

## Natural Antioxidants: *Piper sarmentosum* (Kadok) and *Morinda elliptica* (Mengkudu)

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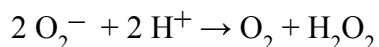
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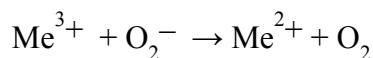
### ABSTRACT

The antioxidant activity of two edible medicinal plants commonly used in Malaysian traditional medicine i.e. *Piper sarmentosum* (kadok) and *Morinda elliptica* (mengkudu) were tested for antioxidant activity. The methanolic leave extracts of kadok and mengkudu, at 250ug/ml, were tested using the Xanthine/Xanthine Oxidase (X/XOD) Superoxide Scavenging assay. Both extracts showed high superoxide scavenging assay, 88% and 80% respectively compared to superoxide dismutase (SOD) standard. The crude extracts were further fractionated using column chromatography and tested for superoxide scavenging activity, to obtain antioxidant active fractions. Two active fractions were obtained from kadok, PsFr6-71.3%, PsFr7-71.3%, and one active fraction from mengkudu, MeFr3-86.6%. These active fractions were compared against 14 phenolic compound standards. After a series of HPLC analysis of samples and standards, a natural antioxidant compound was identified in kadok and mengkudu i.e. Naringenin (4',5,7-Trihydroxyflavanone) with 75.7% superoxide scavenging activity. Naringenin is a highly potent natural antioxidant that has been reported in the raw materials of larch and grapefruit extracts. Thus, kadok and mengkudu which contain Naringenin, could be used as antioxidant dietary supplements.

### INTRODUCTION

Superoxide anion ( $O_2^-$ ) is a toxic by-product formed by the univalent reduction of ground-state molecular oxygen (Michael, Jennifer & Meyrick, 1987). It is produced from a variety of sources including  $\gamma$ -irradiation (Cerutti, 1985), enzyme-substrate reactions such as xanthine-xanthine oxidase (Brawn & Fridovich, 1981) and chemicals such as paraquat (Moody & Hassan 1982); Bagley, Krall & Lynch, 1986), phorbol esters (Birnboim, 1982) and bleomycin (Burger, Peisach & Horwitz, 1981). Ultraviolet and solar radiation can interact with naturally occurring cellular metabolites to produce  $O_2^-$  (Cunningham *et al.*, 1985a; b; Peak & Foot, 1986). Biological macromolecules exist in a medium enriched in transition metal cations ( $Me^+$ ), which may catalyze the reduction of  $O_2^-$  to the more highly reactive oxygen species hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $\bullet OH$ ) by the Fenton reaction :





Superoxide anion and its reduction products  $\text{H}_2\text{O}_2$  and  $\bullet\text{OH}$  produce a variety of effects on tissue macromolecules and have been implicated as participants in a variety of disease states. Enzymatically produced  $\text{O}_2^-$  causes lipid peroxidation, depolymerization of polysaccharides and strand scission of purified bacterial DNA (Demopoulos, Pietronigro & Seligman, 1980, 1983; Brawn & Fridovich 1981). Superoxide anion and its reduction products may also be involved in the etiology of inflammatory arthritis (McCord, 1987), sunburn erythema (Danna *et al.*, 1984), aging and cancer (Ames *et al.*, 1981).

The mechanisms by which active oxygen species contribute to disease states have been widely studied. Superoxide anion and its reduction products have been implicated as causes of mutation in bacteria (Weitzman & Stossel, 1981; Levin *et al.*, 1982) and eukaryotic cells (Cunningham & Lokesh 1983; Cunningham, Ringrose & Lokesh, 1984), chromosome aberrations (Sofuni & Ishidate, 1984), and transformation in mammalian cells (Troll & Wiesner 1985; Cerutti 1985).

Since superoxide anions are produced as toxic by-products of regular biochemical and metabolic reactions in the human biological system, a daily intake of natural antioxidant superoxide scavenger could prevent oxidative damage. Thus, in this study we investigated the antioxidant potential of *P. sarmentosum* and *M. elliptica* as natural superoxide scavengers.

