Bulgarian Journal of Agricultural Science, 20 (No 4) 2014, 818-823
Agricultural Academy

ANTIOXIDANT AND ANTICANCER ACTIVITIES OF MISTLETOE TEA PREPARED BY HIGH TEMPERATURE EXTRACTION WITH CYCLODEXTRIN

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Abstract

RAHMAWATI, S. I., K. ISHIMARU, DE-XING HOU and N. HAYASHI, 2014. Antioxidant and anticancer activities of mistletoe tea prepared by high temperature extraction with cyclodextrin. *Bulg. J. Agric. Sci.,* 20: 818-823

Indonesian tea mistletoe (*Scurrulla atropurpurea*, BL.) is a medicinal plant that can be used to treat cancer. In our previous study, mistletoe extract that have the greatest functionality was obtained by a high temperature batch extraction (HTBE) with 30% ethanol solvent at temperature 100˚C for 10 minutes under 0.2 MPa pressurize. To optimize the extract solution by extracts other compounds that have high activities but poorly soluble in aqueous solution, we added hydroxypropyl-b-cyclodextrin (b-CD) to the extraction solvent. This study was, especially, to investigate how the addition of b-CD to the extraction influenced the yield of the extract, the antioxidant activities and the total phenolic content of the extract. The extracts were also evaluated for their cytotoxic effects in colon cancer cells and normal cells. The assays showed significant difference for all the treatments, which demonstrate that the addition of b-CD influenced the bioactivities of the extracts. Furthermore, the addition of b-CD extract resulted effective to inhibit the growth of cancer cells.

Key words: High temperature extraction, mistletoe, cyclodextrin, antioxidant, anticancer

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 high temperature batch extraction (HTBE) 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-b-cyclodextrin (b-CD) to the extraction solvent to enhance the solubility of the bioactive compounds in mistletoe. Based on the report by Ohashi et al. (2003), the primary biologically active substance in tea mistletoe that is effective for the treatment of cancer may be octadeca-8, 10, 12-triynoic acid, which is obtained from the ethyl acetate soluble fraction. Unfortunately, octadeca-8, 10, 12-triynoic acid is poorly soluble in aqueous solutions. b-CD is also capable of forming inclusions with many compounds, which improves its chemical stability, increases the apparent aqueous solubility, and results in a higher bioavailability

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without changing its pharmacokinetics properties (Tommasini et al., 2004; Li et al., 2003). Cyclodextrins are a family of cyclic oligosaccharides that are composed of a-(1-4) linked glucopyranose subunits. Among the three types of cyclodextrins, b-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 b-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).

Experimental

Material

We collected dried plant material of tea mistletoe (*Scurulla atropurpurea* [BL] Dans.) in September 2008 from Puncak area of Bogor, Indonesia. We identified the specimen 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. We used only analytical grade reagents in this study.

Extraction

During the pre-study, we used extraction treatments in which we compared b-CD additions and the sample weights (1:1, 1:10 and 1:100 ratios). The best extraction occurred with a b-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 HTBE as explained in a previous report (Rahmawati and Hayashi, 2012).

Extractions with the HTBE method used 30% ethanol at 100˚C for 10 minutes under 0.2 MPa pressure and were then treated or not with b-CD. Moreover, as reported by Li et al. (2009), the use of 0.5% ethanol gave better results because it enabled the formation of b-CD with the triterpenoid complex. We therefore used a HTBE method that consisted of a 0.5% ethanol at 100 ˚C for 10 minutes under 0.2 MPa of pressure with the addition of b-CD. The extraction treatments are shown in Table 1.

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). 1 ml 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™) and Caco-2 colorectal adenocarcinoma cells (HTB-37™) 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

Table 1
Extraction treatments

Extract code	Method	Mistletoe's weight	β-CD	Solvent	Time	Pressure
30	HTBE	0.5 g	-	30% EtOH	10 min	0.2 MPa
30hp	HTBE	0.5 g	0.05 g	30% EtOH	10 min	0.2 MPa
0.5hp	HTBE	0.5 g	0.05 g	0.5% EtOH	10 min	0.2 MPa
trad	decoction	15 g	-	dwater	2 hours	-

*HTBE: high temperature batch extraction

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 mg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37˚C.

