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Examination of the Antioxidant Potential of Pycnogenol under Conditions of Oxidative Stress in *Escherichia coli* Mutants Deficient in HP1 and Superoxide Dismutase Activities

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Pycnogenol (PYC) is believed to have potential as a therapeutic agent against free radical-mediated oxidative stress. It is important, therefore, to understand the interactions between PYC and cellular defenses against oxidative stress. Toward this end, we analyzed the survival rates on the gene expression responses of *E. coli sod katG* mutants to PYC after pre-treatment of PQ or H_2O_2-mediated stress under aerobic conditions. We identified SOD induced by PYC, but not HP1 in *sod katG* mutants. A striking result was the PYC induction of SOD with antioxidant property in single *katG* mutant cells, particularly MnSOD and CuZnSOD. These inductions were further increased with oxidative stress, while HP1 was not induced in these conditions. The effects of pycnogenol treatment on these cells depend in part on its concentration on the stress response. Protective effects of PYC exposure which affected gene expression in cells were consistent with cell survival rates. Our results demonstrate that pycnogenol may alter the stress response gene expression in a specific manner such as SoxRS because PYC induction of single mutant only worked under increased PQ stress. All together our data indicate that SOD activity is essential for the cellular defense against PQ-mediated oxidative stress, suggesting that PYC may not be effective as an antioxidant in only oxidative stress conditions. On the other hand, it was expected that PYC may play a role as a pro-oxidant and if it is available for use, it should be evaluated carefully.

Key words: Pycnogenol, free radicals, SOD, catalase, gene expression, antioxidants.

Considerable evidence suggests that pycnogenol (PYC) has potential as a therapeutic agent in the treatment of numerous diseases (Kelley, 1998; Rice-Evans and Diplock, 1993; Araghi-Niknam, 2000; Spade and Baestrazzi, 2001). Because of their strong antioxidant activities (Rice Evans *et al.*, 1998; Shahidi and Wanasundara, 1992; Noda *et al.*, 1997; Masquelier, 1987) polyphenol - rich plant extracts have been commercialized (Horpaphag Research Sales Ltd. Guernsey, Channel Islands UK) and used in dietary food supplements (Ohnishi *et al.*, 2000; Packer *et al.*, 1999). More recently, the ability of antioxidants to elicit specific cellular responses and alter patterns of gene expression has been demonstrated *in vitro*, suggesting a novel redox-sensitive signal transduction pathway (Jackson *et al.*, 1998; Rimbach *et al.*, 1999; Packer *et al.*, 1999). Since reactive oxygen species (ROS) have been implicated in such biological phenomena such as mutations, cancer (Ames *et al.*, 1981) and aging (von Zglinicki *et al.*, 2001), it is important to understand the potential defensive effects of antioxidants. Methyl viologen (paraquat) is a powerful superoxide radical generator (Yang and Kim, 1999). Therefore, it has been used in numerous studies to study the effects of protective mechanisms both *in vivo* and *in vitro* (Park and Kim, 1998; Tomita *et al.*, 2001; Konstantinova *et al.*, 1999). *E. coli sodA sodB* mutants are sensitive to paraquat because they lack antioxidant defense enzymes (Carlioz and Touati, 1986). The *katG* and *katE* genes of *E. coli* encoding bifunctional enzymes HP1 and HPII that have catalase and peroxidase activities have been cloned (Mulvey *et al.*, 1988), and HP deficient mutants have been characterized (Schellhorn and Hassan, 1988). The response to oxidative stress of *sod katG* double mutants was determined with plasmids expressing SOD and HP1 proteins alone and in combination (Park and Kim, 1998). In this previous study, we found that although HP1 could reduce the H_2O_2 or hydroxyl radicals, it also inhibited the SOD function against oxygen radicals. PYC plays a modulating role in the immune response in macrophages (Park *et al.*, 2000) and in influencing transcriptional regulation of inducible ICAM-1 expression (Bito *et al.*, 2000) including down regulation in a dose - dependent manner (Peng *et al.*, 2000). Moreover, it appears to enhance the antioxidant capacity

