

Antioxidant Evaluation in Malaysian Medicinal Plant: *Persicaria minor* (Huds.) Leaf

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Abstract

Persicaria minor (Huds.) from the family Polygonaceae is a common medicinal plant found in South East Asia. In Malaysia the plant is locally known as kesum. The kesum leaves are often consumed as ulam (raw medicinal plants consumed with daily meals) for preventive health care. In the Malaysian Traditional Medicine System, the decoction of kesum leaf is recommended for digestive disorders and stomach pain. In a previous study (Vimala et al. 1999), preliminary screening of various Malaysian medicinal plants showed that kesum leaf possessed high antioxidant activity (>90%). Thus kesum is a potential and interesting plant for antioxidant evaluation.

In this study, to obtain the antioxidant profile of kesum, the leaf aqueous extract was subjected to 3 antioxidant pathways representing Lipid Peroxidation Inhibition, Superoxide Scavenging and DPPH Free Radical Scavenging. The leaf extract was also subjected to Total Phenolic Content (TPC) and HPLC evaluation.

The kesum leaf aqueous extract was further subjected to cytotoxicity evaluation using WRL-68 human liver and Vero monkey kidney cell lines. The extract was also subjected to microbial load count and heavy metal analysis.

The results of the present study showed that kesum leaf aqueous extract possessed high antioxidant activity which was exhibited as >70% inhibitory activity in all 3 antioxidant assays. Kesum leaf also showed high Total Phenolic Content (2800.6 mg/100g GAE). The HPLC analysis showed the presence of flavanoids, phenolics and oxalic acid in kesum leaf. The leaf extract was found to be free from cytotoxicity (IC₅₀ >300 µg/ml) in WRL-68 human liver and Vero monkey kidney cells. Microbial contamination and heavy metals were not detected.

Thus kesum is a potential antioxidant plant for cultivation and exploitation as food additive. Oxalic acid is a powerful oxidizing agent that binds calcium to aid the digestive system. The scientific findings of this study support the traditional claims of kesum leaf as a medicinal plant for preventive health care and digestive disorders. Thus the kesum leaf is recommended as an antioxidant food additive for preventive health care.

Keywords: *Persicaria minor*, kesum, antioxidant, oxalic acid, polyphenolics, preventive health care

Introduction

Persicaria minor (Huds.), from the family Polygonaceae, is locally known in Malaysia as kesum or laksa leaf. It is a local medicinal plant commonly consumed as ulam for preventive health care. The plant leaves are aromatic and is popularly

used as an ingredient in Malaysian delicacies such as laksa (spicy noodle dish), kerabu (fried herbal rice), tom yam (spicy tangy soup) and asam pedas (spicy tamarind curry). The leaves are often sliced and sprinkled for its strong aroma & flavouring. In the Malaysian Traditional Medicine System, the decoction of the kesum fresh leaves is consumed for indigestion, constipation and as a remedy for stomach disorders and pain (Vimala et al. 2003).

The kesum leaves contain oxalic acid, a reducing agent, that gives its distinctive tangy lemony flavour. The human body produces a small amount of organic oxalic acid every 24 hours and it is excreted through the kidneys. Oxalic acid is a powerful oxidizing acid that rouses the human system into activity. It readily combines with calcium to aid the digestive system and stimulates the peristaltic action of the intestines, thus helping the sluggish prolapsed intestines to regain their normal functions (Eastwood & Nyhlin, 1995).

The oxalic acid enhanced sour quality of kesum leaves also increases the flow of saliva, which in turn aids digestion. Oxalic acid also seems to promote faster blood coagulation time which makes it valuable for internal hemorrhages (Milton et al., 1941). The leaves are nutritious and beneficial to eat in moderate quantities. Cooking the leaves will reduce their content of oxalic acid which is beneficial to reduce its ability to bind minerals (Kohman, 1939).

Previous research showed that the kesum leaf is high in natural plant antioxidants (Vimala et al. 1999) which can help to reduce oxidative damage caused by accumulated access free radicals.

