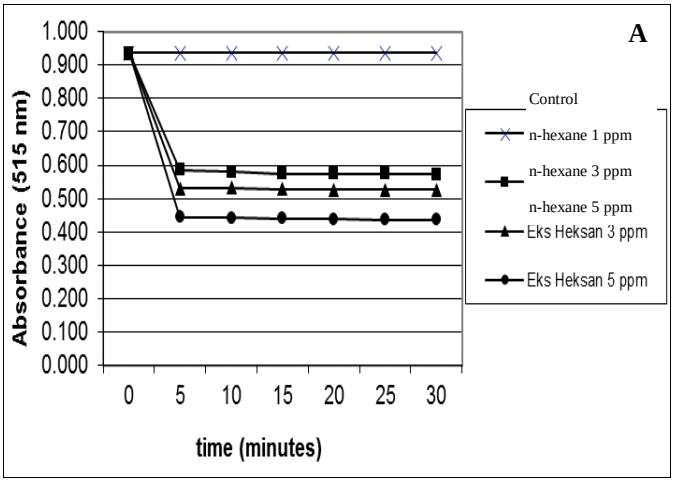
The separation of ethyl acetate extract was done using the eluen mixture of *n*-hexane : ethyl acetate and ethyl acetate : methanol which has gradient composition (Table 2). The TLC analysis using the ratio of eluen mixture of 7 : 3 (ethyl acetate : methanol) resulted only one spot with Rf value of 0.91. Evaporation of this sample yielded white crystal, 0.0032 g weight, which is assigned as fraction B.

The separation of methanolic extract was done using the eluen mixture of *n*-hexane : ethyl acetate and ethyl acetate : methanol which has gradient composition (Table 2). TLC analysis for isolate at vial 10 until 12 showed spots with the same Rf value, thus they can be combined into one vial. After evaporation of this sample, the green solid was obtained and weighed as 0.0031 g, which is called as fraction C.

Antioxidant activity

DPPH is nitrogen centered free radical having an odd electron which gives a strong absorption at 517 nm, its color changes from purple to yellow when DPPH• odd electron paired off in the presence of radical scavenger to form the reduced DPPH-H [21]. The DPPH assay results are indicative of the hydrogen-donating propensity of a test compound. Likewise, the antioxidant activity of plant extracts is also correlated with their reducing powers, which are generally associated with the presence of reductones [22].

Fig. 1 revealed that extract of red betel leaf which has the highest inhibition activity was the raw methanolic extract with IC_{50} value of 3.44 ppm, and that which has the lowest inhibition activity was the raw *n*-hexane extract with IC_{50} value of 43.23 ppm. This phenomenon indicated that polar secondary metabolites in the raw methanolic extract of red betel leaf were bioactive compounds which are highly potential as antioxidant. The nonpolar secondary metabolites from *n*-hexane extract were less potential as antioxidant. Rathee *et al*. reported that antioxidant activity of Piper betel leaf extract from India can be correlated with the total phenolic content and reducing powers of the extracts. HPLC and NMR analysis of these extracts revealed the main constituens of APC (3,4-Dihydroxyallylbenzene) and CHV (3-Hydroxy-4-methoxyallylbenzene) [22].



