Extraction of Indonesian Mistletoe Using Hot Compressed Solvent Extraction and Properties of the Resulted Extracts

インドネシア産ヤドリギ中の機能性物質の高温高圧溶媒抽出と抽出物の機能性に関する研究

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CHAPTER 1
GENERAL INTRODUCTION

Traditional medicine is the sum total of knowledge, skills and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 2000). One of traditional medicines is herbal medicine, which is a medicine that made from plants and contains active compounds. Traditional use of herbal medicines refers to the long historical use of these medicines, therefore the only evidence of their safety and efficacy is embodied in folklore. In other hand, the use of traditional medicine among patients with chronic, painful, debilitating, or fatal conditions, such as HIV/AIDS and cancer, is increasing whereas the use of traditional medicine based on their believes (Richardson and Straus, 2002).

The use of herbal medicine varies widely between and within countries. In Africa, China, and South East Asia countries, 40-90% the population used traditional medicines for their health care or integrated into the heath system (WHO, 2002). While, Australia has 49% of adult population used traditional medicine. Different condition also occurred in western countries (German, France, and USA), which traditional medicine not strongly integrated into the health care system, but up to 40% they have used traditional medicines at least ones (WHO, 2002). However, there is one thing in common that individuals in each countries select traditional medicine are according to their specific beliefs.

Despite the use of herbal medicine over many centuries, only a relatively small number of plant species has been studied for possible medical applications. Safety and efficacy data are available for an even smaller number of plants, their extracts and active ingredients and preparations containing them. Most of cases are not possible to identify the active compounds, the whole herbal medicine may consider as one active compound. However, the safety of that kind of medicine cannot be guaranteed.

Indonesia as tropical country has 30,000 species of plants and 940 species of which include herbal medicine. There were 180 species have been used by traditional herbal medicine industry (Republika, 2009). Therefore, Indonesia has great potentiality to develop herbal medicine as resources for new finding drugs. Unfortunately, the scientific evidences

for those medicine plants were not developed yet, whereas the trend of medicinal plants were greatly increased

One of herbal medicine in Indonesia is mistletoe (Loranthaceae). Mistletoe is one of the unique biodiversity due to their role as a hemiparasite plant. It grows on the branches of trees to get tree nutrients. Despite their hemiparasitic action, they could grow by their self-photosynthesis in an independent manner of tree nutrients (Smith et al., 2001). Mistletoe also considers as an unwanted plant to economically important horticultural plant, however in the other side, mistletoe is known as one of herbal medicine used in traditional therapy. In Indonesia, mistletoes used to treat cancer, kidney malfunction, could decrease high level of serum cholesterol, decrease blood sugar or diabetes, decrease high blood pressure or hypertension and so on.

Mistletoe (Loranthaceae) is grown in Java and Sumatera. It is a woody stem epiphyte, cylindrical twigs brown spotted. Although variable in size and shape, mistletoe has large showy flowers that are bird-pollinated. Indeed, the co-evolutionary relationship with birds has reached a high level, as evidenced also by the seed dispersal mechanism. Many other mistletoe-animal interactions occur, and Watson (2001) has proposed that mistletoe function as keystone resources in many ecosystem, i.e. they are important ecological components that positively affect diversity in habitats (Nickrent and Musselman, 2004).

The most famous of mistletoe is haustorium as an unique part that connecting mistletoe and the host xylem to obtain water and with its dissolved nutrients. But as a hemiparasite, mistletoe still contains chlorophyll to do some photosynthesizes. Haustorium morphology is not extremely variable from their initiation to maturation but also varies with the parasite, the host, as well as age and vigor of host and parasite. Based on report by Devkota and Glatzel in 2007, the species of host plant is may be partly or wholly responsible for the development of haustorium at the attachment site. Furthermore, host-derived secondary metabolite play an important role in the interaction between parasitic weds and their hosts. They are involved in signaling, for example in the induction of parasite germination and the formation of the haustorium, and in plant defense against the parasite (Bouwmeester et al., 2003).

Since secondary metabolite have important role in mistletoe growth, it had diverse compounds such as alkaloids, phenylpropanoids, triterpenes, polysaccharides, peptides, lectins, flavonoids and phytosterols (Fukunaga et al., 1987, 1988; Richter and Popp 1992; Łuczkiewicz et al., 2001) that made research interests on bioactivity of mistletoes have been arisen from pharmacologists. Particularly, polyphenol compounds inside mistletoes, which known as taxonomic markers (Becker and Exner, 1980), are known as an antioxidant

compounds. Antioxidant compounds have ability to inhibit oxidation rate or to neutralize a free radicals (reactive oxygen species/ROS) (Roberts K., 2007).

Under normal condition, ROS are continuously produced during cell metabolism. They are scavenged and converted to non-reactive species by different intracellular, enzymatic and non-enzymatic antioxidant system such as catalase, peroxidase, superoxide dismutase, vitamins and reduced glutathione (Hyman et al., 2005). Overproduction or an ineffective elimination of ROS may induce oxidative stress and cause damage to all types of molecules such as proteins, lipids and nucleic acids (Droge, 2002). Moreover, oxidative damaged caused by ROS may be related to aging and other disease, such as atherosclerosis, diabetes, and cancer. Therefore, polyphenolic compounds inside mistletoe as antioxidant agents have big potential to develop.

In general, mistletoes for medicinal use are subjected to a conventional decoction extracted with water. However, the method could suffer from some issues due to low yield and serious chemical degradation by heat during a long extraction time. In order to overcome these issues, other extraction techniques have been developed. One of those techniques is hot compressed water that resulted good efficiency, reliability and has received increased attention as an important alternative to the conventional separation methods mentioned previously (Huie, 2002)

Hot compressed water is a sample preparation technique that combined elevated temperature and pressure with liquid solvents, particularly water. This method has been found to be safe and efficient and is classified as a hydrothermal reaction (Mustafa and Turner, 2011). During hydrothermal reaction, the rise in temperature decreases permittivity of the water resulting in change of the ionic compounds. High temperature of extraction falls in the super-critical and sub-critical regions. This gives several promising opportunities for separation processes and chemical reactions. Under supercritical conditions, substantial amounts of gases and organic substances can homogeneously be mixed with water, which then can be separated by adjusting the subcritical conditions by forming additional phases. This can be combined with chemical reaction occurring in the homogeneous state leading to integrated processes, which are more effective and competitive (Dinjus and Kruse, 2004).

Water under pressurized condition can be applied to extract polar organic compounds or to decompose lignocellulosic materials to produce valuable compounds such as saccharides and aromatic organic acids. The method has been applied to recover protein, amino acids and phenolic compounds (Sereewatthanawut et al., 2008). Depending on the type

of measurements being carried out, it is important to obtain the fundamental knowledge on the extraction of polyphenol through hydrothermal reaction.

1.2. Objectives

The main purpose of this research is to investigate the optimum preparation of mistletoe as medicinal plant by hot compressed solvent extraction method, also determined chemical and biological properties of the resulted extracts. The chemical properties that were investigated are the total phenolic content, phenolic compounds and antioxidant activities. The biological property that was investigated is cytotoxicity of the extract to colon cancer cells. The experiment were done by several steps, they are:

1. Preliminary study on mistletoe: Polyphenol content by LC-MS
2. Optimization of Hot Solvent Compressed Extraction (HSCE) condition to prepare mistletoe as resources of bioactive compounds.
3. Enhancing polyphenol compounds in the extract by the addition of hydroxypropyl-β-cyclodextrin (β-CD) on solvent at HSCE.
4. Application of the optimum HCSE on various types of mistletoes to extract polyphenolic compounds.

CHAPTER 2
Preliminary Study on Indonesian Tea Mistletoe: Polyphenol-Tea Related Content by LC-MS

2.1. Introduction

Mistletoe is a semi parasitic plant, has been used for therapeutic purposes in human cultures for centuries, and the extracts from mistletoes have been used in the treatment of cancer for long time ago. One of the mistletoe varieties that are often used in Indonesia as a medicine is tea mistletoe. It is known as *benalu teh* in Indonesian.

Tea mistletoe is mistletoe that has haustorium penetrated on tea plant (*Camellia sinensis*) as a host plant. As reported by Devkota and Glatzel in 2007, the species of host plant may be partly or wholly responsible for the development of haustorium at the attachment site. Furthermore, host-derived secondary metabolite, play an important role in the interaction between parasitic weds and their hosts. They are involved in signaling, for example in the induction of parasite germination and the formation of the haustorium, and in plant defense against the parasite (Bouwmeester et al., 2003). Therefore, bioactive compounds of mistletoe and its host plant have a close relationship.

Tea is rich in polyphenolic compounds known as tea flavonoids (mainly catechins), which have strong antioxidant properties (Paquay et al., 2000). Tea has been shown to exhibit a potent antioxidant activity by means of its flavonoids (Suganuma et al., 1999). Catechins, based on flavan-3-ols chemical structure, which show three hydroxyl groups in the B ring (position 3', 4', 5') are called gallo-catechins, while a gallic acid substitution in the position 3 of the C ring, is characteristic for catechin-gallate. The more represented catechin of tea are seven: (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (+)-catechin (C), (-)-epigallocatechin-3-gallate (EGCG), (-)epicatechin (EC), (-)-gallocatechingallate (GCG), (-)-epicatechingallate(ECG). The content of the tea catechin isomers can vary among different tea, depending on the species, the climate, the cultural practices and, in the case of tea extracts, on the conditions and technology used for the storage.

Many studies have been published on the antioxidant properties of catechins and their mechanism. Chen and Chan (1996) reported the following order of catechins antioxidant activity EGC>EGCG>EC>ECG as a result of canola oil oxidation tests. Therefore, the antioxidant ability of different tea extracts, as a consequence, their quality vary according to their tea catechins qualitative and quantitative composition to the concentration and more (Gallina et al., 2000).

Concerning the relation between mistletoe and tea as it host plant on bioactive compounds, Kirana (1996) has investigated thoroughly. In general, the amount of individual

flavonols found in the parasitizing mistletoe was always much higher than those found in the respective host plants. However, between different mistletoes, the amount of individual flavonols varied substantially. The HPLC patterns of individual flavonols of mistletoes were different of those of the respective hosts, thus the flavonols with the highest concentration in the mistletoes were not necessarily the highest in the mistletoes's host.

To fulfill preliminary data about bioactive compounds inside mistletoe, its necessity to determine several compounds that related with the host plant by Liquid Chromatography/Mass Spectrometry (LC/MS).

2.2. Materials and methods

Materials

Dried tea mistletoe (*Scurulla atropurpurea* [BL] Dans.) was obtained on September 2008 from Puncak area, Bogor, Indonesia. The specimen was identified at Herbarium Bogoriense Bogor, Indonesia. The intact plant was separated between leaves and stems. All reagents for this research were analytical grade.

Extraction

Sample with 2 g measured was added to 30 mL of 60% ethanol then extracted by sonication for 60 minutes. After that, the extract was centrifuged at 3000 rpm for 5 minutes, then filtered by 5A filter paper on diameter 110 mm. Prior the injection on LC/MS, the extract was filtered once more through 0.2 μm membrane filter (PTFE) and diluted 3 times using methanol. The injection has volume 2 μL.

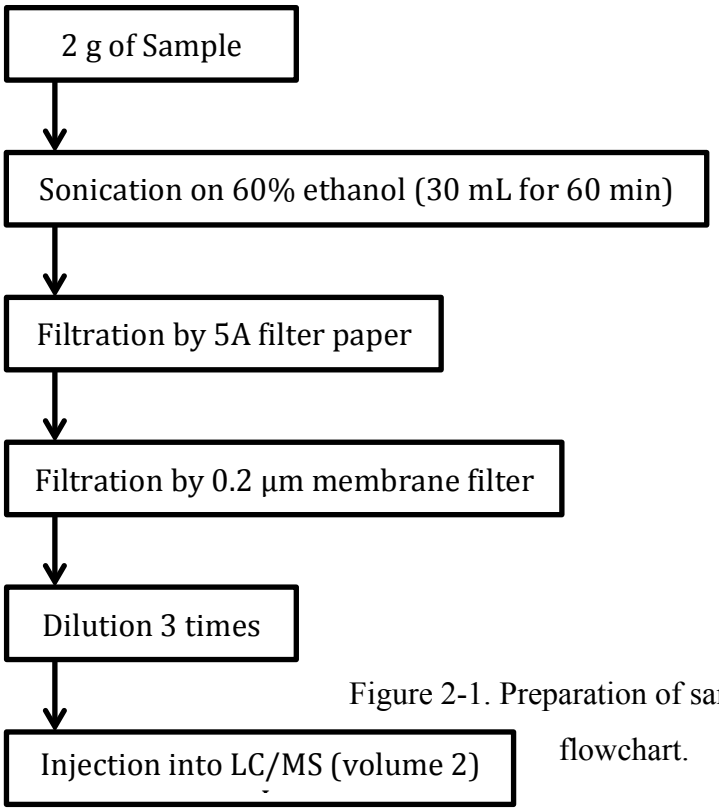

Figure 2-1. Preparation of samples prior to LC/MS analysis flowchart.

LC/MS condition

Experiment was performed with Agilent Zorbax Eclipse plus C18 column (2.1x100 mm i.d. 3.5 µm particle size). The mobile phase was composed of 0.1% formic acid and 2.5 mM ammonium acetate in water:acetonitrile=1:9 (A) and 0.1% formic acid and 2.5 mM ammonium acetate in water:acetonitrile=9:1 (B). The gradient program of which was 0-3 min 100% B, 3-30 min 0% B, 30-45 min 0% B, 45-55 min 100% B, and finally 55-70 min 100% B. The flow rate of the mobile phase was 0.2 mL/min, and the injection volume was 2 µL. The MS condition was under Negative mode, 3500 V ESI needle voltage, and used nitrogen as nebulizer gas.

Table 2-1. Gradient eluent LC/MS

Time (minutes)	Mobile phase A (%)	Mobile phase B (%)
0	0	100
3	0	100
30	100	0
45	100	0
55	0	100
70	0	100

Mobile phase:
A: 0.1% formic acid and 2.5 mM ammonium acetate in water: acetonitrile= 1:9
B: 0.1% formic acid and 2.5 mM ammonium acetate in water: acetonitrile= 9:1

2.3. Result and discussion

Table 2.2. show the concentration of polyphenols in tea mistletoe leaves and stems extracts. Catechin concentrations in both extract were high at 0.01% approximately. If we compared catechin concentration extracts and the one that reported by Li- Kang Ho et al. in 1993, which investigated the chemical component from *Scurrula liquidambaricolus*, catechin

concentration in this sample is very high due to tea mistletoe also producing it own catechin while transported from tea plant as a host plant. In contrary, low concentration of epigallocatechin and methyl-epicatechin-gallate in both sample indicated that both compounds were only transported from tea plant.

From the table, we also can get the information that the polyphenol pattern of stems and leaves extracts was slightly different. Leaves extract has more polyphenol compounds compared with stems extract. In the stem extract, methyl epicatechin-gallate was not detected and the concentration of epicatechin-gallate was lower than leaves extract.

This result showed opposite with the assumption that haustorium contain more secondary metabolites. Abundant of compound can not always make the activities of the extract become stronger. As reported by Murwani (2003), the stems or leaves extract of tea mistletoe increased the sensitivity or susceptibility of WEHI-164 cells to TNFα as shown by decreases in LD_{50} values in TNFα sensitivity assay, showing that the stems extract showed a greater increase than the leaves extract. Although stems extract has less concentration on polyphenolic compounds, but the activities were reported to be higher than leaves extract.

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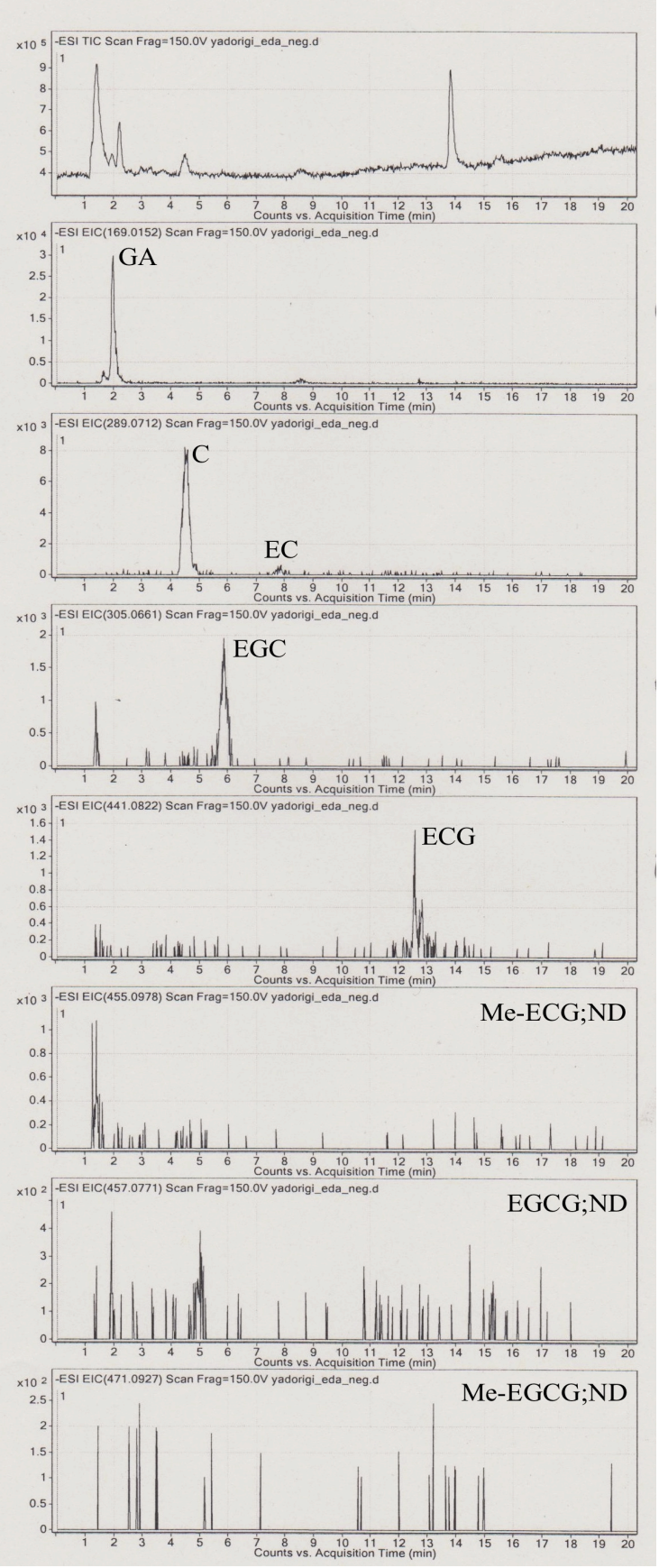
Furthermore, Our method succeeded identify Epigallocatechin in stem extracts, also Epigallocatechin and Methyl-epicatechin-gallate were the first time in tea mistletoe (*Scurrula atropurpurea*). This results were different with the determination of bioactive compound in tea mistletoe that have been done by Ohashi et al., 2003, which they can not determined both Epigallocatechin and Methyl-epicatechin-gallate in tea mistletoe.

