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ANTIOXIDATIVE ACIVITY OF MALAYSIAN HERB EXTRACTS IN REFINED, BLEACHED AND DEODORIZED PALM OLEIN

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ABSTRACT

*Malaysian herbs such as curry leaves (*Murraya koenigii*), kaffir lime leaves (*Citrus hystrix*), pandan leaves (*Pandanus amaryllifolius*) and turmeric leaves (*Curcuma longa*) are often used as flavourings in Malaysian food. They may contain various phytochemicals, which may act as antioxidants or pro-oxidants. In order to investigate the antioxidant capacity, the extracts of these herbs were subjected to antioxidant tests and analysed for polyphenol content. For the accelerated oxidation study, the ethanolic extracts were added to refined, bleached and deodorized (RBD) palm olein and heated to 180°C for 32 hr. The polyphenol content was observed to be highest in turmeric leaves (116.3 mg g^{-1} gallic acid equivalents), followed by curry leaves (109.5 mg g^{-1}), kaffir lime leaves (103.2 mg g^{-1}) and pandan leaves (101.8 mg g^{-1}). In the DPPH assay and the linoleic acid model system, the antioxidative activity was highest in turmeric leaves followed by curry leaves, kaffir lime leaves and pandan leaves. However, in heated oil, the highest activity was observed in the sample containing curry leaf and kaffir lime leaf extracts at 0.4% with activities comparable to that of BHT ($p<0.05$). Based on the oxidative stability index (OSI), each of the herbs was capable of retarding oxidation significantly even at 0.1% compared to BHT ($p<0.05$). The results of this study suggest that these Malaysian herbs have great potential as heat-stable antioxidants, and could be used as alternatives to existing synthetic antioxidants.*

Keywords: antioxidant assays, Malaysian herb extracts, RBD palm olein, accelerated oxidation study.

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INTRODUCTION

Deep fat frying influences the qualities of the fried food product such as flavour, texture, shelf-life and nutritional attributes. Palm olein is one of the most common frying media, used extensively both at home and on a commercial scale. As frying is usually carried out at high temperatures and in the presence of food, complex chemical and physical changes such as oxidation, hydrolysis and polymerization take place, leading to the formation of secondary products, which affects both the oil and the finished product qualities (Andrikopoulos *et al*., 2003).

Synthetic antioxidants such as propyl gallate, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl-hydroquinone (TBHQ) are often added to protect oils and fats by minimizing or retarding oxidation. However, retardation of oxidation occurs only at ambient temperature; thus, they are useful during storage and shipping of oils and fats, but are less effective at the frying temperature due to their volatility.

It has been reported that the dietary administration of BHT to rats caused fatal haemorrhages in pleural and peritoneal cavities, as well as organs such as epialidymis testes and

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pancreas (Deshpande *et al*., 1996). BHA, too, exhibited toxic and carcinogenic effects. These antioxidants are permitted for use within the legal limits in the food industries because of their effectiveness and low cost.

Natural antioxidative substances which are perceived to be safer, healthier and less subject to hazards than synthetic antioxidants are under extensive study owing to recent consumer interest in natural products (Cuvelier *et al*., 1994).

Plant extracts, especially those obtained from herbs and spices, have been proposed for stabilizing frying oils. According to Kaur and Kapoor (2002), herbs contain numerous phenolic compounds which may act as antioxidants because of their redox properties, allowing them to act as reducing agents, hydrogen donors, single oxygen quenchers and metal chelators. A number of studies have been conducted on the capability of natural antioxidants in retarding lipid oxidation in oil during frying (Che Man and Jaswir, 2000; Naz *et al*., 2005), in retarding thermal oxidation of heated oil (Chen and Chan, 1996; Khan and Shahidi, 2001; Nogala-Kalucka *et al*., 2005; Shyamala *et al*., 2005), and in improving food quality (Jaswir *et al*., 2000; Tanabe *et al*., 2002).

Currently, the use of natural antioxidants in industry is somehow limited due to a lack of knowledge on their molecular composition and amounts of active ingredients in the source material, and the limited availability of relevant toxicity data (Shahidi *et al*., 1994). The natural antioxidants found in herbs are safe because herbs have been used for centuries as common food ingredients. This study aims to evaluate the potential of Malaysian herbs as antioxidants, so that they could be used in the food industries.

MATERIALS AND METHODS

Herbs and Oil

Four types of herbs – curry leaves (*Murraya koenigii*), kaffir lime leaves (*Citrus hystrix*), pandan leaves (*Pandanus amaryllifolius*) and turmeric leaves (*Curcuma longa*) – were obtained from the local market, as was refined, bleached and deodorized (RBD) palm olein. Linoleic acid methyl ester and α-α-diphenyl-β-hydrazyl (DPPH) radical were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents were obtained from Merck (Darmstadt, Germany) and Fischer (USA).

