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Protective Effect of *Phyllanthus polyphyllus* on Human Umbilical Vein Endothelial Cells against Glycated Protein-Iron Chelate Induced Toxicity

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Protective effect of alcohol extract of *Phyllanthus polyphyllus* on Human Umbilical Vein Endothelial Cells (HUVEC) against glycated protein (GFBS)-iron chelate ($FeCl_3$) induced toxicity was studied. HUVEC incubated in glycated protein either alone or combined with iron chelate showed a significant ($p<0.001$) elevation of lipid peroxidation (LPO) accompanied by depletion of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and Glutathione Reductase (GR), in addition to increased microsomal cytochrome c reductase and decreased Glutathione S-Transferase (GST). Treatment of HUVEC with *P. polyphyllus* at a concentration of 25 and 50 μg significantly decreased the level of LPO and altered antioxidants, cytochrome c reductase and GST levels to near normal in a dose dependent manner. These findings suggest a protective effect of *P. polyphyllus* on HUVEC against glycated protein-iron chelate induced toxicity. This effect has a greater potential in preventing diabetic angiopathies.

Key words: *Phyllanthus polyphyllus*, HUVEC, glycated protein-iron chelate, lipid peroxidation, antioxidant enzymes

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INTRODUCTION

Vascular endothelial cells and vascular muscle cells are the two major components of the vessel wall. Since endothelial cells are situated within the intimal layer of the vessel wall, abnormal levels of glucose in blood may trigger initial damage leading to functional and structural alterations, which are potentially responsible for diabetic complications (Xu *et al*., 2004). High glucose concentration has been reported to be toxic *in vitro* for endothelial cells, as represented by retarded cell proliferation (Curcio and Ceriello, 1992) disturbed cell cycle (Lorenzi *et al*., 1985) increased DNA damage (Lorenzi *et al*., 1986) and slightly accelerated cell death (Lorenzi *et al*., 1985). Such pathological changes may play important role in causing microvascular lesions that are common and prominent in diabetes. Thus prevention of hyperglycemia-triggered endothelial cell lesions may prevent diabetes associated microvascular complications. The Maillard or Browning reaction between reducing sugars and protein is proposed to be involved in the pathophysiological procedure of aging and diabetic complications (Brownlee *et al*., 1985).

The scavenging function of antioxidant enzymes is reduced by glycation of their free amino terminals to produce Advanced Glycated End-product (AGE) variants of these enzymes. High blood glucose level causes binding of glucose covalently and non enzymatically to various proteins *in vivo* to generate AGEs (Szalecsky *et al*., 1999). Glycation can alter the structure as well as the function of physiological proteins. Therefore, hyperglycemia may modify the properties of the enzymatic antioxidant defense proteins and increase the susceptibility of diabetic patient to vascular damage due to excessive production of free-radical species.

The levels of AGE increased in the serum of patients with diabetes. AGEs have been linked with retinopathy, nephropathy and neuropathy, as well with large vessel disease (Nessar 2005; Sara *et al*., 2003; Thomas *et al*., 2003). The mechanisms leading to micro- and macro-angiopathy are thought to occur by an interaction of AGE with a receptor (known as RAGE) expressed on the cell surface membrane of vascular endothelial cells (Sensi *et al*., 1995). It is therefore interesting to speculate that an inhibition of AGE synthesis, or its antagonism at the level of the RAGE receptor, could play a role in preventing the genesis of vascular diabetic complications.

Oxidative stress has emerged as a strong pathogenic cofactor in the development of longterm complications of type II diabetes, such as atherosclerosis, nephropathy and retinopathy (Jian and Ajay, 2004). The H_2O_2 is the most stable of the Reactive Oxygen Species (ROS) and is produced from the dismutation of superoxide and form the divalent reduction of oxygen by various oxidases. H_2O_2 is detoxified by catalase and by glutathione peroxidases. In general, the toxicity caused by superoxide and by H_2O_2 in a biological systems is believed to be due to the conversion of these ROS to more potent oxidants, such as the hydroxyl radical or ferryl or perferryl species, with the oxidizing power of the hydroxyl radical. This conversion is either catalyzed or actually requires the presence of transition metals, such as iron or copper and it is believed that the toxicity of iron in biological systems is due to iron-catalyzed production of the above-mentioned potent oxidants by Fenton or Haber-Weiss types of reactions (Jagetia *et al*., 2004; Halliwell and Gutteridge, 1983). Results from numerous studies have shown that iron potentiates the toxicity of drugs, redoxcycling agents and prooxidants, whereas removal of iron protects the system against toxicity. Although identification of the low molecular weight, nonheme iron pool in cells has proved elusive, cellular sources of iron clearly potentiate oxidative stress.