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of endothelial cells by increasing the expression of nuclear factor kappa B (Wei *et al.*, 1999). Although there have been reports concerning the effectiveness of PYC against elevation of ROS on immunomodulatory (Cheshier *et al.*, 1996), anti-inflammatory (Blazso *et al.*, 1997) responses and anticarcinogenic (Huynh *et al.*, 2000), at present a comprehensive understanding is lacking. Furthermore, PYC can protect cellular systems by increasing endogenous antioxidant enzymes such as SOD and catalase (Rong *et al.*, 1994-95) and by increasing levels of endogenous antioxidants such as glutathione (Rong *et al.*, 1994-95; Sen *et al.*, 1999) and α-tocopherol (Virgili *et al.*, 1998). Nevertheless, Moini *et al.* (1999) have reported that PYC reduces cytochrome c activity. This suggests that PYC can competitively inhibit the activity of the mitochondrial electron transport chain by possible donation of electrons to the iron of the heme group. Moreover, a horseradish peroxidase-H_2O_2 system has been used to generate free radicals with some phenolic compounds (Schwartner *et al.*, 1996) and glutathione (Harman *et al.*, 1986). Recently it has been shown that free radicals can be generated after PYC oxidation (Guo *et al.*, 1999). Therefore doubts still exist about PYC as a free radical scavenger. In the present study, we investigated the efficacy of PYC in mediating paraquat(PQ)-induced oxidative stress in SOD and catalase deficient *E. coli* cells, and examined the protective effects of PYC against induced free radicals.

Materials and Methods

Bacterial strains and Plasmids

The bacterial strains and plasmids used in this work are listed in Table 1. Single mutant cells, UM255 and QC 779 cells were employed to determine the effects of PYC on expression levels of SOD and catalase respectively. Furthermore, plasmid pYK8, carrying an *sad-katG* fusion gene, was transferred into UM1AB *sod katG* mutant cells. Then the pYK8 that carries the coherent fusion gene on pYK3 and thus a *Hind*III fragment of pBT22 was inserted into a single *Hind*III site of pYK3 (Park and Kim, 1998).

Regents, growth conditions and response to oxidative stress

PYC (US patent # 4,698,360) isolated from the French marine pine (*Pinus maritima*) was donated from Horphag Asia PTE Ltd. (Singapore). Standard human CuZnSOD, PQ, nitro blue tetrazolium, and other chemicals were purchased from Sigma Chemical Co., USA. Pre-cultures in LB inoculated from single colonies were grown at 37°C in the presence of appropriate antibiotics when resistance markers were present (kamamycin 40 μg/ml; chloramphenicol 20 μg/ml; ampicillin 40 μg/ml). Ove- rnight cultures were diluted 1:100 into 50 ml of fresh LB in 250 ml Erlenmeyer flasks, and were monitored for optical density at 600 nm. When the cells were grown (250 rpm) to an OD_{600}=0.1 at 37°C, PQ (0.1 mM) was added. To investigate the effects of PYC, cells were then washed with fresh LB medium, and different concentrations (5, 50, 100 150 μg/ml) of PYC were added to the medium. All cells were grown for 6 h at 37°C prior to use in enzyme essays. Culture density was monitored at OD_{600} every hour. For disc inhibition assays, 100 μl of cells were spread on LB plates and after 30 minutes, Whatman #1 filter paper disks with 20 μl of 5, 50, 100 μg/ml PYC were placed in the center of the plates. Sensitivity was quantified by measuring the diameter of the zone of inhibition after 48 h.

Crude enzyme assays

Cells were disrupted by five cycles of freezing, thawing and vortexing successively at 4°C. Cell debris was removed by 15 min centrifugation at 15,000 g at 4°C. Protein was measured in cell free extracts by the method of Lorwy *et al.*, (1951). SOD measurement was carried out under the same growth conditions by the xanthine oxidase and cytochrome c assay (McCord and Fridovich, 1969), while catalase activity was determined by measuring the rate of hydrogen peroxide decomposition at 240 nm (Beers and Sizer, 1952).