Free radicals are the most important intermediates in biological oxidation-reduction systems. Free radicals occur continually over a long period of time and accumulate age dependently. Accumulated access free radicals gradually induce cell and tissue injury, thereby increasing age related disorders (Hiramatsu et al. 1997). Cancer, inflammation, diabetes, cardiovascular diseases (CVD), neurological diseases, atherosclerosis, aging and many other symptoms have been shown to be related to free radicals (Parker et al., 1992). Antioxidants are substances that can fight and destroy excess free radicals and repair oxidative damage in biomolecules. They inhibit or delay the oxidation of biomolecules by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu et al., 1998).

Thus, antioxidants are important for the defence of living cells against oxidative damage (Zainol et al, 2002).

Antioxidants prolong the life span of cells by protecting the cell membrane against free radical mediated damage, thus helping to retard the aging process. Antioxidants may indeed extend the quality and the length of life and reduce the incidence of many disease processes (Chatterjee, 1994). Since inhibition of reactive free radicals are of critical importance for the maintenance of a healthy life, much attention is given to evaluation and identification of antioxidant rich tropical medicinal plants. Natural plant antioxidants act as therapeutic agents that scavenge reactive oxygen species (Hiramatsu et al, 1997).

Thus the current study was started to evaluate a commonly used Malaysian medicinal plant, the kesum leaf, to explore its antioxidant properties and scientifically support its traditional claims for preventive health care.



Fig. 1 : Persicaria minor

Methodology

Sample Collection

Kesum leaves were collected from Selayang, Selangor, Malaysia. The leaves were separated, washed and chopped into small pieces.

Aqueous Extraction

Aqueous extraction was carried out according to the protocol IHM MBIO 2.10. 100 g plant sample was extracted using the reflux system in a ratio of sample to water (1:10) at 80°C for 1 hour. The extract was filtered using filter paper Whatman 4. The filtered extract was concentrated using a rotary evaporator at 80°C to obtain a minimum Brix value of 15%. The concentrated extract was then freeze dried at -50°C using a vacuum freeze dryer. The powdered extract was stored at 4°C prior to antioxidant evaluation.

Antioxidant Evaluation

1. Lipid Peroxidation Inhibitory Activity - Autoxidation of Linoleic Acid

The autoxidation assay is performed based on the method of Osawa and Namiki (1981) with slight modification. The assay evaluates the inhibitory activity of the sample on lipid

peroxidation (oxidation of fatty acids) caused by hydrogen peroxides.

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 of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of distilled water is 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 are added 9.7 ml of 75% ethanol and 0.1 ml of 2×10^{-2} M ferrous chloride in 3.5 % hydrochloric acid to the reaction mixture. The absorbance of the red colour developed is measured at 500 nm. Antioxidant activity is judged from the decrease of the absorbencies relative to negative control, incubated without plant sample. Butylated Hydroxy Toluene BHT (200 µg) was used as a positive control.

2. Superoxide Scavenging Activity - Xanthine/Xanthine Oxidase

This assay is performed based on the method of Chang et al. (1996) with slight modification. The assay system evaluates the scavenging activity of the sample against superoxide free radical anions.

NBT solution (100 ml of 4.1 mM/l) is prepared by adding 3.15 g TrisHCL, 0.1 g MgCl₂, 15.0 mg 5-bromo-4-chloro-3-indolyl phosphate and 34.0 mg 4-nitro blue tetrazolium chloride to 100 ml of distilled water. The reaction mixture (100 ml) is prepared by dissolving 0.53 g Na₂CO₃ (pH 10.2), 4.0 mg EDTA and 50.0 mg xanthine in 0.025 mM NBT solution. The mixture is kept refrigerated at 4°C

The negative control contains reaction mixture (999 ml) which is transferred into a microcuvette and placed in a 25°C cell holder of a spectrophotometer to auto zero. Generation of superoxide is started by adding 1.0 ml of XOD (20 U/ml). After thoroughly mixing by manual shaking for 5 s, the optical density (OD) measurements were taken at 560 nm for 120 seconds using a spectrophotometer (Lambda 2S). A sample solution containing 1mg/ml plant extract dissolved in ethanol was prepared. 200 µl of the sample solution was added to 799 µl of the reaction mixture and similarly measured as above. Superoxide dismutase (SOD; 6×10^{-3} U/ml) was used as a positive control.