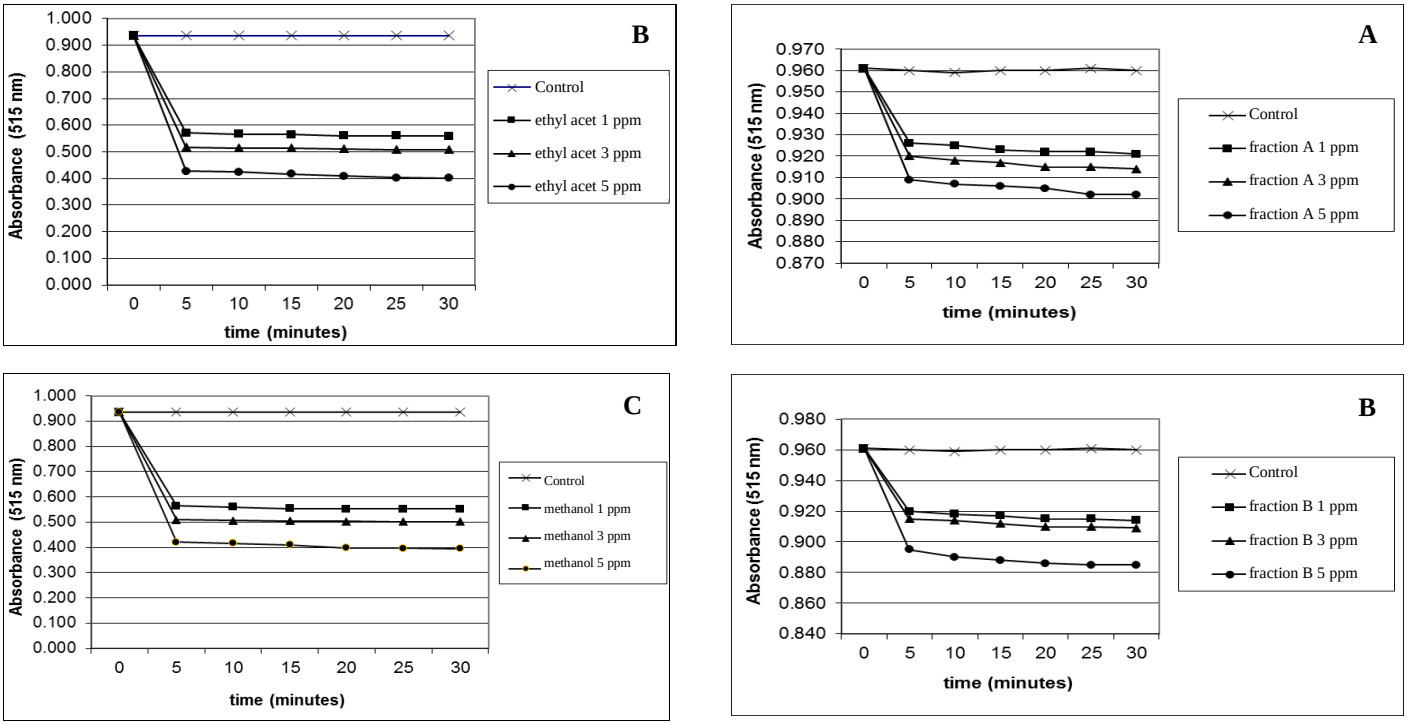


Fig 1: Analysis of antioxidant activity of n-hexane (A), ethyl acetate (B), and methanol (C) extract using DPPH method

Antioxidant activity of raw ethyl acetate and methanolic extract, compared to the fraction obtained after further separation, were higher than that of fraction B and C (Fig. 2 and 3). It was probably due to the chemical components which are present in the ethyl acetate and methanolic extract were absent in the fraction A and B. Among the fraction A, B, and C, fraction B has the highest inhibition activity, thus the further elucidation structure was performed for fraction B.

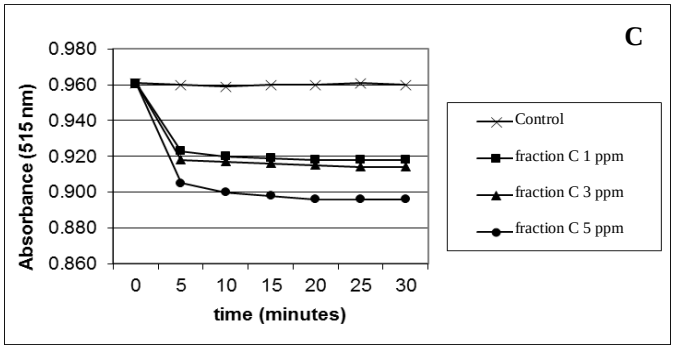


Fig 2: Analysis of antioxidant activity of fraction A, B, and C using DPPH method

