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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation　Study on Bioactive Compounds Found in *Litsea cubeba* Fruits
(*Litsea cubeba* 果実に含まれる生理機能性物質に関する研究)

学位論文要約：
Dissertation Summary

Litsea cubeba (Lour.) Persoon is a plant belonging to the Lauraceae family. This plant is a native species for mountainous area in Himalaya, Southern China, Taiwan, Japan and South East Asia. All parts of *L. cubeba* are used for many purposes, such as food, spice, folk medicine, fodder, timber and shelterbelt. But the specialty of *L. cubeba* is essential oil. The fruits contained high content of essential oil (5% maximum yield), which comprises geranial and neral as the major components. The fruit essential oil is also produced commercially and used in aromatherapy and several industrial purposes (Oyen and Dung, 1999). Many studies have already proven for bioactivities of *L. cubeba*, for example, antioxidant, antimicrobial, antifungal and cytotoxicity. However, there is still no report that confirmed about the active antioxidant compounds in *L. cubeba*. In addition, most of the past research on chemical profile of *L. cubeba* fruit only focused on the essential oil portion.

In this research, the focused bioactivities are anticancer and antioxidant activities. Carcinogenesis and oxidative stress are actually closely related. Oxidative stress is caused by free radicals, which are molecule with unpaired valence electron that can oxidize biomolecules and degrade cellular organelles such as cell membrane, genetic materials and essential proteins or lipids. Alteration of these molecules is a cause of many diseases, including cancer (Hussain *et. al.*, 2003). More than half of human cancers are caused by mutation of the p53 genes that control cell cycle arrest, program cell death (apoptosis) and DNA repair (El-Deiry, 2001; Bai *et. al.*, 2006; Amundson *et. al.*, 1998). Nowadays several clinical practices, such as surgery, radiotherapy and chemotherapy, are applied to cure cancer depends on stage of cancer development. Combination of chemotherapy and other methods is usually used in treatment of late cancer stage (Siegel, 2012). The origin of most presently used chemotherapeutic drugs is traditional medicines or poisons. The major mechanism of these drugs is to suppress cancer cell proliferation and to restore cell cycle arrest. For example Taxol isolated from pacific yew (*Taxus baccata*) and Vinca alkaloids isolated from Madagascar periwinkle (*Catharanthus roseus*) interfere with microtubule arrangement during the anaphase in mitotic cell division, resulting in the M phase cell

cycle arrest and apoptosis (Johnson *et. al.*, 1963; Stephenson, 2002). Camptothecin (CPT) isolated from *Camptotheca acuminate* induces cell cycle arrest in the S phase by inhibiting topoisomerase, which regulates DNA topology during synthesis of new DNA strand. CPT is an important precursor of many clinical chemotherapy drugs, such as topotecan and irinotecan (Du, 2003). The similar anticancer mechanism is occurred with podophyllotoxin and its derivatives (Moraes *et. al.*, 2002). This class of anticancer drug is called topoisomerase inhibitor.

Extraction and isolation of bioactive compounds or crude extract portions in this research relied on the principle of solvent chemical properties and chromatography. Several spectroscopic methods were applied for chemical identification, such as UV/VIS, infrared and nuclear magnetic resonance (NMR) spectroscopy. Generally, solvents can be categorized as non-polar, aprotic polar and protic polar solvents, in which molecular attraction strength is differed. A solute can be dissolved in a solvent if the solvent-solute intermolecular attraction force is greater than that of the solvent-solvent and the solute-solute themselves (Reichardt, 2003). In order to obtain a specific bioactive portion, the choice of solvent is very important. Liquid chromatography is the method applied for chemical isolation, based on interaction between solute, stationary phase and mobile phase. Interaction with stationary phase restrains each composition from moving, and interaction with mobile phase assists the compositions to elute from the stationary phase (Scott, 2003).