We 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 ml of medium containing 5 x 10^4 cells and then incubated for 48 hours. Media (100 ml) containing several concentrations of extract (1mg/ml-10 mg/ml medium) were added to the wells and incubation continued for another 48 hours. The cells were treated with 100 ml of neutral red at a concentration of 25 mg 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.

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 HTBE 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 30hpmethod and resulted in 188-mg/g dry of tea mistletoe. The HTBE yield showed the influence of ethanol concentration and the addition of HP-b-CD. However, the treatments with or without addition of b-CD in 30% ethanol did not result in significantly different yields ($p > 0.05$).

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 2). The concentration of ethanol in the HTBE has an important role in the extraction of phenolic compounds. Because the p value of the HTBEs 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 b-CD (Garcia-Rio et al., 2006). It is therefore possible that b-CD binds ethanol better compared to phenolic compounds.

Casazza et al. (2011) reported that the use of the HTBE methodology resulted in an extract that was rich in polyphenol. HTBE is also more user-friendly, allows for a shorter

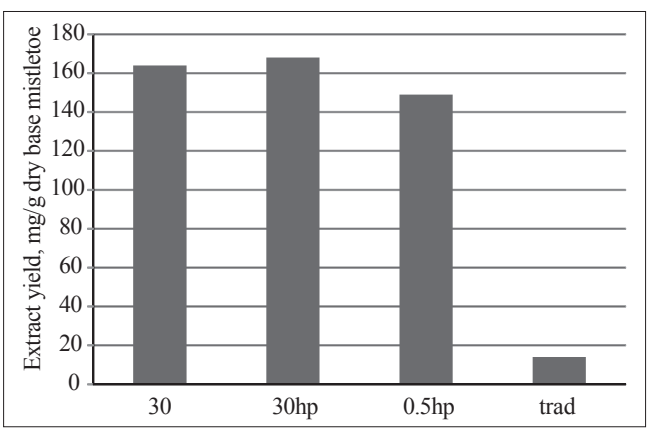

Fig. 1. Yield of several treatment extractions

extraction time and requires significantly less organic solvent (Huie, 2002). The use of hot liquid solvents in HTBE 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 monocomponent solvent system (Yilmaz et al., 2006).

Antioxidant activity

The highest antioxidant activity was obtained with the 30 extract, and the lowest activity was found with the traditional extract (Figure 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). Herrero 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).