The level of glycated protein is known to increase in diabetic patients and the Fructosamine Value (FV) of serum is regarded as an indicator of blood glucose level. Yagi *et al*. (1985) have reported that chinoform-ferric iron chelate causes lipid peroxidation in cultured neural retinal cells and Nishigaki *et al*. (1998) have shown that glycated protein-iron chelate provokes lipid peroxidation in endothelial cells, these facts explain at least in part the mechanism of atherogenesis in diabetic patients.

Phyllanthus polyphyllus Linn. (Euphorbiaceae) is a deciduous shrub or small tree found mostly in hill areas of South India and Ceylon. It is popularly known as Sirunelli in Tamil. Leaves are traditionally used for liver diseases by tribes of Kolli hills, Tamilnadu, India (Gamble, 1993; Mathew, 1983). Phytochemical studies reveal the presence of benzenoid, 4-0-methyl galic acid, together with three arylnapthalide lignans, namely phyllamyricin, justicidin B and diphyllin. Extracts of this plant show dose dependent inhibition of inflammatory mediators such as LPS/INF-γ stimulated peritoneal excluded macrophages (Rao *et al*., 2006), monoacetylated triterpene arabinosides and terpenes found have cytotoxic activity against human nasopharyngeal carcinoma (KB) and small cell lung cancer (NCI-H187) (Youkwan *et al*., 2005). In this paper we have demonstrated that *Phyllanthus polyphyllus* produce a significant protective effect against glycated protein-iron chelate induced toxicity in human umbilical vein endothelial cells *in vitro*.

MATERIALS AND METHODS

Plant material and extraction: Leaves of *Phyllanthus polyphyllus* Linn. were collected in and around Kolli hills, Salem District and authenticated by Dr. G. Murthy, Botanical Survey of India, Coimbatore, Tamilnadu, India. A voucher specimen (EPP-03) has been kept in our laboratory for future reference. The leaves were dried in the shade and pulverized. The powder was treated with petroleum ether for dewaxing as well as to remove chlorophyll. The powder was then packed into soxhlet apparatus and subjected to hot continuous percolation using methanol (95% v/v) as solvent. The extract was concentrated under vacuum and dried in a vacuum desiccator (yield 4.1 % w/w).

Cells and chemicals: HUVEC and endothelial medium (EG2) were purchased from Kurabo (Japan). Cytochrome c reductase was purchased from Sigma (St. louis, USA). Catalase and glutathione S-transferase assay kit was obtained from Cayman chemical company (USA). Glutathione peroxidase and glutathione reductase assay kit was purchased from Oxi research (USA) and Trevigen Inc (USA), respectively. Superoxide dismutase was obtained from Dojindo laboratories (Japan). Protein assay kit was obtained from Bio-RAD, USA. Cell culture flask was from Nunc, Denmark. All other chemicals used are of analytical grade and water was purified through Millipore Milli Q system (Millipore Co., USA)

Serum glycation: For glycation of Fetal Bovine Serum (FBS), 20 mL of FBS was incubated with 50 mM glucose in 1 mL of 67 mM phosphate buffer (pH 7.4) at 37°C for 25 days under sterile conditions. After incubation, the mixtures was dialysed against the same buffer at 4°C. The dialysate was used as glycated FBS after concentration by using aquacide II (Calbiochem-Novabiochem Corp., USA). The extent of glycation of FBS was assayed with a commercial kit (Nippon Roche, Japan) and expressed as Fructosamine Value (FV). The FV of GFBS prepared were 12 mM and that of original FBS was 0.20 mM.

Cell culture and experiments: HUVEC were grown in endothelial medium-2 (EG2), supplemented with 10% (v/v) fetal bovine serum, gentamicin sulphate (50 mg mL^{-1})/amphotericine-B (50 μg mL^{-1}) in addition to human recombinant fibroblast growth factor-B (hFGF-B; 5 μg mL^{-1}), human recombinant epidermal growth factor (hEGF; 10 μg mL^{-1}), hydrocortisone (1 mg mL^{-1}) and heparin (10 mg mL^{-1}). Cells were grown in 25 cm^2 flasks. The cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C, until reaching 80% confluence and used for experiments between passage 3 and 4.