Spectroscopy is the study of interaction between electromagnetic radiation (EMR) and matters. These interactions help indicate specific properties of the analytes. In this study, compound identification was conducted mostly by absorption spectroscopy. In the UV/VIS spectroscopy, analytes are exposed to EMR at 200-800 nm of wavelength. Absorption of UV/VIS radiation causes valence electrons to change the molecular orbital. The UV/VIS spectrum displays the absorption maxima (λmax), which indicates the pattern of electron transition. The absorption maximum is a specific value of each specific chemical. Infrared spectroscopy is the method to determine the functional groups and types of bond in a molecule, based on molecular vibration. Only vibration patterns that alter dipole moment of the analyte can be observed in IR spectroscopy. The energy required for absorption of each functional group is presented as frequency or wavenumber. Each functional group has specific band pattern and absorption region. NMR spectroscopy is a form of absorption spectroscopy. The basic principle is that: an analyte can absorb EMR in the radio wave region in a magnetic field under appropriate conditions. Any atom nuclei containing odd number of proton can generate magnetic field and have specific magnetic moment, which can be described as quantum spin numbers. The most widely used atoms for identification of

organic compounds are ^{1}H and ^{13}C. Other than typical 1-D NMR analysis, correlation NMR or 2-D NMR is an important technique to analyze a compound structure by detecting correlation signals between ^{1}H-^{1}H or ^{1}H-^{13}C. Mass spectrometry is a non-absorption spectroscopy that determines molecular weight and empirical formula. There are three basic steps in MS: 1) ionization (molecule bombardment by various methods such as Electron Ionization, Chemical Ionization, Electron Spray Ionization and Matrix Assisted Laser Desorption Ionization), 2) ion differentiation (separation of ions by their specific properties, using various mass analyzer types such as Quadrupole ion trap and Time of Flight), and 3) detection (Harvey, 2000; Hart et. al., 1995; Silverstein and Webster, 1998).

The first experiment is to evaluate antioxidant potential of individual volatile components in *L. cubeba* fruit essential oil. The oil was extracted by using 7:3 pentane/dichloromethane. The components were quantified and identified by using GC/FID and GC/MS (column: DB-Wax fused silica column, 60 m x 0.25 mm). Antioxidant activity was determined by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and β-carotene bleaching assay. DPPH Assay was conducted to evaluate the free radical stabilization capability. β-carotene bleaching assay determined the potential of test samples to quench peroxyl radicals, generated from fatty acid oxidation, in competition with β-carotene in the system.

In total 19 volatile compounds were able to be identified. The major compounds found in *L. cubeba* fruit essential oil are geranial (*trans*-citral: 45.62%) and neral (*cis*-citral: 30.49%). This data is consistent with other reports on compound identification of this essential oil. Geranial and neral were found to possess weak antioxidant activity, determined by both DPPH (IC_{50}: 452.7 and 560.3 mM at 60 min) and β-carotene bleaching assays (β-carotene remained: 68.27 and 62.72% after 120 min, with 5 mM of compounds). It was found that the components which exhibited significantly high antioxidant activity were ocimene and α-terpinene, which are trace compounds (0.774% and 0.062%, respectively). Ocimene exhibited the strongest DPPH radical scavenging activity (IC_{50}: 34.51 mM at 60 min), but this compound assisted in β-carotene bleaching. However, the similar effect was also observed in ascorbic acid. It was reported before that this potent antioxidant compound showed pro-oxidant effect in β-carotene-linoleic system. Therefore, it could be assumed that ocimene might possess the similar antioxidant effect with ascorbic acid. For α-terpinene, this compound exhibited DPPH radical scavenging activity with IC_{50}: 47.59 mM (60 min) and averagely 83.26% of β-carotene was remained in the β-carotene-linoleic system after 120 min.

There were a few more components that exhibited mild antioxidant activity which are: *β*-caryophyllene (0.674%) and nerol (0.486%) that did not exhibit activity on DPPH radical scavenging but had small effect on quenching peroxyl radical (62.40 and 58.42% of *β*-carotene remained at 120 min). *β*-pinene (0.499%) and geraniol (0.583%) exhibited mild DPPH radical scavenging activity (IC_{50}: 835.5 and 810.1 mM at 60 min) and also mild activity on quenching peroxyl radical (52.71% and 55.33% *β*-carotene remained at 120 min). The rest of the volatile components which are: *α*-pinene (1.227%), sabinene (1.937%), 2-methyl-3-buten-2-ol (0.277%), 6-methyl-5-hepten-2-one (0.221%), limonene (5.398%), 1,8-cineole (0.440%), *β*-citronellal (1.409%), *β*-myrcene (4.398%), linalool (1.741%), *β*-phellandrene (0.102%) and *β*-citronellol (0.178%) did not exhibit significant antioxidant activity.