Polyacryamide gel electrophoresis

SOD and catalase enzymes were resolved by 7.8% and 10% non-denaturing polyacrylamide gels, respectively (Kim, 1990). Gels were visualized for SOD by the nitro blue tetrazolium method (Beauchamp and Fridovich, 1971) and for hydroperoxidase activity by the peroxidase diaminobenzidine method (Claiborne and Fridovich, 1979). All gels were compared with image master VDS (Pharmacia Biotech, USA).

Effect of PYC on E. coli strains under oxidative stress

Table 1. *Esherichia coli* K-12 strains and plasmids used in this study

Strains/plasmids	Phenotype	Genotype	Source
CSH7	Sod$^+$, Hp$^-$	*lacY rpsl, thi-1*	Loewen (1984)
UM1AB	Sod$^-$, Hp$^-$	UM1but(*sodA::MPR13*)$_{25}$(*sodB::Kan*)1-Δ2	Schellhorn and Hassan (1988)
UM255	Sod$^-$, Hp$^-$	*pro leu rpsL hsdM hsdR endI* KL16-99 × UM254-*thy*$^-$ *lacY katG2 katE12::Tn10 recA*	Mulvey *et al.* (1988)
QC779	Sod$^-$, Hp$^+$	*LacU*169 *rps*(*sodA::MPR13*)$_{25}$(*sodB::Kan*)1-Δ2	Carlioz and Touati (1986)
pYK8	Sod$^+$, Hp$^+$	pYK3 encoded *sodC* and *katG*, Amp^r	Park and Kim (1998)

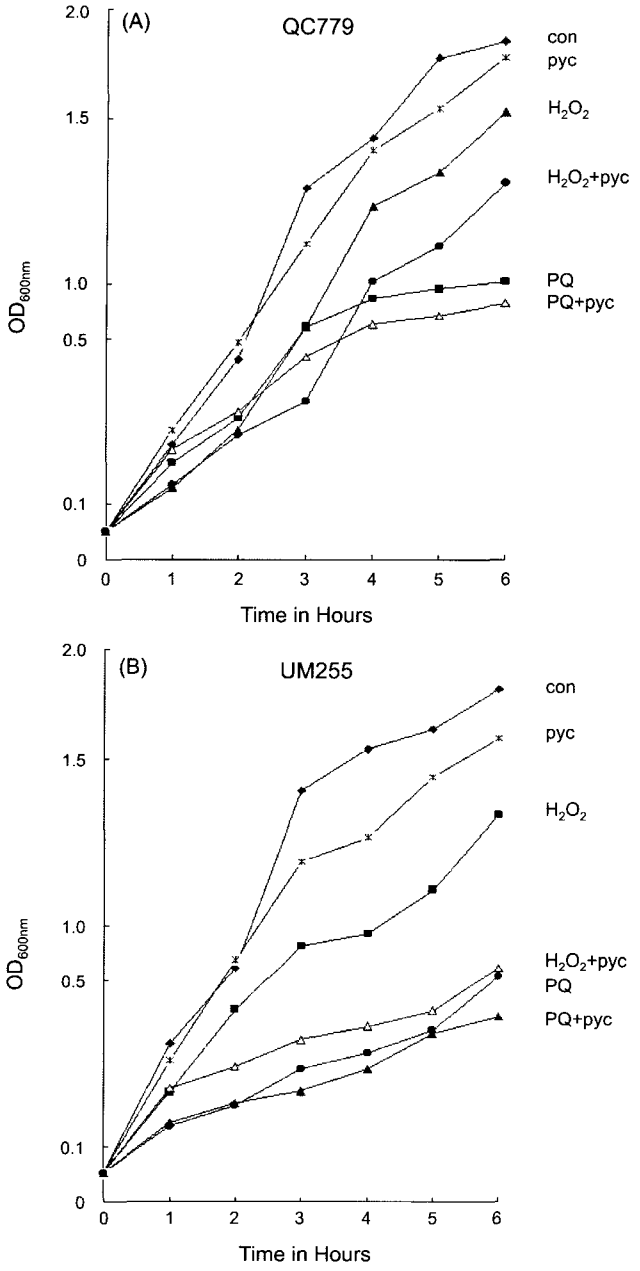

Fig. 1. The effects of PYC on Survival rates of *sod* (QC779) and *katG* (UM255) mutants under oxidative stress. Details are described in Materials and Methods.