The experimental design was formed by 10 groups with 5 flasks in each group. Group I was treated with 15% of FBS. The group II, III and IV were treated with GFBS (70 μM), FBS+$FeCl_3$ (20 μM) and GFBS+ $FeCl_3$, respectively. Other 6 groups were treated similar to group II, III and IV and to them *P. polyphyllus* extract was added at doses of 25 and 50 μg respectively (Nishigaki *et al.*, (1998)). After 24 h incubation, the cells were collected from all the experimental groups by detaching from the flasks using cell scraper-M (Sumitomo bakelite Co., Japan) with phosphate buffer and centrifuged at 1000 ×g for 10 min at 4°C. The cell pellets were suspended with cold phosphate buffer and homogenized by using sonicator (Misonix incorporated, USA). The mixture was again centrifuged at 10,000×g for 15 min at 4°C. A portion of the supernatant was used for the assay of lipid peroxidation and antioxidants enzymes activities. Another portion of supernatant was subjected to centrifugation at 100,000×g for 60 min at 4°C for microsomal separation and was used to assay of cytochrome c reductase and glutathione S-transferase.

Protein assay: Protein content was quantified by means of the BIO-RAD reagent protein assay kit, following the method of Lowry *et al.* (1951).

Lipid peroxidation assay: Lipid peroxidation was determined by using the assay described in Kyowa kit (Japan) manual. In brief, the LPO assay is based on the reaction of a chromogenic reagent, N-methylcarbamoyl-3,7-dimethyl amino-10H-phenothiazine with ascorbic acid oxidase and cumene hydroperoxide at 30°C. The stable chromophore with maximal absorbance at 675 nm and LPO activity were expressed as nmolMDA/ml/mg protein

Catalase activity: Catalase (CAT) activity was determined using Cayman chemical company kit. After addition of 10 mM H_2O_2, cell lysates were incubated for 20 min and mixed with chromogen substrate followed by addition of stopping reagent. The colour developed during incubation was detected at 540 nm using microplate reader (ImmunoMini NJ). The rate of absorbance was converted to nmol min^{-1} mL^{-1} of enzyme activity, determined from the linear regression of standard curve using CAT.

Superoxide dismutase activity: SOD enzymatic activity was determined by using the method described in Dojindo laboratories SOD kit manual. In brief, cell lysates were treated with water soluble tetrazolium salt (WST) and SOD activity (% inhibition) was determined by measuring the absorbance at 450 nm using microplate reader.

Glutathione peroxidase activity: Glutathione peroxidase activity was determined using the assay described in Oxi research GPx - 340 kit. After addition of 350 μL of NADPH to cell lysates, the reaction was initiated by the addition of tert-butyl hydroperoxide. The changes in absorbance were recorded at 340 nm using spectrophotometer (Jenway, UK).

Glutathione reductase activity: Glutathione reductase activity was determined using the assay described in Trevigen kit. In brief, cell lysates were treated with oxidized glutathione and changes were recorded at 340 nm after the addition of nicotinamide adenine dinucleotide phosphate (NADPH). The glutathione reductase activity was expressed in terms of the oxidation of NADPH mU mL^{-1}.

Cytochrome c reductase assay: This assay is based on colorimetric using Sigma kit and all reagents required for performing the assay are included within the assay kit. The assay was performed according to the manufacturer's instructions. In brief, cell lysate was treated with cytochrome c reductase in presence of NADPH. The reduction of cytochrome c was measured at 550 nm.

Glutathione S-transferase assay: Glutathione S-transferase assay was done using Cayman chemical company kit. This assay is based on measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione in the microsomal fraction. The conjugation is accompanied by an increased absorbance measured at 340 nm. The GST activity was expressed as nmol min^{-1} mL^{-1}.

Statistical analysis: The results are expressed as mean±SD and the statistical significance was analyzed by one way ANOVA followed by Tukey multiple comparison test (GraphPad InStat, USA). $p<0.05$ were considered significant.

RESULTS

Lipid peroxidation: The lipid peroxide levels were measured as MDA concentration and expressed as nmol mL^{-1} mg^{-1} protein (Table 1). Treatment of HUVEC cells with GFBS-iron chelate elevated lipid peroxidation levels significantly when compared with FBS treatment group ($p<0.001$). The treatment of HUVEC cells with 25 and 50 μg of *P. polyphyllus* significantly ($p<0.001$) reduced the iron chelate induced lipid peroxidation.

Antioxidant enzymes: GFBS-iron treatment significantly reduced the SOD and catalase activity in HUVEC when compared with the control group; however, treatment of HUVEC with *P. polyphyllus* elevated the cellular SOD and catalase levels in a dose dependent manner when compared with control group (Table 1).