The second experiment (Trisonthi *et. al.*, 2013) focused on anticancer activity. In this part, the active chemical portions in *L. cubeba* fruit were evaluated for their capability to induce apoptosis in cancer cells. HeLa cell was used as a cancer model. This cell line is a human cancer cell, derived from cervix tissue of a patient with cervical cancer. The cause of this cancer is infection by human papilloma virus strain 18 and this type of cell line exhibited low p53 expression. There were 5 cell based assay applied for evaluation: WST-8 assay is for cytotoxicity; BrdU incorporation assay is to evaluate inhibition of DNA replication; Determination of LDH release is to quantify cell membrane rupture; Nuclei staining with DAPI is for observation of apoptotic bodies indicated by changes in nuclei morphology; and cleavage of caspase-3/-7 pro-fluorescent substrate is for determination of activated caspase-3/-7 in treated cells populations.

Firstly, fresh *L. cubeba* fruit powder was extracted with hexane to obtain the essential oil equivalent portion. The fruit powder residue from hexane extraction was extracted again with methanol to obtain the methanol extract, which comprises higher polar compounds that cannot be dissolved by hexane. The residue from methanol extract was extracted again with water to obtain the water extract. It was found that the methanol extract exhibited the greater cytotoxicity (IC_{50}: 7.75 μg/mL) than the essential oil (IC_{50}: 42.02 μg/mL) and the water extract did not exhibit the activity. The methanol extract was then fractionated by using Amberlite XAD-7 column chromatography with 20-100% methanol as mobile phase to obtain 8 fractions.

Fractions from the methanol extract as well as the essential oil portion were evaluated for the effect on cell viability, cell proliferation and cell membrane rupture in comparison with Etoposide. It was found that the essential oil exhibited no effect on HeLa cell proliferation. This result suggests that cytotoxicity of this portion

might be caused by traumatic cell death. Fraction 5A and 5B from the methanol extract exhibited the highest activity among all the test samples (IC_{50}: 8.62 and 8.39 μg/mL, 48 h). The effect on cell proliferation (IC_{50}: 4.89 and 3.26 μg/mL, 48 h) is almost as high as etoposide (IC_{50}: 2.09 μg/mL, 48 h) and the result on LDH release suggest that the cells treated with these fractions entered the late apoptosis stage sooner than the etoposide treated population.

Enzymes in caspase family have a major role in apoptosis. Caspase-3/-7 initiates apoptosis by cleavage of cytoskeleton and cell survival proteins. Caspase-3/-7 is activated by caspase-8 and -9 by two different pathways. In extrinsic pathway, caspase-8 is activated when the death receptor on cell membrane is stimulated. And for the intrinsic pathway, cellular stress induces mitochondria to release cytochrome C which later binds to apoptosome to activate caspase-9. The highest level of caspase-3/-7 was found in the population treated with fraction 5B and the second highest was that of 5A. It was also found that level of caspase-3/-7 in essential oil treated population was not different from the spontaneous activated caspase-3/-7 in untreated population. According to the result on nuclei staining with DAPI (Figure 1), at 24 h, nuclei content in the untreated population was intact with a few condensed chromatins in the anaphase. In the fraction 5A and 5B treatment, several fragmented nuclei were observed. And at 48 h, most of HeLa cells exposed to fraction 5A and 5B exhibited apoptosis characteristics, while that in the untreated population are still normal.