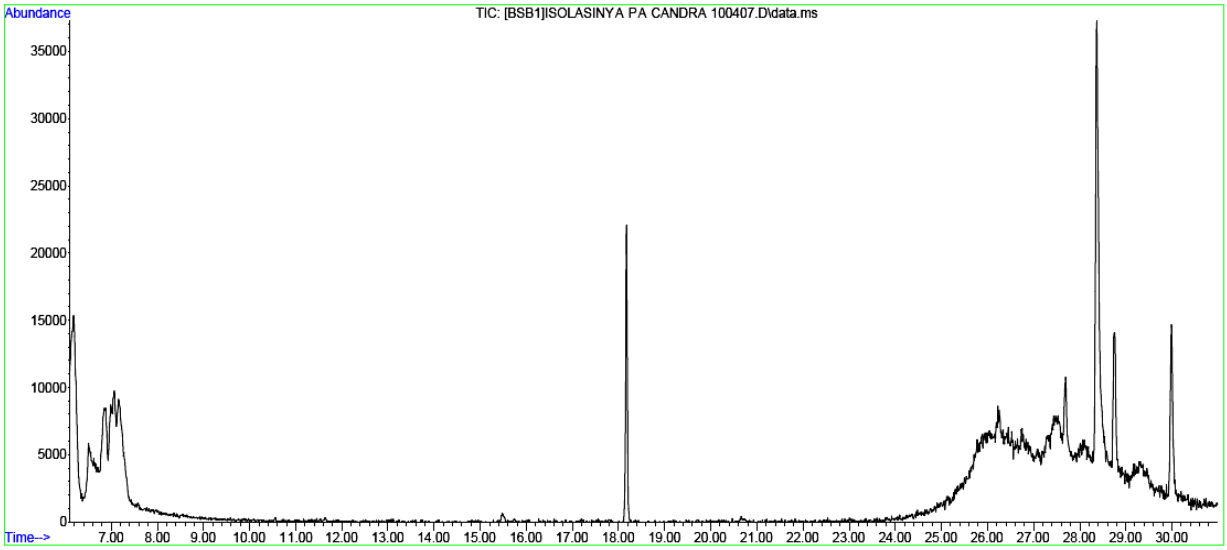


Fig 3: GC Chromatogram of fraction B

GC-MS analysis

Identification of chemical compounds in fraction B was performed by GC-MS. The chromatogram showed several peaks with different time retention (Fig. 4). Due to the large number of peaks in the chromatogram, the discussion was focused on peak which has the highest antioxidant activity. Since the peak at retention time of 18.171 minutes has high antioxidant activity, thus the further structure elucidation was performed for this peak. Mass spectrum of this peak was shown in Fig. 5 which revealed that the parent peak has m/z = 220, and the fragmentation of parent ion yielded peaks at m/z of 205 and 189.

GC-MS method was proved to be an excellent tool to provide information about prediction of the chemical structures of phytocomponents from extract of plants [23-24]. The prediction of the chemical structure of the red betel extract can be drawn from their GC-MS spectrum (Fig. 4 and 5). Based on the fragmentation route, the molecular formula which has molecular mass of 220 is $C_{13}H_{16}O_3$. According to the database (library search report NIST05), the high similarity (91%) is achieved for the compound with chemical name of 6, 7-dimethoxy-2,2-dimethyl-1-benzopyran. The base peak with m/z = 205 was obtained from fragmentation of parent ion which loses its methyl radical. Ion with m/z = 189 yielded from fragmentation of parent ion which removes its methoxy group. The fragmentation of this compound may proceed as shown by the scheme below (Fig. 6).

The 6,7-dimethoxy-2,2-dimethyl-1-benzopyran compound is classified as chromene compound. The biosynthesis route for this compound can be predicted as a combination of shikimate pathway and mevalonate one. The biosynthesis of the compound may proceed via reaction as below (Fig. 7). The reaction pathways involved epoxydation of C=C double bond, followed by cyclization of degraded epoxy group resulting in ring with five C atoms and an oxygen atom, and OH group with meta position of the O atom.

This benzopyran derivative probably has significant correlation with the antioxidant activity, since it can exert antioxidant action by breaking the free radical chains as well as reacting with certain precursors of peroxide and preventing peroxide formation. This compound has structure similarity as the main compound (allylpyrocatechol and chevibetol) in three varieties of Piper betel leaves extracts from India,[22] thus we predicted that the mechanism of antioxidative activity of the benzopyran derivative was also similar with that of allylpyrocatechol and chevibetol.