## **MATERIALS AND METHODS**

### **Plant materials**

The leaves of *P. sarmentosum* were collected from FRIM ethnobotanic garden while the leaves of *M. elliptica* were collected from Port Dickson.

### **Xanthine/xanthine oxidase (X/XOD) superoxide scavenging assay**

All chemicals for the assay were purchased from SIGMA.

NBT solution (100 ml of 4.1 mM/L) was prepared by adding 3.15 g TrisHCL, 0.1 g  $\text{MgCl}_2$ , 15.0 mg 5-bromo-4-chloro-3-indolylphosphate and 34.0 mg 4-nitro blue tetrazolium chloride to 100 ml of distilled water.

The reaction mixture (100 ml) was prepared by dissolving 0.53 g  $\text{Na}_2\text{CO}_3$  (pH 10.2), 4.0 mg EDTA and 2.0 mg xanthine in 0.025 mM NBT solution. The mixture was kept refrigerated at 4 °C.

The reaction mixture (999ul) was transferred into a microcuvette and placed in a 25 °C cell holder

of a spectrophotometer. Generation of superoxide is initiated by adding  $1 \times 10^{-3}$  U/ml of XOD. The optical density (OD) measurements were taken at 560nm for 120 seconds using a Lambda 2S spectrophotometer.

The reaction mixture (979ul) was transferred into a microcuvette and placed in a 25°C cell holder of a spectrophotometer. SOD (1.16U/ml) was added into the reaction mixture and thoroughly mixed. XOD ( $1 \times 10^{-3}$  U/ml) was then added to start the generation of oxyradicals. OD was taken at 560 nm for 120 seconds at intervals of 10 seconds.

Methanolic crude extracts of the plants were dissolved in the reaction mixture at a concentration of 250ug/ml. The stock solution (5ul) was added to 994ul of the reaction mixture and placed in a cell holder to autozero. XOD ( $1 \times 10^{-3}$  U/ml) was then added and after thoroughly mixing, similarly measured for the XOD and SOD curves.

### Extraction and separation of active fractions

The samples, 3 kg of *P. sarmentosum* and *M. elliptica*, were dried, pulverized and soxhlet extracted with 3x500ml MeOH. The extracts were evaporated to dryness under partial vacuum. The methanolic extracts obtained were subjected to superoxide scavenging assay to determine their antioxidative activity. The isolation scheme of the active compound from *P. sarmentosum* is shown in Figure 1. The active methanolic extract of *P. sarmentosum* was absorbed in celite and then subjected to a medium pressure column chromatography [glass column, Ø5.5 cm x 47.0 cm, packing material, silica gel 60F, solvent system (isocratic), 40% EtOAc in *n*-hexane], and 7 fractions were obtained. The fractions were tested for antioxidant activity. The active fractions 6(PsFr6) and 7(PsFr7) were subjected to HPLC analysis.