The GFBS-iron chelate significantly reduced the GPx levels in HUVEC when compared with the control group ($p<0.001$). Treatment of HUVEC with 25 and 50 μg of *P. polyphyllus* significantly altered the GPx levels to near normal when compared with GFBS-iron treated group (Table 1).

The concentration of glutathione reductase has been expressed as mU mL^{-1} (Table 1). Treatment of HUVEC with GFBS-iron depleted GSH concentration significantly ($p<0.001$) when compared with the FBS treated group. Treatment of HUVEC cells with 25 and 50 μg of *P. polyphyllus* significantly elevated the cellular glutathione levels when compared to GFBS-iron treated group ($p<0.001$).

Cytochrome c reductase: Cytochrome c plays an important role in cell death. It is released from the mitochondria in response to apoptotic signals (Skulachev, 1998). In the present study, the protective effect *P. polyphyllus* on glycated protein-iron chelate induced toxicity in HUVEC, was evaluated by using cytochrome c reductase kit. As shown in Table 2,

Table 1: Effect of *P. polyphyllus* on lipid peroxidation and antioxidants level in HUVEC against glycated protein-iron chelate induced toxicity

Treatments	LPO (nmol MDA mL^{-1} mg^{-1}) protein	SOD inhibition (%)	Catalse (nmol min^{-1} mL^{-1})	Gpx (mU mL^{-1})	GR (mU mL^{-1})
FBS	61.35±1.05	94.48±1.94	23.78±1.15	0.92±0.007	53.27±2.90
GFBS	131.33±2.19[a]	83.06±3.81	16.36±1.08[a]	0.70±0.006[a]	40.00±1.46[a]
FBS + $FeCl_3$	91.06±1.36[a]	87.96±2.42	18.85±0.97[b]	0.75±0.005[a]	47.25±1.77
GFBS + $FeCl_3$	149.00±3.92[a]	78.44±2.36[a]	12.38±0.52[a]	0.68±0.002[a]	35.42±1.26[a]
GFBS+*P. polyphyllus*(25 μg)	101.45±2.51[a,d]	86.28±1.23	18.85±0.74[b]	0.89±0.002[a,d]	46.66±0.88
FBS + $FeCl_3$+ *P. polyphyllus* (25 μg)	70.66±2.02[d]	89.76±1.74	20.20±0.69	0.78±0.005[a,d]	49.00±1.15[e]
GFBS+$FeCl_3$+*P. polyphyllus* (25 μg)	119.72±3.72[a,f,$]	81.76±1.32[b]	16.65±0.54[a,$]	0.83±0.002[a,d,$]	43.56±1.10[b,$]
GFBS+*P. polyphyllus* (50 μg)	81.94±1.28[a,d]	89.20±1.14	19.96±0.87	0.92±0.002[d]	50.14±1.70[e]
FBS + $FeCl_3$+ *P. polyphyllus* (50 μg)	67.18±1.20[d]	91.45±1.26	22.85±0.94	0.95±0.003[a,d]	51.86±1.47[d]
GFBS + $FeCl_3$+*P. polyphyllus* (50 μg)	103.33±1.84[a,d,$]	84.26±1.57[c]	18.24±0.76[b,d,$]	0.85±0.006[a,d,$]	47.33±0.88

LPO- lipid peroxidation; SOD- Superoxide dismutase; Gpx -Glutathione peroxide; GR- Glutathione Reductase, Average of 5 determinations; Values are expressed as mean±SEM, [a]$p<0.001$; [b]$p<0.01$; [c]$p<0.05$ Vs FBS; [d]$p<0.001$; [e]$p<0.01$; [f]$p<0.05$ Vs GFBS; [$]$p<0.001$ Vs GFBS+ $FeCl_3$

Table 2: Effect of *P. polyphyllus* on Cytochrome c reductase and glutathione *S*- transferase in HUVEC against glycated protein-iron chelate induced toxicity