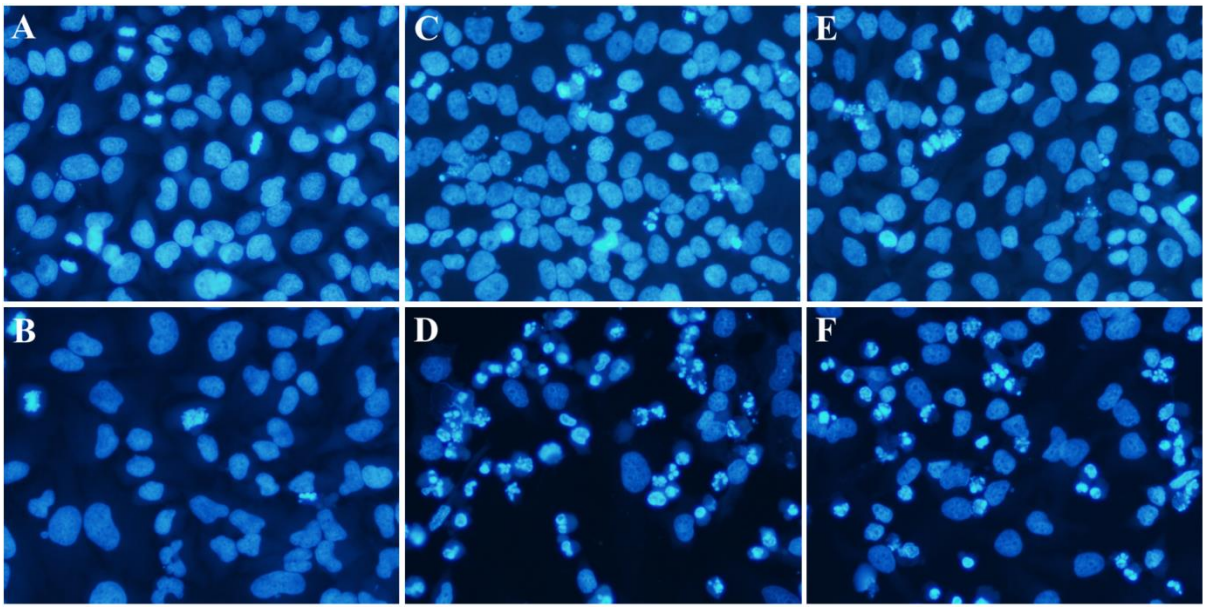

Figure 1 DAPI stained HeLa cells after being treated with DMSO 0.1% for 24 h (A) and 48 h (B), RME-5A 10 μg/mL for 24 h (C) and 48 h (D) and RME-5B 10 μg/mL for 24 h (E) and 48 h (F)

In conclusion, the methanol soluble portion in Litsea cubeba fruit was more cytotoxic than the essential oil. Therefore, there is possibility that some anticancer compounds can be recovered from the organic waste from *Litsea cubeba* fruit essential oil production. The essential oil neither inhibits DNA replication nor promotes caspase-3/-7 activation in HeLa cell. This suggested that essential oil may cause traumatic cell death rather than apoptosis. The methanol extract was fractionated by using ion exchange polymer stationary phase, which absorb compounds by dispersion force and acid-base attraction. The active portions were eluted by 80-100% methanol. Therefore, the active compounds could be moderate to non-polar or basic compound such as alkaloids.

The last part of this research (Trisonthi *et. al.*, 2014) is about isolation and identification of anticancer compounds from methanol extract of *L. cubeba* fruit. As The effective fractions from experiment 2 comprise large number of compositions that made difficulty in compound isolation. As for the solution, the methanol extract of *L. cubeba* fruit residue was fractionated again by normal phase chromatography, using silica gel which is more suitable for compounds with low polarity. Gradient elution was conducted by using mixture of hexane and diethyl ether to give 20 fractions. The last fraction was washed from column by using 10% acetic acid in ethanol. The effective cytotoxic compounds were selected by cytotoxicity screening test and isolated by preparative HPLC. The cell based assays were conducted with mostly the same methods as in the second experiment, except that: Hoechst 33342 and PI were used for nuclei staining and amount of activated caspases was determined by using specific fluorescent inhibitor for caspase-8, caspase-9 and caspase-3/-7.