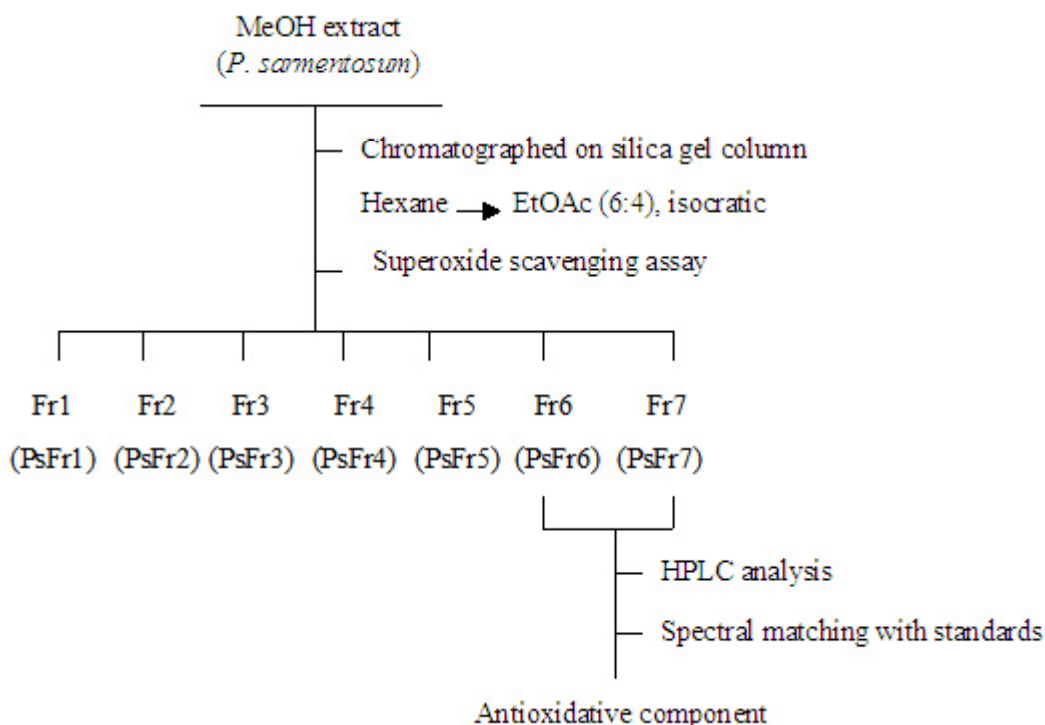


Figure 1. Isolation and fractionation of antioxidative component from *P. sarmentosum*

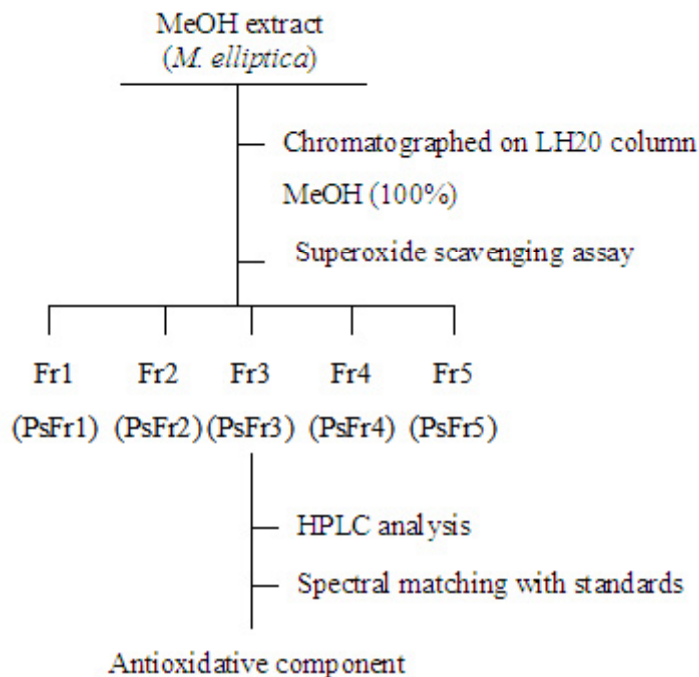


Figure 2. Isolation and fractionation of antioxidative component from *M. elliptica*

As for *M. elliptica*, the isolation scheme of the active compound is shown in Figure 2. The active methanolic extract was subjected to medium pressure column chromatography (glass column, Ø2.5 cm x 47.0 cm, packing material, sephadex LH20, solvent system 100% MeOH), and 5 fractions were obtained. The active fraction 3(MeFr3) was subjected to HPLC analysis.

### HPLC analysis

Acetonitrile and MeOH were HPLC grade and purchased from MERCK, Germany. Phosphoric acid was of analytical grade. The HPLC system comprised of a Waters 600E pump, a Waters 996 photodiode array detector and a Millennium 32 software. The detection wavelength was set at 365nm. A Delta Pak C4 (150x3.9mm, id 5µm) was used for the chromatographic separation. The mobile phase used for the separation was water (solvent A) and acetonitrile (solvent B) each containing 0.05% H<sub>3</sub>PO<sub>4</sub> at flow rate of 0.8 ml/min at room temperature (25°C). The volume of injection was 20µl.

