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# Influence of ethanol concentration on the extraction of monacolin K, γ-amino butyric acid (GABA) and antioxidant activity from angkak produced from germinated brown rice as a substrate

**Kitisart Kraboun**[1,*]

**Abstract**

The present study estimated the influence of ethanol concentration on the extraction of monacolin K, Y-amino butyric acid (GABA) and antioxidant activity from angkak produced from germinated brown rice as a substrate. The various concentrations (60%, 70%, 80%, and 95%) of ethanol were used as solvents in the extraction of angkak. The monacolin K, GABA and citrinin contents and trolox equivalent antioxidant capacity (TEAC), DPPH radical scavenging activity of angkak were investigated by using various *in vitro* assays. The pigment extract of angkak extracted with 95% of ethanol concentration led to the highest pigment intensity, monacolin K, GABA and citrinin contents which were 400.92 unit/g substrate, 45.96 mg/kg dry weight, 100.38 mg/kg dry weight and 10.48 µg/kg dry weight, respectively; whereas, the lowest $IC_{50}$ values of DPPH and ABTS were 0.041 and 0.45 mmol Trolox/mL, respectively.

**Keywords:** Influence, Antioxidant, Ethanol, Germinated brown rice, Angkak

## 1. Introduction

HMG−CoA reductase (3−hydroxy−3−methyl−glutaryl−coenzyme A reductase, officially abbreviated HMGCR) is a kind of enzymes and the rate limiting reaction of cholesterol biosynthesis. It is the rate-controlling enzyme (NADH-dependent, EC 1.1.1.88; NADPH-dependent, EC 1.1.1.34) of the mevalonate pathway, the metabolic pathway that produces cholesterol and other isoprenoids. Normally in mammalian cells this enzyme is suppressed by cholesterol derived from the internalization and degradation of low density lipoprotein (LDL) via the LDL receptor as well as oxidized species of cholesterol. Competitive inhibitors of the reductase induce the this enzyme is thus the target of the widely available cholesterol-lowering drugs known collectively as the statins (Chen *et al*., 2011).

[1] Division of Food Safety Management and Technology, Department of Science, Faculty of Science and Technology, Rajamangala University of Technology Krungthep, Bangkok 10120, Thailand.

* Corresponding author, e-mail: kitisart.k@mail.rmutk.ac.th

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This type of medication is usually an option for Coronary Heart Disease (CHD) treatment. Lovastatin has been well known as a strong HMG-CoA reductase inhibitor widely known among people (see Figure 1). Lovastatin or monacolin K can be found not only a synthesis drug but also a natural product. It has been investigated that monacolin K occurred in angkak was a cholesterol lowering agent in a dog after 3 weeks with the treatment of 8 mg/kg weight per day **(**Lee *et al*., 2007**)**.