Brine Shrimp Lethality Test (BSLT)

BSLT analysis of the red betel leaf extract was performed using shrimp larvae of *Artemia salina Leach*. This study used 10 shrimp larvae for each type of extracts and this step was done triplicate.

Table 4 showed that all of the extracts have toxic effect toward shrimp larvae of *Artemia salina Leach*, as shown by the value of % mortality. LC_{50} value was calculated using probite analysis, and the data was shown in Table 5. The data from Table 5 can be further analyzed to obtain linier curve which fit to the equation of $Y = 4.465 X – 4.486$ for extract of n-hexane, $Y = 2.27 X – 1.456$ for extract of ethyl acetate, and $Y = 1.965 X – 2.626$ for methanolic extract. In this equation, Y is probite value and X is log (concentration of extract). By substituting the X with real value of log (concentration of extract), the LC_{50} value of each extract can be calculated, as listed in the Table 6.

In the drug development process, a large number of crude plant-derived extracts were firstly screened for their cytotoxic activity before they were further assayed using cancer cell lines or higher animals. Many researches showed that Brine Shrimp Lethality Test (BSLT) has a good correlation with various tumour cell lines. The cytotoxic activity of extract in BSLT is determined by 50% brine shrimps mortality response (LC_{50}). The plant extract was considered toxic to brine shrimps if it had $LC_{50} < 1000$ μg/mL [25]. The toxicity level would show potency of extracts as anticancer.

The degree of lethality was directly proportional to the concentration of the extract. Maximum mortalities (100%) were observed at a concentration of 1000 ppm in hexane extract, ethyl acetate extract, and methanolic extract (Table 5). Based on the results, the brine shrimp lethality of the three types of extracts were found to be concentration-dependent. This results were in agreement with the previous study [26].

Table 6 revealed that the highest toxicity effect (lowest LC_{50} value) of the raw extract of red betel leaf was achieved by using methanol as extracting agent, and the lowest toxicity effect (highest LC_{50} value) was obtained when the *n*-hexane was applied as extracting agent. Elsyana *et al*. [27] explained that flavonoids and triterpenoids were toxic for brine shrimps in a concentration-dependent manner, so it might have contributed to brine shrimps mortality. Since benzopyran compound found in red betel extract is derivative of flavonoid, it clearly explained the high toxocity of methanolic extract. In the other hand, Elsyana *et al*. [27] also reported that the *n*-hexane extract of clove mistletoe have exhibited the highest cytotoxic activity compared to other extracts and fractions that also contained flavonoid and triterpenoids. Thus we assumed that in our study, the *n*-hexane extract might not contain other active compounds or other constituents which might have had a synergistic effect on cytotoxic activity.

It is well known that the toxicity of secondary metabolites of the plants is related to self-defence mechanism of the plant against predator. The mechanism probably works by protecting the plant's organ which is possible to be a target for predator, or by inhibiting division of cells which are attacked by pathogen microorganism. The lower the LC_{50} value of the bioactive compound, the more potential the compound can be applied as a major parts of the drug system since it has high self-defence capability or high protecting ability [27].

Table 4: The result of BSLT analysis of hexane, ethyl acetate and methanolic extract of red betel leaf (*Piper cf. arcuatum* Blume)

Sample	Concentration of extract (ppm)	The number of dead larvae	The number of surviving larvae	% mortality
n-hexane extract	10	0	30	0
	100	9	21	30.00
	1000	30	0	100.00
ethyl acetate extract	10	8	22	26.67
	100	11	19	36.67
	1000	30	0	100.00
methanolic extract	10	15	15	50.00
	100	23	7	76.67
	1000	30	0	100.00
Blank (seawater)		0	30	0

Table 5: Probite analysis of red betel (*Piper cf. arcuatum* Blume) leaf extract of hexane, ethyl acetate, and methanol