### Phenolic compound standards

The phenolic standards used were purchased from MERCK. Antioxidant testing and HPLC analysis were carried out on each phenolic standard compound : Flavonone, Naringenin (4',5,7-Trihydroxyflavanone), Hesperitin (3',5,7-Trihydroxy-4'-methoxy-flavanone), Taxifolin / Dihydroquercetin (3,3',4',5,7-Pentahydroxyflavanone), Flavone, Fisetin (3,3',4',7-Tetrahydroxyflavone), Quercetin (3,3',4',5,7-Pentahydroxyflavone), Phenol, 2,5-

Dimethylphenol, 2,6-Dimethoxy-phenol, Pyrocatechin/pyrocatechol (1,2-Dihydroxybenzene), Catechin/catechol, 2,4,4'-Trihydroxychalcone, Curcumin

## RESULTS AND DISCUSSION

The crude methanolic extracts of *P. sarmentosum* and *M. elliptica* were tested for superoxide scavenging activity using the X/XOD superoxide scavenging assay. As shown in Table 1, the methanolic extracts of *P. sarmentosum* and *M. elliptica* showed high superoxide scavenging activity of 87.6 % and 82.0 % respectively, compared to the controls.

The active methanolic extracts of *P. sarmentosum* and *M. elliptica* were solvent partitioned and chromatography fractionated. The fractions were then tested for superoxide scavenging activity. As shown in Table 2, two fractions of *P. sarmentosum* showed high superoxide scavenging activity, PsFr6-71.3 %, PsFr7-71.3 % and one fraction of *M. elliptica* showed high superoxide scavenging activity, MeFr3-86.6.

Table 1. Superoxide scavenging activity of the crude leaf extracts of *P. sarmentosum* and *M. elliptica*

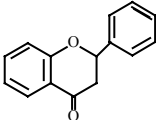
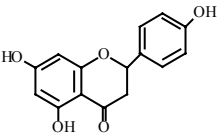
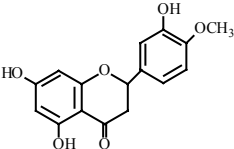
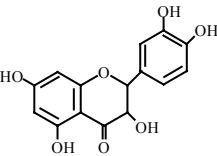
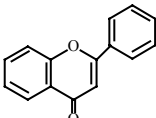
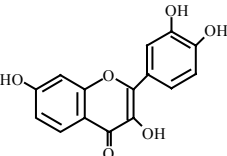
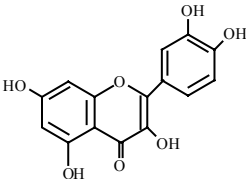
Plant Sample 250ug/ml	Extract	Superoxide Scavenging Activity (%)
Control (-)		0.0
Control (+)		100.0
<i>P. sarmentosum</i>	Methanol	87.6
	Water	70.6
<i>M. elliptica</i>	Methanol	82.0
	Water	59.1


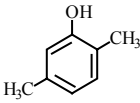
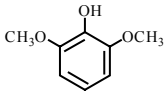

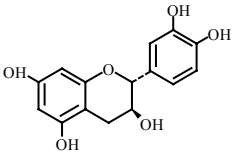
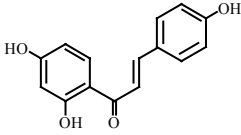
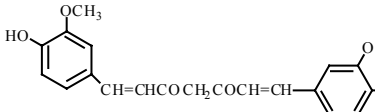
Table 2. Superoxide scavenging activity in the fractions of *P. sarmentosum* and *M. elliptica*

Fractions	Superoxide Scavenging Activity (%)
<i>P. sarmentosum</i> (kadok)	
Fr1	28.8
Fr2	42.4
Fr3	41.5
Fr4	32.3
Fr5	52.4
Fr6	71.3
Fr7	71.3
<i>M. elliptica</i> (mengkudu)	
Fr1	75.5
Fr2	60.9
Fr3	86.6
Fr4	10.4
Fr5	66.6

Among the 14 phenolic compound standards tested for superoxide scavenging activity, 9 compounds showed high antioxidant activity, above 70 % (Table 3).