Table 2-2. Polyphenol-tea related concentration on tea mistletoe by LC/MS analysis

Compounds	Concentration (%)	
	Leaves	Stems

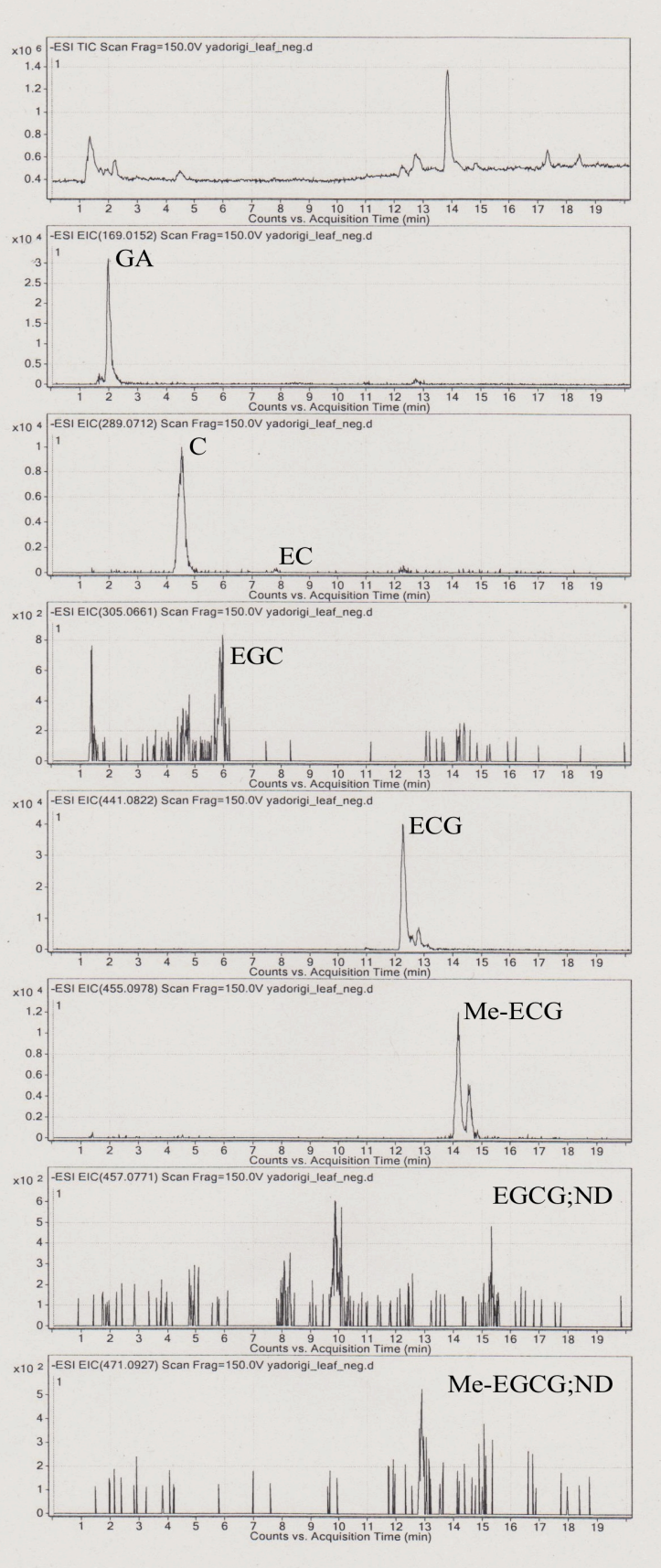
Gallic acid (GA)	0.0006	0.0006
Catechin (C)	0.01	0.01
Epicatechin (EC)	ND	ND
Epigallocatechin (EGC)	0.0002	0.0002
Epicatechin gallate (ECG)	0.002	0.0001
Methyl epicatechin gallate (Me-ECG)	0.002	ND
Epigallocatechin gallate (EGCG)	ND	ND
Methyl epigallocatechin gallate (Me-EGCG)	ND	ND

*ND: Not Detected (minimum detection 2 ppm)



Annotation:
GA: Gallic acid;
C: Catechin;
EC: Epicatechin;
EGC: Epigallocatechin;
ECG: Epicatechin gallate;
Me-ECG: Methyl epicatechin gallate;
EGCG: Epigallocatechin gallate;
Me-EGCG: Methyl epigallocatechin gallate;
ND: Not Detected (minimum detection 2 ppm).

Figure 2-2. HPLC-QTOFMS spectrum of tea mistletoe stem extract



Annotation:
GA: Gallic acid;
C: Catechin;
EC: Epicatechin;
EGC: Epigallocatechin;
ECG: Epicatechin gallate;
Me-ECG: Methyl epicatechin gallate;
EGCG: Epigallocatechin gallate;
Me-EGCG: Methyl epigallocatechin gallate;
ND: Not Detected (minimum detection 2 ppm).

Figure 2-3. HPLC-QTOFMS spectrum of tea mistletoe leaves extract.

2.4. Conclusion

This experiment showed that the bioactive content inside tea mistletoe has a close relationship with their host plant, tea. However, the concentration of each compounds were differ between each part either with the host plant. Considering the bioactive content inside tea mistletoe that has close relationship with tea, this fact make interesting to explore more about the bioactive compounds especially polyphenol inside tea mistletoe.

CHAPTER 3

The Effects of Different Solvent, Temperature and Time by Hot Compressed Solvent Extraction on Antioxidant Activity of Tea Mistletoe (*Scurulla atropurpurea* [BL] Dans.)

3.1. Introduction

Mistletoe is a semi-parasitic plant that is considered as an unwanted plant to

economically important horticultural plant, however in the other side, mistletoe has been known as one of medicinal plant used in traditional therapy. One of mistletoe often used in Indonesia as medicine is tea mistletoe. It's locally called as *benalu teh*. It is sold by kiosk along the road in Puncak, West Java, and also has been cultivated by people in small-scale.

Tea mistletoe is grows in Java and Sumatera. It is a woody stem epiphyte, cylindrical twigs brown spotted. It has single leaf type, across, oval with spiked tip, plat perimeter and has area of 5 – 10 cm length and 2.5 – 5 cm width. The upside of a leaf is shine and the bottom is vague. It has composite flower in an umbrella type, 2 – 8 flowers in a leaf base or stem, corolla has four part curved and red stamen 2-3 mm, its fruits are brown of 8 mm in length. Its seed is small and black (Sunaryo *et al*., 2007). It consists epiphytic and hemi parasitic plants which adhere to branches and twigs of tree by means of haustoria (a kind of root system) which penetrate into the host in order to absorb water and nutrient (Ribeiro *et al.*, 1999).

The main active principles in *tea mistletoe* include bioactive lipid, alkaloids, flavonoids and others component (Ohashi *et al*., 2003). Flavonoids inside *benalu teh* consist of quercetin and its glycoside, which have strong antioxidant activity (Ikawati *et al*., 2008).

Antioxidant is a compound that has ability to inhibit oxidation rate or to neutralize a free radicals (Roberts K., 2007). Oxidative damaged was caused by free radicals that may be related to aging and disease, such as atherosclerosis, diabetes, and cancer. So that antioxidant inside *benalu teh* has big potential to develop.

In the context of extraction, the traditional extraction using method by boiling in the water until the solution one-third remains which is not effective from time and energy that consumed. Instead different technologies may be applied to obtain extracts with a high concentration of selected active principles. Moreover, solvents of various polarities and temperatures can be utilized to adjust the selectivity of it (Valle *et al*., 2005). On this research I used hot compressed solvent extraction (HCSE) to keep the solvent in liquid phase even on high temperature by adding the pressure higher than vapor pressure. By this technology it is expected to obtain better result, than by the traditional method.

Ethanol, a polar solvent, effectively extracts flavonoids and their glycosides from raw materials (Bazykina *et al*., 2002). The solubility of these compounds can be further enhanced using a mixed solvent over a limit compositional range (Cacace and Mazz 2003). Particularly, mixtures of alcohol and water have revealed to be more efficient in extracting phenolic constituent than the corresponding mono-component solvent system (Yilmaz and Romeo, 2006).

Temperature and time of extraction is important parameter to be optimized even in order to minimize energy cost of the process. In fact, an increase in the working temperature extraction enhances both the solubility of the solute and the diffusion coefficient, but beyond a certain value phenolic compounds can be denaturized (Yilmaz and Romeo, 2006).

This research`s compare the traditional extraction with batch rector extraction, also to examine the extraction with different treatments of solvent, temperature and time to obtain antioxidant compounds inside the tea mistletoe. Then, I analyze the antioxidant activities of the extracts by radical scavenging activities and total phenolic content. The best extraction condition is further used for HPLC analysis to figure the flavonols content in the extract.

3.2. Materials and methods

Materials

Dried *benalu teh* (*Scurulla atropurpurea* [BL] Dans.) was obtained on September 2008 from Puncak area, Bogor, Indonesia. The specimen was identified at Herbarium Bogoriense Bogor, Indonesia. The intact plant was grinded by Willey mill 1029- A type Yoshida Seisakusho Co. with screen size 1 mm into powder as the range size of 200-500 μm. All reagents for this research were analytical grade.

Extraction

Extraction was examined by traditional method (=decoction/boiling) and batch reactor that has specified tube reactor made from 1/2 inch SUS316 stainless tube with volume 6 mL and mantle heater made from stainless vessel (fig.1). Traditional extraction started with measured 15 g of sample then added with water 300 mL, next boiled under bunsen burner until the volume 100 mL remain (1-2 hours). While 0.5 g of samples were then extracted by HCSE with the treatments different solvent 5 mL water or ethanol (30, 50, 70 and 99 %) at temperature 100^0 C for 10 minutes and 0.2 MPa of additional pressure was applied by adding N_2 gas for preventing from phase change of solvent at higher temperature of boiling point. Next the reactor was immersed into oil bath for heating. After predefined time heating, the reactor was immersed into cooling bath. So the necessary time to increase and decrease the solvent temperature were about 120 and 30 second, respectively. In addition, solvent temperature is measured by K-type thermocouple that located inside the reactor. So the temperature was measured directly, accurately and without delay. Then another treatment

applied with 40 ˚C-180˚C temperatures using the best solvent treatment whiles other conditions were static. Last the treatment of different time 10, 15 and 20 minutes were used the best solvent and temperature condition while pressure static. After cooling down, the extracts were filtered by 2 step filtration using paper filter 90 mm then 0.2 µm syringe filter, next evaporated and concentrated by centrifugal evaporation (EYELA CVE 2000) in vacuum condition at temperature 80 ˚C. Finally the extracts were lyophilized to give an amorphous powder. For chemical analysis sample, the dry extract was dissolved with water in 1 mg/mL (w/v) concentration.

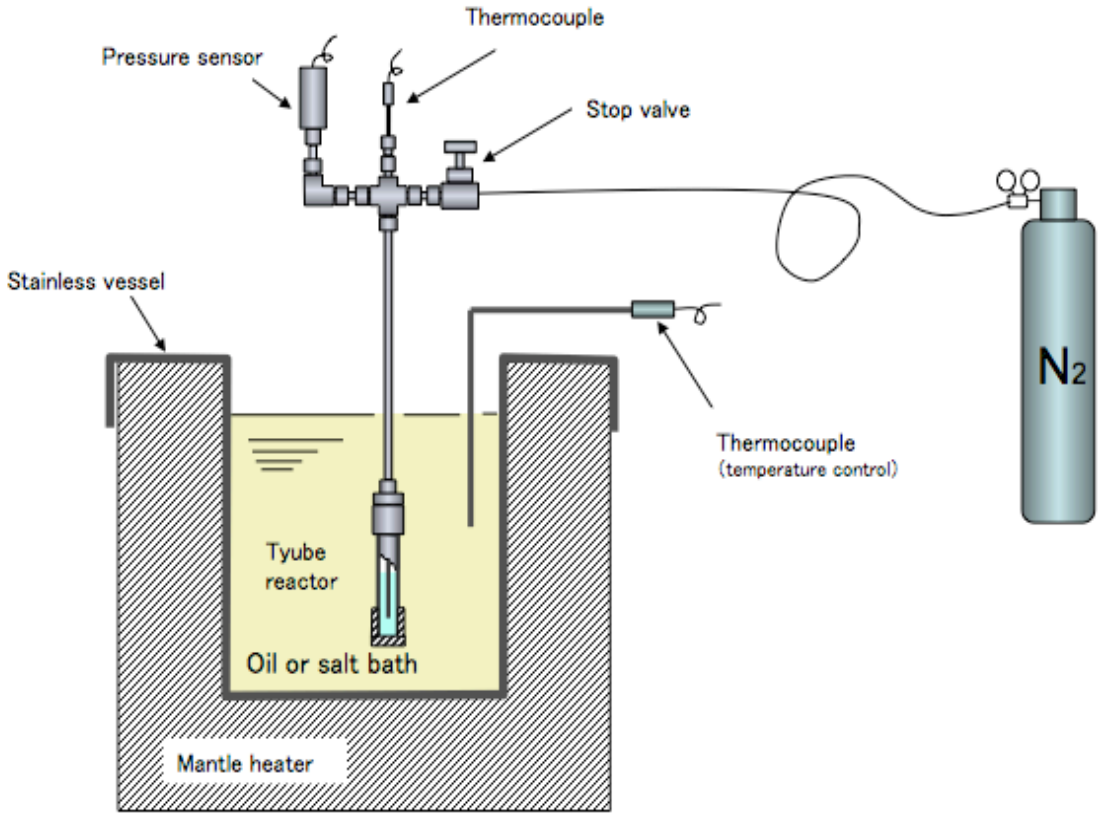


Figure 3-1. Hot compresses solvent extraction by batch reactor extraction type.

Antioxidant activities (ABTS assay)

Antioxidant activity of extracts was measured as the ability of ABTS or 2,2'-azino-bis(3-6-sulfonic acid-Ethylbenzthiazoline) diammonium salt radical cation reduction capability. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{+}$) was produced by reacting 5 mL ABTS stock solution with 140 mM potassium persulfate 88 µL and allowing the mixture to stand in the dark at room temperature for 12–16 h. Next the $ABTS^{+}$ solution was diluted with ethanol 155 mL. Each samples (1 mg/mL; w/v) and trolox as standard (0.2 mM) were diluted in ethanol until 1.5 ml and then mixed with 1.5 mL $ABTS^{+}$ solution. After that stand in the water bath 25 ˚C for 10 minutes, absorbance at 630 nm was measured by UV-VIS JASCO V-530 spectrophotometer (Pellegrini *et al*., 1999).

Total phenolic content

Total phenolic content is based on the method of Slinkard and Singleton (1977) with slight modification in the volumes that have been reduced. First made gallic acid stock solution was to result standard curve. From each calibration solution, sample, or blank, pipette 20 µL into separated cuvettes. Next, were added 1.58 mL water and 100 µL of the Folin-Ciocalteu reagent (Sigma Aldrich), and mixed well. Waiting for 30 second to 8 minutes, and 300 µL of the sodium carbonate solution was then added, and shaken to mix. The solutions were left at 40 ˚C for 30 minutes and determined the absorbance of each solution at 765 nm against the blank.

HPLC analysis

The authentic standards quercetin and rutin, were purchased from Sigma Aldrich Japan, and Quercetin 4'-*O*-β-glucopyranoside (Quercetin 4'-glucoside) from Polyphenols Laboratories Co, Norway. An Intelligent HPLC Pump 880-PU was used with column Ascentis Sigma Aldrich Amine $_{18}$ (length x I.D, 250 mm x 5.0 mm i.d) and a column heater Sugai U-620 type VP50 (36 ˚C). Mobile phase were 0.2% formic acid and methanol with flow rate 1 mL/min and concentration gradient elution 25 – 70% ended at 50 minutes and

changed linearly. Flavonols were detected by Hitachi L-4000 UV detector (360 nm). First dry extract was dissolved with solvent used in the extraction in 1 mg/mL (w/v) concentration. Then the extracts were injected to HPLC through 20 µL sample loop, after filtered by 0.2 µL syringe filter.

3.3. Result and discussion

This experiment showed the extract yield from different extractions and treatments is 32-136 mg/g dry base tea mistletoe (w/w). The traditional extraction method yielded 52 mg/g dry base tea mistletoe. On the other hand, the using of mixture ethanol and water as solvent increased the extract yield compared with traditional extraction that using water as solvent extraction. Moreover, batch reactor extraction could increase the extract yield on shorten time, only 10 minutes, at 100^0 C and above compared with the traditional one. All data of extract yield are shown in Figure 3-2.

Conventional extraction methods have several drawbacks; e.g. they require a long extraction time and result in low yields of extraction such as traditional extraction. Another attractive alternative to obtain antioxidants is the use of batch reactor. It is used a polarity solvent extraction with high temperature (above 100 ˚C). Batch reactor system also use the addition of pressure to maintain the solvent in liquid state.

The highest activities of antioxidant and total phenol content at 30% ethanol (Figure 3-3) were obtained. The radical scavenging activities with the treatment of different temperature were in the range 140-160 µg trolox equivalent/g dry base tea mistletoe. The highest total phenolic content was obtained by 30% ethanol solvent extraction treatment, its 2.6 mg equivalent gallic acid/g dry tea mistletoe. The water as solvent extraction only resulted approximately a half value of total phenolic content that produced by 30% ethanol solvent treatment, it was 1 mg equivalent gallic acid/g dry tea mistletoe.

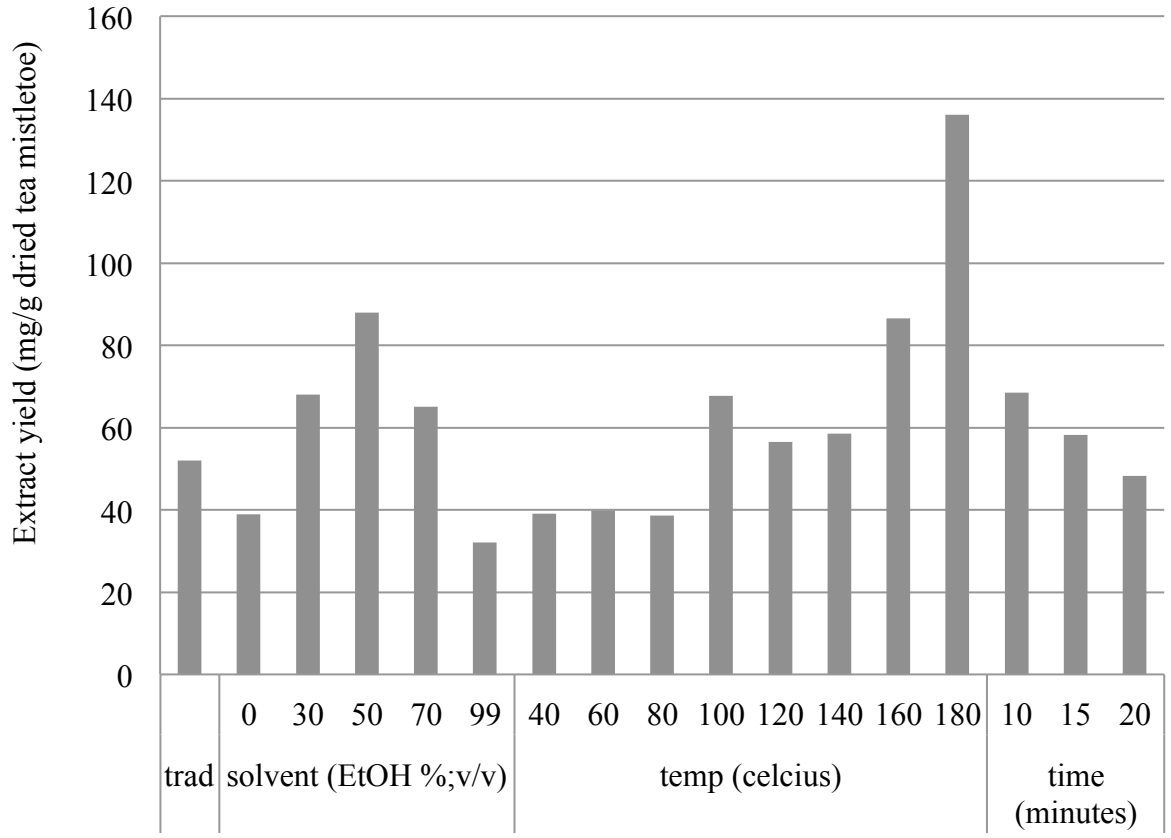


Figure 3-2. Extractions yield of all treatments. Trad (traditional extraction; boiling in the water until one-third remain) and batch reactor extraction with treatment solvent (solvent 0, 30, 50, 70, and 99% EtOH at temperature 100 ˚C for 10 minutes under pressure 0.2 MPa), temperature (temperature 40 ˚C -180 ˚C with solvent 30% EtOH, for 10 minutes under pressure 0.2 MPa) and time (time 10-20 minutes with solvent 30% EtOH at temperature 100 ˚C under pressure 0.2 MPa).