Treatments	Cyt C reductase (Unit mL^{-1})	GST (nmol min^{-1} mL^{-1})
FBS	10.89±0.35	8.61±0.75
GFBS	28.66±0.98[a]	3.97±0.15[a]
FBS + $FeCl_3$	17.66±1.32[a]	4.63±0.16[a]
GFBS + $FeCl_3$	34.76±1.08[a]	3.30±0.10[a]
GFBS + *P. polyphyllus*(25 μg)	19.33±1.24[a,c]	6.92±0.27[c]
FBS + $FeCl_3$+ *P. polyphyllus* (25 μg)	15.00±0.97[c]	7.75±0.34[c]
GFBS + $FeCl_3$ + *P. polyphyllus* (25μg)	26.47±1.45[a,#]	5.45±0.26[a,$]
GFBS + *P. polyphyllus*(50 μg)	16.15±0.85[c]	7.86±0.54[c]
FBS + $FeCl_3$+ *P. polyphyllus* (50 μg)	13.84±1.74[c]	8.47±0.58[c]
GFBS + $FeCl_3$ + *P. polyphyllus* (50 μg)	19.30±1.68[a,c,$]	6.54±0.37[b,d,$]

GST- Glutathione S-transferase; Average of 5 determinations; Values are expressed as mean±SEM, [a]$p<0.001$; [b]$p<0.05$ Vs FBS; [c]$p<0.001$; [d]$p<0.01$ Vs GFBS, [$]$p<0.001$; [#]$p<0.05$ Vs GFBS+ $FeCl_3$

cytochrome c reductase activity was inhibited by *P. polyphyllus* in a concentration-dependent manner.

Glutathione S-transferase: Treatment of GFBS-iron with HUVEC significantly ($p<0.001$) reduced GST levels when compared with FBS treatment group. Treatment of HUVEC with *P. polyphyllus,* marginally increased the microsomal GST levels when compared with GFBS-iron chelate treated group (Table 2).

DISCUSSION

Endothelial dysfunction has been proved to mediate the initiation and development of many kinds of diabetic angiopathies. Disorders of contraction and relaxation, the basic function of endothelium is caused by decreased NO release. Since endothelial cells were the intimal layer of vessel wall, it was proposed to be the prominent target of AGEs and other toxic factors in diabetes state. Hyperglycemia may exert toxic effect on endothelium through direct path by formation of AGEs.

Well-established risk factors for the development of atherosclerosis such as hyperlipidemia, hyperglycemia, hypertension, hyperhomocysteinemia and local hemodynamic stress are known to mediate elevated levels of ROS in the vasculature (Ross, 1999). The molecular and cellular changes involved in linking these diverse risk factors to a common mechanism are unclear. Increase in ROS may affect four fundamental mechanisms that contribute to atherosclerosis: Endothelial cell dysfunction, Vascular Smooth Muscle Cell (VSMC) growth, monocyte migration and oxidation of LDL (Alexander, 1995).

Lipid peroxidation has been used as an indirect measure of oxidative stress. The endproducts of stable aldehydes react with thiobarbituric acid (TBA) to form thiobarbituric acid-malondialdehyde adduct (Berliner and Heinecke, 1996). In previous studies, similarly reported that increase LPO level in HUVEC when incubated with glycated protein and iron (Jagetia *et al*., 2004; Nishigaki *et al*., 1998). Treatment of HUVEC with *P. polyphyllus* significantly ($p<0.001$) reduced lipid peroxidation. Hence it is likely that *P. polyphyllus* has an antioxidant property.

Glutathione a tripeptide and an essential bio-factor synthesized in all living cells. It functions mainly as an effective intracellular reductant (Beckman and Ames, 1998). It protects cells from free radical-mediated damage caused by drugs and also by ionizing radiation. It forms an important substrate for GPx, GST and several other enzymes, which are involved in free radical scavenging. *P. polyphyllus* inhibits GFBS-iron chelate induced reduction in glutathione levels in HUVEC .

The *P. polyphyllus* arrested the GFBS-iron induced decline in GST and reduced iron chelate induced damage. GST acts like a peroxidase and removes stable peroxides from the system, resulting in reduction in the peroxide-induced damage (Rahman and MacNee, 1999). Superoxide and hydrogen peroxide are important byeproducts in usual cellular energy metabolism. Both are not highly toxic as such, but uncompartmentalized iron chelate can initiate the formation of OH radical and influence lipid peroxidation via Fenton/Haber-Weiss reactions (Prohaska, 1980). Cells are equipped with an impressive repertoire of antioxidant enzymes, such as superoxide dismutase, which hasten the dismutation of O_2^- to H_2O_2 and catalase and glutathione peroxidase, which convert H_2O_2 to water (Fridovich, 1986). SOD brings the first line of defense against free radicals by dismutating toxic superoxide into a less toxic hydrogen peroxide. SOD works in conjugation with other H_2O_2 removing enzymes. Selenium containing GPx decomposes H_2O_2 and other peroxides and initiates free radical chain reaction. Catalase heme enzyme brings the decomposition of high amounts of H_2O_2 and other peroxides. SOD, GPx and catalase, by a concerted action, protect the oxidative attack of superoxide and hydrogen peroxide in the cells. *P. polyphyllus* elevates cellular catalase and SOD levels accompanied by an arrest of iron-chelate induced depletion of SOD, GPx and catalase in HUVEC.