Table 3: Phenolic standards: antioxidant activity and retention time

Phenolic Compound Standard	Structure	Antioxidant activity (% inhibition)	Retention time (min)	
			254nm	365nm
<b>Flavanone</b>		-	-	-
Naringenin (4',5,7-Trihydroxyflavanone)		75.7	12.678	12.678
Hesperitin (3',5,7-Trihydroxy-4'-methoxyflavanone)		91.7	11.998	12.005
Taxifolin / Dihydroquercetin (3,3',4',5,7-Pentahydroxyflavanone)		90.9	11.212	11.218
<b>Flavone</b>		-	-	-
Fisetin (3,3',4',7-Tetrahydroxyflavone)		66.0	8.554	8.554
Quercetin (3,3',4',5,7-Pentahydroxyflavone)		98.1	10.864	10.864

Phenol		-	-	-
2,5-Dimethylphenol		62.8	2.055	1.884
2,6-Dimethoxyphenol		96.2	4.616	4.628
Pyrocatechin/pyrocatechol (1,2-Dihydroxybenzene)		89.8	11.893	11.892
Catechin/catechol		87.0	12.280	12.280
2,4,4'-Tryhydroxychalcone		91.0	1.926	1.912
Curcumin		94.2	22.518	22.518
- nil / not detected				

They are Naringenin (4',5,7-Trihydroxyflavanone), Hesperitin (3',5,7-Trihydroxy-4'-methoxyflavanone), Taxifolin/Dihydroquercetin (3,3',4',5,7-Pentahydroxyflavanone), Quercetin (3,3',4',5,7-Pentahydroxyflavone), 2,6-Dimethoxyphenol, Pyrocatechin/pyrocatechol (1,2-Dihydroxybenzene), Catechin/catechol, 2,4,4'-Tryhydroxychalcone and Curcumin.

The active fractions of *P. sarmentosum* and *M. elliptica* were analysed using the HPLC system. The HPLC profiles were then matched with Standard Phenolic Compounds and their retention time were compared. As shown in Figures 3 & 4, fraction 6 and 7 of *P. sarmentosum* showed peaks at 12.575 and 12.566 min respectively which were comparable to the Naringenin peak, 12.678 min. As for the *M. elliptica*, active fractions, fraction 3 was found to have a peak at 12.640 min, comparable to Naringenin at 12.678 min.

