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Determination of antioxidant activities of dried kacangma (*Leonurus sibiricus)* extract in three bioassay systems

[Penentuan aktiviti antipengoksidaan ekstrak kacangma *(Leonurus sibiricus)* kering dalam tiga sistem bioasai]

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Keywords: *Leonurus sibiricus*, antioxidant activity, extraction, radical scavenging, xanthine oxidase superoxide

Abstract

Kacangma (*Leonurus sibiricus* L.) is a potential medicinal and culinary herb of Sarawak and its biological activities have not been fully studied. Antioxidant activities of ethanol and aqueous extracts of dried kacangma were studied under three bioassay systems, namely, auto-oxidation of linoleic acid lipid system (LP), xanthine oxidase superoxide scavenging activity (XOD) and 1,2-diphenyl-2-picrylhydrazyl radical scavenging activity (DPPH). Based on the antioxidant activity range, antioxidant activity of ethanol extract was high in LP system (72%), moderate in XOD system (70%) and low in DPPH system (49%). On the other hand, antioxidant activity of aqueous extract was found high in all three bioassays with mean percentages of 73, 76 and 78% for LP, XOD and DPPH respectively. Thus, the results indicated that extraction using water was more efficient than ethanol in extracting antioxidant compounds from dried kacangma.

Introduction

Free radicals are by-products from biological functions in the body such as food metabolism and biochemical reactions. They are created when cells use oxygen to generate energy or enter the body from a polluted environment. These by-products are generally reactive oxygen species (ROS) such as superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and hypochlorous acid (HOCl) that resulted from the cellular redox process (Aruoma 1998; Vimala et al. 2003; Amarowicz et al. 2004). At high levels, free radicals generate oxidative stress that can damage cell structures, including lipids, proteins and DNA (Pham-Huy et al. 2008). Oxidative stress is found to be associated with a wide range of diseases such as cancer, rheumatoid arthritis, cataract, cardiovascular and autoimmune disorders, and the premature onset of aging symptoms such as skin wrinkling and chronic fatigue (Dorman et al. 2003; Willcox et al. 2004; Pham-Huy et al. 2008).

The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced *in situ* as natural antioxidation enzymes (such as superoxide dismutase, glutathion peroxidase and catalase), or externally supplied through diet

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and/or supplements (such as α-tocopherol, ascorbic acid, carotenoids and phenolic compounds). These antioxidants act as free radical scavengers by preventing and repairing damages caused by ROS (Mau et al. 2002; Murakami et al. 2006; Pham-Huy et al. 2008). Even though majority of the food industries today have used synthetic antioxidants effectively, consumers are more health conscious and prefer natural phytochemicals to synthetic chemicals. Epidemiological evidence strongly suggests that foods containing phytochemicals with antioxidant potential have strong protective effects against major diseases and hinder aging symptoms. Thus, the commercial development of plant materials as sources of antioxidants to enhance health and food preservation is of current interest (Rice-Evans et al. 1997).

Kacangma (*Leonurus sibiricus* Linn.) is a herbaceous shrub from the mint family Lamiaceae with quadrangle erect branches. As a popular traditional medicinal herb of Sarawak, the kacangma leaves are chopped, sun-dried and used as culinary ingredient as well as folk remedy for post-natal care especially for women to reduce body pain, emmenagogue and to hasten the contraction of uterus after delivery (Chai et al. 1989). Although the role of kacangma as a potential medicinal herb has been recognized by the Malaysian Ministry of Agriculture (MOA 1995, Paulus and Lau 2004), the biological properties of kacangma such as its antioxidant activity have not been fully studied. Hence, this experiment was conducted to determine the antioxidant activities of ethanol and aqueous extracts of dried kacangma herb using three bioassay systems i.e. auto-oxidation of linoleic acid lipid system (LP), xanthine oxidase superoxide scavenging activity (XOD) and 1,2-diphenyl-2-picrylhydrazyl radical scavenging activity (DPPH).

Materials and methods

Raw materials

Dried kacangma leaves were obtained by drying the freshly harvested kacangma herbs grown in MARDI Kuching. Kacangma of pink-flowered variety at 70-day maturity was used for producing dried leaves. The aerial parts of the plant, which consists of leaves and young stems, were chopped and oven-dried at 60 °C in a force-air oven for 5 – 6 h until a final moisture content of below 6% (w/w). The dried herb was then ground into powder and stored in airtight containers (Chua et al. 2006).