Alcoholic solvent has been commonly employed to extract phenolics from natural sources. It gave high yield of total extract even though it was not highly selective for phenols. Particularly, mixtures of alcohol and water have revealed to be more efficient in extracting phenolic constituent than the corresponding mono-component solvent system (Yilmaz and Romeo, 2006).

The treatments of different temperature were shown similar value on the antioxidant activities (Figure 3-4). It is assumed that the active compound inside tea mistletoe already extracted at lower temperature. So it's not necessary to use high temperature. On the other hand fast and efficient extraction is achieved by applying high pressure and elevated temperature. It was found that higher temperature treatments resulted in higher recovery rates (Pineiro *et al*., 2004).

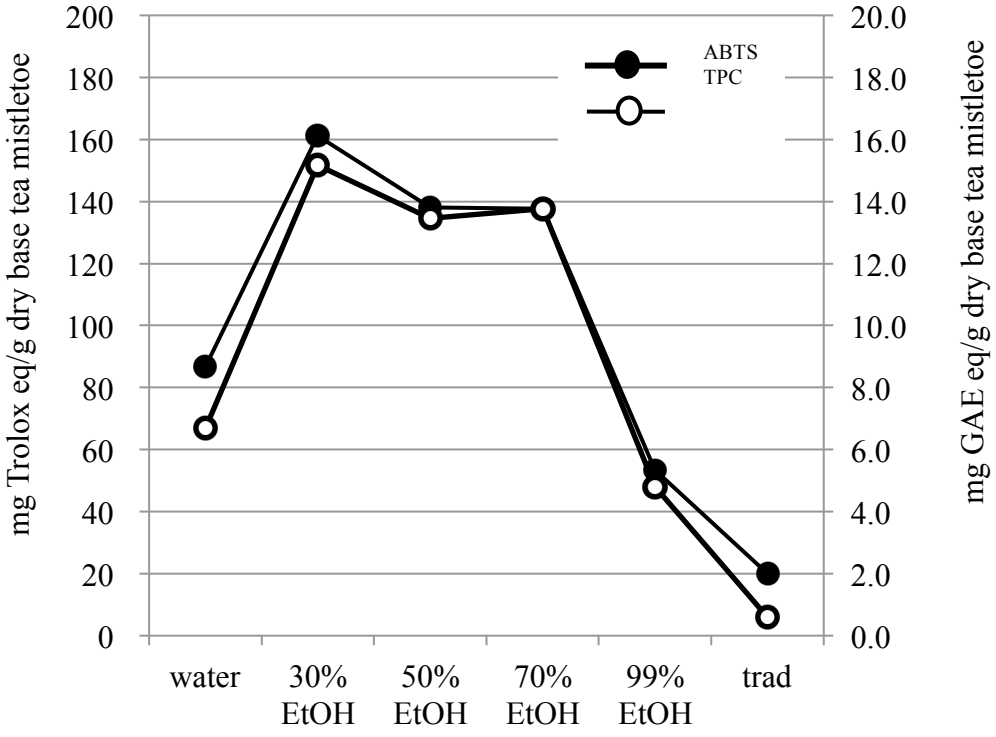


Figure 3-3. Radical scavenging activities (mg Trolox eq/g dry base tea mistletoe) and total phenolic content (mg GAE eq/g dry base tea mistletoe) values from different treatment of solvents (0, 30, 50, 70, 99% EtOH) at temperature 100 ˚C for 10 minutes under pressure 0.2 MPa and traditional extraction (trad).

Total phenolic content from treatment of different temperature showed increasing value by increasing temperature until temperature 100 ˚C, but then it decreased by temperature 140 ˚C, and it slightly increased again at 160 ˚C (Figure 3-4). It is slightly different in total phenolic content between temperature 100 ˚C and 160 ˚C. The value of total phenolic content on temperature 100 ˚C was 3 mg gallic acid equivalent/g dry tea mistletoe.

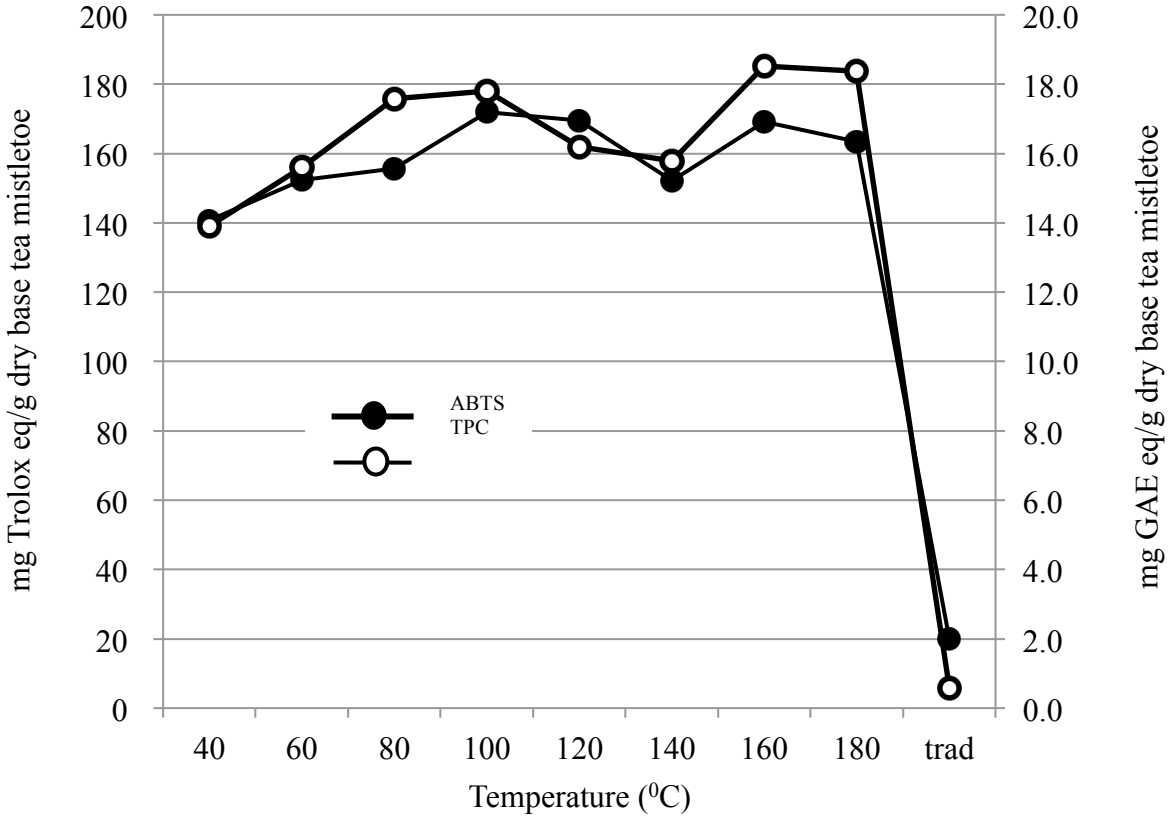


Figure 3-4. Radical scavenging activities (mg Trolox eq/g dry base tea mistletoe) and total phenolic content (mg GAE eq/g dry base tea mistletoe) values from different treatment of temperature (40˚C-180˚C) with solvent 30% EtOH, for 10 minutes under pressure 0.2 MPa and traditional extraction (trad).

The increasing value again on 160 ˚C after decreasing moment start on 120 ˚C can

cause by temperature extraction is too high. So not only flavonoid compound was extracted but also decomposed the hemicelluloses and lignin in the tea mistletoe. Moreover, at temperature 100 ˚C obtained the optimum extract of flavonoid compound inside the tea mistletoe thus on high temperature solvent inside the batch reactor kept on the liquid state due to the addition of pressure. Then solvent's polarity was decreased and antioxidant solubility was increase, as temperature increased (Kruse and Dinjus, 2006).

The different time treatment (Figure 3-5) showed that the 10 and 15 minutes have same value on radical scavenging activities it were 168µg trolox equivalent/g dry base tea mistletoe and total phenolic content it's were 2.6 mg gallic acid equivalent/g dry tea mistletoe. Then on 20 minutes all the analysis value was decreased, it's become 145µg trolox equivalent/g dry base tea mistletoe for radical scavenging activities and 1.4 mg gallic acid equivalent/g dry tea mistletoe for total phenolic content. The different time treatment showed that the longer of extraction time made the chemical analysis value decreases. It caused by phenolic compound gradually denaturized by association of increasing time and temperature. The shorter time of extraction can minimize the damaged of phenolic compound. Chemical analysis data result can be seen in the Table 3-1.

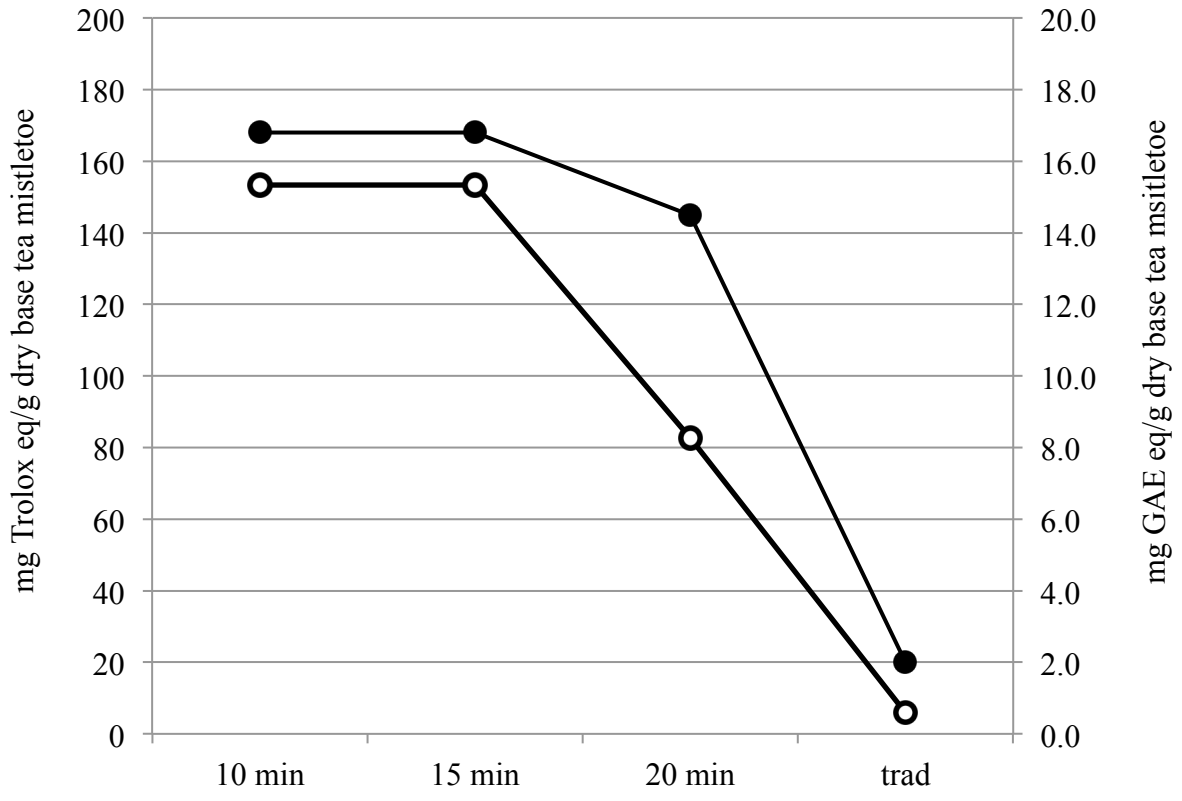


Figure 3-5. Radical scavenging activities (mg Trolox eq/g dry base tea mistletoe) and total

phenolic content (mg GAE eq/g dry base tea mistletoe) values from different treatment of time 10-20 minutes with solvent 30% EtOH at temperature 100 ˚C under pressure 0.2 MPa and traditional extraction (trad).

Tabel 3-1. ABTS (mg Trolox eq/g dry base tea mistletoe) and TPC (mg GAE eq/g dry base tea mistletoe) values from different treatment of solvents, temperatures and times extraction condition.

Assay	Extraction Treatment															
	Solvent (ethanol; v/v)					Temperature (˚C)								Time (minutes)		
	0%	30%	50%	70%	90%	40	60	80	100	120	140	160	180	10	15	20
ABTS	87	161	138	138	53	141	152	156	172	170	152	169	163	168	168	145
TPC	1.1	2.6	2.3	2.3	0.8	2.4	2.6	3.0	3.0	2.7	2.7	3.1	3.1	2.6	2.6	1.4

The chemical analysis data showed that the optimum extraction condition was 30% ethanol solvent at temperature 100 ˚C for 10 minutes under 0.2 MPa. After that the flavonoid content inside it was analyzed by HPLC (Figure 3-6). Rutin was the highest flavonoid compound in the extract; it has 0.026% (w/w). Then followed by quercetin-4-glucoside that has 0.003% (w/w) in the extract (Figure 3-7).

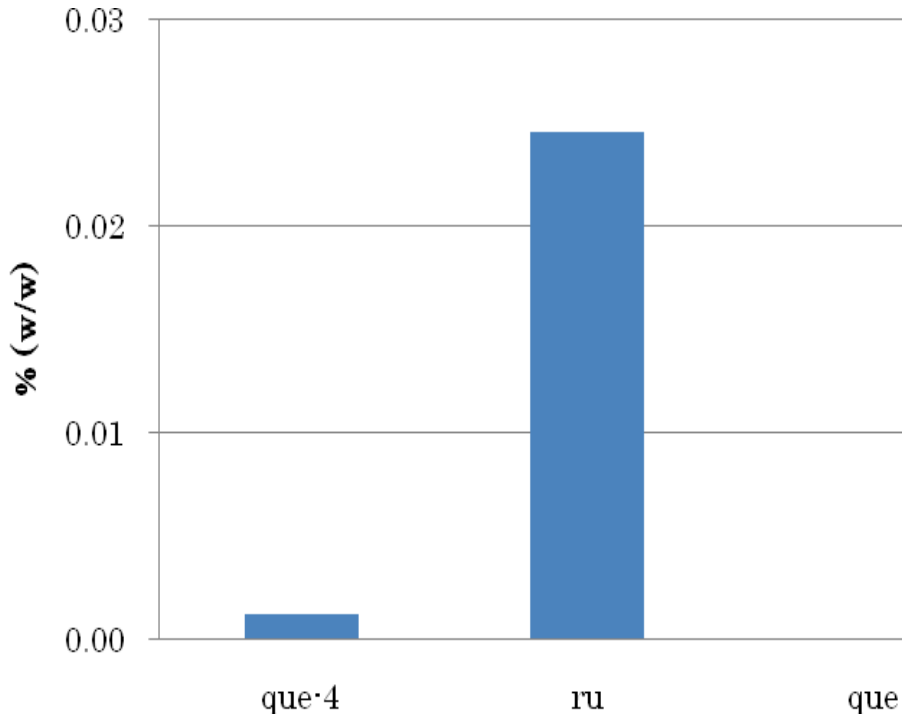


Figure 3-6. Flavonoid content from tea mistletoe extract with extraction condition 30% ethanol solvent at temperature 100 ˚C for 10 minutes under 0.2 MPa

The main compound of the extract was rutin (Figure 3-8) that determined by HPLC analysis. Higher water composition in the solvent can aid in the extraction of glycoside flavonoid, although due to complexity of heterosidic combinations, certain groups of flavonoids, such as flavones and flavanols, are not generally characterized as intact compound but in the form of their aglycones (Tsao and Deng 2004).