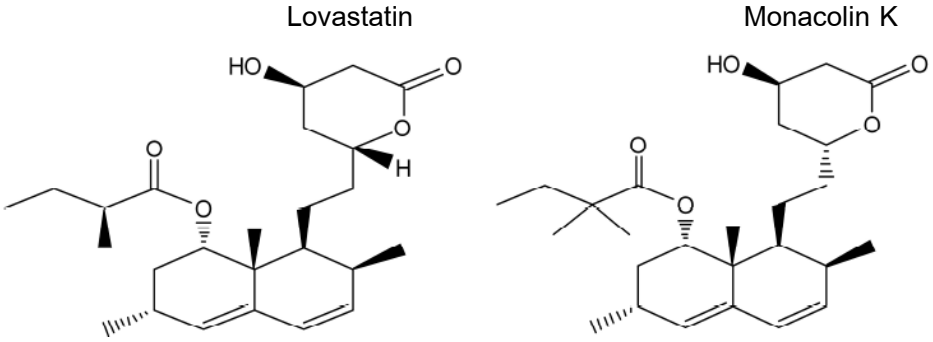


**Figure 1** Structure of monacolin K and lovastatin

There are many organic solvents used to recover antioxidants from angkak, such as methanol, hexane, chloroform, ethyl acetate and acetone. Moreover, methanol ($CH_3OH$) is better for the extraction of *Monascus* pigments and antioxidants from the cell and has been generally found to be more efficient in extraction of lower molecular weight polyphenols. However, those solvents were not permitted for use in food and packaging materials (Turkmen *et al.,* 2006). Ethanol ($C_2H_5OH$) is used often to recover the significant substances because of low price, non-harmful volatile, and non-toxic solvent in a lot of researches (Carvalho *et al*., 2007; Do *et al*., 2014; Singgih *et al*., 2014). However, there are lack of reports leading to appropriate ethanol concentration in order to the recovery of GABA, monacolin K and citrinin contents together to the overall antioxidant activities of angkak. Therefore, the objectives of this work were to investigate the effects of solvents on the extraction of GABA, monacolin K and citrinin contents from angkak and to investigate the antioxidant activities of the extracts by *in vitro* methods, such as ABTS radical scavenging assay and DPPH radical scavenging activity.

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## 2. Materials and Methods

### 2.1 Microorganism

Lyophilised *Monascus purpureus* TISTR 3090 was purchased from the Thailand Institute of Scientific and Technological Research (TISTR). The strain was cultivated on Potato Dextrose Agar (PDA; Merck, Darmstadt, Germany) at 25 °C for 7 days. After a pure culture was obtained, the mycelium was reinoculated into PDA slant and incubated at 25 °C for 7 days or until $10^6$ spores/mL before being used for angkak production.

### 2.2 Conventional fermentation method and 2-step fermentation of angkak

Conventional fermentation, brown rice (*Oryza sativa*) seeds were germinated at 48 h. Then, a 100 g of germinated brown rice (GBR) was put into a flask 500 mL and sterilized in an autoclave at 121 °C for 15 min and then left until cool down. Preparation of *M. purpureus* spore suspension was obtained from a pure culture on PDA slant, which incubated at 25 °C for 7 days or until $10^6$ spores/mL (counting spores using a hemocytometer), diluted with sterile water 5 mL for being equal to $10^6$ spores/mL. About 5 mL of $10^6$ spores/mL of *M. purpureus* was inoculated into sterilized GBR and incubated at 25 °C for 12 days. Fermentation of step 2, angkak produced from GBR with a period time for 48 h was then reinoculated with the same volume and spore suspension contents and continuously fermented with the same condition as the conventional method for another 12 days. Then, the product was dried in an oven at 40 °C for 24 h. A fine powder (20 mesh) was obtained using a mill (Retsch ultracentrifugal mill and sieving machine, Haan, Germany) (Kongbangkerd *et al*., 2014).

### 2.3 Sample extraction for antioxidant activity assay

The extraction method described by Yang *et al*. (2006) was used with some modifications. About 10 g of blended dried angkak were put in an Erlenmeyer of 250 mL and then added by 100 mL of ethanol solution. Concentration of ethanol solution used was varied i.e. 60, 70, 80, and 95% (v/v). After that MFR was agitated in a shaker incubator (New Brunswick Scientific, Edison M.J, USA) for 24 h at 170 rpm. The filtrates were separated under filtered vacuum through Whatman no. 4 filter paper. The residue was then extracted with two additional 100-mL portions of methanol as described above. The combined methanolic extracts were then evaporated at 40 °C to dryness. The dried product was used for analysis of antioxidant activities.