Diabetes-associated hyperglycemia causes intracellular oxidative stress, which contributes to

vascular dysfunction (Baynes, 1991). The effects of hyperglycemia on endothelial cell function can be mediated by several pathways: (a) production of ROS (b) accumulation of sorbitol (c) nonenzymatic glycoxidation of macromolecules and (d) direct activation of protein kinase C (Cines *et al*., 1998). Glycoxidation of proteins and lipids occur ubiquitously in patients with diabetes and it is irreversible. Its consequences are especially relevant to long-term vascular dysfunction. The initial glycoxidation of proteins results in the formation of early glycation products. The interaction of AGEs with cell surface receptors (RAGEs) has been shown to generate ROS and to decrease the levels of reduced gluthatione. GSH is a major endogenous antioxidant, the level of which declines during cardiovascular diseases (Sian, 1994). Several groups have shown that GSH depletion causes various biochemical and pathological changes(Jagetia *et al*., 2004). Jagetia *et al*. (2004) have reported similarly that iron overload caused a significant elevation in the LPO accompanied by reduction in SOD, catalase, GPx and glutathione reductase. The presence of *P. polyphyllus* might have taken the burden of free radicals upon itself, thereby sparing the depletion of GSH. This could have helped the HUVEC to overcome the iron chelate-induced insult. The antioxidant activity of *P. polyphyllus* has been found to be similar to that of GSH.

Two mechanisms have been proposed to explain how hyperglycemia causes increased ROS formation (Wolff and Dean, 1987; Hunt *et al*., 1993). One mechanism involves the transition metal catalyzed autoxidation of protein-bound Amadori products which yields superoxide and hydroxyl radicals and highly reactive dicarbonyl compounds. The other mechanism involves the transition metal-catalyzed autoxidation of free sugars, which also yields dicarbonyl compounds and superoxide and hydroxyl radicals. Autoxidation of protein-bound Amadori products appears to be the most important source of free radicals under near physiologic conditions (Hunt *et al*., 1988).

Xenobiotic enzymes like cytochrome c reductase, are toxic due to their redox-cycling capability. Thus interact with synergistically iron chelate to increase the catalytic effectiveness of iron in generating reactive oxygen intermediates. Microsomes isolated from HUVEC when incubated with GFBS-iron show an elevated production of superoxide and H_2O_2. In the presence of iron, there is an increased production of reactive oxygen intermediates, which can cause lipid peroxidation, chemiluminescence, oxidation of OH-chemical scavengers and inactivation of metabolic enzymes. Increased oxygen radical production by microsomes after incubation with the toxicant is due to inefficient coupling between the reductase and cytochrome; increased content of enzymes such as cytochrome c reductase, which comprise the mixed-function oxidase system and increased oxidase activity of the iron chelate-inducible cytochrome isoenzyme. Treatment of HUVEC cells with 25 and 50 μg of *P. polyphyllus* significantly decreases microsomal cytochrome c reductase levels when compared with the GFBS-iron treated group ($p<0.001$) (Syu-ichi *et al*., 2003).

The demonstration that an ROS-dependent process plays a central role in the generation of intracellular AGEs and that inhibition of oxidant pathways prevents intracellular AGE formation have important implications for pharmacologic attempts in preventing diabetic complications. Currently, AGE formation can be reduced both by lowering hyperglycemia (Hammes *et al*., 1995) and by blocking reactive AGE precursors with drugs such as aminoguanidine (Brownlee, 1995). Both interventions are effective in preventing diabetic complications in animal models. Blocking intracellular AGE formation by antioxidants offers an additional strategy for the potential prevention of diabetic complications and this point deserves further exploration.

Alcohol extract of *P. polyphyllus* has the capacity to protect the endothelium *in vitro* against the insult induced by reactive oxygen species. The alcohol extract has antioxidant properties in a cellular environment, at concentrations 25 and 50 μg and it is devoid of toxicity to the endothelial cells. In conclusion, the results suggest that the leaves of *P. polyphyllus* have a beneficial effect in diabetic patients, since it prevents oxidative damage to endothelial cells and so it may prevent or slow-down the development of vascular complications.

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