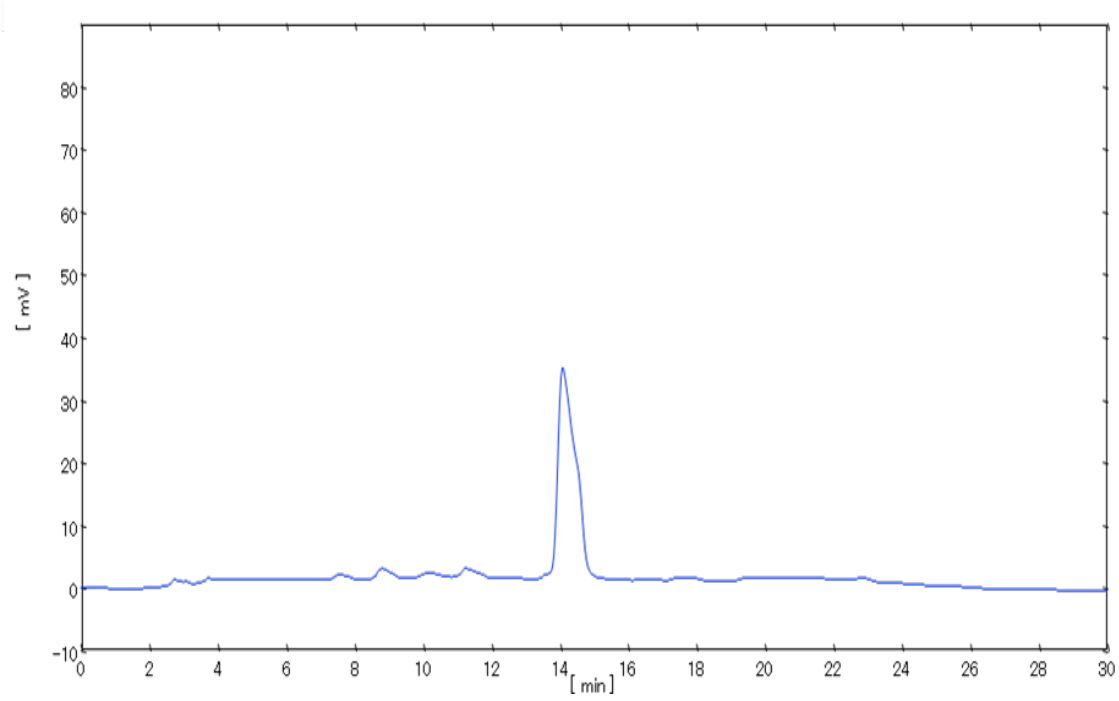


Figure 3-7. Chromatogram of extract tea mistletoe with extraction treatment 30% ethanol solvent at temperature 100 ˚C for 10 minutes under 0.2 MPa; a) rutin

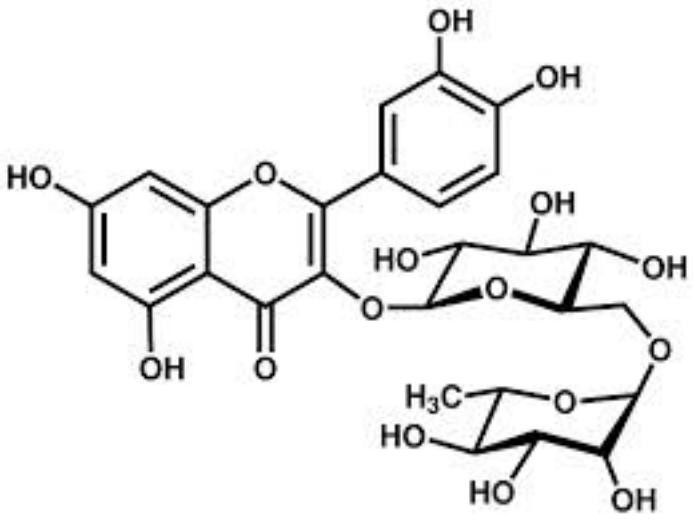


Figure 3-8. Chemical structure of rutin

Tea mistletoe has flavonoid compound inside the leaf and their stem. As a semi parasitic plant, tea mistletoe still does their photosynthesis. But on their stem was also found the flavonoid compound that cause by haustorium. Haustorium serves several functions for the parasite: they attach the parasite and the host, they invade host tissue through a combination of enzymatic and physical processes, and they serve as the physiological conduct through which the parasite robs the host plant of water and nutritional resources (Albrecht *et al*., 1999). Many of active haustorium-inducing molecules are common constituents of plant cell, where they function in lignin biosynthesis, host defense and other specialized physiological processes, including phenolic compound such as flavonoid.

3.4. Conclusion

Tea mistletoe as a semi parasitic plant has a potentiality as a nutraceutical resource, because it has a flavonoid compound. Batch reactor extraction gave a better result than traditional extraction on extract yield, radical scavenging activities and total phenolic compound. Furthermore the best extraction is treated with solvent 30% ethanol at temperature 100 ˚C for 10 minutes. The main flavonoid compound of the extract is rutin, as nutraceutical compound reported.

CHAPTER 4

Antioxidant and Anticancer Activities Effect of Tea Mistletoe Prepared By Hot Compressed Solvent Extraction with Cyclodextrin

4.1. Introduction

Mistletoes are parasites of the closely related Loranthaceae or Viscaceae families. Mistletoes have haustoria that originate as root tissue and penetrate into the stems or roots (in Loranthaceae) of the host plant (Smith et al. 2001). Like other plant and animal parasites, mistletoes also live in an intimate association with their hosts, derive nutrition from the host and share a life-long association with a single individual host. Mistletoes have recently been described to be both agricultural pests and a threatened species in different parts of the world (Norton and Reid, 1997). However, mistletoes are also known to be medicinal plants that can be used in traditional therapies. One of the mistletoe varieties that are often used in Indonesia as a medicine is tea mistletoe. It is known as *benalu teh* in Indonesian.

A previous study showed that a hot compressed solvent extraction (HCSE) methodology resulted in a more effective extraction than the traditional extraction process, higher antioxidant activity and greater total phenolic content. Furthermore, the optimal extraction treatment involves the use of 30% ethanol as the solvent and extraction at 100 °C for 10 minutes under 0.2 MPa of pressure. The main flavonoid compound that is found in the extract is rutin (Rahmawati and Hayashi, 2012).

We added hydroxypropyl-β-cyclodextrin (β-CD) to the extraction solvent to enhance the solubility of the bioactive compounds in mistletoe. β-CD is capable of forming inclusions with many compounds, which improves its chemical stability, increases the apparent aqueous solubility, and results in a higher bioavailability without changing its pharmacokinetics properties (Tommosini et al. 2004; Li et al. 2003). Cyclodextrins are a family of cyclic oligosaccharides that are composed of α-(1-4) linked glucopyranose subunits. Among the three types of cyclodextrins, β-CD is the most accessible, is the lowest priced and is generally the most useful (Valle 2004).

This work was investigated the influence of the addition of β-CD on the extraction treatment protocol, which was determined by measuring the yield of the extract, the antioxidant activity and the total phenolic content. The extracts were also evaluated for cytotoxic effects in colon cancer lines (HT-29 and Caco2 cells) and a normal cell line (Balb/c 3T3 mouse cells).

4.2. Materials and methods

Material

Dried plant material of tea mistletoe (*Scurulla atropurpurea* [BL] Dans.) was collected in September 2008 from Puncak area of Bogor, Indonesia, and was identified at the Herbarium Bogoriense in Bogor, Indonesia. Intact mistletoe was ground into a powder by using a Willey mill (1029- A type Yoshida Seisakusho, Japan) with a screen size of 1 mm. Samples were kept in a controlled, low-humidity dry cabinet until they were needed. Only analytical grade reagents were used in this study.

Extraction

During the pre-study, I used extraction treatments in which we compared β-CD additions with the sample weights (1:1, 1:10 and 1:100 ratios). The best extraction occurred with a β-CD to sample weight ratio of 1:100 based on the total phenolic content (TPC) and the antioxidant activity (ABTS assay) value (data not shown). The main study used an extraction protocol that employed the traditional method (=decoction/boiling) and HCSE as explained in a previous report (Rahmawati and Hayashi, 2012).

Extractions with the HCSE method used 30% ethanol at 100 ˚C for 10 minutes under 0.2 MPa pressure and were then treated or not with β-CD. Moreover, as reported by Li et al. (2009), the use of 0.5% ethanol gave better results because it enabled the formation of β-CD with the triterpenoid complex. I therefore used a

HCSE method that consisted of a 0.5% ethanol at 100 ˚C for 10 minutes under 0.2 MPa of pressure with the addition of β-CD. The extraction treatments are shown in Table 4-1.

Table 4-1. Extraction treatments

Extract code	Extraction method	Sample weight	β-CD	Solvent	Time	Pressure
30	HCSE	0.5 g	-	30% EtOH	10 min	0.2 MPa
30hp	HCSE	0.5 g	0.05 g	30% EtOH	10 min	0.2 MPa
0.5hp	HCSE	0.5 g	0.05 g	0.5% EtOH	10 min	0.2 MPa
Trad	Decoction	15 g	-	dH_2O	2 hours	-

*HCSE: hot compressed solvent extraction

Analysis of total phenol content

Total phenol content was measured using the Folin-Ciocalteu reagent, which was described by Javanmardi et al. (2003). To 50 µL of each extract, 2.5 mL of a 1/10 dilution of Folin-Ciocalteu reagent and 2 mL of 7.5% (w/v) Na_2CO_3 were added and then incubated at 45 ˚C for 15 minutes. We measured the absorbance at 765 nm using a UV-Vis JASCO V-530 spectrophotometer. As a standard, several concentrations of gallic acid were used, and the results were expressed as milligrams of gallic acid equivalent per gram of dry base mistletoe (mg GAE/g dry mistletoe).

Antioxidant activity (ABTS assay)

The antioxidant activities of the extracts were measured with the ABTS assay proposed by Re et al. (1999), with a slight modification. The ABTS powder was first dissolved in water and then reacted with potassium persulphate to produce the ABTS radical cation ($ABTS^+$). The mixture was then allowed to stand in the dark at room temperature for 12-16 hours before it was used. The $ABTS^+$ solution was then diluted with ethanol to an absorbance at 734 nm of 0.70 (+0.02). One milliliter of diluted $ABTS^+$ solution was added to 1 ml of 1 mg/mL H_2O solution. The trolox equivalent antioxidant activity was determined as mM TEAC/g dry mistletoe, using the absorbance at 734 nm that was measured 6 minutes after mixing.

Cytotoxicity assay

Human HT-29 (HTB-38^{TM}) and Caco-2 colorectal adenocarcinoma cells (HTB-37^{TM}) were purchased from Summit Pharmaceuticals International. Normal cells Balb/c 3T3 (IF050070) fibroblast mouse cells were purchased from the Japan Health Science Foundation. Caco-2 and Balb/c cells were cultivated in 175 cm^2 flasks in DMEM medium, while HT-29 cells were cultured in McCoy's medium. The media were supplemented with 10% FBS (Fetal Bovine Serum), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 ˚C.

I used the neutral red assay to determine the cytotoxicity (O'hare and Atterwill, 1995). The individual wells of a 96-well plate were inoculated with 100 μL of medium containing 5 x 10^4 cells and then incubated for 48 hours. Media (100 μL) containing several concentrations of extract (1μg/mL-10 mg/mL medium) were added to the wells, and incubation continued for another 48 hours. The cells were treated with 100 μL of neutral red at a concentration of 25 μg of neutral red dye per ml of medium for 3 hours in an incubator. The cells were rinsed with D-PBS and added fixation solution. Following an additional brief agitation, the absorption at 540 nm was measured in a microtiter plate reader (immunoMini NJ-2300 InterMed, Japan) using the blank as the reference.

The viability was calculated relative to the untreated cell control [y_0], which was set to 100% viability. A lysis control [y_{100}] was defined as an absorbance of 0, which showed 100% cell death. The resulting curves were fit, and IC50 values were calculated as the concentration [c] at which the viability of the cells reached 50% using the Hill equation with four parameters. The Hill coefficient is defined as [H].

$$y = y_0 + \frac{(y_{100} - y_0)[c]^H}{[IC_{50}]^H + [c]^H}$$

Statistical analyses

The results of the performed assays are presented as the mean ± standard error of the mean (S.E.M), which was determined in triplicate under the respective experimental conditions. The significance of the differences in the results was determined by using a simple one-way analysis of variance (ANOVA), with Statplus® software on a Mac. P values < 0.05 were considered to be significant.

4.3. Results and discussion

Extract yield

As shown in Figure 1, all of the extraction yields were significantly different than one another ($p < 0.001$). The lowest yield of extract was obtained with the traditional extraction process. Furthermore, compared with the HCSE methods, which resulted in 10-folds higher yields of extract, the traditional extraction can be considered to be ineffective. The traditional extraction method required a long extraction time but resulted in a low yield of extract. The highest yield was obtained with the 30hp method and resulted in 188-mg/g dry of mistletoe. The HCSE yield showed the influence of ethanol concentration and the addition of β-CD. However,

the treatments with or without addition of β-CD in 30% ethanol did not result in significantly different yields ($p > 0.05$).

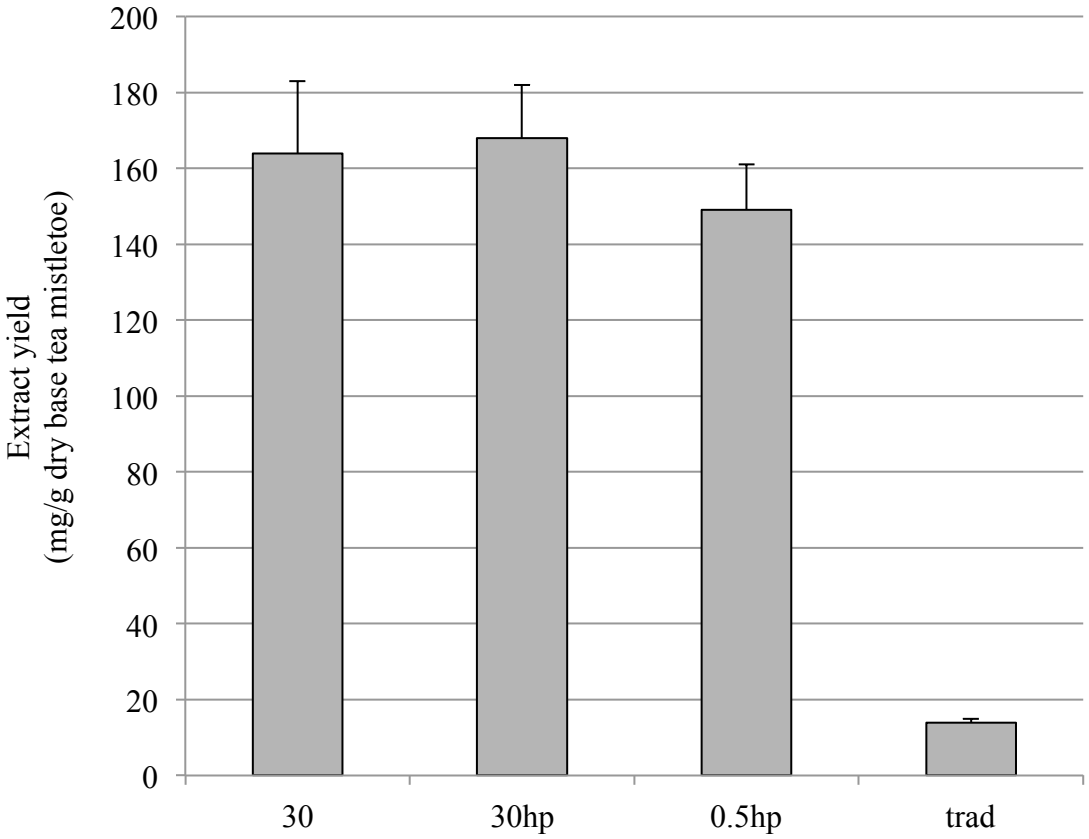


Figure 4-1. Yield of several treatment extractions. 30: HCSE using 30% EtOH, a 10 min extraction and 0.2 MPa of pressure; 30hp: HCSE using 30% EtOH, a 10 min extraction and 0.2 MPa of pressure with the addition of β-CD; 0.5hp: HCSE using 0.5% EtOH, a 10 min extraction and 0.2 MPa of pressure with the addition of β-CD; trad: Traditional extraction procedure.

Benthin et al. (1999) reported that high temperature-pressurized extraction was superior to other extraction methods for medicinal crude herbs analysis in terms of recovery, extraction time and solvent consumption. Moreover, alcoholic solvents provide high yields of the total extract but were not selective for a number of compounds. The addition of cyclodextrin also increased the yield because the potential guest list for molecular encapsulation in cyclodextrin is quite varied. These

compounds include straight or branched chain aliphatics, aldehydes, ketones, alcohols, organic acids, fatty acids, aromatics, gases, and polar compounds, such as halogens, oxyacids and amines (Schmid 1989). Thus, the highest yield can be obtained with the 30hp procedure.

Total phenolic content (TPC)

The highest TPC value was produced with the 30 extraction procedure. In contrast, the traditional extract had the lowest TPC value (Figure 4-2). The concentration of ethanol in the HCSE has an important role in the extraction of phenolic compounds. Because the p value of the HCSEs for all of the treatments was bellow 0.001, so TPC values were determined to be significantly different. Meanwhile, the 30hp extract was resulted in a lower TPC value than the 30 extract. However, the TPC values were not significantly different. These results may be due to the solubility of the phenolic compounds and their influence on the compound-cyclodextrin binary complex and the competitive binding of ethanol to molecules of β-CD (Garcia-Rio et al. 2006). It is therefore possible that β-CD binds ethanol better compared to phenolic compounds.

Casazza et al. (2011) reported that the use of the HCSE methodology resulted in an extract that was rich in polyphenol. HCSE is also more user-friendly, allows for a shorter extraction time and requires significantly less organic solvent (Huie 2002). The use of hot liquid solvents in HCSE can partly overcome strong analyte-matrix interactions, including with more polar compounds. Moreover, alcohols also play an important role in extracting phenolic compounds. Alcoholic solvents have been commonly employed to extract phenolics compounds from natural sources. Mixtures of alcohol and water have been shown to be more efficient in extracting phenolic compounds than the corresponding mono-component solvent system (Yilmaz et al. 2006).

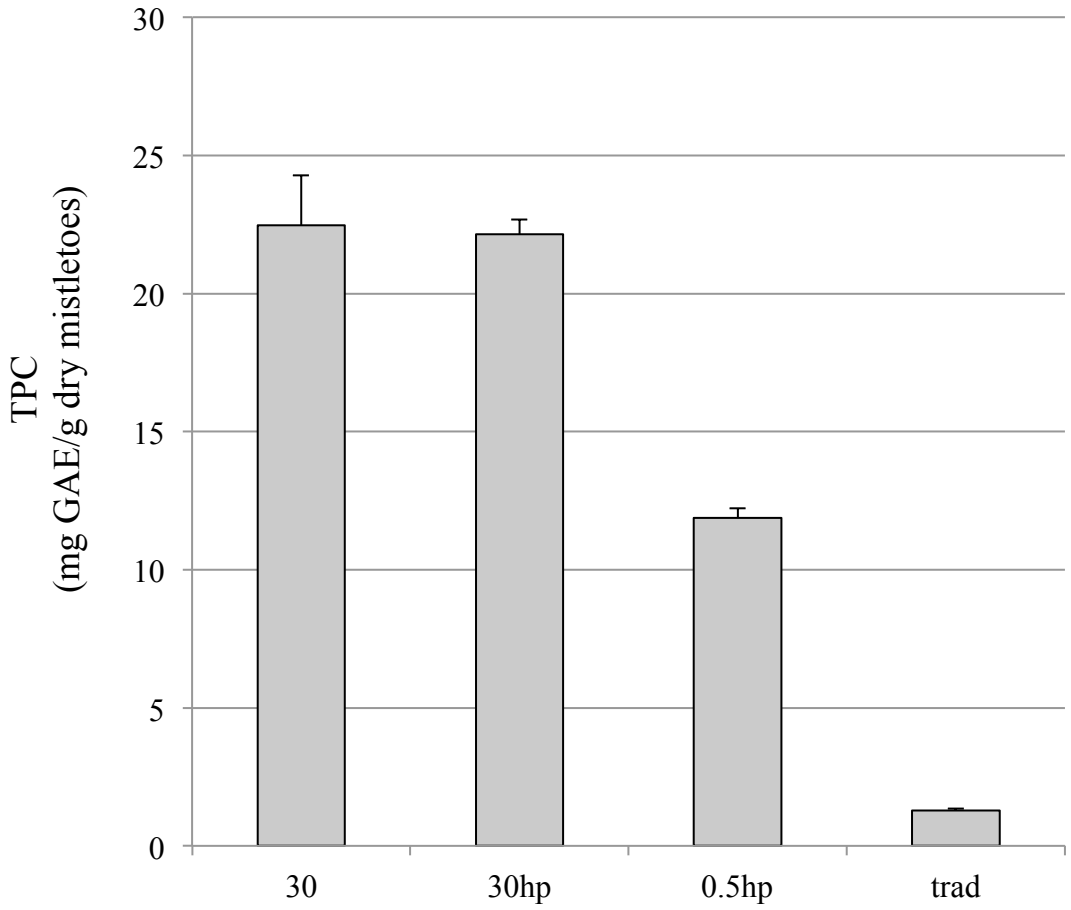


Figure 4-2. Total phenolic content (TPC) of several treatment extractions. 30: HCSE using 30% EtOH, a 10 min extraction and 0.2 MPa of pressure; 30hp: HCSE using 30% EtOH, a 10 min extraction and 0.2 MPa of pressure with the addition of β-CD; 0.5hp: HCSE using 0.5% EtOH, a 10 min extraction and 0.2 MPa of pressure with the addition of β-CD; trad: Traditional extraction procedure.