SOD (QC779, Sod^-Hp^-) and catalase mutant cells (UM255, Sod^+Hp^-) are sensitive to oxyradical-generating medium and hydrogen peroxide, respectively (Park and Kim, 1998). Interestingly, the sensitivity of *katG* mutant cells (UM255) to PQ was enhanced by PYC (Fig 1). Moreover, the sensitivity of *sod* (QC779) mutant cells to hydrogen peroxide was enhanced by PYC. UM1AB mutants (Sod^-Hp^-) grew only under anaerobic conditions (Schellhorn and Hassan, 1988). However, when UM1AB cells were transformed with plasmid pYK8 encoding the *sod* or *katG* genes, they grew as wild-type on LB medium

Table 2. Sensitivity of *sod* and *katG* mutant strains against PYC treatment after oxidative stress

Oxidative stress	Zone of inhibition (mm)			
	Wild type [UM1AB(pYK8)][a]	Sod^+Hp^- (CSH7)	Sod^-Hp^+ (UM255)	Sod^+Hp^+ (QC779)
PYC	15 ± 1.2	17 ±1.5	20 ± 1.8	16 ± 2.1
PQ	15 ± 2.1	18 ± 1.2	25 ± 1.3	19 ± 1.8
PQ+PYC	18 ± 1.6	22 ± 1.3	27 ± 1.6	20 ± 1.2
H_2O_2	17 ± 1.2	28 ± 1.2	20 ± 1.2	18 ± 2.1
H_2O_2+PYC	18 ± 1.4	30 ± 1.6	22 ± 1.3	20 ± 1.2

Data reperesent mean ± SEM.
a: represents average data (± SEM) of both wild type and [UM1AB (pYK8)] cells.

under aerobic conditions. When plasmid encoding the *sod* or *katG* genes were transformed into double mutant cells, the survival patterns were more resistant compared to their single or double mutants against oxidative mediated stress (data not shown). The PYC effect on sensitivity of *sod katG* mutant cells against pre-treatment of oxidative stress showed a consistency with their survival rates as shown in Table 2. In particular, PYC effects are not strongly dose - dependent in this system, and its most effective concentration was 5 μg and more sensitive to the cells. The results suggested that PYC is not effective against PQ-induced oxidative stress in suggest a dose-dependent manner. Furthermore, it was expected that PYC played a role as a prooxidant and thus inhibited the survival rates (Fig. 1).

Expression of SOD and catalase affected by PYC under oxidative stress

Single *Sod* mutant (QC779, Sod^-Hp^+) and HP1 mutant (UM255, Sod^+ Hp^-) cells express catalase and SOD respectively. Catalase activity was strongly induced in QC779 cells under treatment with hydrogen peroxide, while SOD activity was induced in UM255 cells under treatment with PQ. SOD enzyme activity in *katG* cells was increased by treatment with PYC. PYC treatment following pre-treatment with PQ or hydrogen peroxide in Sod^+ Hp^- cells increased MnSOD 11 fold and CuZnSOD activity 65 fold (Table 3, Fig. 2). However, the catalase activity in Sod^-Hp^+ cells was not induced by PYC as shown in Fig. 2 and Table 2. The results suggest that PYC did not reduce the damage against oxidative mediated damage when SOD was increased. This means that PYC may not be an effective antioxidative agent against oxygen free radicals. Furthermore, when pYK8 was transferred to Sod^-Hp^- mutant cells, data show that PYC induced MnSOD activity slightly compared to the control (Fig. 3). CuZnSOD activities of SOD mutant cells were increased more at only 5 μg PYC, while the increased concentration of PYC did not much effect it (Fig. 4). It means that the regulatory region of the *sod* gene may be regulated by appropriate PYC concentration or other factors. Furthermore, it was also interpreted that PYC may

Table 3. SOD and catalase activity of Sod^+Hp^- (QC779) and Sod^-Hp^+ (UM255) mutant cells by PYC under oxidative stress