There are 5 cytotoxic compounds discovered. Compound **1-3** were found in fraction 11 and 12 eluted by 50% diethyl ether. Compound 4-5 were found in the wash fraction. Compound 1 was completely identified and it is a newly discovered compound. Compound **4** and **5** are trace compounds, which the collected amount is still not enough for NMR analysis. The chemical properties of these compounds are shown as followed:

Compound **1**: pale yellowish oil; $[\alpha]_D^{20}$ +274.025 (c = 0.02, $CHCl_3$); λ_{max} 216, 324 nm; υ_{max} 3597, 3516–3393 (broad), 3034, 3010, 2978, 1670, 1629, 1561 cm^{-1}; *m/z* 299.2017 $[M-OH+H]^+$ (calcd. 299.2011 for $C_{20}H_{28}O_2$), *m/z* 339.1911 $[M+Na]^+$ (cald. *m/z* 339.1936 for $C_{20}H_{28}O_3Na^+$)

Compound **2**: pale yellowish oil; λ_{max} 217, 313 nm; *m/z* 335.2223 $[M+H]^+$ (cald. *m/z* 335.2222 for $C_{20}H_{31}O_4$)

Compound **3**: colorless oil; λ_{max} 216 nm

Compound **4**: white powder; λ_{max} 286 nm; *m/z* 476.4203 $[M+H]^+$ (cald. *m/z* 476.4216 for $C_{29}H_{54}N_3O_2$)

Compound **5**: white powder with λ_{max} at 282 nm; *m/z* 490.4265 $[M+H]^+$ (cald. *m/z* 490.4260 for $C_{31}H_{56}NO_3$)

The chemical structure of compound **1** was determined by 1-D (^{1}H, ^{13}C and DEPT) and 2-D NMR (^{1}H-^{1}H COSY, HETCOR, HMBC and NOESY) spectroscopy. The complete planar structure of 1 is shown in Figure 2 and the 2-D NMR correlations are shown in Figure 3. There had been no report on compound with the similar skeleton, according to the search on several chemical databases. Therefore, this compound has been named as cubelin. There are some moieties that can be related with structures of neral and geranial, so it was proposed that cubelin might be biosynthesized from conjugation of these compounds (Figure 4).

Among compounds from the 50% diethylether fraction, compound **1** exhibited the strongest activities. At 24 h, the cytotoxicity was stronger than etoposide but still weaker than CPT. This compound also showed positive result in BrdU incorporation assay, which suggest possibility of apoptosis induction. Compound **4** and **5** exhibited very high activities, which can be compared to that of CPT. The result on LDH release suggests that compound **1**, **2**, **4** and **5** could induce apoptosis sooner than both of the standard anticancer compounds.

For the nuclei morphological changes of treated cells (Figure 5), the Hoechst 33342 is a membrane permeable nuclei binding dye, while PI is membrane impermeable. Only dead cells with membrane rupture are PI positive. The cells were also stained with caspase-3/-7 fluorescent inhibitor to reveal presence of caspase-3/-7. HeLa cells exposed to 15 ug/mL of compound **1** for 24 h clearly exhibited apoptotic characteristics. Several cells were also positive to PI. More secondary necrotic cells were identified in the cell population treated with compound 4 and 5 at 2.5 μg/mL concentration. Presence of cells that are positive to caspase-3/-7 inhibitor indicated that compound **1**, **4** and **5** could induce apoptotic cell death.

Quantification of activated caspases is presented as folds of spontaneous caspase activation in untreated population. It was found that activity of compound **1** was only slightly lower than etoposide. From 6-30 h, caspase-8 level was increased from 1.7 to 4.5 folds. Amount of caspase-9 was quite stable at first, but it increased sharply to 4.2 folds at 24 h. Amount of caspase-3/-7 also increased gradually as the exposure time increased. Compound **4** and **5** exhibited even stronger activity than CPT on activation of caspase-8, -9 and caspase-3/-7. The maximum level of all caspases was observed at 24 h. Compound **4** activated 23 folds of caspase-8 and -9 and activated 10 folds of caspase-3/-7, while compound **5** activated 25, 30 and 13 folds of caspase-8, -9 and -3/-7, respectively. As compound **1**, **4** and **5** promoted activation of both caspase-8 and -9, this result suggest that these compounds can initiate apoptosis by both intrinsic and extrinsic pathways.