Antioxidant activity

The highest antioxidant activity was obtained with the 30 extract, and the lowest activity was found with the traditional extract (Figure 4-3). All of the extracts antioxidant activities were significantly different than one another ($p < 0.001$). The different activities may be due to method of extraction and the concentration of ethanol. Several factors, including the temperature, the pressure of the extraction and mixture with other solvents, can influence the efficiency of extraction (Mustafa and

Turner 2011). Herero et al. (2004) reported that extraction yield increased as the polarity of the organic solvent was increase. Thus, ethanol extracts showed a high antioxidant activity and a high extraction yield. Using a mixture of organic solvent and water also improves the recovery of phenolic compounds that can increase antioxidant activity. Water is usually important to break matrix and matrix-analyte (hydrogen) bonding (Mustafa and Turner 2011).

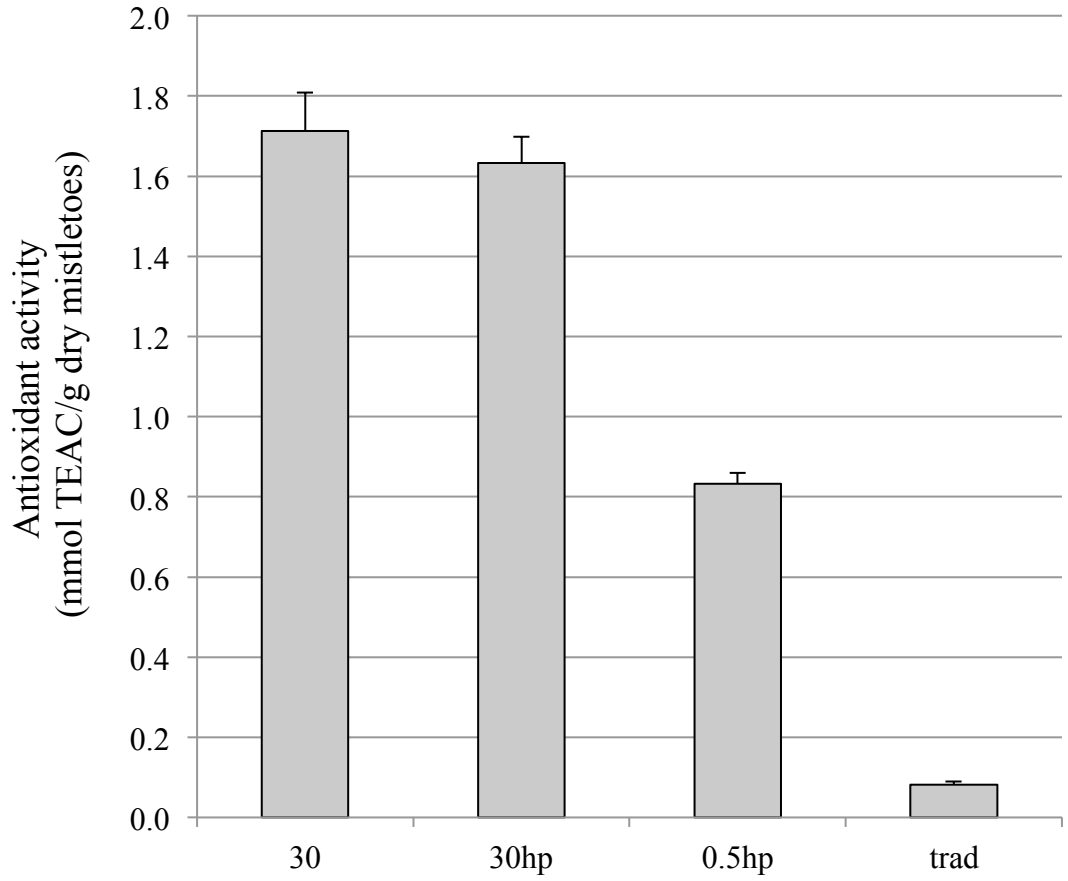


Figure 4-3. Antioxidant activity (ABTS assay) of several treatment extractions. 30: HCSE using 30% EtOH, a 10 min extraction and 0.2 MPa of pressure; 30hp: HCSE using 30% EtOH, a 10 min extraction and 0.2 MPa of pressure with the addition of β-CD; 0.5hp: HCSE using 0.5% EtOH, a 10 min extraction and 0.2 MPa of pressure with the addition of β-CD; trad: Traditional extraction procedure.

In contrast, the antioxidant activities of the 30 and 30hp extracts were not significantly different from one another. These results indicated that the addition of β-CD did not influence the antioxidant activity or the TPC of the extract. The most notable feature of cyclodextrins is their ability to form solid inclusion complexes (host-guest complexes) by a molecular complexation mechanism with a very wide range of solid, liquid and gaseous compounds. The binding of guest molecules within the host cyclodextrin is not fixed or permanent but is rather in a dynamic equilibrium. Furthermore, the affinity of cyclodextrin for the substances and the stability constant decrease with increasing temperatures, which negatively impacts the stability of the complex and to the final degree of solubility. The final effect of temperature on solubility depends on the prevalence of one effect rather than the other (Tommasini et al. 2004).

Phenolic compounds have attracted the interest of many researchers because they are powerful antioxidants and can protect the human body from oxidative stress. The antioxidant activity of phenolics is mainly due to their redox properties (Vicas et al. 2012). Most of extracts that contain a high amount of polyphenols also exhibit high antioxidant activity (Wong et al. 2006). This observation is demonstrated by a strong correlation between TPC value and antioxidant activities, which can be observed in Figure 4-4.

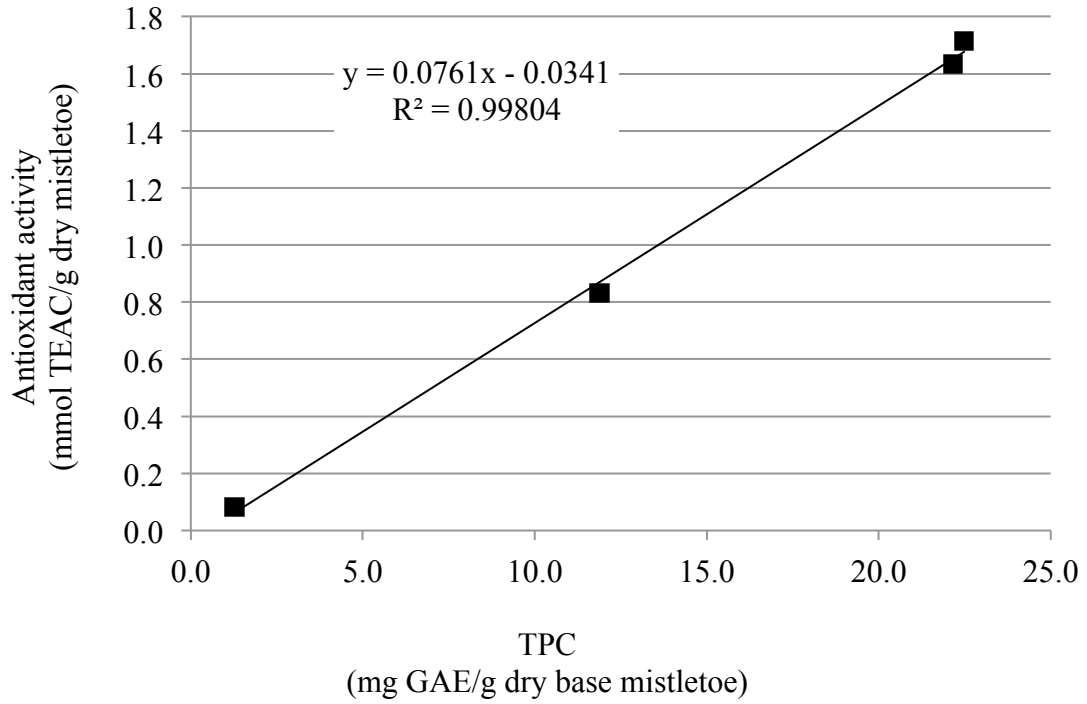


Figure 4-4. Correlation between TPC values and antioxidant activities.

Cytotoxicity assay

The lowest IC_{50} value for cancer cells and normal cells was obtained with the 30hp extract (Figure 4-5). This extract led to an effective 50% decrease in cancer cells growth at a concentration of approximately 107 µg/mL. The extract also inhibited normal cell growth by 50% at a concentration of 518 µg/mL, which was much higher than in cancer cells. From these data we can conclude that the 30hp extract has a high inhibitory growth activity in colon cancer cells but is still safety in normal cells. On the other hand, the IC_{50} value of the 30 extract was higher than the 30hp extract. However, the IC_{50} value of the 30 and 30hp extracts in the Caco-2 cell lines was not significantly different ($p > 0.05$). The IC50 value of extract lacking the addition of β-CD (826 µg/mL) in normal cells was also higher than with the addition of β-CD.

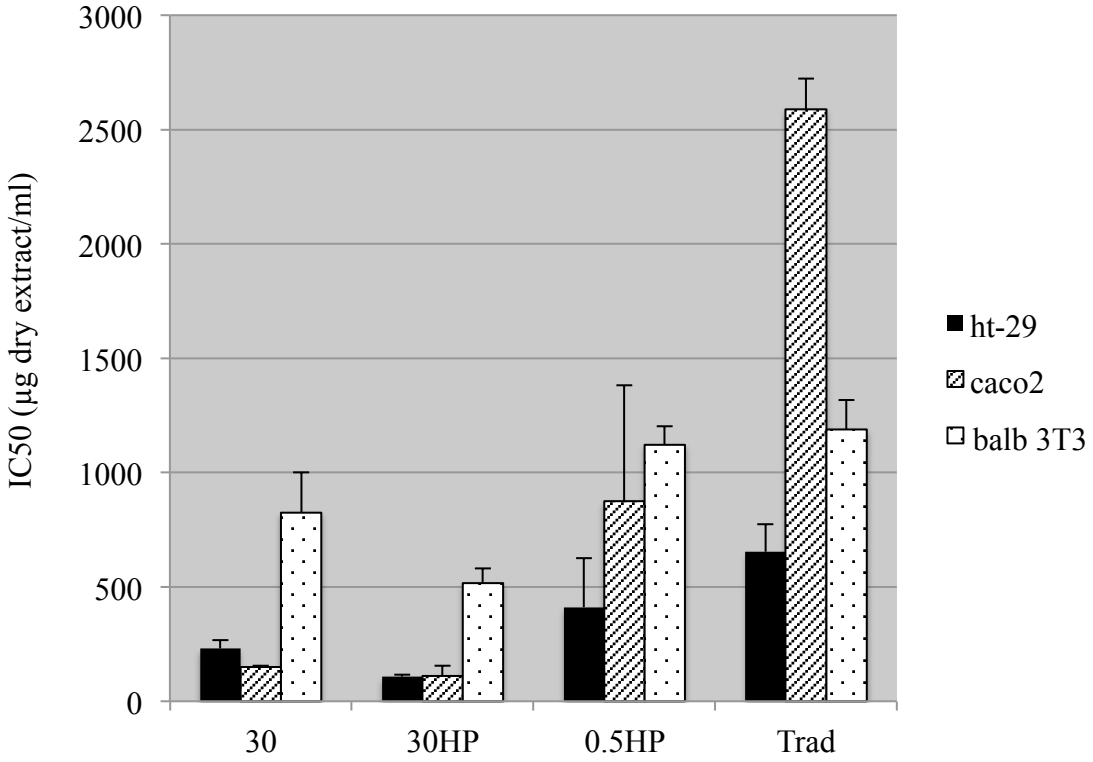


Figure 4-5. IC_{50} values of extracts on neutral red uptake in the colon cancer cell lines Ht-29 and Caco-2 and the normal mouse fibroblast cell line Balb/c 3T3. 30: HCSE using 30% EtOH, a 10 min extraction and 0.2 MPa of pressure; 30hp: HCSE using 30% EtOH, a 10 min extraction and 0.2 MPa of pressure with the addition of β-CD; 0.5hp: HCSE using 0.5% EtOH, a 10 min extraction and 0.2 MPa of pressure with the addition of β-CD; trad: Traditional extraction procedure.

The cytotoxic effects of the extract containing β-CD was strong, which can be observed in the 30hp extract data. This result may be due to the presence of a bioactive compound that is extracted by β-CD but not with one of the phenolic classes. Thus the addition of β-CD may have extracted another bioactive compound that has a high toxicity in cancer cells. However, further study is needed to identify the specific compound.

The 0.5hp and traditional extracts resulted in IC50 values that were greater than approximately 400 µg/mL for all of the cell lines. Neutral red uptake in the normal cells showed that these extracts did not generate significant damaged at concentrations bellow 1 mg/mL. Furthermore, these extracts are considered to be less

effective in inhibiting the growth of cancer cells. This result is particularly true in the Caco-2 cell line in which concentrations of up to 876 μg/mL for the 0.5hp extract and 2 mg/mL for the traditional extract were needed to decrease growth by 50%.

The use of 0.5% and 30% ethanol in the HCSE significantly influenced all of the assays, although 0.5% ethanol resulted in lower values. It can be concluded that the use of 0.5% ethanol is less effective in extracting bioactive compounds from tea mistletoe. However, in comparison with the traditional extract, the 0.5hp extract resulted in higher values in all of the assays. Thus, the used of the HCSE indeed gives better results than with the traditional extraction method.

Neutral red used to be as a vital stain. The neutral red uptake assay provides a quantitative estimation of the number of viable cells in a culture. Live cells will incorporate neutral red into their lysosome. As cells begin to die, their ability to incorporate neutral red diminishes. Thus loss of neutral red uptake corresponds to loss of cell viability. Moreover, neutral red is one of the most used cytotoxicity tests with many biomedical and environmental applications (Repetto, et al., 2008).

4.4. Conclusion

All of the assays showed significant differences for all of the treatments, which indicates that the addition of β-CD influenced the bioactivities of the extracts. Hot compressed solvent extraction using 30% ethanol for 10 minutes under 0.2 MPa of pressure with the addition of β-CD provided the greatest extract yield. The antioxidant activities and total phenolic contents of the extracts generated with and without the addition of β-CD were not significantly different. Furthermore, the lowest IC50 for cancer cells cytotoxicity was obtained with the 30hp extract. At a concentration of approximately 518 μg/mL, normal cell were also damaged by this extract. However, the cytotoxic concentration in normal cells is 5-fold higher than in cancer cells. In future studies, I will perform isolation and separation assays to identify the bioactive compounds in the extract of tea mistletoe that are generated with and without the addition of β-CD.

CHAPTER 5
Antioxidant Activity and Phenolic Content of Mistletoes Extracts by Hot Compressed Solvent Extraction Method

5.1. Introduction

Mistletoes are a hemiparasite that grows on the branches of trees to get tree nutrients. Despite their hemiparasitic action, they could grow by their self-photosynthesis in an independent manner of tree nutrients (Smith et al., 2001). Research interests on bioactivity of mistletoes have been arisen from pharmacologists, since mistletoes had diverse compounds such as alkaloids, phenylpropanoids, triterpenes, polysaccharides, peptides, lectins, flavonoids and phytosterols (Fukunaga et al., 1987; 1988; Richter and Pop, 1992).

Mistletoes have been used as a folk medicinal source in Indonesia, since one of the typical Indonesian mistletoes, *Scurrula atropurpurea*, has been reported to show anti-cancer effect (Ohashi et al., 2003). In general, mistletoes for medicinal intake are subjected to a conventional decoction extraction with water. However, the method could suffer from some issues due to low yield and serious chemical degradation by heat during a long extraction time. In order to overcome these issues, a pressurized liquid-extraction could be applied for the extraction, but any heat-degradation of mistletoes phenolic compounds still occurred (Huie, 2002).

Recently, we have developed a high-temperature batch extraction (HTBE) method using ethanol as a solvent, by which a rapid (<60 min) extraction was achieved even at high temperatures to avoid any degradation of phenolic compounds. Under HTBE conditions in 30% ethanol at 100 ˚C for 10 min, the method provided higher extraction yield of polyphenolic compounds in mistletoe (*Scurrula atropurpurea*) as well as high antioxidant activity, compared to conventional decoction method (Rahmawati and Hayashi, 2012). This finding suggested the proposed method would be useful for the extraction of antioxidant polyphenolic compounds with less degradation during extraction. Thus, in this study, we tried to apply the HTBE method for diverse mistletoes species to get an insight on the relationship between phenolic compounds and antioxidant activity among the species.

5.2. Materials and Methods

Materials

Six mistletoes used in this study (Table 5-1) were collected from the West and Central Java regions in Indonesia in 2011. The specimens of mistletoes were identified at the Herbarium Bogoriense in Bogor, Indonesia. The mistletoes were dried in an oven at 40˚C, and were ground into powder with a 1 mm-particle size by a Willey mill (1029-A, Yoshida Seisakusho Co., Tokyo, Japan). The powder samples were kept in a controlled dry cabinet (Auto Dry Tokyo Living Co., Tokyo, Japan) prior to use. The 2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)/ ABTS was purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan). The (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Gallic acid, catechin, epicatechin, rutin, and quercetin were obtained from Sigma-Aldrich Japan Co. (Tokyo, Japan). Quercetin 4'-*O*-β-glycopyranoside (que-4-gluc) was purchased from Polyphenols Lab. (Sandnes, Norway). All other chemicals were of analytical-reagent grade and used without further purification.

Table 5-1. Mistletoe species used in this study

Code	Mistletoe	Host plant	Local name	Origin
TP	*Scurrula atropurpurea*	Tea (*Camellia sinensis*)	*Benalu teh*	Puncak, West Java
TC	*Scurrula oortiana*	Tea (*Camellia sinensis*)	*Benalu teh*	Ciater, West Java
KE	*Scurrula parasitica*	Orange jessamine (*Murraya paniculata*)	*Benalu kemuning*	Sukorejo, Central Java
BE	*Dendrophthoe pentandra*	Star fruit (*Averrhoa carambola*)	*Benalu belimbing*	Sukorejo, Central Java
RA	*Dendrophthoe pentandra*	Kapok (*Ceiba pentandra*)	*Benalu randu*	Sukorejo, Central Java
KO	*Macrosolen cochinchinensis*	Coffee (*Coffea arabica*)	*Benalu kopi*	Sukorejo, Central Java

Code based on local name of mistletoes

Extraction

Extraction for powdered mistletoe samples was performed by an HCSE method according to our previous report (Rahmawati and Hayashi, 2012). Namely, 5 mL of 30% ethanol was added into a 6 mL-batch reactor (1/2-inch SUS316 stainless tube) containing 0.5 g of sample. After that, a 0.2 MPa-pressure by nitrogen gas was applied to the reactor, followed by the shaking at 100 ˚C. At this condition, solvent temperature rapidly (within 1.5 min) reached to a desired temperature of 100±0.3 ˚C, and then the temperature was kept for 10 min for extraction. After the 10 minutes of extraction, the reactor was immediately cooled down to the room temperature within 60 second. The solution was centrifuged at 10,000 x g at 4 ˚C for 15 min. The supernatant was then concentrated by a rotary evaporator (Eyela N-N series, Tokyo

Rikakikai Co., Tokyo, Japan). The concentrate was finally subjected to a lyophylization. The powered extract was dissolved in ultra-pure water at a concentration of 1 mg/mL (w/v) for further experiments.