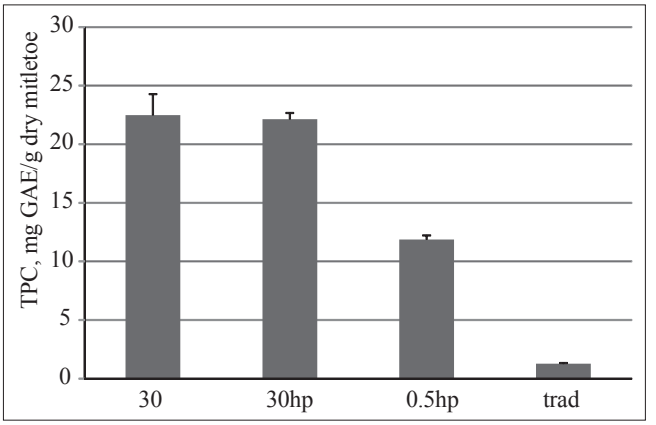

Fig. 2. Total phenolic content (TPC) of several treatment extractions

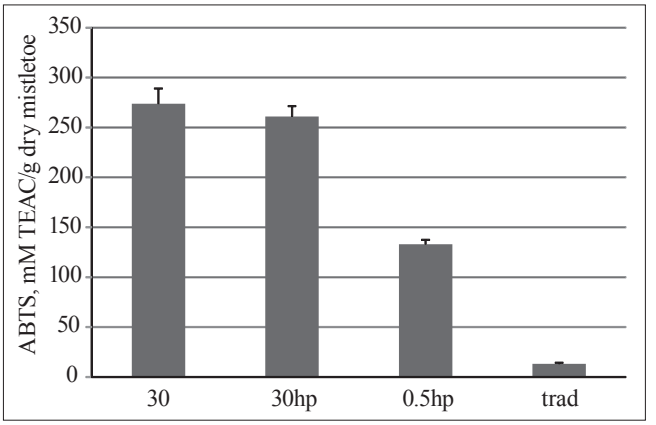

Fig. 3. Antioxidant activity (ABTS assay) of several treatment extractions

Abbreviations for extraction treatments are the same as in Table 1. Data represent the mean ± SD (n=3). Bars are standard deviations for each treatment ($P<0.05$)

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 b-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.

Cytotoxicity assay

The lowest IC50 value for cancer cells and normal cells was obtained with the 30hp extract (Figure 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 IC50 value of the 30 extract was higher than the 30hp extract. However, the

IC50 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 b-CD (826 µg/ml) in normal cells was also higher than with the addition of b-CD.

The cytotoxic effects of the extract containing b-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 b-CD but not with one of the phenolic classes. Thus the addition of b-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.

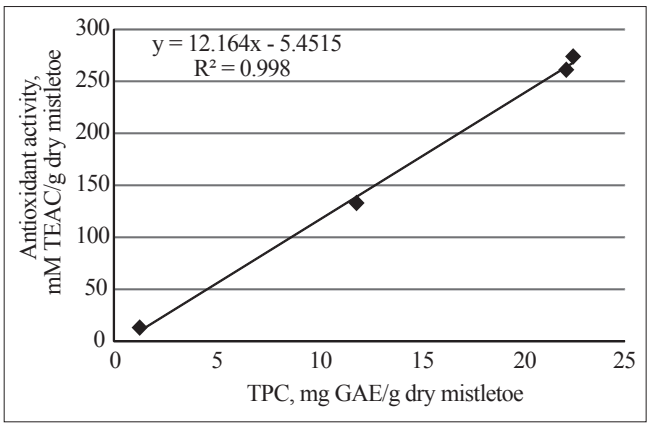

Fig. 4. Correlation between TPC values and antioxidant activities

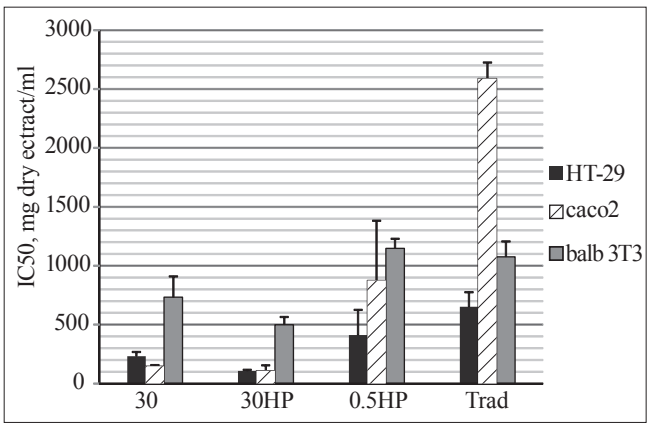

Fig. 5. IC_{50} values of extracts on neutral red uptake in the colon cancer cell lines Ht-29 and Caco-2 and the normal mouse fibroblast cell line Balb/c 3T3

The 0.5hp and 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, 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 HTBE 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 HTBE indeed gives better results than with the traditional extraction method.

Conclusion

All of the assays showed significant differences for all of the treatments, which indicates that the addition of b-CD influenced the bioactivities of the extracts. High temperature batch extraction using 30% ethanol for 10 minutes under 0.2 MPa of pressure with the addition of b-CD provided the greatest extract yield. The antioxidant activities and total phenolic contents of the extracts generated with and without the addition of b-CD were not significantly different. Furthermore, the lowest IC50 for cancer cells cytotoxicity was obtained with the 30hp extract. At a concentration of appoximately 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, we 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 b-CD.

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Received February, 28, 2014; accepted for printing June, 2, 2014.