3. DPPH Free Radical Scavenging - DPPH (1, 2-diphenyl-2-picrylhydrazyl)

is a stable free radical. Antioxidant reducing activity on DPPH radical is estimated according to the method of Blois (1958). Scavenging of DPPH represents the free radical reducing activity of antioxidants based on a one-electron reduction which determines the Antioxidant Potential (AOP) of the test sample, showing its effectiveness, prevention, interception and repair mechanism against injury in a biological system.

A sample solution containing 1mg/ml plant extract dissolved in ethanol was prepared. 200 µl of the sample solution was added to 200 µl of DPPH (1mM) dissolved in ethanol and

600 µl of absolute ethanol in a 5 ml screw capped amber bottle. The mixture is mixed using a vortex (2800 rpm) and left to stand at room temperature for 10 minutes. The absorbencies of the resulting solution is measured using a spectrophotometer (Lambda 2S) at 520 nm.

Chemical Analysis

1. Total Phenolic Content (TPC)

Determination of TPC was performed using Folin-Ciocalteu reagent according to the method of Singleton and Rossi, 1965, with slight modifications. A 1.0 mg quantity of plant sample was extracted for 2 hours with 1.0 ml of 80.0% methanol containing 1.0% hydrochloric acid and 1.0% of distilled water at room temperature on a shaker (200 rpm). The mixture was centrifuged at 6000 rpm for 15 minutes and the supernatant decanted into vials. The supernatant was used for the determination of TPC.

A 200.0 ml of supernatant extract was mixed with 400.0 ml of Folin-Ciocalteu reagent (0.1 ml/0.9 ml) and allowed to stand at room temperature for 5 minutes. Then, 400.0 ml of sodium bicarbonate (60.0 mg/ml) solution was added and the mixture was allowed to stand at room temperature for 90 minutes. Absorbance was measured at 725 nm. Samples were expressed as gallic acid equivalents (GAE-TPC mg/100 g) based on a gallic acid standard curve.

2. HPLC Analysis

In HPLC analysis, acetonitrile, acetic acid, purified water and methanol used for sample/standard preparation and analysis were of HPLC grade and filtered using 0.45 µm filter paper. HPLC profiling based on retention time were carried out on phenolic compound standards. 10.0 mg of kesum leaf extracts were dissolved in 10.0 ml of diluent. The water extracts were dissolved in distilled water and the methanolic extracts were dissolved in methanol, to obtain a final concentration of 1.0 mg/ml (1000.0 µg/ml) samples for the analysis. The HPLC system comprised of, pump: Shimadzu LC-10AT, UV-Vis detector: Shimadzu SPD-10AVP with Class VP software, wavelength: 280 nm, chromatographic separation column: Luna Phenomenex C18 (250 mm x 4.6 mm, ID 5.0 mm), mobile phase: A (1% acetic acid in H₂O v/v) & B (acetonitrile) - sonicated for ½ an hour, flow rate: 1.0 ml/minute at room temperature (25°C), injection volume: 10 µl, run time: 25 minutes, run mode: gradient, interval between analysis: 5 minutes, LOD & LOQ determined by calculation (calibration slope)].