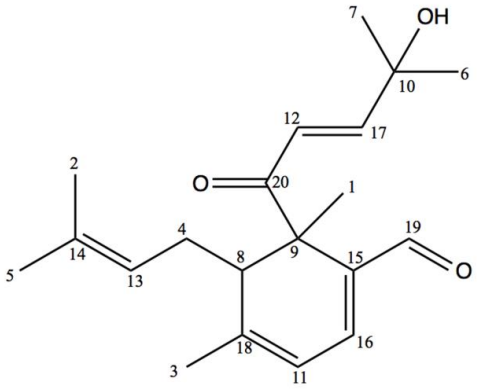

Figure 2 Chemical structure of cubelin (**1**)

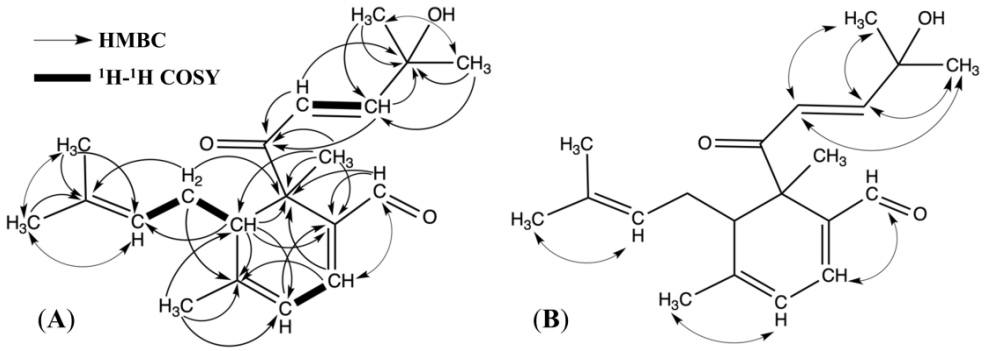

Figure 3 HMBC and ^{1}H-^{1}H COSY correlations (**A**) and NOESY correlations (**B**) of cubelin