### 2.4 Pigment intensity

A 1 g of angkak was extracted with 5 mL methanol using a rotary shaker at 170 rpm for 1 h. The extract was then filtered through Whatman no.4 filter paper to remove suspended solids and the supernatant was analysed by a spectrophotometer (Thermo spectrophotometer

model Genesys 20) against a methanol blank. The pigment concentration was measured at 500 nm (Yongsmith *et al*., 2000). Pigment intensity was calculated from the following equation.

$$\text{Pigment intensity} = \frac{A_{500} \times \text{dilution factor} \times \text{Volume of methanol}}{\text{(Unit /g dry weight) Weight of sample (g)}}$$

**2.5 ABTS radical scavenging assay**

For ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay, antioxidant activity of angkak extracts against $ABTS^+$ radical was evaluated spectrophotometrically by a slightly modified method of Re *et al*. (1999). ABTS radical scavenging assay is based on the scavenging of $ABTS^+$ radical converting into a colourless product. The degree of decolourisation induced by a compound is related to that induced by Trolox, giving the 'ABTS value'. The $ABTS^+$ radical was produced by the reaction between 2 mL of 7 mM ABTS solution and 40 µL of 2.45 mM potassium persulphate solution and stored in the dark at room temperature for 16 h. Before usage, the $ABTS^+$ solution was diluted to get an absorbance of 0.700 ± 0.025 at 734 nm with ethanol. For the assay, the resulting solution was mixed with 300 µL of sample of each pigment extract (1–20 mg/mL). The absorbance was read at 30°C after exactly 6 min. The obtained absorbance of samples was compared with a standard curve from the corresponding readings of Trolox (0.4–0.04 mM). ABTS radical scavenging assay was estimated as Trolox equivalents by interpolation to 50% inhibition ($IC_{50}$).

**2.6 DPPH radical scavenging activity**

The scavenging activity (H/e-transferring ability) against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was measured spectrophotometrically by following Velazquez *et al*. (2003).

Each 1–20 mg/mL of aliquot of 40 µL appropriately diluted extracts was mixed with 200 µL of 0.02 mM DPPH solution and methanol 4 mL. Samples were kept for 15 min at 25 °C and the absorbance was measured at 517 nm. The absorbance of a blank sample containing the same amount of solvent was also measured. The extent of decolourisation is calculated as a percentage reduction of absorbance, and this is determined as a function of concentration and calculated relative to the 0.1–0.01 mM of equivalent Trolox concentration. The radical scavenging activity is expressed in mmol of equivalent Trolox per gram of sample (mmol Trolox /mL) with interpolation to 50% inhibition ($IC_{50}$).

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**2.7 Monacolin K analysis**

An 0.5 g sample was extracted with 25 mL of 70% ethanol at 50 °C for 2 h, followed by filtration through a 0.2 µm membrane and the extract was analysed by HPLC. The HPLC system consisted of Shimadzu LC−10AT VP Liquid Chromatograph, a FCV−10AL VP pump, an LDC Analytical SpectroMonitor 3100 detector set at 238 nm and an LDC Analytical CI−4100 integrator. A chromatography column Ascentis C18, 5 µm, 250×4.6 mm was connected to a 20 µL loop injector. An isocratic mobile phase of acetonitrile : water in the ratio of 65:35 (by vol.) was used. The flow rate and temperature were 1.0 mL/min and 28 °C, respectively (Chayawat *et al*., 2009). Monacolin K dissolved in 70% ethanol was used as a standard.

**2.8 γ-amino butyric acid (GABA) analysis**

One gram of dried angkak powder was extracted with 5 mL water at 60 °C for 2 h with vigorously shaking. After 12,000 × g cencentrifuging for 20 min at 4 °C, 400 µL aliquot of supernatant (or standard solution of GABA) was vacuum-dried. The residue was dissolved in 50 µL ethanol-water-triethylamine (2:2:1) solution, and the mixture was then evaporated to dryness under vacuum until dry and redissolved again in 40 µL ethanol−water−triethylamine-phenylisothiocyanate solution (6:1:1:1). The final mixture was allowed to react for 20 min at room temperature to form phenylisothiocyanate-GABA (PTC-GABA) (Wang *et al*., 2004).