Measurement of total phenol content

Total phenol content (TPC) was measured according to a method by Javanmardi et al. (2003). To an aliquot (50 µL) of sample solution (conc. 1 mg/mL) 2.5 mL of a 1/10-fold diluted Folin-Ciocalteu reagent and 2 mL of 7.5% (w/v) Na_2CO_3 solution were added. After incubation at 45 ˚C for 15 min, an absorbance at 765 nm was measured as TPC. Gallic acid was used as standard to calculate TPC. The calculated TPC was represented as mg-gallic acid equivalent per gram of extract (mg GAE/g extract).

Measurement of antioxidant activity

Antioxidant activity was measured by an ABTS assay (Re et al., 1999) with a slight modification. Briefly, ABTS solution dissolved in water was reacted with potassium persulfate so as to produce ABTS cation radicals ($ABTS^{\cdot+}$) The resulting solution was then allowed to stand in the dark at room temperature 12-16 h prior to use for antioxidant assay. The $ABTS^{\cdot+}$ solution was diluted with ethanol to adjust an appropriate radial concentration of 0.70±0.02 at 734 nm. Antioxidant activity was determined by monitoring the absorbance at 734 nm (530 UV-Vis spectrophotometry, JASCO, Tokyo, Japan) after incubating 1 mL of the $ABTS^{\cdot+}$ solution with 1 mL of sample solution or standard solution for 6 min at 30 ˚C. The trolox equivalent of µmol TEAC/g extract was used as an index of antioxidant activity.

Measurement of phenolic compounds in mistletoe extract by HPLC

A high-performance liquid chromatography (HPLC, Shimadzu 6A series, Shimadzu Co., Kyoto, Japan) was performed to measure phenolic compounds in mistletoes with an TSK gel ODS-80 Ts column (4.6 mm x 250 mm, Tosoh Co., Kyoto, Japan). Step-wised gradient elution was performed using a mobile phase of acetonitrile containing 0.1% formic acid at 40˚C at a flow rate of 0.6 mL/min at 280 nm: 10% to 20% acetonitrile (10 min), 20% to 40% (10 min), and 40% to 80% (10 min). The measurement of phenolic compounds in mistletoes was performed by corresponding elution of standard phenolic compounds (gallic acid, catechin, epicatechin, rutin, que-4-gluc, quercetin). The quantification of each compound in mistletoes was made by calibration curves of standard solution ranged from 5 to 100 μg/mL (Table 5-2).

Table 5-2. Calibration curves for the determination of the content of phenolic compounds in mistletoes.

Standard	RT (min)	Linear regression	r^2
Gallic acid	8.18	y=0.0014x-0.3081	0.996
Catechin	18.58	y=0.0044x-0.9253	0.986
Epicatechin	21.62	y=0.0042x-1.3559	0.975
Rutin	24.81	y=0.0029x+1.1182	0.999
Que-4-gluc	27.73	y=0.0016x+3.3666	0.966
Quercetin	32.32	y=0.0018x+1.16	0.999

RT (min), retention time on an TSK gel ODS-80 Ts column; y, concentration [μg/mL]; x, detected peak area

Statistical analysis

The results of the performed assays are presented as the mean ± standard error of the mean (S.E.M), as determined by triplicate and under the respective experimental conditions. After that, the results were statistically analyzed by a simple one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison using SPSS software 17th version. The differences was considered significantly different if $p < 0.05$ (α=0.05).

5.3. Results and Discussion

Measurement of polyphenolic compounds in mistletoe extract

Fig. 5-1 to 5-7 shows the HPLC chromatograms of mistletoe extracts by HCSE method, together with 6 phenolic standards. By the comparison of elution profiles of each standard with those of sample mistletoe extracts, in TP mistletoe there were five phenolic compounds, i.e., gallic acid, catechin, epicatechin, rutin and que-4-gluc that found to be naturally occurring. In TC mistletoe there were gallic acid, catechin, rutin and que-4-gluc; In KE, we found gallic acid, catechin, rutin, que-4-gluc and quercetin; and in RA mistletoe there were gallic acid, epicatechin, rutin and que-4-gluc. In contrast, only gallic acid and que-4-gluc (also rutin for KO mistletoe) were detected in BE and KO mistletoes extracts.

Yield of extraction by HCSE method

The RA extract showed a high extraction yield of 300±12 mg/g mistletoe material by HTBE method among the 6 mistletoe extracts in the descending order of RA>BE>KE>TC>KO>TP mistletoe extracts (Figure 5-8). This indicates that the extraction by the HCSE method was greatly affected by mistletoe characteristics or phenolic contents in each mistletoes.

Total polyphenolic content in mistletoe extract

In order to get information on polyphenolic contents in mistletoe extracts, along with the efficient HCSE-extraction from the extracts (Figure 5-8), TPC in each mistletoe extract by HCSE method was determined by the Folin-Ciocalteu method. As shown in Figure 5-9, TC had the highest polyphenolic contents in the HCSE-extract (280 ± 24 mg GAE/g extract), indicating that most compounds present in TC extract by HTBE method were polyphenolic compounds. Although the TPC values of BE and RA were lower than other extract, their content in extract was estimated to be over 35%. Therefore, the HCSE method under the present extraction condition using 30% ethanol would be useful for extracting natural polyphenolic compounds from mistletoes.

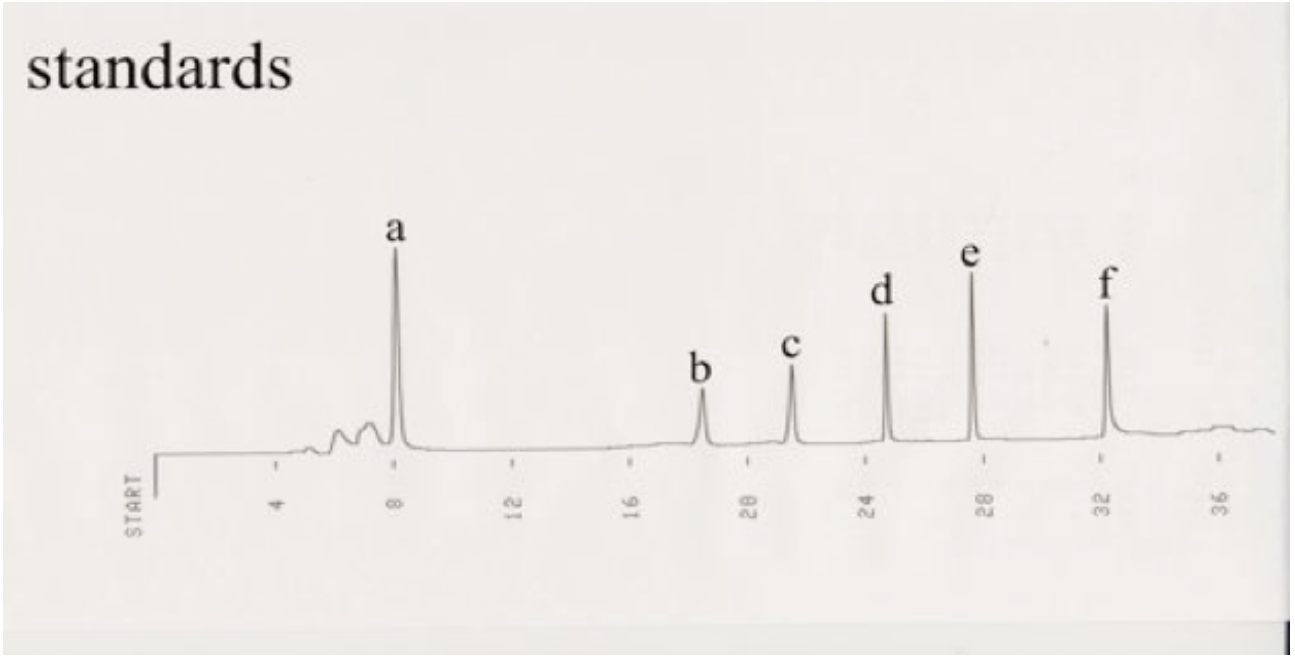


Figure 5-1. HPLC chromatogram of standard poyphenolic compounds
"a" to "f" corresponds to standard polyphenolic compounds, gallic acid, catechin, epicatechin, rutin, que-4-gluc and quercetin, respectively. HPLC separation of polyphenol compounds was performed on an TSK gel ODS-80 Ts column at 0.6 mL/min at 40 ˚C, while monitoring at 280 nm.

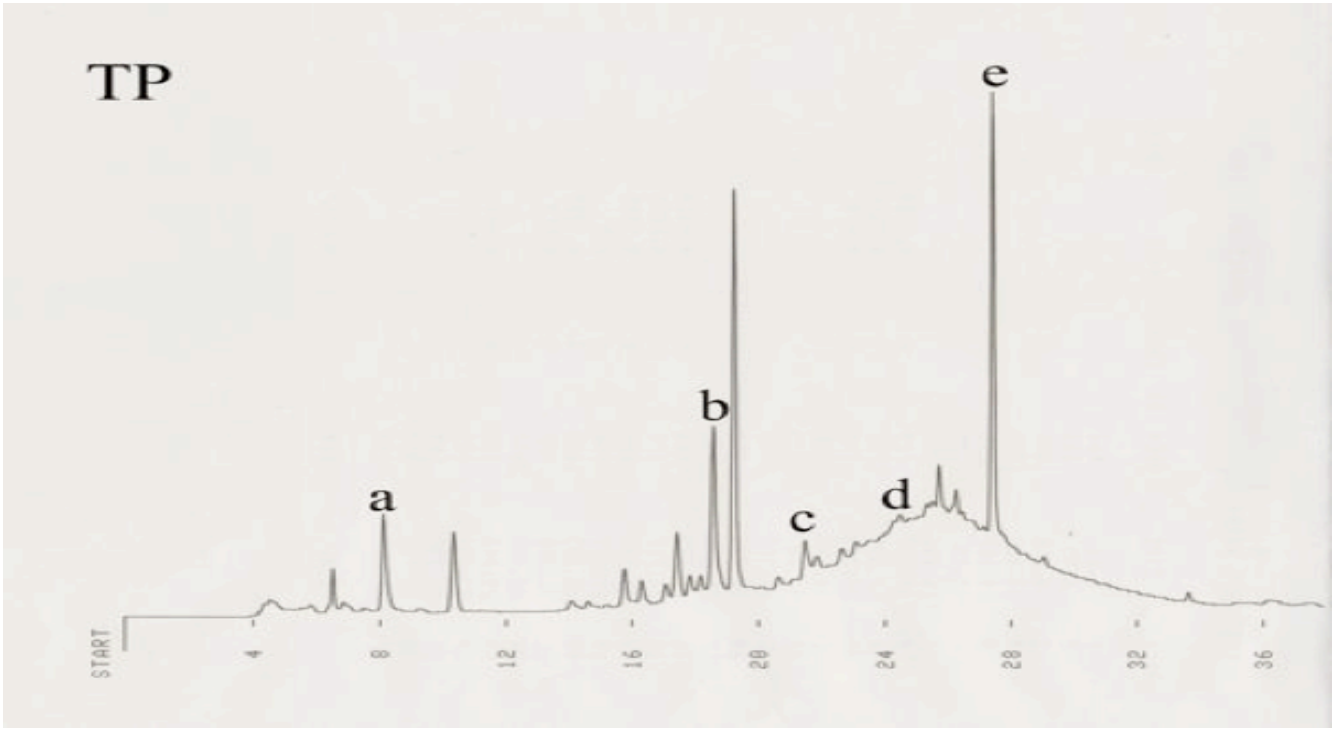


Figure 5-2. Typical HPLC chromatogram of tea mistletoe from puncak (TP) extracts by HTBE method. “a” to “f” corresponds to standard polyphenolic compounds, gallic acid, catechin, epicatechin, rutin, que-4-gluc and quercetin, respectively. HPLC separation of mistletoe extracts was performed on an TSK gel ODS-80 Ts column at 0.6 mL/min at 40 ˚C, while monitoring at 280 nm.

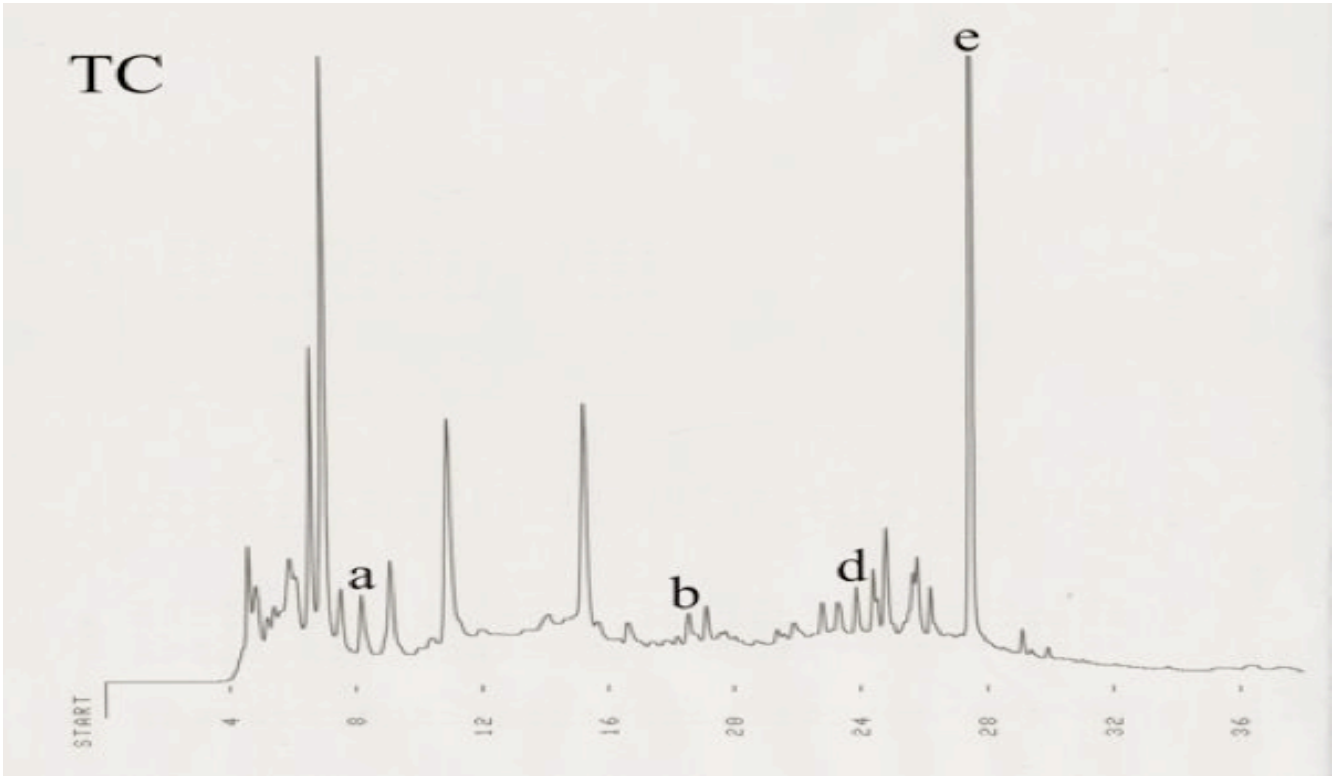


Figure 5-3. Typical HPLC chromatogram of tea mistletoe from ciater extracts by HTBE method. “a” to “f” corresponds to standard polyphenolic compounds, gallic acid, catechin, epicatechin, rutin, que-4-gluc and quercetin, respectively. HPLC separation of mistletoe extracts was performed on an TSK gel ODS-80 Ts column at 0.6 mL/min at 40 ˚C, while monitoring at 280 nm.

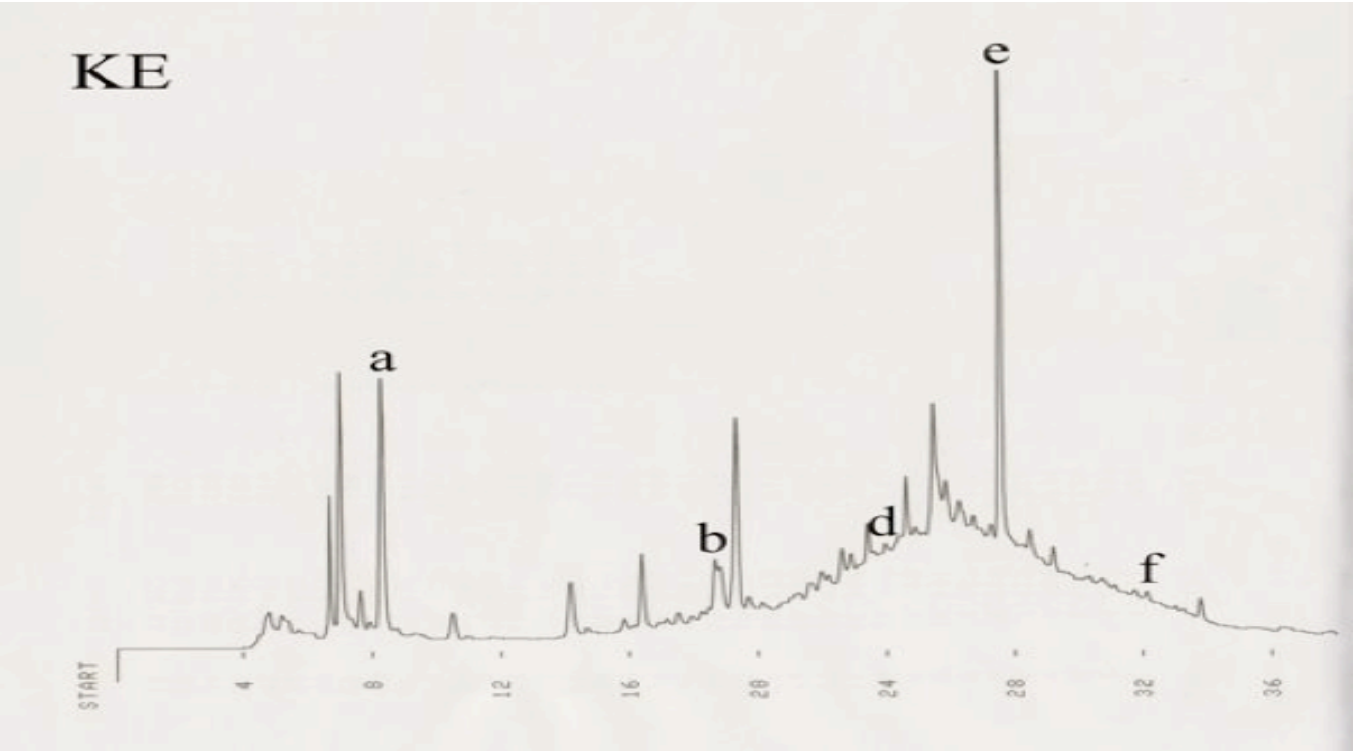


Figure 5-4. Typical HPLC chromatogram of orange jessamine mistletoe extracts by HTBE method. “a” to “f” corresponds to standard polyphenolic compounds, gallic acid, catechin, epicatechin, rutin, que-4-gluc and quercetin, respectively. HPLC separation of mistletoe extracts was performed on an TSK gel ODS-80 Ts column at 0.6 mL/min at 40 ˚C, while monitoring at 280 nm.