Cells		Enzyme activity (SOD[a]/catalase[b])					
		Control	PQ	PYC	PQ+PYC	H_2O_2	H_2O_2+PYC
QC779	MnSOD	16.3	17.2	182.6	213.5	22.2	43.2
	CuZnSOD	0.5	0.5	32.5	34.4	0.3	2.3
	FeSOD	6.7	7.2	7.3	7.2	5.6	5.8
UM255	HPI	11.3	9.3	11.4	9.1	83.5	83.7
	HPII	0.8	0.7	0.6	0.6	21.4	21.9
	HPIII	4.9	5.0	5.1	5.0	6.8	6.9

a: Units per milligram.
b: Micromoles per minute per milligram of protein.

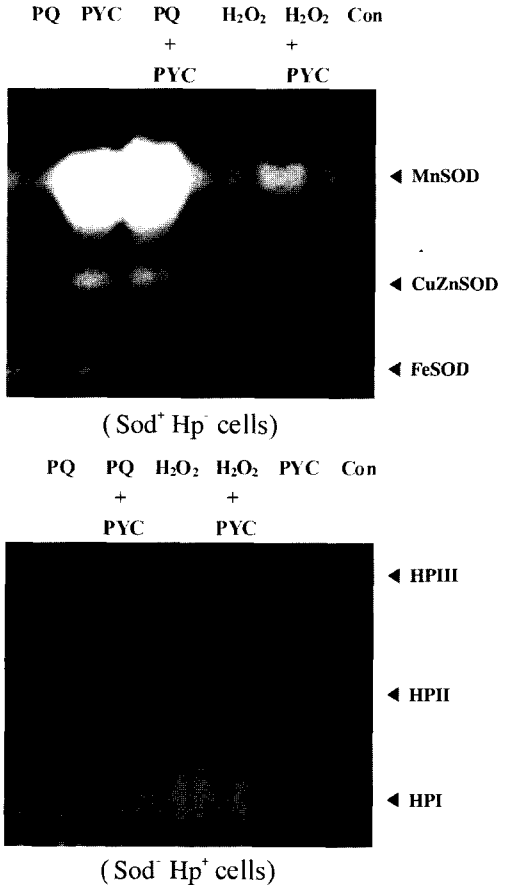

Fig. 2. Effects of PYC on the expression of SOD and catalase in mutants under oxidative stress Each lane was loaded with 50 μg protein and separated from non-denaturing gels (7.8%). Detail was described in the Materials and Methods.

induce the antioxidant enzymes against oxidative stress generated from the prooxidant (Fig. 2, Table 3).

Discussion

PQ mediated oxidative stress induced MnSOD in *E. coli* by virtue of its ability to increase the intracellular flux of oxygen radicals, while H_2O_2 induced catalase (Kao and Hassan, 1985). As a possible antioxidant, PYC can protect cellular systems by increasing the endogenous antioxidant enzymes (Rong *et al.*, 1994-1995). Nevertheless, the antioxidant nature of PYC has been questioned because of its inhibitory role in the electron transport chain and its potential for generating free radicals (Moini *et al.*, 1999; Guo *et al.*, 1999). In the present study, PYC did not protect against the deleterious effects on survival of Sod^-Hp^+ and Sod^+Hp^- cells under oxidative-mediated stress. Nevertheless, single mutant cells for HP1- induced SOD activity by PYC after pre-treatment of PQ and H_2O_2 or PYC only respectively. Therefore, we expected that PYC may protect the cellular damage from induced - SOD but not catalase. However, the growth patterns did not recover with PYC treatment. Moreover, PYC treated cells after pre-treatment with PQ or hydrogen peroxide were more sensitive compared with their PQ or hydrogen peroxide treatment. Therefore it is possible that PYC could generate the free radicals. This was further supported by the failure of the recovery of growth rates from the induced SOD after PYC treatment. Cells carrying the *sod* and *katG* genes induced SOD activity only in these conditions, and thus the cellular damage was not decreased and still was deleterious compared to wild type cells. In general, cells expressing SOD induced by antioxidants reduced cellular damage more against both PQ and H_2O_2 compared to controls in the presence of radical generated chemicals. When the cells were treated with PYC (5 μg/ml) alone without oxidative stress, the cell survival rates show a slightly lower pattern compared to the control. This pattern was more sensitive with increasing