Sample	Concentration of extract (ppm)	% mortality	Log (concentration of extract)	Probite value
hexane extract	10	0	1	0.00
	100	30.00	2	4.40
	1000	100.00	3	8.93
ethyl acetate extract	10	26.67	1	4.39
	100	36.67	2	4.67
	1000	100.00	3	8.93
methanolic extract	10	50.00	1	5.00
	100	76.67	2	5.74
	1000	100.00	3	8.93

Table 6: LC_{50} value of hexane, ethyl acetate, and methanolic extract red betel (*Piper cf. arcuatum* Blume) leaf

Sample	LC_{50} (ppm)
extract of hexane	133.20
extract of ethyl acetate	36.41
extract of methanol	16.15

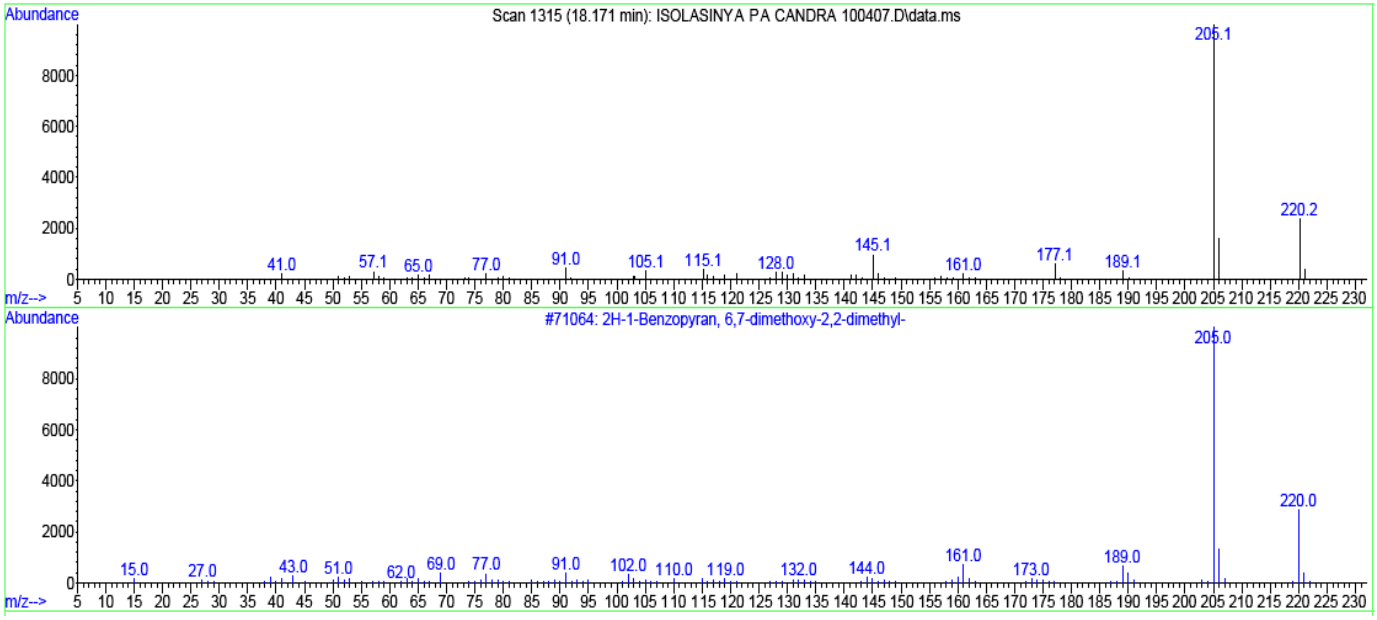


Fig 4: Mass spectrum of fraction B at peak with retention time of 18.171 minutes

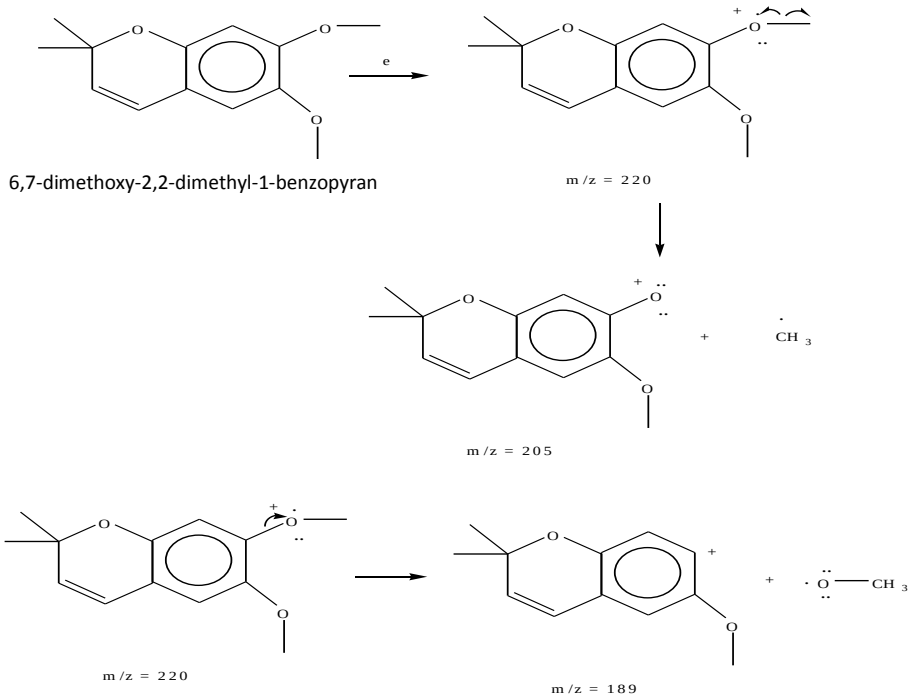


Fig 5: Fragmentation route of 6, 7-dimethoxy-2, 2-dimethyl-1-benzopyran