Preparation of kacangma extracts

The extraction is conducted based on the method of de Souza et al. (2004). Ethanol extract of kacangma was prepared by soaking 100 g powdered kacangma in 600 ml ethanol for 48 h at room temperature. The solvent was then filtered and evaporated using a rotary evaporator (Heidolph WB2001) at 36 – 40 °C. The dried crude extract was weighed and dissolved in 99.5% ethanol to provide a series of stock solutions of 10, 25, 50 dan 100 mg/ml prior to antioxidant testing.

To obtain the aqueous extract, 100 g powdered kacangma was refluxed with 1,000 ml water for 3 h at 100 °C. The solvent was then filtered, concentrated to 1/3 of the initial volume and freeze dried. The freeze dried extract was weighed and dissolved in distilled water to provide a series of stock solutions of 10, 25, 50 dan 100 mg/ml prior to antioxidant testing.

Auto-oxidation of linoleic acid lipid system

The auto-oxidation assay is performed according to the method by Osawa and Namiki (1981) with slight modifications by Vimala et al. (2003). The assay evaluates the inhibitory activity of the samples against lipid peroxidation (oxidation of fatty acids) caused by hydrogen peroxides. This system is also called ferric thiocyanate method (FTC).

A sample solution containing 4.0 mg plant extract in 4.0 ml of 99.5% ethanol, 4.1 ml of 5.0% linoleic acid in 99.5% ethanol, 8.0 ml 0.05M phosphate buffer (pH 7.0) and 3.9 ml of distilled water was placed in a columnar vial (diameter 40 mm, height 75 mm) with a screw cap and incubated in the dark at 40 °C for one week. To 0.1 ml of this sample solution, 9.7 ml of 75.0% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 0.002 M ferrous chloride in 3.5% hydrochloric acid were added to the reaction mixture. After 3 min, the absorbance of the red colour was measured at 500 nm. Antioxidant activity was determined from the decrease of the absorbance relative to the negative control (solution without kacangma extract) which was incubated without plant sample. Butylated hydroxy toluene (BHT) 4.0 mg was used as positive control.

Xanthine oxidase superoxide scavenging system

This assay was conducted according to the methods of Chang et al. (1996) and Vimala et al. (2003) with slight modifications. The XOD superoxide scavenging activity was determined by means of spectrophotometric measurement of the product on the reduction of nitro blue tetrazolium (NBT) (100 ml of 4.1 mM/litre) which was prepared by adding 3.15 g trizma hydrochloride (TrisHCl), 0.1 g $MgCl_2$, 15.0 mg 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and 34.0 mg 4-nitro blue tetrazolium chloride to 100 ml distilled water. Measurement was made spectrophotometrically at 560 nm in a 1.0 ml volume cuvette after the addition of XOD at 25 °C.

The reaction mixture in a total volume of 1.0 ml contained 0.53 g (50 mM) sodium carbonate (pH 10.2), 0.04 g (0.1 mM) EDTA, 50.0 mM NBT solution and 0.05 g (2.5 mM) xanthine. The reagent is kept refrigerated at 4 °C. The reaction mixture in a 1.0 ml volume cuvette was inserted into the spectrophotometer holder; attached to a refrigerant set at 25 °C, to obtain the auto zero base line. Then, 0.1 µl XOD was added to the reaction mixture, covered with disposable parafilm and inverted 5 times in order to mix the content. The cuvette was then inserted into the spectrophotometer holder and measurement was started. OD was measured at 560 nm for 100 s. For screening of kacangma extracts, samples at a concentration of 250 µg/ml was added to the reaction mixture before setting to zero and the addition of XOD.

1,2-diphenyl-2-picrylhydrazyl radical scavenging system

The effect of plant extracts on DPPH radical was estimated according to the method of Blois (1958) with slight modifications by Brand-Williams et al. (1995). Scavenging of DPPH, a stable free radical, represents the free radical reducing ability of antioxidants based on a one-electron reduction. Scavenging of DPPH determines the antioxidant potential (AOP) of the test sample, which shows its effectiveness, prevention, interception and repair mechanism against injury in a biological system caused by free radicals.