Preparation of Herb Extracts

The herbs were washed thoroughly in tap water and dried in a hot air oven at 45°C for 24 hr. The dried leaves were then finely powdered using a blender. The dried powder was extracted in ethanol (99.8%, analytical grade) under reflux for 8 hr at 50°C, at a 1:10 ratio (weight/volume) of powder to ethanol. This was followed by filtration using filter paper. The solvent was then removed using a rotary evaporator. The extraction process was repeated three times.

Determination of Polyphenols

The total content of phenolics in the extract was determined using a modification of the method described by Matthaus (2002). Ten milligrams of the herbs were dissolved in 10 ml methanol (analytical grade). A 200 µl aliquot of the resulting solution was added to 1 ml of Folin-Ciocalteau reagent, then 0.8 ml of 0.2% Na_2CO_3 was added and the volume made up to 10 ml using water-methanol (4:6) as the diluting fluid. After 30 min, the absorbance of the sample was measured at 765 nm using a spectrophotometer (Ultra-spec 2000, Pharmacia Biotech, Sweden). The concentration was calculated using gallic acid as a standard (10-50 µg ml^{-1}), and the results were expressed as gallic acid equivalents per gram extract.

Free Radical Scavenging Activity

The activity of the extracts against the DPPH radical was evaluated using the method described by Blois (1958). Solution of DPPH (0.1 mmol $litre^{-1}$) in methanol was prepared and 4 ml of this solution were added to 1 ml of the herb extract solution (at 50 and 100 ppm). After 20 min, the absorbance was measured at 517 nm using the spectrophotometer (Ultra Spec 2000, Pharmacia Biotech, Sweden). Radical-scavenging activity was expressed as the inhibition percentage, and was calculated using the following formula:

$$\% \text{ Radical-scavenging activity} = \frac{(\text{Control OD-sample OD})}{\text{Control OD}} \times 100$$

where OD = optical density.

Linoleic Acid Model System

The linoleic acid model system was used in the determination of antioxidative activities of the extracts (Lingnert *et al*., 1979). The substrate, consisting of 10 mM of linoleic acid ester, was emulsified with an equal amount of Tween 20 in sodium phosphate pH7 buffer. The emulsion was then homogenized for about 1 min. Extract samples of 10 µl (at 1000, 2000, 3000 ppm) were mixed with 5 ml of the emulsion. The control was the emulsion without addition of the sample. The samples were incubated at 50°C for 20 hr, then 0.2 ml of the solution was diluted in 5 ml methanol, and absorbance was measured at 234 nm before and after oxidation.

The activity of the antioxidant (AOA) was calculated according to the following equation:

$$AOA = \frac{\Delta A\ 234\ nm\ C - \Delta A\ 234}{\Delta A\ 234\ nm\ C}$$

where ΔA 234 = increase in absorbance at 234 nm during incubation in the sample;
ΔA 234 nm C = the corresponding absorbance increase in the control.

Antioxidative Activity of Herbs in Oil

The herb extracts were added to palm olein at 0.1%, 0.2%, 0.3% and 0.4% (w/w) and the mixtures were stirred. Samples containing BHT and the control were also prepared under the same conditions. All samples were heated at frying temperature (180°C) for 0, 8, 16, 24 and 32 hr. Samples were collected, cooled to 60°C before flushing with nitrogen gas, and then kept at -20°C until the day of analysis.

Analysis of Oil Quality

Changes in the quality of the oil such as the concentration of free fatty acids, the anisidine value and the peroxide value were analysed using the MPOB Test Method (2005). Oxidative stability index (OSI) was analysed according to the American Oil Chemists' Society Official Method Cd 12b-92 (1993).

Statistical Analysis

Each analysis was done in triplicate. The MINITAB 14 software was used to analyse data for ANOVA, standard deviation and Duncan's multiple range test.

RESULTS AND DISCUSSION

Polyphenol Content

As plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it is necessary to determine their total amount in the selected plant extracts. Crude estimations of the amount of phenolic compounds present in an extract were done using Folin-Ciocalteau phenol reagent. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phomolybdic acids present in the reagent.

Table 1 indicates the polyphenolic contents of the herbs after refluxing with ethanol. Turmeric leaves had the highest amount of polyphenols followed by curry leaves, kaffir lime leaves and pandan leaves. Generally, extracts that contain high polyphenol contents also exhibit high antioxidant activity.

TABLE 1. TOTAL POLYPHENOLIC CONTENT OF DIFFERENT HERBS

Herb	Polyphenol content (mg GAE/g extract)*
Curry leaves (*Murraya koenigii*)	109.5 ± 0.3
Kaffir lime leaves (*Citrus hystrix*)	103.2 ± 0.1
Pandan leaves (*Pandanus amaryllifolius*)	101.8 ± 0.4
Turmeric leaves (*Curcuma longa*)	116.3 ± 0.2

Notes: Each value is expressed as mean ± standard deviation (n=6).
*GAE: gallic acid equivalents.