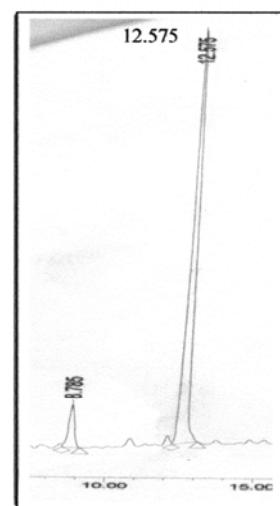


Figure 3. HPLC analysis profile of active fraction, PsFr6

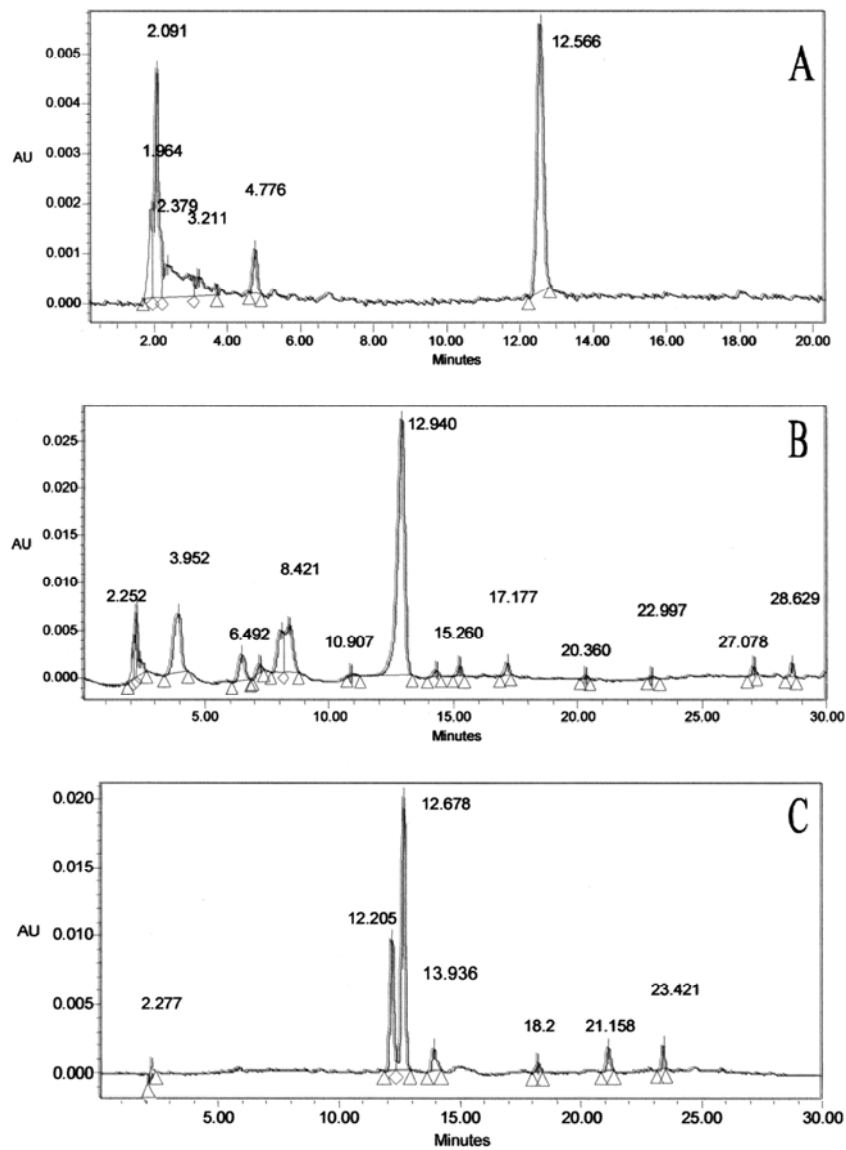


Figure 4. HPLC analysis profiles of active fractions, A: PsFr 7, B: MeFr3, C: Naringenin

## DISCUSSION

From this study, we found that both the methanolic leaf extracts of *P. sarmentosum* and *M. elliptica* possessed a natural antioxidant superoxide scavenger, Naringenin. As shown in Figure 5, Naringenin belongs to the flavonoid group, 4',5,7-Trihydroxyflavanone. The Naringenin compound showed high superoxide scavenging activity, 75.7 %.

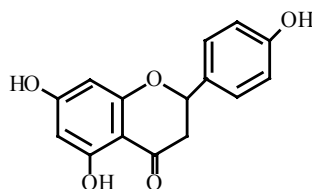


Figure 5: Naringenin, 4',5,7-Trihydroxyflavanone, AA : 75.7%

Malaysian traditional medicine for diabetes, hypertension and joint aches. The fruits and young leaves are often consumed raw as ulam (Burkill, 1966).

Over recent years, considerable epidemiological evidence has been gathered to suggest an association between consumption of fruits and vegetables and a reduced risk of certain chronic diseases such as cardiovascular disease (Rimm *et al.*, 1996), stroke (Gillman *et al.*, 1995), hypertension (Ascherio *et al.*, 1992, cataract (Leske *et al.*, 1998), muscular degeneration (Seddon *et al.*, 1994, cancer (Steinmetz & Potter, 1996) and DNA damage (Ames, 1998).

Fruits and vegetables are a rich source of many food factors including vitamins, minerals and phytochemicals which may act as antioxidants (Lampe, 1999). The antioxidant activity of fruits and vegetables is often assumed to be of greatest importance in combating a number of degenerative diseases, as free radical-related damage has been implicated in causing many of these conditions (Ames, 1998). Thus a daily consumption of five or more servings of antioxidant-rich food (Baghurst *et al.*, 1992) will provide the body with the essential antioxidants needed to prevent degenerative diseases, prematured aging symptoms, chronic fatigue and general disability.