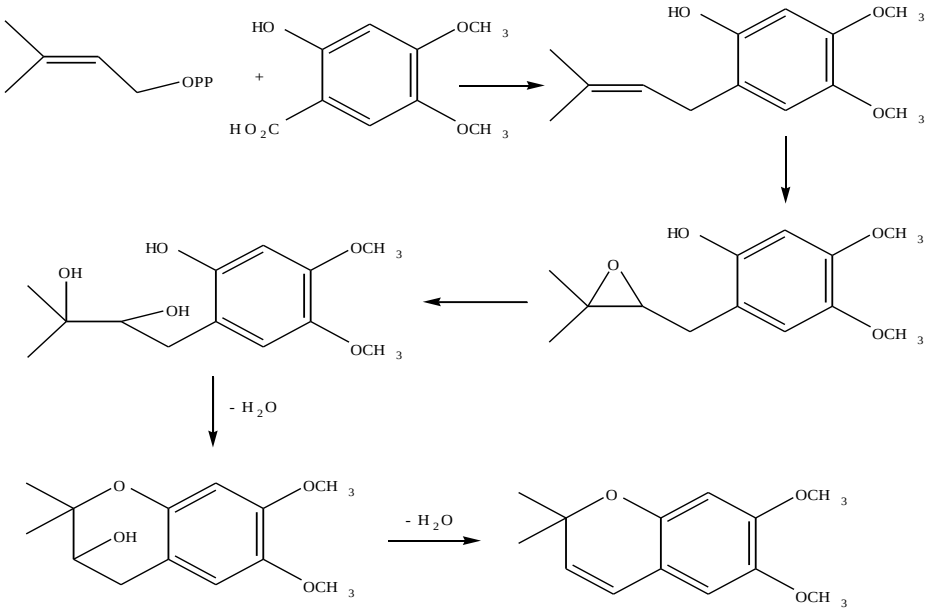


Fig 6: The proposed mechanism of biosynthesis of 6, 7-dimethoxy-2, 2-dimethyl-1-benzopyran

Conclusion

Based on GC-MS analysis, the methanolic extract of red piper betel contains major component of 6,7-dimethoxy-2,2-dimethyl-1-benzopyran, a chromene compound. The biosynthesis route was predicted to be a combination between shikimate pathway and mevalonate one. The results revealed that the methanolic extract has the highest antioxidant activity compared to *n*-hexane and ethyl acetate extract. The BSLT analysis showed that the methanolic extract has the highest toxicity level or lowest LC_{50} value (16.15 ppm) compared to the other type of extract.

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