Free Radical Scavenging Activity

The DPPH assay is based on the reduction of the DPPH radical in methanol or the capability of stable free radicals to react with H-donors, which then causes an absorbance drop at 517 nm. *Figure 1* shows that at 100 ppm the free radical scavenging activity was highest in BHT; followed by turmeric leaves, curry leaves, kaffir lime leaves and pandan leaves. At 50 ppm, the highest activity after BHT was from kaffir leaves, followed by turmeric, curry and pandan leaves. The activity increased significantly with an increase in extract concentration.

At 100 ppm, the activity of turmeric leaves was in fact comparable to BHT. This indicated that turmeric leaves contained compounds which were responsible for retarding free radical activity. The observations were similar to what was reported by Ningappa *et al.* (2007), where the activity of a synthetic antioxidant was observed to be higher than the sample. The better activity of curry leaves compared to kaffir lime leaves (at 100 ppm) has been reported earlier by Wong *et al.* (2006). Numerous studies have been done on compounds from the turmeric rhizome. To date, the potent phenolic antioxidant compound/s from turmeric leaves have not been reported.

Antioxidative Activities in Linoleic Acid Model System

The autoxidation rate of linoleic acid was determined by measuring the increase in conjugated diene and the decrease in linoleic acid content in the samples. The autoxidation of linoleic acid is accompanied in the early stage by the formation of hydroperoxides that exhibit absorption at 234 nm. *Figure 2* shows the antioxidative activity in the linoleic acid model system. The order of antioxidative activity was: BHT> turmeric leaves> curry leaves> kaffir lime leaves> pandan leaves.

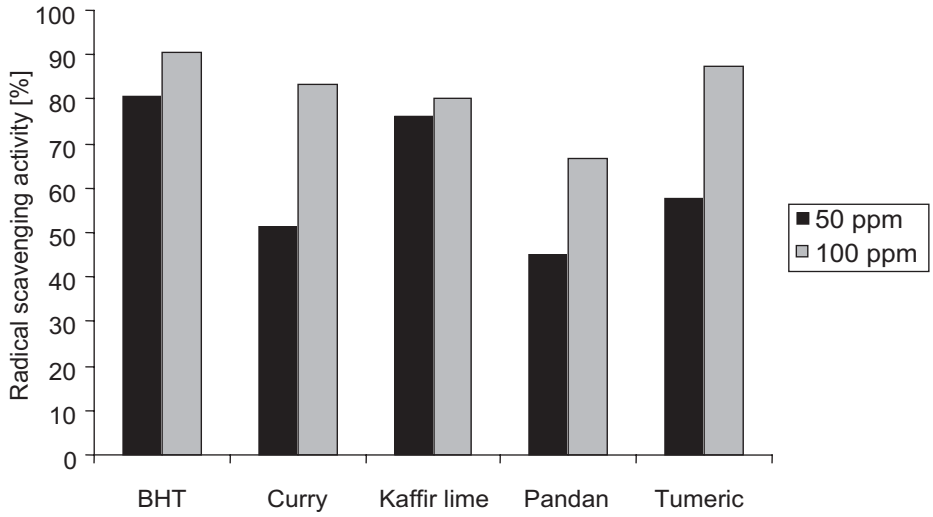

Figure 1. Antiradical activity of the herb extracts against the DPPH radical.

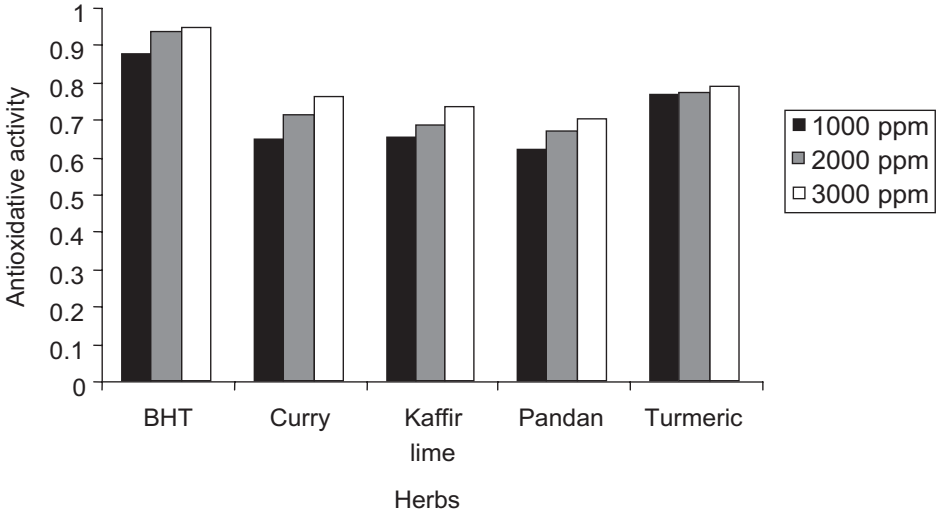

Figure 2. Antioxidative activities of herbs in the linoleic acid model system.