Four ml kacangma extract (0.5 mg/ml) was added to 1.0 ml of DPPH (1.0 mM in methanol solution) in a 5.0 ml amber bottle with screw cap. The mixture was shaken and left to stand at room temperature for 10 min until purple cromogen was formed. The absorbance of the resulting solution was measured spectrophotometrically at 520 nm. Ascorbic acid (5.0 µg/ml) was used as the positive control. Ethanol (99.0%) substituting DPPH served as a blank to obtain the auto zero base line.

To determine the kacangma extract concentration required to achieve optimal inhibition, DPPH dose-response study was conducted. A range of 10 concentrations were used from 0.0 to 2.0 mg/ml as shown in *Table 1*. These concentrations were obtained by ranging the 20 mg/ml plant stock solution against methanol.

Table 1. Preparation of kacangma stock solutions for DPPH dose-response study

No.	Dose (mg/ml)	Plants stock solution (20 mg/ml) (X)	Methanol (99.0%) (4 ml – X)	Total (ml)
1	0.00	0.00	4.00	4.00
2	0.01	10.0	3.99	4.00
3	0.05	0.50	3.95	4.00
4	0.15	0.15	3.85	4.00
5	0.25	0.25	3.75	4.00
6	0.50	0.50	3.50	4.00
7	1.00	1.00	3.00	4.00
8	1.25	1.25	2.75	4.00
9	1.50	1.50	2.50	4.00
10	2.00	2.00	2.00	4.00

Statistical analysis

Data were analysed using analysis of variance (ANOVA) at 5% level of probability (p <0.05). Significance was determined using Duncan Multiple Range Test (DMRT) on all possible pairs of treatment means using the Statistical Analysis System (SAS 1994). All values were expressed as group mean ± standard deviation of mean.

Results and discussion

Effects of kacangma extract on antioxidant activities

Antioxidants in kacangma were extracted in both aqueous and ethanol using three bioassay systems: auto-oxidation of linoleic acid lipid system (LP), xanthine oxidase superoxide scavenging activity (XOD) and 1,2-diphenyl-2-picrylhydrazyl radical scavenging activity (DPPH). The results of the antioxidant activity are shown in *Figure 1* as determined according to the antioxidant activity range suggested by Vimala et al. (2003) (*Table 2*). These results showed that the activities varied between the ethanol and aqueous extracts. Higher activities were observed in the aqueous extracts of kacangma indicating that water was a more efficient solvent for extraction of antioxidant compunds (*Figure 1*). The results also indicated the

Table 2. Antioxidant activity range and status

Antioxidant activity range (%)	Activity status
0	Nil
1 – 39	Low
40 – 69	Moderate
70 – 100	High

(Source: Vimala et al. 2003)

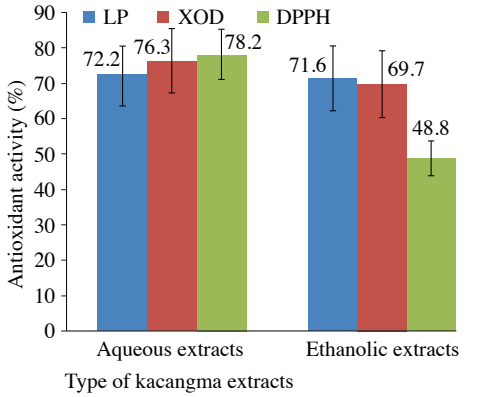

Figure 1. Antioxidant activities of aqueous and ethanol extracts of dried kacangma. LP: auto-oxidation of linoleic acid lipid system; XOD: xanthine oxidase superoxide scavenging activity; DPPH: 1,2-diphenyl-2-picrylhydrazyl radical scavenging activity

possible influence of extracting solvent on natural antioxidants such as ascorbic acid and phenolic compounds. Previous study on nutritional value of kacangma (Chua et al. 2005) showed that the ascorbic acid content of the 70-day pink-flowered kacangma was 7.51 mg/100 g, whereas total polyphenol content ranged from 4.24 mg/g to 10.91 mg/g depending on the drying temperatures. Drying temperature at 60 °C was found to produce herb with higher polyphenol.

Studies conducted by Sulaiman et al. (2011) and Kchaou et al. (2013) showed that the antioxidant compounds in plant samples varied among different solvent extractions. For instance, the recovery of phenolic contents is influenced by the polarity of extracting solvents and the solubility of the compounds in the solvent used for the extraction process. A mixed polarity solvent may be required to extract more phenolic contents. For example, addition of water up to 50% in acetone was found to increase extraction of total phenolic compounds.