Procedure of HPLC analysis described by Wang *et al.* (2004) was slightly modified. Briefly, the dry residue containing PTC−GABA was dissolved by adding 400 µL µmobile phase that consisted of 80% solution A (aqueous solution of 8.205 g sodium acetate, 0.5 mL triethylamine, 0.7 mL acetic acid, and 5.0 ml acetonitrile in 1000 ml distilled water, pH 5.8) and 20% solution B (acetonitrile-water, 60:40, pH 5.8). Chromatographic separation was conducted on a Shim-pack VP-ODS C18 column (4.6 × 150 mm *i.d*., 5 µm). The eluent was pumped at a flow rate at 0.6 mL/min. Temperature of column oven was 46°C and UV detection wavelength was set at 254 nm.

**2.9 Citrinin analysis**

Citrinin analysis was described by Lim *et al*. (2010). A 1 g sample was extracted with a solution (acetone:ethyl acetate = 1:1, v/v) at 65 $^{o}$C for 90 min under vigorous shaking. The supernatant was obtained by centrifugation at 1,600 × g for 10 min followed by filtration through a 0.45 µm PTEE filter unit (National Scientific, Rockwood, TN). The citrinin was determined by HPLC using a chromatography column Ascentis C18 column (4.6 × 250 mm). The mobile phase consisted of methanol/acetonitrile/ 0.1% phosphoric acid (3:3:4, v:v:v) and the analysis was performed with a fluorescence detector set at excitation and emission

wavelengths of 330 and 500 nm, respectively. The flow rate was 0.6 mL/min and the sample was spiked to confirm the presence of citrinin.

**2.10 Statistical analysis**

All determinations were performed in triplicate and results were expressed as the mean ± standard deviation calculated using spreadsheet software Microsoft Excel. This was carried out in a completely randomized experimental design (CRD) and the data were analyzed by an analysis of variance ($p \leq 0.05$) and means were separated by Duncan's multiple range test. The results were processed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA) for Windows.

## 3. Results and Discussion

### 3.1 The Influence of ethanol concentration on the extraction of pigment intensity, monacolin K, GABA and citrinin contents

Pigment intensity and monacolin K, GABA and citrinin contents of pigment extracts of angkak extracted with 60, 70, 80 and 95 % of ethanol concentration are shown in Table 1. The pigment intensity and monacolin K, GABA and citrinin contents increased with the ethanol concentrations ($p \leq 0.05$). The highest pigment intensity and monacolin K, GABA and citrinin contents of the pigment extract of angkak extracted with 95% of ethanol concentration were 400.92 unit/g substrate and 45.96 mg/kg dry weight,100.38 mg/kg dry weight and 10.48 µg/kg dry weight, respectively. Whereas, the lowest pigment intensity and monacolin K, GABA and citrinin contents were obtained from the pigment extract of angkak extracted with 60% of ethanol concentration. This was in agreement with Singgih *et al.* (2014) who reported that 40, 50, 60, 70, 80 and 95% of ethanol concentration were studied to extract angkak. It was found that the highest contents of monacolin K (1,760 µg/g) were achieved when 95% of ethanol concentration used to extract angkak. This is a cause of the lower level polarity of ethanol 95% v/v. As well, monacolin K, GABA and citrinin are more soluble in ethanolic solution; therefore, the higher ethanol concentration increased the yield of monacolin K, GABA and citrinin (Arabshahi *et al*., 2007).

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**Table 1** Pigment intensity, monacolin K, GABA and citrinin of pigment extracts from angkak extracted with 60 70 80 and 95% of ethanol concentration

| Ethanol concentration (%) | Pigment intensity (unit/g substrate)* | Monacolin K (mg/kg dry weight) | GABA (mg/kg dry weight) | Citrinin (µg/kg dry weight) |
|---|---|---|---|---|
| 60 | 150.17 ± 4.00$^{a}$ | 15.98 ± 2.00$^{a}$ | 31.11 ± 2.52$^{a}$ | 3.78 ± 0.52$^{a}$ |
| 70 | 180.21 ± 5.30$^{d}$ | 20.98 ± 1.10$^{b}$ | 40.87 ± 3.87$^{b}$ | 4.58 ± 0.44$^{b}$ |
| 80 | 228.22 ± 6.19$^{c}$ | 30.04 ± 3.10$^{c}$ | 58.49 ± 4.66$^{c}$ | 6.37 ± 0.61$^{c}$ |
| 95 | 400.92 ± 6.14$^{b}$ | 45.96 ± 2.40$^{d}$ | 100.38 ± 6.40$^{d}$ | 10.48 ± 1.59$^{d}$ |