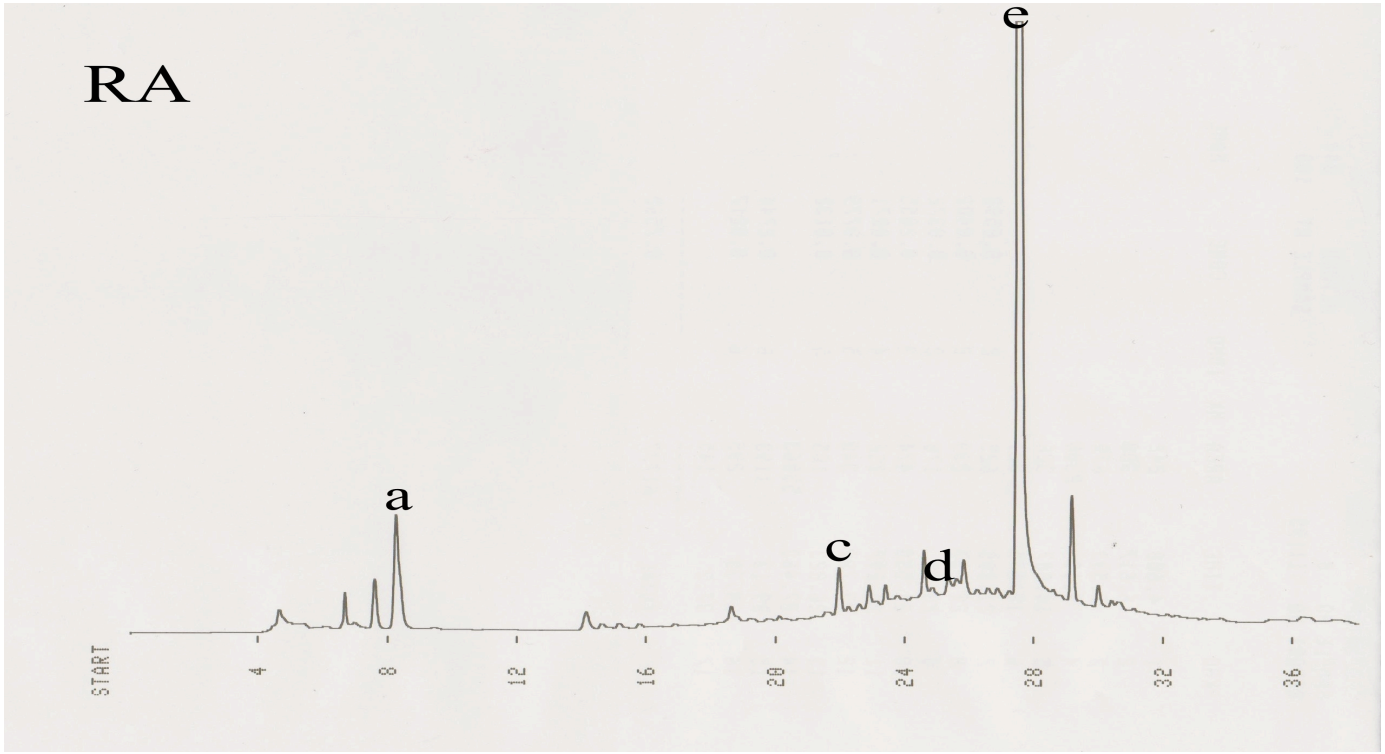


Figure 5-5. Typical HPLC chromatogram of kapok mistletoe extracts by HTBE method.
"a" to "f" corresponds to standard polyphenolic compounds, gallic acid, catechin, epicatechin, rutin, que-4-gluc and quercetin, respectively. HPLC separation of mistletoe extracts was performed on an TSK gel ODS-80 Ts column at 0.6 mL/min at 40 ˚C, while monitoring at 280 nm.

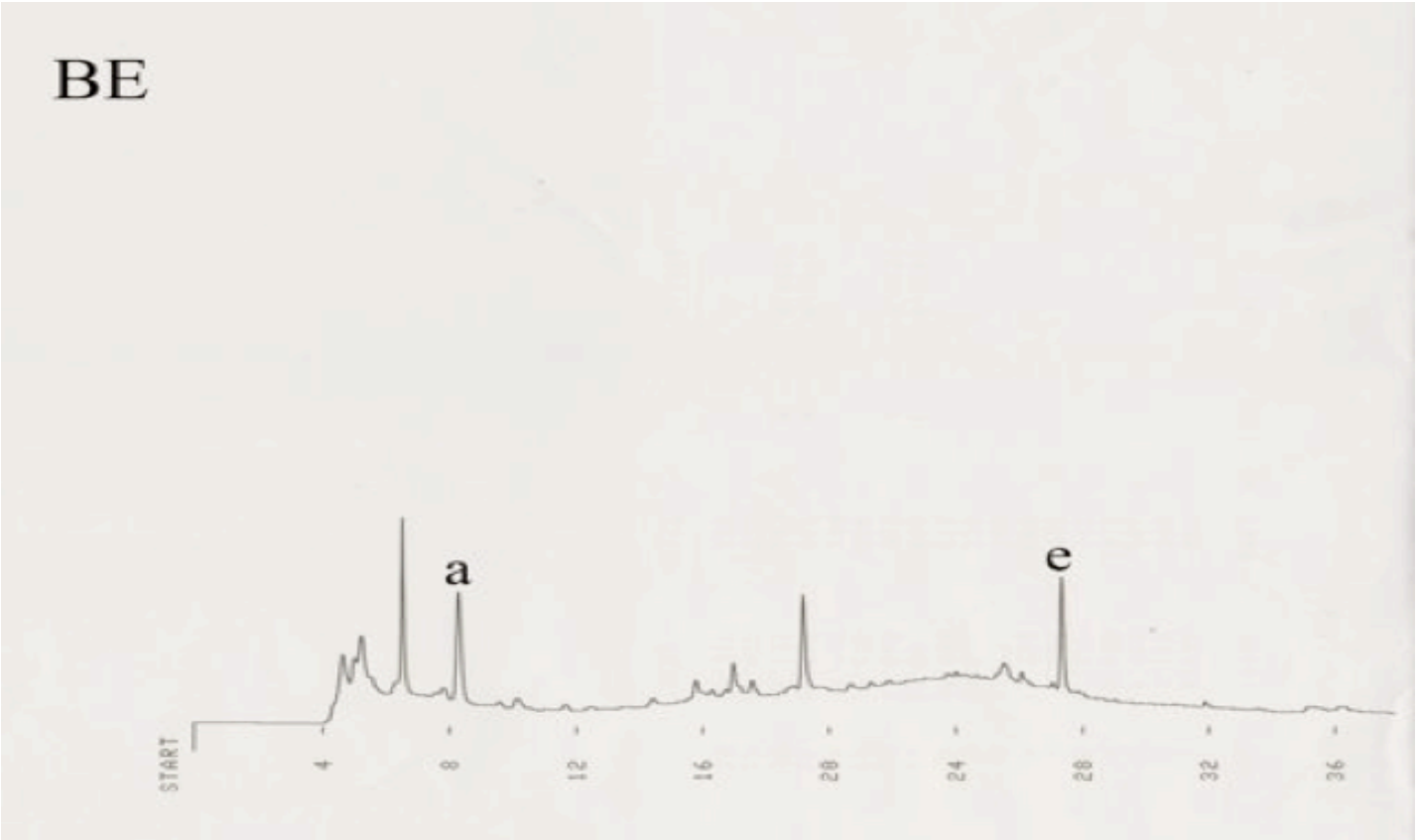


Figure 5-6. Typical HPLC chromatogram of star fruit mistletoe extracts by HTBE method.
"a" to "f" corresponds to standard polyphenolic compounds, gallic acid, catechin, epicatechin, rutin, que-4-gluc and quercetin, respectively. HPLC separation of mistletoe extracts was performed on an TSK gel ODS-80 Ts column at 0.6 mL/min at 40 ˚C, while monitoring at 280 nm.

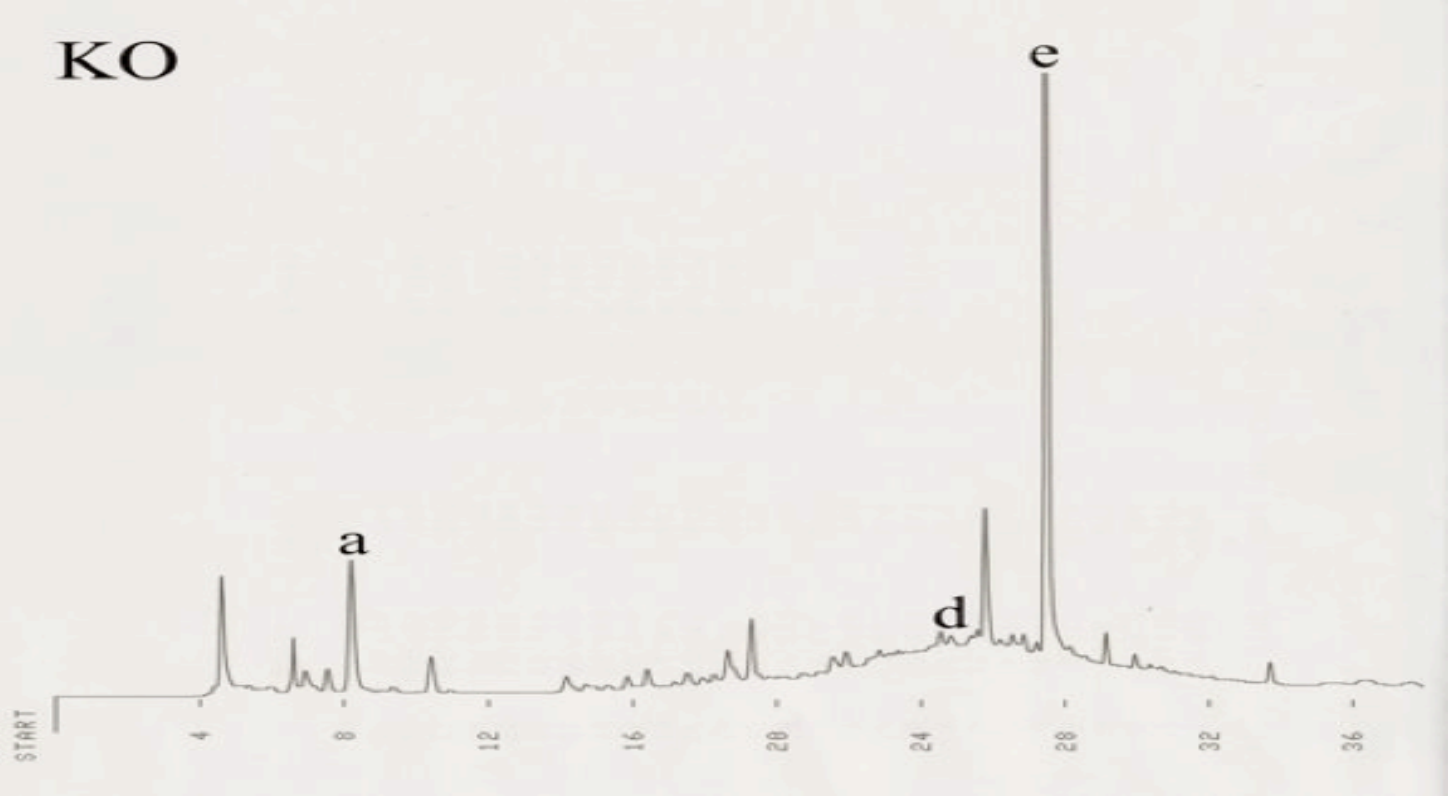


Figure 5-7. Typical HPLC chromatogram of coffee mistletoe extracts by HTBE method.
"a" to "f" corresponds to standard polyphenolic compounds, gallic acid, catechin, epicatechin, rutin, que-4-gluc and quercetin, respectively. HPLC separation of mistletoe extracts was performed on an TSK gel ODS-80 Ts column at 0.6 mL/min at 40 ˚C, while monitoring at 280 nm.

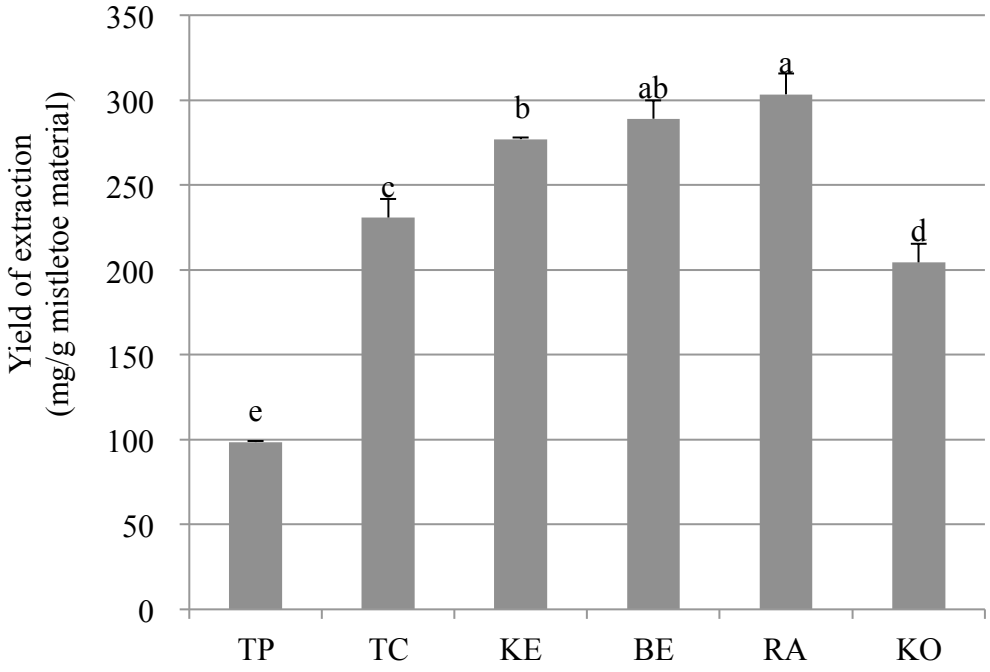


Figure 5-8. Results for extraction yield of mistletoe extracts by HTBE method. Six mistletoes were extracted by HTBE method using 30% ethanol under 0.2 MPa pressure at 100 ˚C for 10 min. Data represent the mean ± SD (n=3). Bars with different letters of the alphabet are significantly different (P<0.05). Abbreviations for mistletoe extracts are the same as in Table 5-1.

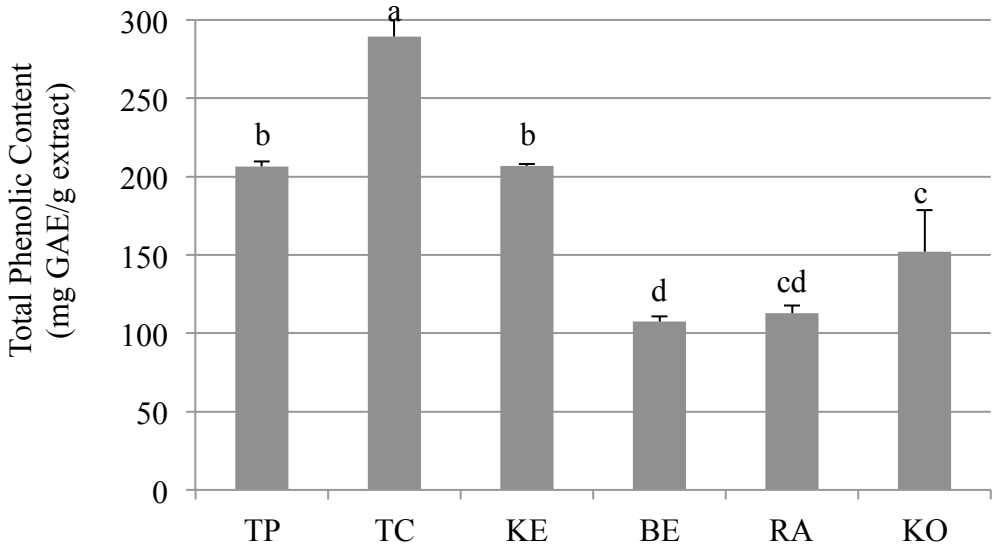


Figure 5-9. Results for total phenolic contents of mistletoe extracts by HTBE method. Six mistletoes were extracted by HTBE method using 30% ethanol under 0.2 MPa pressure at 100 ˚C for 10 min. Total phenolic content (mg gallic acid (GAE) equivalent/g extract) represent the mean ± SD (n=3). Bars with different letters of the alphabet are significantly different (P<0.05). Abbreviations for mistletoe extracts are the same as in Table 5-1.

Poyphenolic profiles in mistletoe extract by HPLC analysis

The HCSE-aided mistletoe extracts were applied to reversed-HPLC to make clear natural compounds responsible for high TPC values in the extracts (Figure 5-3). As shown in Figure 5-1 to 5-7, we could successfully detected some polyphenolic compounds in each extract by the aid of their standard elution on an TSK gel ODS-80 Ts column, as similar to other reports (Ohashi et al., 2003; Vicas et al., 2011). Figure 5-10 summarized the contents of polyphenolic compounds detected by HPLC. As a result, que-4-gluc was commonly and predominantly present in all the 6 mistletoe extracts in this study; RA (38 mg/g extract) as well as TC (24 mg/g extract) and KO (26 mg/g extract) had the high content of que-4-gluc among them. In contrast, the contents of other polyphenolic compounds such as gallic acid, catechin, epicatechin rutin and quercetin differed from mistletoe species, suggesting that the nutritional compositions of mistletoes may be distinct from their growing conditions (Łuczkiewicz et al., 2001).