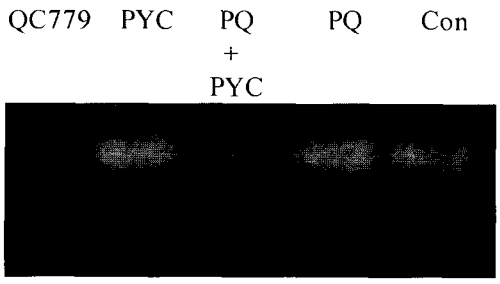

Fig. 3. The effects of PYC on MnSOD expression in the *sod katG* mutant transformed with pYK8.

PYC concentrations. This could be interpreted by PYC damaging the cell with increasing oxidative stress. It may generate free radicals because of PYC oxidation through electron spin resonance study (Guo *et al.*, 1999). On the other hand, it should be noted that the PYC may function as a prooxidant because of generation of free radicals as shown in Fig. 2 and Table 3.

In addition, the particular target of PYC on reactive oxygen species may exist in the *sod* gene because wild type or cells which encoded the *sod* gene responded only to PYC, and *sod* mutant cells did not affect catalase activity in this condition. It means that PYC involves superoxide radicals rather than hydroxyl radicals. Nevertheless, Cho *et al.* (2001) previously reported that PYC exerted strong scavenging activities against ROS generated by H_2O_2 in RAW 264.7 cells. It might be caused by the expression of proinflammatory cytokines, but not by induction of the catalase.

We demonstrated that MnSOD was found to be more important than hydroperoxide in preventing oxygen-mediated stress except for the case of H_2O_2 treatment in the presence of SOD (Park and Kim, 1998). Nevertheless, a balance between both enzymes may be necessary for an effective defense against oxygen mediated stress. Therefore, PYC may induce SOD for appropriate balance because cells were previously damaged via an oxygen radicals generated by PQ pre-treatment. It is further supported by induced-MnSOD expression consistent with survival rates. Furthermore, we should note that a high concentration of PYC is toxic to the cells because the possible damaging effect was observed *in vitro* DNA strand breakage through Penton reaction in our laboratory. Therefore, its possible damaging effects as an antioxidant should not be ruled out at this time, and it should be further studied *in vivo* eukaryotic disease model caused by generated oxygen radicals.

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References

Ames, B. N., R. Cathcart, E. Schwiers, and P. Hochstein. 1981. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc. Natl. Acad. Sci. USA* 78, 6858-6862.

Araghi-Niknam, M., S. Hosseini, D. Larson, P. Rohdewald, and R. R. Watson. 2000. Pine bark extract reduces platelet aggregation. *Integr. Med.* 2, 73-77.

Beauchamp, C. and I. Fridovich. 1971. Superoxide dismutase: Improved assays and applicable to acrylamide gels. *Anal. Biochem.* 44, 276-287.

Beers, R. F. and I. W. Sizer. 1952. A spectrophotometetric method for measuring the breakdown of hydrogen peroxidase by catalase. *J. Biol. Chem.* 44, 276-287.

Bito, T., S. Roy, C. K. Sen, and L. Packer, 2000. Pine bark extract pycnogenol down regulates IFN-gamma-induced adhesion of T cells to human keratinocytes by inhibiting inducible ICAM-1 expression. *Free. Radic. Biol. Med.* 28, 219-227.

Blazso, G., M. Gabor, and P. Rohdewald. 1997. Anti-inflammatory and superoxide radical scavenging activities of procyanidines containing extract from *Pinus pinaster Ait.* after oral and cutaneous application. *Pharmazie.* 52, 380-382.

Carlioz A. and D. Touati. 1986. Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? *EMBO J.* 5, 623-630.

Cheshier, J. E., S. Ardestani-Kaboudanian, B. Liang, M. Araghiniknam, S. Chung, L. Lane, A. Castro, and R. R. Watson. 1996. Immunomodulation by pycnogenol in retrovirus-infected or ethanol-fed mice. *Life Sci.* 58, PL87-96.