Oxidation of unsaturated fatty acids in biological membranes leads to the formation and propagation of lipid radicals, uptake of oxygen, rearrangement of the double bonds in unsaturated lipids, and eventual destruction of membrane lipids, which produce breakdown products such as malondehyde and 4-hydroxyalkanals (Siriwardhana *et al.*, 2003). Addition of phenolic compounds at certain concentrations markedly slowed down the rate of conjugated diene formation (Chimi and Cillard, 1991). In the absence of phenolic compounds, the concentration of linoleic acid decreased dramatically because it was rapidly being oxidized. The antioxidant effectiveness of those compounds seemed to be related to their stability in quenching the peroxyl radical.

Peroxide Value, Anisidine Value and Free Fatty Acid

The peroxide value is a measure of the degree of initial oxidation of fats and oils. *Figure 3* shows that an increase in concentration of the herbs could significantly ($p<0.05$) reduce the oil oxidation. This is in agreement with Morteza-Semnani *et al.* (2006) who reported that the antioxidative effect of the species, *Phlomis bruguieri* and *Stachys laxa*, increased as their concentrations increased in sunflower oil. In samples treated with turmeric extract, it was observed that at 0.1% to 0.3%, the peroxide value decreased after a certain number of hours of heating, indicating the breakdown of peroxides and the formation of oxidation secondary products such as

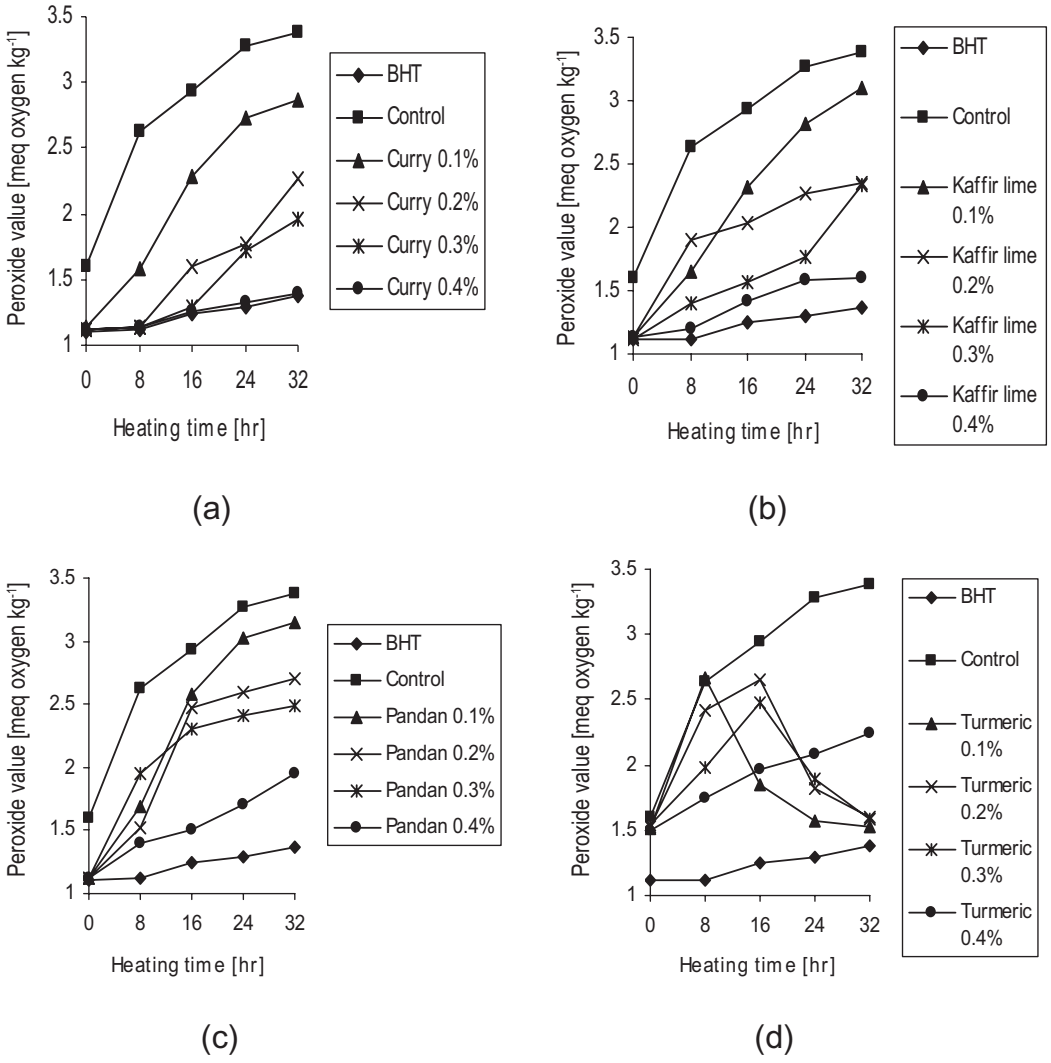

Figure 3. Changes in peroxide value (PV) over time in RBD palm olein added with extracts of (a) curry leaves, (b) kaffir lime leaves, (c) pandan leaves, and (d) turmeric leaves at concentrations of 0.1%, 0.2%, 0.3% and 0.4%, respectively.

ketones, aldehydes, hydrocarbons, epoxides, *etc*., which could be determined using the anisidine test. This also indicated that extract concentrations of 0.1% to 0.3% were not effective in preventing oxidation.