Doctors and nutritionist have long known that antioxidants are needed by the human body for optimal well being, especially for maintaining a healthy body system and defense mechanism against cell damage (Rohana *et al.*, 2002). The term, antioxidant is used to describe a dietary component that can function to decrease tissue damage by reactive oxygen (Vimala & Ilham, 1999).

## CONCLUSION

Naringenin, a naturally occurring antioxidant superoxide scavenger was found in the methanolic leaf extracts of *P. sarmentosum* and *M. elliptica*. Thus these plants could be considered as antioxidant food. Therefore if consumed daily, they could scavenge excess free-radicals in the human biological system and could prevent oxidative related diseases. Antioxidant food supplies the body with the essential antioxidant nutrients needed to enhance the immune system, eliminate excess free radicals and to keep the oxidative stress state in balance. Thus the leaves of *P. sarmentosum* and *M. elliptica* can help to maintain energy, general ability and fitness even as we age.

## REFERENCES

- Ames BN (1998). Micronutrients prevent cancer and delay aging. *Toxicology Letters* 102-103 : 5-18
- Ames BN, Cathcart R, Schwiers E, Hochstein P (1981). Uric acid provides an antioxidant defense in human against oxidant and radical-caused aging and cancer; a hypothesis. *Proc. Natl. Acad. Sci. USA* 78:6858-62.
- Ascherio A, Rimm EB, Giovanucci EL, Colditz GA, Rosner B, Willet WC, Sacks F & Stampfer MJ (1992). A prospective study of nutritional factors and hypertension among US men. *Circulation* 86: 1475-1484.
- Baghurst KI, Hertzler AA, Record SJ & Spurr C (1992). The development of a simple dietary assessment and educational tool for use by individuals and nutrition educators. *Journal of Nutrition Education* 24: 165-172.
- Bagley AC, Krall J & Lynch RE (1986). Superoxide mediates the toxicity of paraquat for Chinese hamster ovary cells. *Proc Natl Acad Sci (USA)* 83 : 3189-3193.
- Birnboim HC (1982). DNA strand breakage in human leukocytes exposed to a tumour promoter, phorbol myristate acetate. *Science* 215 : 1247-1249.
- Brawn K & Fridovich I (1981). DNA strand scission by enzymically generated oxygen radicals. *Arch Biochem Biophys* 206 :414-419.
- Burger RM, Peisach J & Horwitz SB (1981). Activated bleomycin : a transient complex of drug, iron and oxygen that degrades DNA. *J. Biol. Chem.* 256 : 11636-11644.
- Burkill IH (1966). A dictionary of the economic products of the Malay Peninsula. Kuala Lumpur: Ministry of Agriculture and Cooperative. 2<sup>nd</sup> ed. 1&2 : 132-2347.
- Cerutti PA (1985). Prooxidant states and tumour promotion. *Science.* 227 : 375-381.
- Cunningham ML & Lokesh BR (1983). Superoxide anion generated by potassium superoxide is cytotoxic and mutagenic to Chinese hamster ovary cells. *Mutation Res.* 121 : 299-304.
- Cunningham ML, Johnson JS, Giovanazzi SM & Peak MJ (1985a). Photosensitized production of superoxide anion by monochromatic (290-405nm) ultraviolet irradiation of NADH and NADPH coenzymes. *Photochem. Photobiol.* 42 : 125-128.
- Cunningham ML, Krinsky NI, Giovanazzi SM & Peak MJ (1985b). Superoxide anion is generated from cellular metabolites by solar radiation and its components. *J. Free Rads. Biol. Med.* 1 : 381-385.