Cho, K. J., C. H. Yun, L. Packer, and A. S. Chung. 2001. Inhibition mechanisms of bioflavonoids extracted from the bark of *Pinus maritima* on the expression of proinflammatory cytokines. *Ann. N.Y. Acad. Sci.* 928, 141-156.

Claiborne, A. and I. Fridovich. 1979. Purification of the orthodianisidine peroxidase from *Escherichia coli* B. *J. Biol. Chem.* 254, 116640-116648.

Guo, Q., B. Zhao, and L. Packer. 1999. Electron spin resonance study of free radicals formed from a procyanidin-rich pine (*Pinus maritima*) bark extract, pycnogenol. *Free. Radic. Biol. Med.* 27, 1308-1312.

Harman, L. S., D. K. Carver, J. Schreiber, and R. P. Mason. 1986. One- and- two electron oxidation of reduced glutathione by peroxidases. *J. Biol. Chem.* 261, 1642-1648.

Huynh, H. T. and R. W. Teel. 2000. Selective induction of apoptosis in human mammary cancer cells (MCF-7) by pycnogenol. *Anticancer. Res.* 20, 2417-2420.

Jackson, M. J., A. McArdle, and F. McArdle. 1998. Antioxidant micronutrients and gene expression. *Proc. Nutr. Soc.* 57, 301-305.

Kao, S. M. and H. M. Hassan. 1985. Isolation of paraquat-resistant mutants of *Escherichia coli*: lack of correlation between resistance and the activity of superoxide dismutase. *FEMS Microbiol. Letters.* 28, 93-97.

Kelly, F. J. 1998. Use of antioxidants in the prevention and treatment of disease. *J. Int. Fed. Clin. Chem.* 10, 21-23.

Kim, Y. G. 1990. Superoxide dismutase: gene replacement studies. Doctoral dissertation. UNM, Albuquerque, NM. USA. p. 1-58.

Konstantinova, S. G. and E. M. Russanov. 1999. Studies on paraquat-induced oxidative stress in rat liver. *Acta. Physiol. Pharmacol. Bulg.* 24, 107-111.

Loewen, P. C. 1984. Isolation of catalase-deficient *Escherichia coli* mutants and genetic mapping of KatE, a locus that affects catalase activity. *J. Bacteriol.* 157, 622-626.

Lorwy, H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265-275.

Masquelier, J. 1987. Plant extract with a proanthocyanidins content as therapeutic agent having radical scavenger effect and use thereof. US Patent 4698360. p 1-11.

McCord, J. M. and I. Fridovich. 1969. The pathophysiology of

superoxide: roles in inflammation and ischemia. *Can. J. Physiol. Pharmacol.* 60, 1346-1352.

Moini, H., A. Arrovo, J. Vava, and L. Packer. 1999. Bioflavonoid effects on the mitochondrial respiratory electron transport chain and cytochrome c redox state. *Redox. Rep.* 4, 35-41.

Mulvey, M. R., P. A. Sorby, B. L. Triggs-Raine, and P. C. Loewen. 1988. Cloning and physical characterization of *katE* and *katF* required for catalase HPII expression in *Escherichia coli*. *Gene.* 73, 337-345.

Noda, Y., K. Anzai, A. Mori, M. Kohno, M. Shinmei, and L. Packer. 1997. Hydroxyl and superoxide anion radical scavenging activities of natural source antioxidants using the computerized JES-FR30 ESR spectrometer system. *Biochem. Mol. Biol. Int.* 42, 35-44.

Ohnishi, S. T., T. Ohnishi, and G. B. Ogunmola. 2000. Sickle cell anemia: a potential nutritional approach for a molecular disease. *Nutrition* 16, 330-338.

Packer, L., G. Rimbach, and F. Virgili. 1999. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. *Free. Radic. Biol. Med.* 27, 704-724.

Park, H. Y. and Y. G. Kim. 1998. Response of CuZnSOD and HP1 conjugated gene in *Escherichia coli* double mutants to oxidative stress. *J. Microbiol.* 36, 171-178.