It was generally observed that all extracts lowered the peroxide value significantly ($p<0.05$) when compared to the control. At 0.1% and 0.2%, the peroxide lowering effect was not significantly different amongst the herbs. The activity of curry leaves at 0.4% was not significantly different from BHT. At 0.4% concentration, the extracts of curry leaves and kaffir lime leaves exhibited high antioxidant activities, which were comparable to BHT. Hindered phenols (caffeic acid, vannilic acid and ferrulic acid) and crude tea extract were reported to be capable of lowering the peroxide value and the anisidine value at 0.02% concentration in oil (Naz *et al*., 2005). Inhibition of peroxide formation is through donation of the hydrogen atom from the OH group of phenolic compounds to the lipid radical which in turn produces a stable product.

The anisidine value is a measure of the secondary product of fat and oil oxidation, which results from the breakdown of hydroperoxides. Based on *Figure 4*, it was observed that the effect of different concentrations of extracts in lowering the anisidine value was not significant. However, the effects were significant when compared to the control sample. Curry leaves at 0.2%, 0.3% and 0.4% and kaffir lime leaves at 0.4% showed comparable results with BHT. Rao *et al.* (2007) reported that curry leaves at 1% level gave better protection to ghee than BHA and BHT.

Free fatty acids (FFA) represent the degradation of the oil quality during heating due to oxidation and hydrolysis. Based on *Figure 5*, the effect of concentration in lowering FFA formation can be observed. For curry leaf, kaffir lime leaf and pandan leaf extracts, the FFA value at 0.1% was significantly different from those at 0.3% and 0.4%. In samples