Cytotoxicity Evaluation

Sulforhodamine B (SRB) Cytotoxicity Assay in WRL-68 human liver and Vero monkey kidney cell lines

The SRB cytotoxicity assay was carried out according to the method of Skehan et al., 1990 on two cell lines i.e. WRL-68 (normal human liver cells) and Vero (normal African green monkey kidney cells). Cell lines were grown as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM)

supplemented with 5% of fetal bovine, 0.125% of gentamycin, and 1% of penicillin-streptomycin and amphotericin B. Cells serum were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Adherent cells at logarithmic growth phase were harvested and diluted to the density of 2,000 cells/well. Cells were seeded (100 µl/well) in 96-well flat-bottom microplates and allowed to resume growth by incubating overnight at 37°C. After an overnight incubation, cells were treated with plant extracts at concentrations 1–625 µg/ml. Paclitaxel was used as positive control and the control wells were treated with the vehicle used to dissolve the extract. Background control wells contain the same volume of complete culture medium, without cells. Each concentration of the extract was tested in triplicates, and experiment was performed twice. After 72 hours of exposure, the toxic endpoints were determined by SRB assay.

SRB assay began with the fixation of living cells to the bottom of the well. In each well the media was removed and added 100 µl of cold (4°C) 10% trichloroacetic acid (TCA). The microplates were incubated for 30 minutes at 4°C. Media was removed and microplates were washed 5 times with deionised water and left to dry at room temperature for 24 hours. 70 µl of SRB dye (0.4% w/v in 1.0% of acetic acid) was added into each well and left for 30 minutes at room temperature. Unbound SRB was removed by washing 5 times with 1% of acetic acid and left for 1 hour for air drying. Bound SRB was solubilised with 100 µl of 10 mM unbuffered Tris-base solution. The microplates were left on a plate shaker for 30 minutes (350 rpm), before reading the absorbance on a microplate reader at 492 nm, subtracting the background measurement at 620 nm.

Safety Evaluation

Microbial load count and heavy metal analysis were carried out by the Quality Control Laboratory and Soil Chemistry Laboratory respectively, at the Forest Research Institute of Malaysia (FRIM).

Results

Antioxidant Activity

The kesum leaf freeze dried aqueous extract (200 µg/ml) was evaluated for antioxidant properties using 3 bioassay systems representing Lipid Peroxidation Inhibition, Superoxide Scavenging and DPPH Radical Scavenging. As shown in Table 1, kesum leaf aqueous extract exhibited high antioxidant activity in all 3 pathways. It showed 98.3±0.4% lipid peroxidation inhibitory activity, 77.9±1.0% superoxide scavenging activity and 98.8± 0.5% DPPH radical scavenging activity.

Chemical Analysis

The kesum leaf aqueous extract was further subjected to chemical analysis to determine the total phenolic content and to obtain a HPLC profile on polyphenols. As shown in Table 1,

the kesum leaf aqueous extract showed high total phenolic content (2800.6 ± 2.6 mg/100g GAE). The HPLC profile (Figure 1) showed the presence of phenolics at 254 nm &

flavonoids at 330 nm which are major antioxidant compounds in plants. In Fig 2, the HPLC profile showed the presence of oxalic acid at 200nm, which aids in digestion.

Table 1: Antioxidant activity of *P. minor* (kesum) leaf aqueous extract (200 ug/ml)

Plant Sample	Lipid Peroxidation Inhibition (%)	Superoxide Scavenging (%)	DPPH Radical Scavenging (%)	Total Phenolic Content mg/100g GAE
Persicaria minor leaf aqueous extract (200 ug/ml)	98.3 ± 0.4	77.9 ± 1.0	98.8 ± 0.5	2800.6 ± 2.6