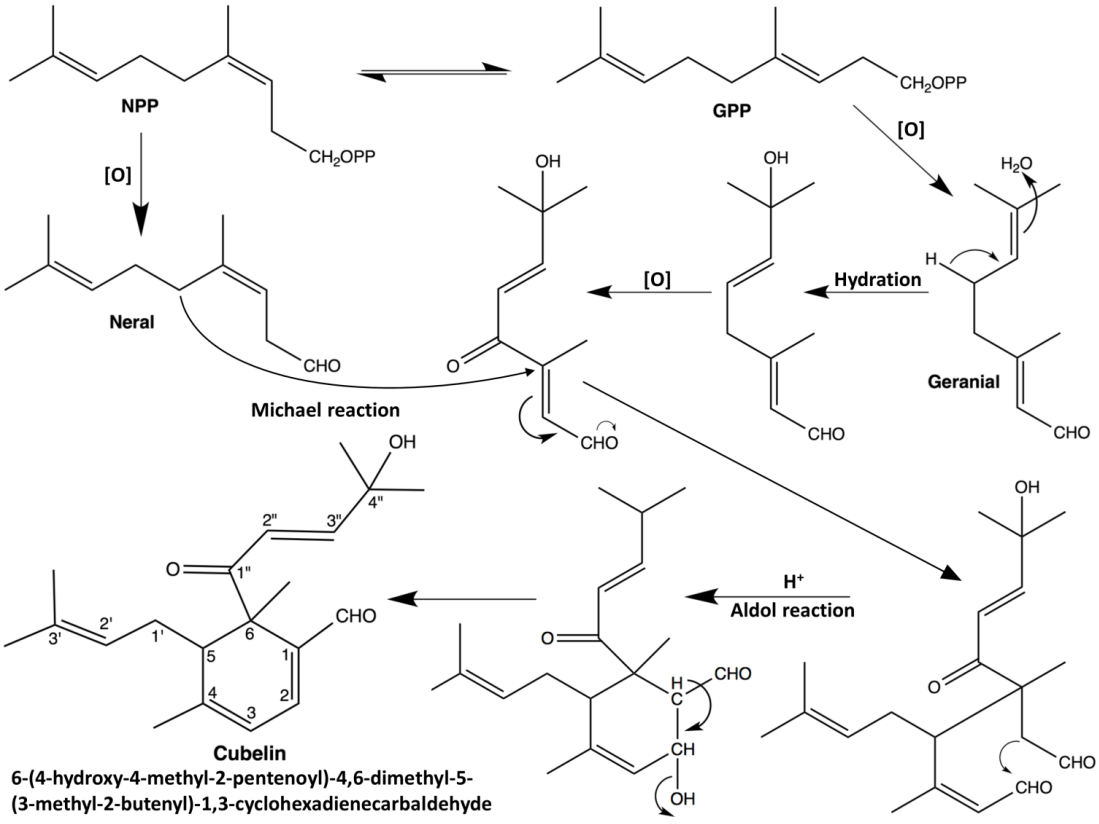

Figure 4 The proposed biosynthesis pathway of cubelin

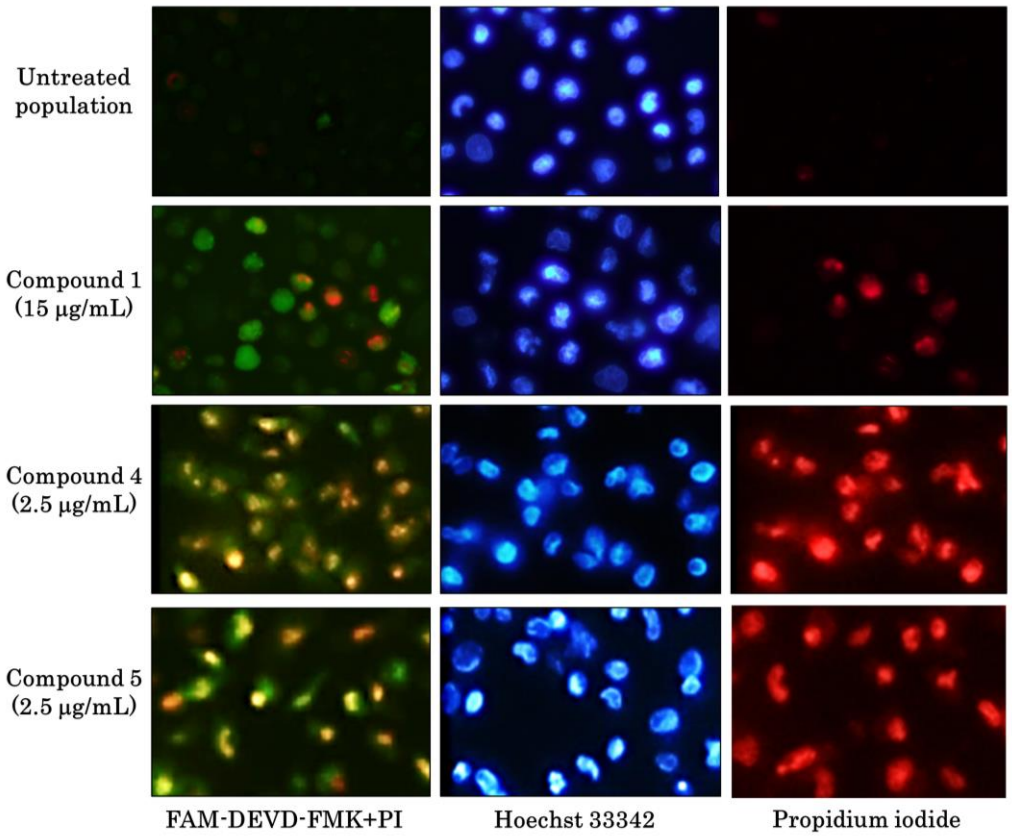

Figure 5 HeLa cells stained with Hoechst 33342, PI and caspase-3/-7 fluorescent inhibitor, after being exposed to compound 1, 4 and 5 for 24 h and the untreated population was exposed to 0.1% DMSO

In conclusion, five cytotoxic compounds were found in methanol extract of *Litsea cubeba* fruit. Compound 1, 4 and 5 were proven for apoptosis inducing activity in HeLa cells by both intrinsic and extrinsic pathways. Compound 1 exhibited slightly lower activity than etoposide and compound 4 and 5 exhibited the stronger activity than CPT. Compound 4 and 5 are trace compounds, approximately 2 mg can be obtained from the present material, which is still inadequate for structure elucidation. For compound 1 or cubelin, the complete structure has already been identified and it is a newly discovered diterpenoid compound. In total, 64 mg of this compound can be isolated from 2 kg of fresh fruit. So, there is a possibility that tremendous amount of this compound can be recovered from the waste material from *Litsea cubeba* essential oil production.

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