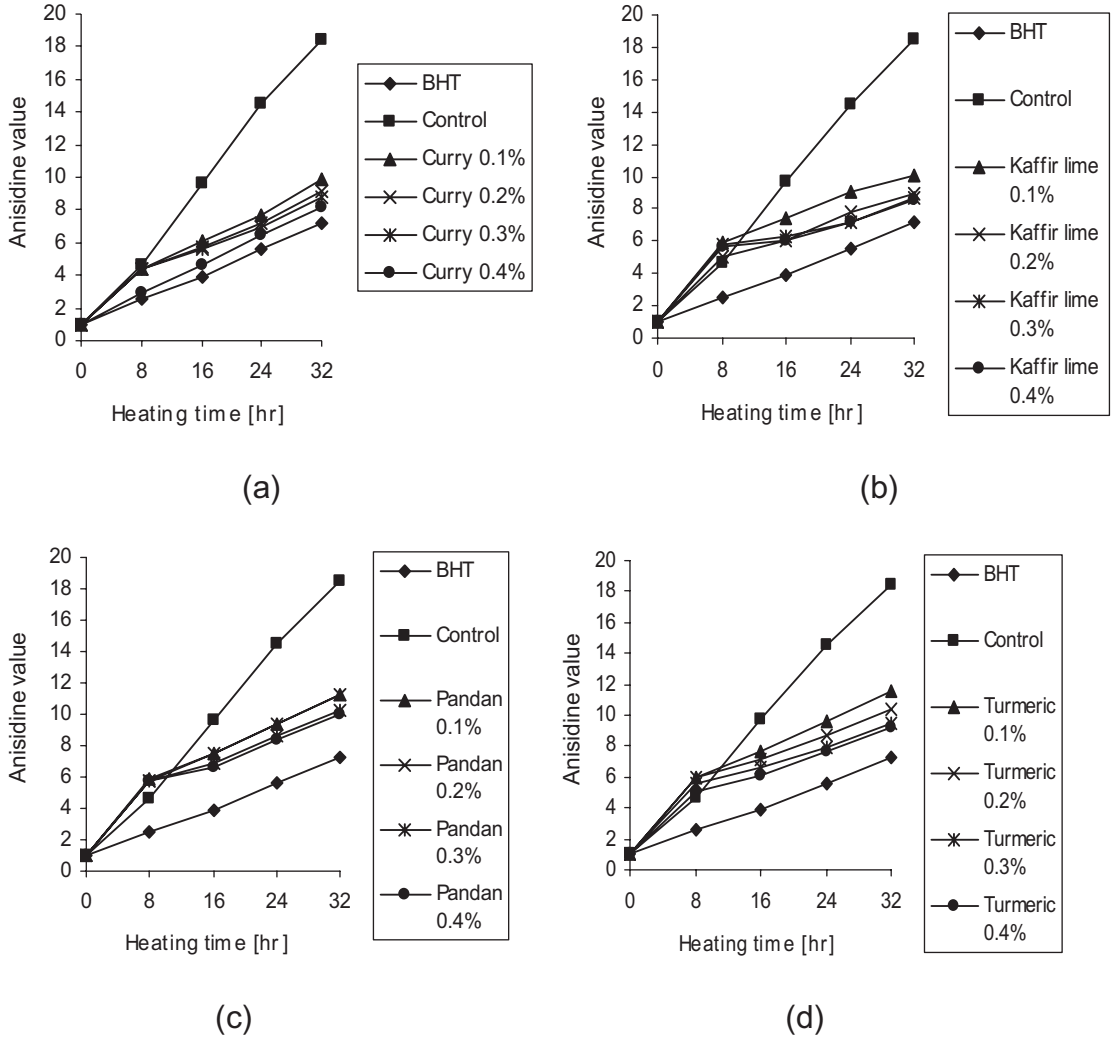

Figure 4. Changes in anisidine value (AV) over time in RBD palm olein added with extracts of (a) curry leaves, (b) kaffir lime leaves, (c) pandan leaves, and (d) turmeric leaves at concentrations of 0.1%, 0.2%, 0.3% and 0.4%, respectively.

treated with turmeric leaves, the concentration difference did not give significant effects in hindering the formation of FFA. It was observed that turmeric leaf extract at 0.1% concentration resulted in a significantly lower formation of FFA compared to extracts of other herbs added at a similar concentration. At 0.2%, 0.3% and 0.4% concentrations, there was no significant difference amongst the four herbs studied. Compared to the control, formation of FFA was significantly lower in palm olein added with the various herb extracts. This finding is in agreement with Che Man and Jaswir (2000) who reported that addition of rosemary and sage oleoresin extract at 0.4% was able to retard the formation of FFA in RBD palm olein when compared to the control. Farag *et al.* (2003) reported that total and free polyphenols obtained from both olive leaves and fruits of *Kronakii cultivar* possessed antihydrolytic activity which increased with concentration.

The first step in lipid oxidation is the donation of a hydrogen atom from the active methylene group to form a free radical (Farag *et al.*, 2003). The reaction can be accelerated by the addition of a radical source, by light or by raising the temperature. It appears that there is a relationship between antioxidant efficiency and the chemical composition of phenolic compounds. According to Nakatani (2000), there were 26 compounds from *Curcuma longa* (rhizome) and three carbazoles isolated from *Murraya koenigii*. Turmeric leaves contain the antioxidative enzyme, superoxide dismutase (Dixit *et al*., 2005). Curry leaves, pandan leaves and kaffir lime leaves contain various carotenoids and tocopherols (Lee *et al*., 2004). Berhow *et al*. (1996) reported that *Citrus hystrix* contains various flavone and flavonal compounds. These compounds and some other polyphenols might contribute to their antioxidative activities even at high temperature. Phenolics transfer the hydrogen atom to lipid peroxyl radicals and form the aryloxyl