Park, Y. C., G. Rimbach, C. Saliou, G. Valacchi, and L. Packer. 2000. Activity of monomeric, dimeric, and trimeric flavonoids on NO production, TNF-alpha secretion, and NF-kappaB-dependent gene expression in RAW 264.7 macrophages. *FEBS Lett.* 465, 93-97.

Peng, Q., Z. Wei, and B. H. Lau. 2000. Pycnogenol inhibits tumor necrosis factor-alpha-induced nuclear factor kappa B activation and adhesion molecule expression in human vascular endothelial cells. *Cell. Mol. Life. Sci.* 57, 834-841.

Pietta, P., P. Simonetti, C. Gardana, A. Brusamolino, P. Morazzoni, and E. Bombardelli. 1998. Relationship between rate and extent of catechin absorption and plasma antioxidant status. *Biochem. Mol. Biol. Int.* 46, 895-903.

Rice-Evans C. A. and A. T. Diplock. 1993. Current status of antioxidant therapy. *Free. Radic. Biol. Med.* 15, 77-96.

Rice-Evans, C. A., N. J. Miller, and G. Paganga, 1998. Structure-antioxidant activity relationship of flavonoids and phenolic acids. in Rice-Evans, C and I. Packer, eds. Flavonoids in health and disease. p.199-209. New York. Marcel Dekker.

Rimbach, G., F. Virgili, Y. C. Park, and L. Packer. 1999. Effect of procyanidins from *Pinus maritima* on glutathione levels in endothelial cells challenged by 3-morpholinosydnonimine or activated macrophages. *Redox. Rep.* 4, 171-177.

Rong, Y., L. Li, V. Shah, and B. H. Lau. 1994-95. Pycnogenol protects vascular endothelial cells from t-butyl hydroperoxide induced oxidant injury. *Biotechnol. Ther.* 5, 117-126

Schellhorn, H. E. and H. M. Hassan. 1988. Response of hydroperoxidase and superoxide dismutase deficient mutants of *Escherichia coli K-12* to oxidative stress. *Can. J. Microbiol.* 34, 1171-1176.

Schwartner, C., C. Michel, K. Stettmaier, H. Wagner, and W. Bors. 1996. Marchantins and related polyphenols from liverwort: physico-chemical studies of their radical-scavenging properties. *Free. Radic. Biol. Med.* 20, 237-244.

Sen, C. K., S. Roy, and L. Packer. 1999. Flow cytometric determination of cellular thiols. *Methods Enzymol.* 299, 247-258.

Shahidi, F. and P. K. Wanasundara. 1992. Phenolic antioxidants. *Crit. Rev. Food. Sci. Nutr.* 32, 67-103.

Spade, L. and E. Balestrazzi. 2001. Treatment of vascular retinopathies with Pycnogenol. *Phytother. Res.* 15, 219-223.

Tomita, M., T. Okuyama, T. Ishikawa, K. Hidaka, and T. Nohno. 2001. The role of nitric oxide in paraquat-induced cytotoxicity in the human A549 lung carcinoma cell line. *Free. Radic. Res.* 34, 193-202.

Virgili, F., D. Kim, and L. Packer. 1998. Procyanidins extracted from pine bark protect alpha-tocopherol in ECV 304 endothelial cells challenged by activated RAW 264.7 macrophages: role of nitric oxide and peroxynitrite. *FEBS Lett.* 431, 315-318.

von Zglinicki, T., A. Burkle, and T. B. Kirkwood. 2001. Stress, DNA damage and ageing - an integrative approach. *Exp. Gerontol.* 36, 1049-1062.

Wei, Z., O. Peng, B. H. Lau, and Y. Shah. 1999. *Ginkgo biloba* inhibits hydrogen peroxide-induced activation of nuclear factor kappa B in vascular endothelial cells. *Gen. Pharmacol.* 33, 369-375.

Yang, M. K. and Y. G. Kim. 1999. Protective role of germanium-132 against paraquat-induced oxidative stress in the livers of senescence-accelerated mice. *J. Toxicol. Environ. Health A.* 58, 289-297.