- Cunningham ML, Ringrose PS & Lokesh BR (1984). Inhibition of the genotoxicity of bleomycin by superoxide dismutase. *Mutation Res.* 135 : 199-202.
- Danna K, Horio T, Takigawa M & Imamura S (1984). Role of oxygen intermediates in UV-induced epidermal cell injury. *J Invest Dermatol* 83 : 166-168.
- Demopoulos HB, Pietronigro DD & Seligman ML (1983). The development of secondary pathology with free radical reactions as a threshold mechanism. *J. Am. College Toxicol.* 2 : 173-184.
- Demopoulos HB, Pietronigro DD, Flamm ES & Seligman ML (1980). The possible role of free radical reactions in carcinogenesis. *J. Environ. Pathol. Toxicol.* 3 : 272-304.
- Donald RB & Cristobal M (2001). Antioxidant Activities of flavonoids. *Seminar Series*, January 2: 1-3. Oregon State University, USA.
- Gillman MW, Cupples LA, Gagnon D, Posner BM, Ellison RC, Castelli WP & Wolf PA (1995). Protective effects of fruits and vegetables on stroke in men. *Journal of American Medical Association* 273: 1113-1117.
- Lampe JW (1999). Health effects of vegetables and fruits: assessing mechanisms of action in human experimental studies. *American Journal of Clinical Nutrition* 70: 475S-490S.
- Leske MC, Chylack LT, He Q, Wu SY, Schoenfeld E, Friend J & Wolfe J (1998). Antioxidant vitamins and nuclear opacities: the longitudinal study of cataract. *Ophthalmology* 105: 831-836.
- Levin DE, Hollstein M, Christman MF, Schweirs EA & Ames BM (1982). A new *Salmonella* tester strain (TA102) with A-T base pairs at the site of mutation detects oxidative mutagens. *Proc Natl Acad Sci (USA)*. 79 : 7445-7449.
- McCord, JM (1987). Oxygen derived radicals: a link between reperfusion injury and inflammation. *Federation Proceedings* 46: 2402-2406.
- Michael LC, Jennifer GP & Meyrick JP (1987). Single-strand DNA breaks in rodent and human cells produced by superoxide anion or its reduction products. *Mutation Res* 184 : 217-222.
- Moody CS & Hassan HM (1982). Mutagenicity of oxygen free radicals. *Proc Natl Acad Sci (USA)* 79 : 2855-2859.
- Peak JG, Peak MJ & Foote CS (1986). Observations on the photosensitized breakage of DNA by 2-thiouracil and 334nm ultraviolet radiation. *Photochem. Photobiol* 44 : 111-116.

- Rimm EB, Ascherio A, Giovanucci E, Spiegelman D, Stampfer MJ & Stalenhoef AH (1996). Vegetable, fruit and cereal fibre intake and risk of coronary heart disease among men. *Journal of the American Medical Association* 275: 447-451.
- Rohana S, Vimala S, Abdull Rashih A & Mohd Ilham A (2002). Engkabang (*Shorea macrophylla*) : antioxidant evaluation. Chang, YS, M Mastura, S Vimala & MY Nurhanan (Eds.). In *Proceedings of the Seminar: Medicinal and Aromatic Plants – Towards Modernisation of Research and Technologies in Herbal Industries*. pp. 120–123 pp. 120–123. Kuala Lumpur: Forest Research Institute Malaysia.
- Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, Burton TC, Farber MD, Gragoudas ES, Haller J, Miller DT, Yanuzzi LA & Willet W (1994). Dietary carotenoids, vitamins A, C and E, and advanced age-related muscular degeneration. Eye Disease Case-Control Study Group. *Journal of the American Medical Association* 272: 1413-1420.
- Sofuni T & Ishidate Jr M (1984). Induction of chromosomal aberrations in cultured Chinese hamster cells in a superoxide-generating system. *Mutation Res* 140 : 27-31.
- Steinmetz KA & Potter JD (1996). Vegetables, fruit and cancer prevention: a review. *Journal of the American Dietetic Association* 96: 1027-1039.
- Troll W & Wiesner R (1985). The role of oxygen radicals as a possible mechanism of tumour promotion. *Am Rev Pharm Toxicol* 25 : 509- 528.
- Vimala S & Mohd Ilham A (1999). Malaysian tropical forest medicinal plants: a source of natural antioxidants. *Journal of Tropical Forest Products* 5(1): 32–38.
- Weitzman SA & Stossel T.P. (1981). Mutation caused by human phagocytes. *Science* 212 : 546-547.