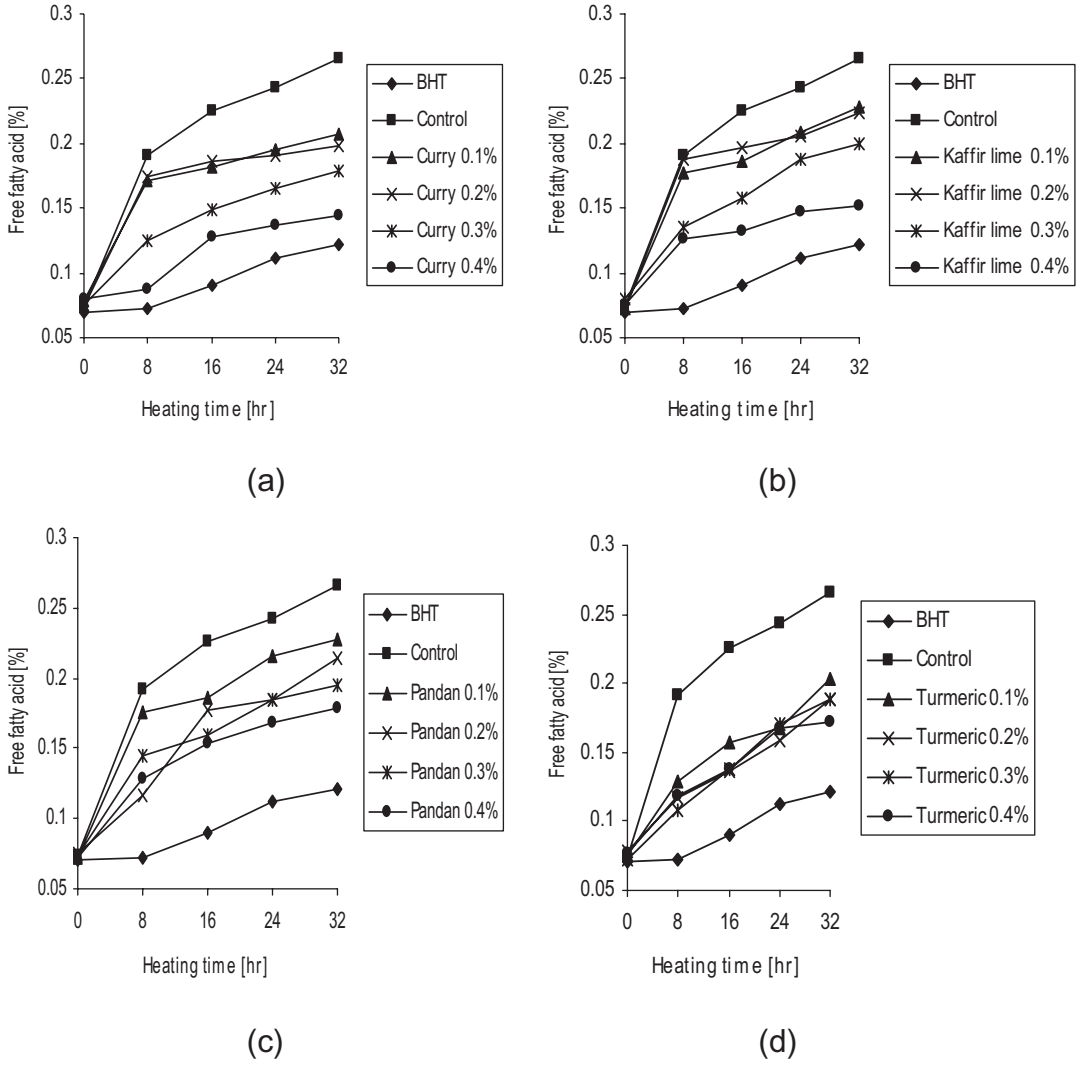

Figure 5. Changes in free fatty acid (FFA) over time in RBD palm olein added with extracts of (a) curry leaves, (b) kaffir lime leaves, (c) pandan leaves, and (d) turmeric leaves at concentrations of 0.1%, 0.2%, 0.3% and 0.4%, respectively.

which, being incapable of acting as a chain carrier, couples with another radical thus quenching the radical process. They are particularly effective antioxidants for polyunsaturated fatty acids (Ruberto *et al*., 2001). For turmeric leaves, the existence of the antioxidative enzyme, superoxide dismutase, would not be helpful in retarding oxidation at high temperature. However, there might be some other compounds that exhibit antioxidative activity, which have yet to be studied. Previously, studies on turmeric concentrated more on the rhizomes, where antioxidative activity is due to the existence of curcuminoids (Cai *et al*., 2004).

In this study, the ethanolic extract was chosen due to its safety aspect in food (Food Act 1983) and its best stabilization effect in oil, even at high temperature (Krings *et al*., 2000; Juntachote and Berghofer, 2005). The antioxidative compounds are supposed to possess more polar characteristics. More studies should be undertaken to identify the main compounds responsible for retarding oxidation.

Oxidative Stability Index

The length of time before rapid acceleration of oxidation is a measure of the resistance to oxidation, and is commonly referred to as the 'induction period' or OSI (AOCS, 1993). The development of oxidative rancidity is accelerated so that the useful life of an oil containing the material may be determined. From there, the effects of various levels and types of anti- or pro-oxidants could be studied.

Figure 6 shows the OSI analysis of the different herbs compared to the control and BHT. All herb extracts showed significantly higher OSI than the control and RBD palm olein added with BHT. With the kaffir lime leaf extract, the difference in OSI readings among the different levels of extract added was not significant. There was no significant difference in OSI between RBD palm olein added with 0.1% and 0.2% pandan leaf extracts, neither was there a significant difference between RBD palm olein added with 0.3% and 0.4% pandan leaf extracts.