Antioxidant activities based on the bioassay systems

The LP system was based on auto-oxidation of linoleic acid to determine inhibition of plant extracts towards lipid oxidation by hydrogen peroxide. The XOD system assessed the inhibition rate of the free radical, superoxide anion, while the DPPH system involved cleaning DPPH free radicals to determine the antioxidant potential (AOP) of the plant extracts (Vimala et al. 2003).

In the LP system, both aqueous and ethanol extracts of kacangma showed high antioxidant activity i.e. 72.7% and 71.6% respectively (*Figure 1*). This means that both media of extraction are effective in the inhibition of lipid oxidation and have the potential to be used for preventing rancidity caused by lipid oxidation in most food products.

Based on the XOD system, ethanol and aqueous extracts of kacangma showed moderate (69.7%) and high (76.3%) activity respectively. Aqueous extract of kacangma has higher ability to scavenge superoxide free radical anions. The scavenger effect of free radicals in the aqueous extract may be due to certain water soluble phytochemicals such as phenolic acids and flavonoids.

Meanwhile, in the DPPH system, the aqueous extract showed very high activity (78.2%) whereas the ethanol extract had low activity (48.8%). This means that aqueous extract of kacangma is more appropriate for repairing damages to biological systems because it is more effective in inhibiting free radicals compared to the ethanol extract (Vimala et al. 2003).

Different results of these assays suggested that the antioxidant activities of extracts do not depend upon only one particular compound. A study by Scartezzini et al. (2006) showed that the antioxidant activity of aqueous extract of processed fruit is due to ascorbic acid content for only 60% or less. Other compounds such as hydrolysable tannins can also exhibit a strong antioxidant activity in both *in vitro* and *in vivo* systems (Bhattacharya et al. 2000). Besides, radical-scavenging activity differs not only by the concentration of antioxidant compounds but also with degree of hydroxylation and polymerisation as suggested by Kchaou et al. (2013). In this study, low antioxidant activity of the ethanol extract in the DPPH system may be related to oil fraction interference as reported in a study by Prasad et al. (2010).

Antioxidant activity in food products is usually determined using LP system. However, more detailed qualitative and quantitative analyses of the compounds with antioxidant activity will be necessary to elucidate the antioxidant activity of kacangma. It is suggested that other analyses be carried out especially for plant samples to measure activities during early and maturity stages (Schwarz et al. 2001; Kaur and Kapoor 2002). This allows for comparisons to determine the characteristics of the antioxidant mechanism and at the same time, evaluate the practical uses of

food product as antioxidant diet (Frankel et al. 1996; Lampi et al. 1997; Matkowski and Piotrowska 2006). The methods of analyses towards this end include the thiobarbituric acid test (TBA), electron spin resonance spectroscopy (ESR) (Halliwell and Gutteridge 2007) and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid test (ABTS) (Li et al. 2012).

The DPPH dose-response study was carried out to determine the dosage of kacangma extract required to achieve optimal inhibition. As shown in *Figure 2*, the dosage needed to achieve 100% inhibition of free radicals was approximately 0.5 mg/ml.

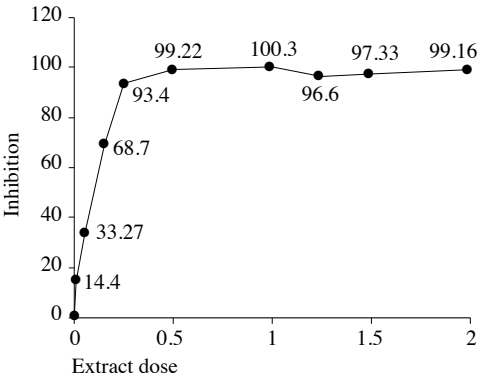

Figure 2. DPPH dose-response study of dried kacangma extracts

Comparison of kacangma antioxidant potential with other plants

Vimala et al. (2003) reported on antioxidant activity in a variety of local vegetables and herbs. To determine the antioxidant potential of kacangma, a comparison was made between aqueous extract of kacangma with aqueous extract of 10 other local culinary plant species, as shown in *Table 3*, in which the antioxidant activities were measured using the same bioassay systems i.e. LP, XOD and DPPH.