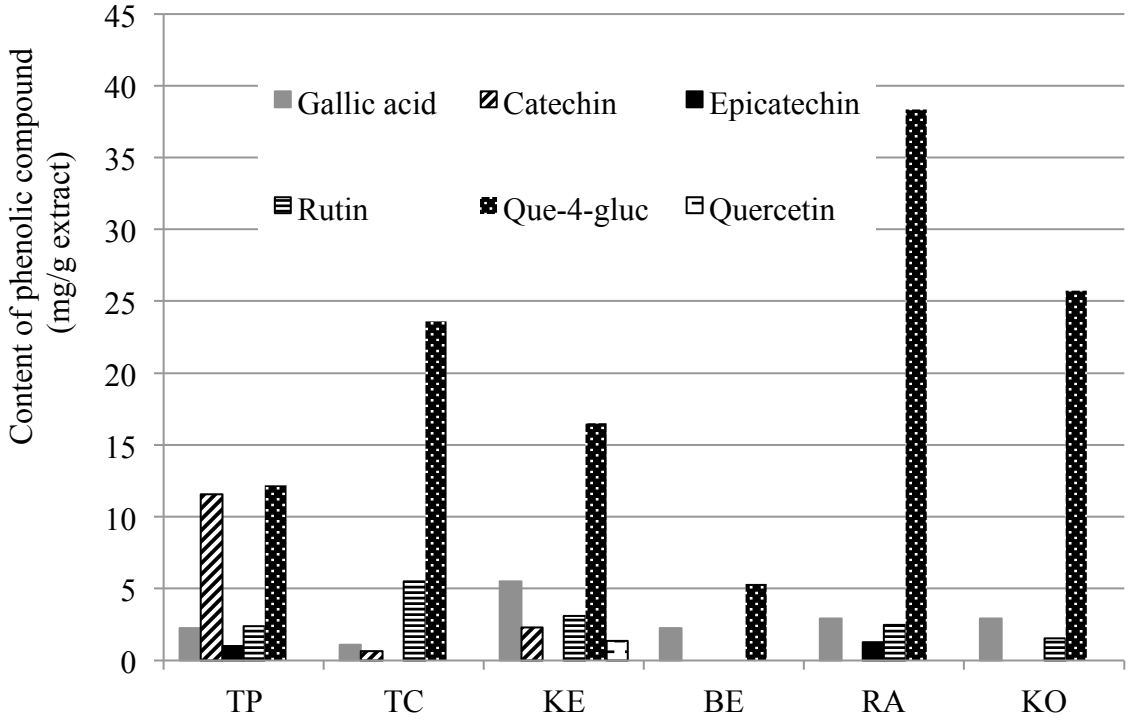


Figure 5-10. Profiles of phenolic contents in 6 mistletoe extracts by HCSE method. Abbreviations for mistletoe extracts are the same as in Table 5-1.

Antioxidant activity

Considering the high contents of polyphenolic compounds in HCSE-aided mistletoe extracts, we next evaluated their antioxidant activity by ABTS method. As shown in Figure 5-11A, TP, KE, and TC extracts evoked high ABTS radical scavenging activity of >40 μmol TEAC/g extract, as similar to those for antioxidant activity per mistletoe material (Figure 5-11B) except TP. The lower antioxidant activity of TP evaluated by mistletoe material (5.4±1.0 μmol TEAC/g mistletoe material) indicated that TP contained much more inactive contaminants rather than other mistletoe species. In order to clarify the contribution of antioxidant activity of detected polyphenolic compounds to total activity of mistletoe extracts, we evaluated the total antioxidant activity of each polyphenolic compounds based on their contents in extracts. As summarized in Table 5-3, TP and RA extracts showed high total antioxidant activity (22 μmol TEAC/g extract) of polyphenolic compounds present in mistletoe extracts in the descending order of TP, RA>KE>KO>TC>BE mistletoe extracts. This indicated that antioxidant effect of RA extract might result from the contribution of polyphenolic compounds listed in Table 5-3. In contrast, lower contribution of polyphenolic compounds to antioxidant activities of other extracts, in particular TC extract with ca. 20% contribution ratio suggested that other antioxidant compounds must be present in mistletoe extracts, as reported by Haas et al. (2003), who demonstrated the presence of phytochemicals such as triterpenoid in mistletoes. Therefore, further experiments for identification of other antioxidants in HCSE-aided mistletoe extracts are now in progress.

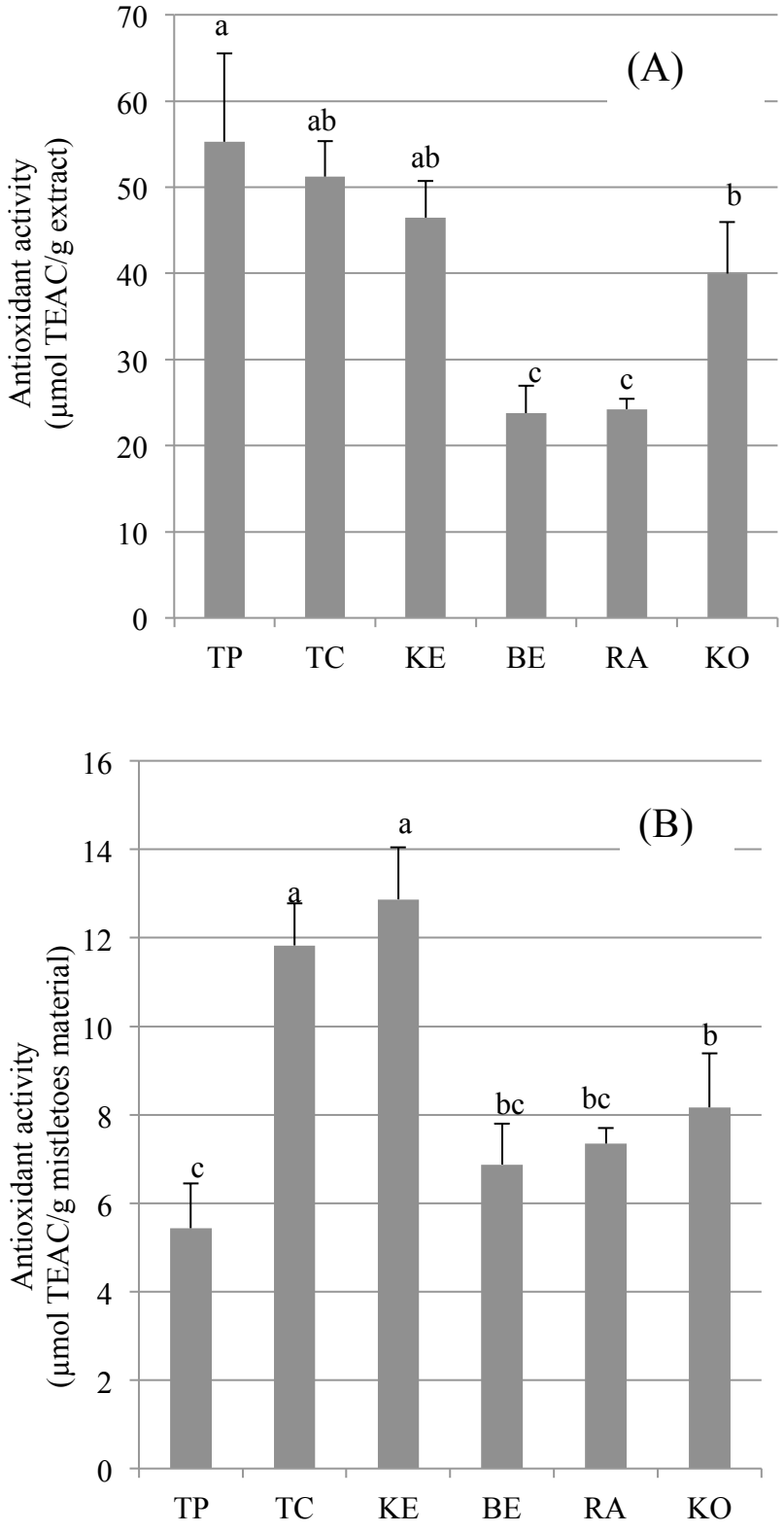


Figure 5-11. Results of antioxidant activities of mistletoe extracts by HTBE method (µmol TEAC/g extract) (A) and the activities of mistletoe materials (µmol TEAC/g mistletoe material) (B).
Data represent the mean ± SD (n=3). Bars with different letters of the alphabet are significantly different (P<0.05). Abbreviations for mistletoe extracts are the same as in Table 5-1.

Table 5-3. Total antioxidant activities of mistletoe extracts (mmol TEAC/g dry extract) estimated from antioxidant activity of each standard (mmol TEAC/mg standard).

Phenolic standards	Antioxidant activity (μmol TEAC/mg for standard or μmol TEAC/g for extract)						
	Standard	TP	TC	KE	BE	RA	KO
Gallic acid	1.5±0.001	3	2	8	3	4	4
Catechin	1.0±0.005	12	1	2	*	*	*
Epicatechin	1.0±0.005	1	*	*	*	1	*
Rutin	0.3±0.025	1	2	1	*	1	*
Que-4-gluc	0.4±0.061	5	9	7	2	15	10
Quercetin	0.8±0.023	*	*	1	*	*	*
Total		22	13	19	6	22	15

*: indicate "not available"
Abbreviations for mistletoes extracts are the same as in Table 5-1.

5.4. Conclusion

In this study, we could clarify the candidates responsible for antioxidant effect of mistletoes by the aid of HTBE extraction method. The predominant antioxidant in the extract was que-4-gluc, even though other antioxidant polyphenolic compounds including gallic acid, catechin, epicatechin, rutin, and quercetin were also found in the mistletoe extracts. Among the 6 mistletoe species used in this study TP as well as KE, TC and KO extracts possessed a high antioxidant activity with >40 mmol TEAC/g extract, together with their high total phenolic contents. Taken together, the present study demonstrated that the HCSE method could be applicable for the extraction of antioxidant compounds from mistletoes, and the resulting antioxidant activity of mistletoes apparently differed from their species.

CHAPTER 6

GENERAL DISCUSSION

Mistletoe, in especially tea mistletoe, is known as one of traditional medicine in Indonesia. People used to consume the mistletoe by decoction for several hours until the solution 1/3 remain. However, decoction as one of the conventional extraction method have several drawbacks; e.g. this method is time consuming, involves high solvent consumption and may have lower extraction efficiencies. In order to overcome the issues, other extraction techniques have been developing. One of those techniques is hot compressed solvent extraction (HCSE), which a developed method of Pressurize Liquid Extraction. HCSE is a sample preparation technique that developed by combined elevated temperature and pressure with liquid solvents rapidly to avoid any degradation of phenolic compounds.

In our experiment, it was proved that the HCSE method gives better result than traditional extraction method on tea mistletoe. The traditional extraction process obtained the lowest yield of extract. Moreover, compared with the HCSE methods, the yields were resulted in 10-folds higher than the extract yield of traditional extraction. Therefore, the traditional extraction can be considered to be ineffective, because of the method required a long extraction time but resulted in a low yield of extract.

The extraction yields of HCSE show the influence of ethanol concentration, which an increasing of ethanol concentration could also increase the extract yield. The use of hot liquid solvents in HCSE can partly overcome strong analyte-matrix interactions, including with more polar compounds such as ethanol. Thus, the HCSE with ethanol solvent could extract more compounds that hide inside the cell wall of the tea mistletoe. Furthermore, ethanol solvent also has ability to dissolve wide ranges of bioactive compounds such as ionic compound; sodium hydoxides, etc, also dissolve non-polar substances, including most essential oils, etc (Martha, 1976).

A solvent of 30% ethanol treatment had the highest antioxidant activities and total phenolic content assays in the experiments. Hence, the mixtures of alcohol and water have revealed to be more efficient in extracting phenolic constituent than the

corresponding mono-component solvent system (Yilmaz and Romeo, 2006). Additionally, the advantages of using 30% ethanol as solvent are; non-toxic, neutral, well absorption, and in ethanol above 20% mold and germ are difficult to grow so it can avoid contamination.

The treatments of different temperature on HSCE method also give different yield of extraction. However the activities of resulted extracts showed similar activities on radical scavenging. This result assumed that the active compound in tea mistletoe already extracted at lower temperature (start from 100˚ C). Hence, it is not necessary to use higher temperature extraction.

From the first experiment we can conclude that the best extraction treatment for tea mistletoe is HSCE method with solvent 30% ethanol, at 100˚ C temperature, for 10 minutes under 0.2 MPa pressurize. The pressurize treatment made the boiling point of the solvent higher, so the solvent can prevent from evaporation. We assumed that the pressurize treatment made the extraction time become shorter than traditional extraction method.

After we proposed the best extraction method, we want to improve the resulted extract activities by added β-CD into the solvent of best HCSE method as our second experiment. The addition of β-CD on HSCE resulted the highest yield of extraction among others treatments. However, the difference of the yields between with and without the addition of β-CD did not show significantly different. The addition of β-CD increased the yield because the potential guest list for molecular encapsulation in cyclodextrin is quite varied, include straight or branched chain aliphatics, aldehydes, ketones, alcohols, organic acids, fatty acids, aromatics, gases, and polar compounds, such as halogens, oxyacids and amines (Schmid 1989).

On the other hand, antioxidant activity and total phenolic content on extract of HSCE with the addition of β-CD also showed not significantly different than without β-CD. It may be due to the solubility of the phenolic compounds and their influence on the compound-cyclodextrin binary complex and the competitive binding of ethanol to molecules of β-CD (Garcia-Rio et al., 2006). Therefore, the addition of β-CD consider as not necessary.

The unique of mistletoe is that they have haustorium as the connection part between mistletoe and host plant. Many of active haustorium-inducing molecules are common constituents of plant cell including phenolic compound such as flavonoid,

where it functions in lignin biosynthesis, host defense and other specialized physiological processes (Albrecht *et al*., 1999). Thus, bioactive compounds that have an important role in radical scavenging activities were from phenolic compounds.

The analysis of flavonoid by HPLC on extracts of tea mistletoe by HSCE showed that the main compounds of the extract were quercetin 4'-glucoside and rutin. The highest concentration of it was obtained by extraction condition with the mixture between water and ethanol. Hence, higher water composition in the solvent can aid in the extraction of glycoside flavonoid. So, due to complexity of heterosidic combinations, certain groups of flavonoids such as flavones and flavanols, are not generally characterized as intact compound but in the form of their aglycones (Tsao, *et al*., 2004).

The result of cytotoxic effects of the extract containing β-CD was the strongest among other treatments. However, the total phenolic content showed insignificantly different between the treatment of with and without β-CD HSCE method. It considered that the phenolic content inside both extracts unchanged, but the toxic effect might be due to the presence of another bioactive compound that is extracted by β-CD and has a high toxicity in cancer cells. However, further study is needed to identify the specific compound.

In spite the influence of the addition of β-CD, the used of HCSE indeed gives better results of toxicity on cancer cells than the extract of traditional extraction method. It may be due to the extraction time of traditional method was very long that made the unwanted compounds also extracted. Therefore, HCSE method with short time is the most suitable, to reduce the viability of cancer cells.

Neutral red used to be as a vital stain. The neutral red uptake assay provides a quantitative estimation of the number of viable cells in a culture. Live cells will incorporate neutral red into their lysosome. As cells begin to die, their ability to incorporate neutral red diminishes. Thus loss of neutral red uptake corresponds to loss of cell viability. Moreover, neutral red is one of the most used cytotoxicity tests with many biomedical and environmental applications (Repetto, et al., 2008). So, its assumed that the active compound inside tea mistletoe give a damaged to the lysosome of cancer cells, that made the cells loss their viability.

On third experiment, we could clarify the candidates responsible for antioxidant effect of mistletoes by the aid of HCSE extraction method in varied type of mistletoes

(*Scurrula*, *Dendrophtoe* and *Macrosolen*). The predominant antioxidant in the extract was que-4-gluc, even though other antioxidant polyphenolic compounds including gallic acid, catechin, epicatechin, rutin, and quercetin were also found in the mistletoe extracts. Moreover, polyphenolic profile in the mistletoe extracts showed that que-4-gluc was commonly and predominantly present in all the 6 mistletoe extracts in this study; *Dendrophtoe pentandra*, as well as *Scurrula oortiana* and *Macrosolen cochinchinensis* had the high content of que-4-gluc among them. In contrast, the contents of other polyphenolic compounds such as gallic acid, catechin, epicatechin rutin and quercetin differed from mistletoe species, suggesting that the nutritional compositions of mistletoes may be distinct from their growing conditions (Łuczkiewicz et al., 2001). In conclusion, the present study demonstrated that the HCSE method could be applicable for the extraction of antioxidant compounds from mistletoes, and the resulting antioxidant activity of mistletoes apparently differed from their species.

SUMMARY

Mistletoes are traditional medicinal plant in Indonesia that can be used to treat cancer. Research interests on bioactivity of mistletoes have been arisen from pharmacologists since mistletoes had diverse compounds such as alkaloids, phenylpropanoids, triterpenes, polysaccharides, peptides, lectins, flavonoids and phytosterols. One of the famous mistletoe in Indonesia is tea mistletoe (*Scurulla atropurpurea*) or *benalu teh* (Indonesian).

In general, mistletoes for medicinal intake are subjected to a traditional extraction by decoction with water. However, the method could suffer from some issues due to low yield and serious chemical degradation by heat during a long extraction time. In order to overcome these issues, we used hot compressed solvent extraction (HCSE). The HCSE is an extraction technique under elevated temperatures by which a rapid (<60 min) extraction at high temperatures to avoid any degradation of bioactive compounds. We also applied pressure to the extraction, higher than solvent vapor pressure to keep the solvent in the liquid phase even on high temperature. Through this method of extraction, we hoped to obtain a better result of the extraction than the traditional extraction method.

Tea mistletoe was extracted using traditional and batch reactor with different solvents (water, 30%, 50%, 70% and 99% ethanol) at different temperatures (40 ˚C–180 ˚C) and for different time periods (10-20 minutes) under additional pressure 0.2 MPa. The resulted extracts were analyzed by antioxidant activity (ABTS) and total phenolic content. Thereafter, identification and quantification of flavonols as bioactive compounds from tea mistletoe were determined by HPLC. The analyses results suggested that the best extraction condition was 30% ethanol solvent with temperature100 ˚C for 10 minutes, with the detected flavonoid predominantly rutin. We considered that suitable water and ethanol mixture, boiled temperature, shorter time of extraction, and also the addition of pressure could extract active compound from mistletoe.

We also optimized the extraction of extract bioactive compounds, by enhance the solubility of the bioactive compounds that are poorly soluble in aqueous solutions using hydroxypropyl-β-cyclodextrin (β-CD) into solvent of extraction. This study

investigated how the addition of β-CD influenced the yield of the extract, the antioxidant activities, the total phenolic content and the cytotoxicity of the extracts on colon cancer cells. The assays showed significant differences for all the treatments, which demonstrate that the addition of β-CD influenced the extracted bioactive compounds. However, this extract showed the cytotoxic effects in colon cancer cells and normal cells. So we suggested that the addition of β-CD is not necessary.

Last, we investigated the biological properties of mistletoe extract prepared by HCSE by determined the cytotoxicity level of the extract on colon cancer cells and normal cells. The cytotoxic effects of the extract containing β-CD were strong within cancer and normal cells, which can be observed in the resulted data. This result may be due to the presence of a bioactive compound that is extracted by β-CD but not extracted without it. Thus the addition of β-CD may have extracted another bioactive compound that has a high toxicity in cancer cells. This bioactive compound may be octadeca-8,10,12-triynoic acid, which based on the report by Ohashi et al. (2003) was the main biologically active substance from tea mistletoe for the treatment of cancer. However, further study is needed to identify the specific compound.

In contrary, traditional extracts resulted in IC50 values that were greater than approximately 400 μg/mL for all off the cell lines. Neutral red uptake in the normal cells showed that these extracts did not generate significant damaged at concentrations bellow 1 mg/mL. Furthermore, this extract is considered to be less effective in inhibiting the growth of cancer cells. Particularly in the Caco-2 cell line in which concentrations of up to 2 mg/mL for the traditional extract were needed to decrease the cells growth by 50%.

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