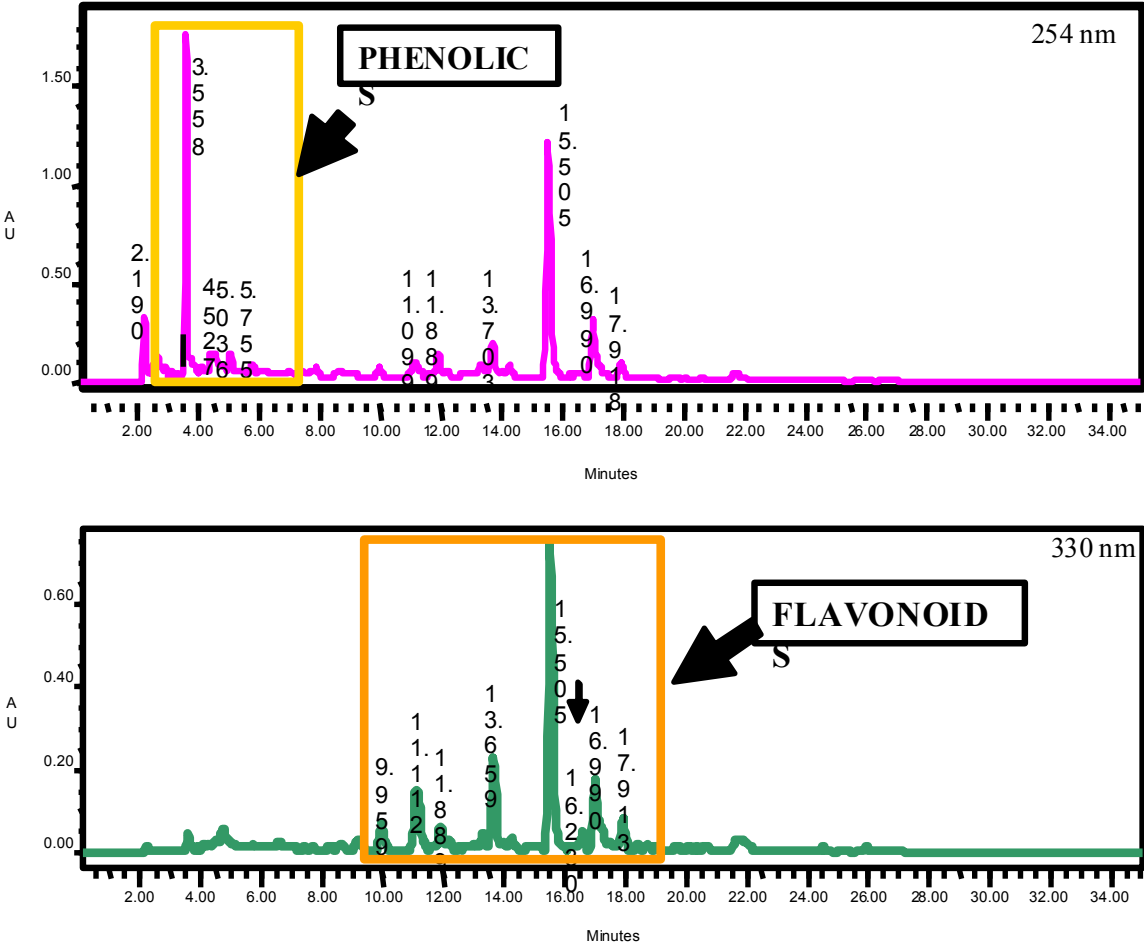


Fig. 1: HPLC profile of *P. minor* (kesum) leaf freeze dried aqueous extract

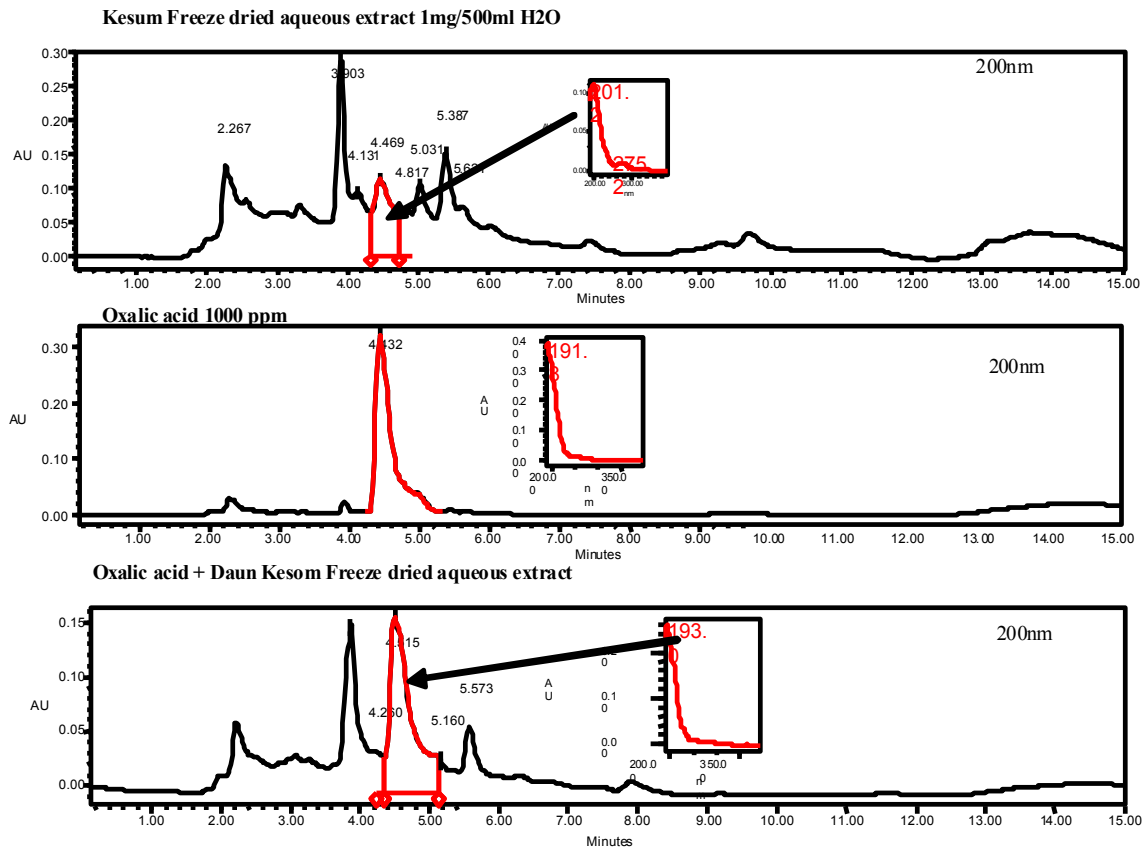


Fig. 2: HPLC profile of *P. minor* (kesum) leaf freeze dried aqueous extract

Cytotoxicity and Safety Evaluation

The *P. minor* leaf aqueous extract was also tested for Cytotoxicity, Microbial Load Count and Heavy Metals.

As shown in Table 2, in the cytotoxicity analysis, the IC50 of the extract was found to be high; 322.95ug/ml in WRL-68 Human Liver Cell Line and 334.70ug/ml in Vero Monkey

Kidney Cell Line, showing that the extract is free from cytotoxicity towards normal cell lines.

The heavy metal analysis showed that the extract was free from plumbum (Pb), mercury (Hg), cadmium (Cd) and arsenic (As). In the microbial load count analysis, both, total aerobic microbial and total mould/yeast were undetectable in the kesum leaf aqueous extract.

Table 2: Cytotoxicity & safety evaluation of *P. Minor* (kesum) leaf aqueous extract

Plant Sample	Cytotoxicity IC ₅₀	Total Microbial Load	Heavy Metal (Pb, Hg, Cd, As)
<i>Persicaria minor</i> (leaf aqueous extract)	WRL-68 Human Liver Cell Line: 322.95ug/ml	Aerobic Microbe: Not Detected	Not Detected
	Vero Monkey Kidney Cell Line: 334.70ug/ml	Mould/Yeast: Not Detected	

Discussion

In Malaysia, the kesum leave is commonly used as an additive ingredient in Malaysian delicacies such as soups, curries and spicy noodle dishes for its aroma and tangy flavouring. The young leaves are also consumed raw, as ulam, with daily meals for preventive health care. In the Malaysian traditional medicine system, the kesum leaves are recommended as a digestive enhancer.

In the present study, the kesum leaf freeze dried aqueous extract was subjected to antioxidant evaluation, chemical analysis, cytotoxicity and safety analysis.