The antioxidant activity of kacangma aqueous extract ranged from 73 – 78% for all three bioassay systems, and is categorised as high activity, even though the percentages are lower than those of the plants tested, for

Table 3. Comparison of antioxidant activity of aqueous extract of kacangma with 10 other local culinary plant species

Plant species	Family	Antioxidant activity of aqueous extract (%) and activity ranges		
		LP	XOD	DPPH
Kacangma (*Leonurus sibiricus*)	Lamiaceae	72.7 (H)	76.3 (H)	78.2 (H)
Basil (*Ocimum basilicum*)	Lamiaceae	92.1 (H)	90.8 (H)	95.5 (H)
Ulam Raja (*Cosmos caudatus*)	Compositae	97.9 (H)	93.8 (H)	97.3 (H)
Bamboo shoot (*Dendrocalamus giganteus*)	Gramineae	80.0 (H)	86.0 (H)	40.5 (M)
Jering (*Achidendron jiringa*)	Leguminoseae	33.9 (L)	93.1 (H)	97.1 (H)
Petai (*Parkia speciosa*)	Leguminoseae	85.4 (H)	89.2 (H)	47.1 (M)
Okra (*Abelmoschus esculentus*)	Malvaceae	84.4 (H)	92.6 (H)	41.5 (M)
Kesum (*Polygonum minus*)	Polygonaceae	98.3 (H)	97.4 (H)	97.5 (H)
Mengkudu (*Morinda citrifolia*)	Rubiaceae	95.5 (H)	69.2 (M)	49.2 (M)
Curry leaf (*Murraya koenigii*)	Rutaceae	97.7 (H)	8.9 (L)	94.1 (H)
Pegaga (*Centella asiatica*)	Umbelliferae	98.2 (H)	86.4 (H)	92.7 (H)

Bioassay system:
LP: auto-oxidation of linoleic acid lipid system
XOD: xanthine oxidase superoxide scavenging activity
DPPH: 1,2-diphenyl-2-picrylhydrazyl radical scavenging activity

Antioxidant activity range:
(L): Low
(M): Moderate
(H): High

example, basil *(Ocimum basilicum)*, ulam raja *(Cosmos caudatus)*, kesum *(Polygonum minus)* and pegaga *(Centella asiatica)* which had percentages in the range of 86 – 98%. Apart from that, inhibition of DPPH free radicals for kacangma aqueous extract was higher than bamboo shoot (*Dendrocalamus giganteus*), petai (*Parkia speciosa*), okra (*Abelmoschus esculentus*) and mengkudu (*Morinda citrifolia*).

Conclusion

Extraction in water is more effective in obtaining antioxidant compounds from kacangma compared to extraction in ethanol. Kacangma ethanol extract was high in LP system, moderate in XOD system and low in DPPH system. On the other hand, the antioxidant activities of kacangma aqueous extract was high in all three systems, specifically 73, 76 and 78% for LP, XOD and DPPH systems respectively.

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Abstrak

Kacangma (*Leonurus sibiricus* L.) ialah herba ubatan dan masakan yang berpotensi di Sarawak yang aktiviti biologinya belum dikaji secara mendalam. Aktiviti antipengoksidaan ekstrak etanol dan air daripada herba kacangma kering telah dikaji menggunakan tiga sistem bioasai iaitu sistem pengoksidaan auto asid linoleik (LP), sistem menghapus sisa xantin oksidase superoksida (XOD) dan sistem menghapus sisa 1,2-difenil-2-pikrilhidrazil (DPPH). Berdasarkan turutan aktiviti antipengoksidaan, ekstrak etanol daripada kacangma kering didapati mempunyai aktiviti antipengoksidaan yang tinggi dalam sistem LP (71.6%), sederhana dalam sistem XOD (69.7%) dan rendah dalam sistem DPPH (48.8%). Sebaliknya, ekstrak air daripada kacangma kering pula didapati mempunyai aktiviti antipengoksidaan yang tinggi dalam ketiga-tiga sistem iaitu masing-masing pada 73, 76 and 78% bagi LP, XOD dan DPPH. Hasil kajian ini menunjukkan pengekstrakan menggunakan air lebih berkesan dalam mengeluarkan bahan antipengoksidaan daripada kacangma kering berbanding dengan etanol.

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