**Note:** Different letters behind means within a column are significantly different ($p \leq 0.05$).

### 3.2 The $IC_{50}$ values of DPPH radical scavenging activity and ABTS radical scavenging assay

The $IC_{50}$ values of DPPH and TEAC of pigment extracts from angkak extracted with 60 70, 80 and 95% are shown in Figure 2. The $IC_{50}$ values of DPPH and ABTS showed a trend to decrease with increasing ethanol concentration ($p \leq 0.05$). As well, the lowest $IC_{50}$ values of DPPH and ABTS were 0.041 and 0.45 mmol trolox /mL, respectively, when 95% of ethanol concentration applied to extract angkak. Nevertheless, using 60% ethanol concentration led to the highest $IC_{50}$ values of DPPH and ABTS which were 0.9 and 2.29 mmol Trolox /mL, respectively. In this study, there were correlations between $IC_{50}$ values of antioxidant activities (DPPH and ABTS) and the pigment intensity and monacolin K, GABA and citrinin contents of the pigment extracts from angkak (Table 1 and Figure 2). With increasing water content in the solvent, monacolin K, GABA and citrinin and antioxidant activities decreased. These results were different from the results of previous studies on black tea and mate tea (Turkmen *et al*., 2006) and bunga kantan inflorescence (Wijekoon *et al*., 2011). The differences between the results of this study and those of other studies may be attributed to several factors: (1) the difference in sample matrix; (2) different solvents used in extraction resulted in differences in compositions and antioxidant activities of the extracts; (3) an extract possessing secondary metabolic compounds that contain a higher number of hydroxyl groups has a higher antioxidant activity; (4) the method and conditions of extraction (temperature and time) also affecting antioxidant activities (Robards, 2003).

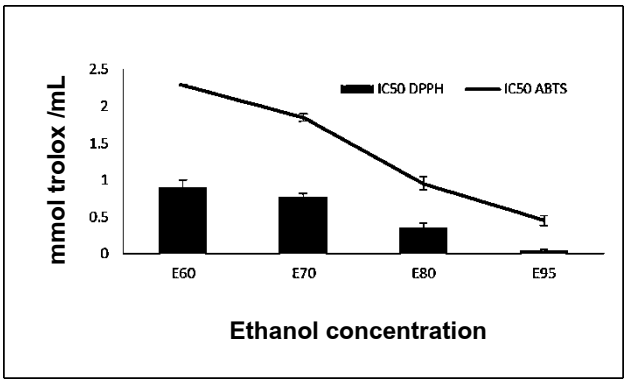


**Figure 2** $IC_{50}$ values of DPPH and ABTS of pigment extracts from angkak extracted with 60, 70, 80 and 95% of ethanol concentration

## 4. Conclusion

The extracted monacolin K, GABA and citrinin increased and $IC_{50}$ values of DPPH and ABTS decreased when increasing ethanol concentration. This may be caused by the lower polarity of organic solvent that facilitates the extraction of all compounds. It was clear that the extract obtained by 95% ethanol showed the highest pigment intensity and monacolin K, GABA and citrinin contents which were 400.92 unit/g substrate and 45.96 mg/kg dry weight, 100.38 mg/kg dry weight and 10.48 µg/kg dry weight, respectively. Whereas, the lowest $IC_{50}$ values of DPPH and ABTS were 0.041 and 0.45 mmol trolox /mL, respectively. The results of this work indicated that a proper extraction solvent was established to be an effective solvent for angkak extraction to obtain *Monascus* pigment to be used as medicine against free-radical-associated oxidative damage.

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