As shown in Table 1, the kesum leaf aqueous extract (200 µg/ml) exhibited high antioxidant activity in all 3 antioxidant pathways. It showed 98.3±0.4% lipid peroxidation inhibitory activity, 77.9±1.0% superoxide scavenging activity and 98.8±0.5% DPPH radical scavenging activity. The antioxidant evaluation shows the great antioxidant potential of kesum leaf as a food additive that could prevent lipid peroxidation and scavenge access free radicals.

An important, primary target of oxidative damage is the unsaturated fatty acid components of cell membranes (Kuroda et al., 1990). Lipid peroxidation not only poses problems in the development of rancidity in processed foods, but also causes serious damage to the human body (Allen and Hamilton, 1983). Lipid peroxidation is known as a free radical chain reaction that takes place inside the human body, and produces many secondary products, such as alkanes, alcohols, acids and carbonyls. These secondary products are themselves highly reactive and they react with other biological components, such as proteins, amino acids, amines and DNA (Osawa, 1995). The peroxidation of unsaturated lipids in living organisms is related to a wide range of diseases and chronic conditions including aging, mutagenesis, cancer, diabetes, cardiovascular diseases and rheumatoid arthritis (Jorgensen and Skibsted, 1993).

Superoxide is universally generated from organic compounds, proteins and cells during metabolism and other normal biochemical functions. In the mitochondria, 3% of oxygen is incompletely reduced to superoxide anion during normal physiological conditions, thus making it the major source of free radicals (Afanas'ev, 1991). The central nervous system (CNS), which comprises about 2% of the body's mass, consumes 20% of all the oxygen. Hence there is a large production of superoxide anion by CNS tissue, and the superoxide production by brain increases as one ages (Sawada and Carlson, 1987).

Superoxide is harmful because it reduces iron-III to iron-II. Superoxide can give rise to strong oxidants such as singlet oxygen and interact with other compounds such as nitric oxide radical or hydrogen peroxide to give rise to the hydroxyl radical and nitrogen dioxide (Juurlink, 1998). Excess production of superoxide anions are known to

damage cells which is one of the several causative factors of many diseases (Toshihiko, 1998) and aging (Harman, 1981). Superoxide has been implicated in a wide range of diseases such as inflammatory disease (McCord, 1987), diabetes (Shah et al., 1983), cardiovascular disease and cancer (Spatz & Bloom, 1992).

DPPH is a stable free radical. Scavenging of DPPH represents the free radical reducing activity of antioxidants based on a one-electron reduction. Scavenging of DPPH free radical determines the free radical scavenging capacity or antioxidant Potential (AOP) of the test sample, which shows its effectiveness, prevention, interception and repair mechanism against injury in a biological system (Lee and Halliwell, 2001).

Thus the kesum leaf, with its high lipid peroxidation inhibitory activity, free radical scavenging capacity and strong antioxidant potential, can prevent the occurrences of oxidative related diseases and slow down the onset of aging symptoms.

In the current study (Table 1), the chemical analysis showed that the kesum leaf aqueous extract had high total phenolic content, 2800.6 ± 2.6 mg/100g GAE, which supports the high antioxidant activity exhibited in various antioxidant pathways. The HPLC profile (Figure 1) showed the presence of phenolics at 254 nm & flavanoids at 330 nm which are major antioxidant compounds in plants. In Fig 2, the HPLC profile showed the presence of oxalic acid at 200nm, which aids in digestion.

Plant polyphenols are potent antioxidants, widely distributed and accumulated in large amount in various plants consumed by human beings. Plant polyphenols inhibit lipid peroxidation (Okuda et al. 1983), scavenge DPPH free radicals (Yoshida et al., 1989) and inhibit xanthine oxidase by superoxide radical scavenging activity (Hatano et al., 1989). Flavonoids are nonnutritive naturally occurring phenols with antioxidant properties, occurring in plant foods (Hertog et al., 1992a; 1993a). Most fruits and vegetables have substantial quantities of flavonoids (Beecher, 1999). Plant flavonoids are health-promoting, disease-preventing dietary antioxidant compounds that have been shown in numerous in vitro and in vivo experiments to have antioxidant activity (Middleton, 1996).