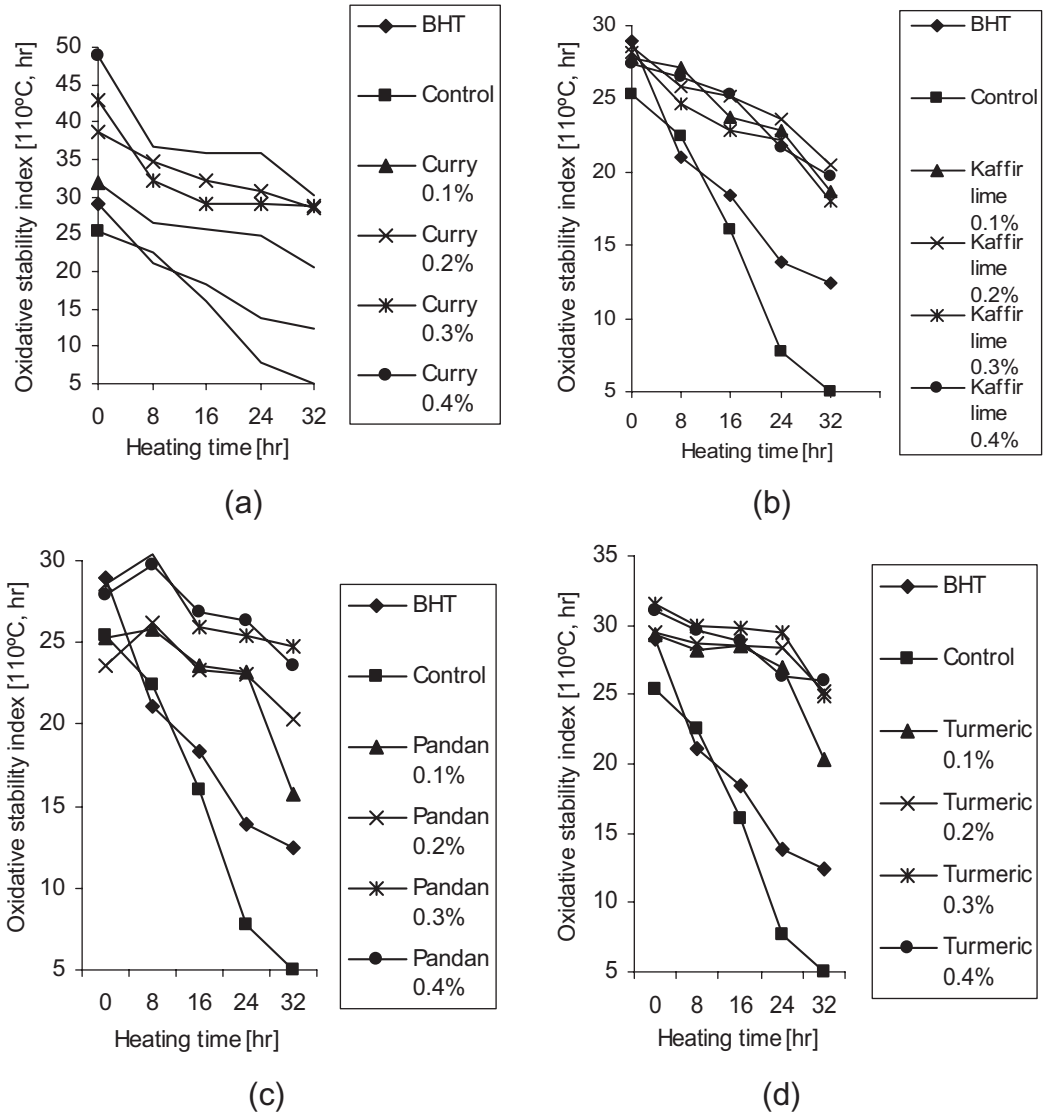

Figure 6. Oxidative Stability Index (OSI) over time in RBD palm olein added with extracts of (a) curry leaves, (b) kaffir lime leaves, (c) pandan leaves, and (d) turmeric leaves at concentrations of 0.1%, 0.2%, 0.3% and 0.4%, respectively.

However, between 0.2% and 0.3% there was a noticeable difference. RBD palm olein added with turmeric leaf extract was significantly different in OSI between the 0.1% and 0.2% extracts. As the concentration increased from 0.2% to 0.4%, the OSI was no longer significantly different. Significant differences in OSI of RBD palm olein added with different levels of curry leaf extracts were noted. The higher the level of curry leaf extract, the higher was the OSI reading. Curry leaf extract at 0.4% showed the highest OSI value compared to other samples. It is interesting to note that the OSI for each herb was significantly higher when compared to both the control and BHT, even at 0.1%. This might be explained by the higher volatility of BHT at high temperature (Frankel, 1993). Rosemary extract at 500 ppm gave better protection in rapeseed oil triacylglycerols in the Rancimat and Oxidograph test compared to BHT at 100 ppm (Nogala-Kalucka *et al.*, 2005).

According to Chu and Hsu (1999), addition of catechin and composites of catechin with other antioxidants resulted in significant increases in the stability of peanut oil when compared to the control. Razali *et al*. (2003) reported that the addition of a combination of additives, *i.e*. TBHQ, citric acid and food grade silicone, to palm superolein improved its performance during five days of frying chicken nuggets. Several reports (Coulter, 1988; Kikugawa *et al*, 1990) indicate that composites of several antioxidants showed synergistic effects in increasing oil stability. It has been reported that curry leaves, kaffir lime leaves and pandan leaves contain tocopherols and carotenoids (Lee *et al*., 2004), that there are alkaloids in pandan (Nonato *et al*., 1993), flavonoids such as eriocitrin, neoeriocitrin, narirutin,

naringin, hesperidin, neohesperidin and didymin in kaffir lime leaves (Berhow *et al*., 1996; Kaur and Kapoor, 2002), and diarylheptanoids in the rhizomes of turmeric (Ravindranath and Satyanarayana, 1980). Interaction between these compounds and existing tocopherols and tocotrienols in RBD palm olein might cause some synergistic effect that enables the oil to last longer.

CONCLUSION

The antioxidative activities of herbs in antioxidant assays show that turmeric leaves have the highest activity, followed by curry leaves, kaffir lime leaves and pandan leaves. However, in the accelerated oxidation studies, curry leaves and kaffir lime leaves at 0.4% concentration showed the best activity compared to the other herb extracts, and was comparable to BHT. Based on the OSI, the herbs are capable of retarding oxidation even at 0.1% compared to the control and BHT. The results suggest that these herbs could be utilized as natural food antioxidants, but more studies are required to optimize their usage.

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