Oxalic acid is a powerful oxidizing acid that rouses the human system into activity. It readily combines with calcium to aid the digestive system and stimulates the peristaltic action of the intestines, thus helping the sluggish prolapsed intestines to regain their normal functions (Eastwood & Nyhlin, 1995).

Thus the results of the present study shows that, *P. minor* leaf aqueous extract is a potential antioxidant plant with digestion enhancing properties. The kesum leaf is high in antioxidant activity, contributed by plant polyphenols and

flavonoids. The oxalic acid in the leaf supports its traditional usage as a digestive enhancer.

The cytotoxicity and safety evaluation in the present study showed that, the kesum leaf aqueous extract is safe for consumption. As shown in Table 2, the extract exhibited high IC₅₀ values; 322.95ug/ml and 334.70ug/ml in WRL-68 Human Liver and Vero Monkey Kidney Cell Lines respectively. Cell death not in excess of 50% of the negative control is considered non-toxic (Elmore et al., 2003). Thus the results show that the kesum leaf is without cytotoxic effect on the WRL-68 human liver and Vero Monkey Kidney cells.

The heavy metal analysis showed that the extract was free from plumbum (Pb), mercury (Hg), cadmium (Cd) and arsenic (As). In the microbial load count analysis, both, total aerobic microbial and total mould/yeast were undetectable in the kesum leaf aqueous extract.

Thus the kesum leave has great potential to be exploited as an antioxidant food additive. Antioxidant food can scavenge access free radicals and keep the oxidative stress state (OSS) in balance. Naturally occurring plant antioxidants are useful for the prevention of oxidative related, age-dependant chronic degenerative disease and aging symptoms (Vimala et al., 2003). However, the leave should only be consumed as an additive, as recommended traditionally, and not in large quantities because the oxalic acid may form oxalate crystals such as calcium oxalate kidney stones.

Conclusion

Persicaria minor or kesum leaves are a common Malaysian food additive which is added as a flavouring ingredient in Malaysian spicy delicacies. The young leaves are also consumed raw, as ulam for preventive health care. The Malaysian traditional medicine system recommends the kesum leaf as a digestive enhancer.

In the present study, the kesum leaf was found to exhibit high antioxidant activity. Kesum leaf aqueous extract exhibited high antioxidant activity in 3 antioxidant pathways, 98.3±0.4% lipid peroxidation inhibitory activity, 77.9±1.0% superoxide scavenging activity and 98.8± 0.5% DPPH radical scavenging activity. It also showed high total phenolic content (2800.6± 2.6 mg/100g GAE). The HPLC profile showed the presence of phenolics at 254 nm, flavanoids at 330 nm oxalic acid at 200nm, which aids in digestion.

The cytotoxicity and safety evaluation in the present study showed that, the kesum leaf aqueous extract is safe, without cytotoxic effect on the WRL-68 human liver and Vero Monkey Kidney cells. The extract exhibited high IC₅₀ values; 322.95ug/ml and 334.70ug/ml in WRL-68 Human Liver and Vero Monkey Kidney Cell Lines respectively. The extract was also found to be free from heavy metal contamination. The heavy metal analysis showed that the extract was free from plumbum (Pb), mercury (Hg), cadmium (Cd) and arsenic (As).

In the microbial load count analysis, both, total aerobic microbial and total mould/yeast were undetectable in the kesum leaf aqueous extract.

The results of the current study indicate that kesum leaf is a potential antioxidant food additive with high content of phenolics and digestive enhancing properties. Thus it has great potential to be exploited and cultivated for the herbal market. Antioxidant food can provide chemoprevention which helps to prevent chronic, degenerative, age-dependant diseases and aging symptoms. Antioxidants can help to maintain health and fitness even as one ages.

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