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Title Page

Pine Bark Extracts: Nutraceutical, Pharmacological and Toxicological Evaluation

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Running title: Physiology and toxicology of pine bark extracts

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Abbreviations: ADRP: adipose differentiation-related protein; CAD: coronary artery disease; CAT: catalase; COX: cyclooxygenase; CVD: cardiovascular disease; CVI: chronic venous insufficiency; DM: diabetes mellitus; DNP: dinitrophenyl; EBV: Epstein–Barr virus; GSH-Px: glutathione peroxidase; ICS: inhalation corticosteroid; LOX: lipoxygenase; LPS: lipopolysaccharide; MDA: malondialdehyde; MMP: matrix metalloproteinase; NF-kB: nuclear factor-kappa B; NOS: NO synthase; OA: oleic acid; PAE: proanthocyanidin-rich extract; PBE: Pinus bark extract; PBMC: peripheral blood mononuclear cells; PMBE: *Pinus massoniana* bark extract; PYC: Pycnogenol; ROS: reactive oxygen species; RPMC: rat peritoneal mast cells; SOD: superoxide dismutase; SPF: specific pathogen free

Section: Drug Discovery and Translational Medicine

Abstract

Proanthocyanidins are among the most abundant constituents in pine bark extracts (PBEs). This review summarizes medical research on PBEs from *Pinus pinaster*, *Pinus radiata*, *Pinus massoniana*, and other less well-characterized species. The precise mechanisms of the important physiological functions of PBE components remain to be elucidated, but there is evidently great potential for the identification and development of novel antioxidant, anti-inflammatory, cardiovascular, neuroprotective, and anti-cancer medicines. Although toxicological data for PBEs are limited, no serious adverse effects have been reported. PBEs may therefore have potential as nutraceuticals and pharmaceuticals and should be safe for use as food ingredients.

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1. Introduction

The bark, needles, and pollen of several species of pine tree have proven to be a useful source of natural products and have been used by mankind for many years. Proanthocyanidins are the main active components in pine bark extracts (PBEs), and are among the most abundant compounds in various pine species. The first use of PBEs dates back to 1535, when the French explorer Jacques Cartier and his crew escaped death by scurvy, a disease caused by a lack of vitamin C, by drinking tea made from the bark of a pine tree. This indicated that one of the active ingredients in PBEs may share a function with, or enhance the function of, vitamin C. Cartier's writing inspired the researcher Jacques Masquelier to identify the active ingredients in PBEs, and he extracted an oligomeric proanthocyanidin from peanut skin, as well as other proanthocyanidins such as Pycnogenol (PYC) from the bark of the European coastal or French maritime pine, *Pinus pinaster*) (Andrea, 2010), which was later patented in the USA.

PBEs were previously considered to be an inconvenient waste product in the timber industry, but are now widely acknowledged as a rich source of natural polyphenols with potential beneficial nutritional, health and medicinal properties (Jerez et al., 2007). PYC, the most widely studied PBE, is now used as a nutritional supplement and phytochemical remedy for various diseases throughout the world, including chronic inflammation, circulatory dysfunction, and asthma (Packer et al., 1999). In addition to PYC, *P. radiata* (Monterey or Insignis pine) and *P. massoniana* bark extracts (PMBE) have also aroused the interest of many researchers. *P. radiata* PBE (trade name Enzogenol) is extracted from 15-30 year old trees, and is formulated with vitamin C. Enzogenol is rich in

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proanthocyanidins, is a richer source of procyanidins than PYC (Jerez et al., 2007), and has antioxidative, anti-inflammatory, anti-cancer, cardioprotective and neuroprotective properties.

P. massoniana Lamb is native to the south and southwest of China, and its bark, needles, pollen, and turpentine have been used in traditional Chinese medicine for the treatment of hemorrhage, rheumatism, arthralgia, inflammation and cancer (Cui et al., 2005[b]). The main bioactive constituents were identified and extracted during the last decade of the 20th century, and the principal ingredient of PMBE is also a procyanidin. PMBE inhibits the migration and growth of cancer cells, and shows similar antioxidant and immunomodulatory properties to PYC and Enzogenol.

In this review, we compiled the findings of recent experimental studies on PBEs from the French maritime pine (trade name PYC: Pycnogenol), *P. radiata* (trade name Enzogenol) and *P. massoniana* (PMBE). All extracts are abundant in proanthocyanidins, and their potential as a source of antioxidant, anti-inflammatory, anti-mutagenic, anti-metastatic, and anti-carcinogenic compounds was assessed, along with their cardioprotective and neuroprotective properties.

2. Production and principal components of PBEs

Methods for preparation of PBEs differ, but all include grinding, washing, and extracting with deionized hot water (95-99°C) for 30 min, removing extracted solids, cooling the raw liquor using a heat exchanger, and concentrating by reverse osmosis to approximately 25% to dissolve solids. Any undisolved solids are continually removed, and the sample is

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freeze-dried, ground, and blended. Quality control is performed to assess purity.

As mentioned previously, PBEs are rich in proanthocyanidins, and more diverse compounds are continually being discovered, such as taxifolin, catechin (Iravani et al., 2011), phenolic acids and stilbenes, protocatechuic acid, and carbohydrates (Frevel et al., 2012). However the primary biologically active components of PYC are the non-conjugated procyanidins B_1 (5-(3', 4'-dihydroxyphenyl)-g-valerolactone) and M1 (formed *in vivo* from catechin polymer by gut microbiota) (Jerez et al., 2007). Interestingly, PYC displays stronger biological activity as a mixture than when separated into its individual components, indicating that the components interact synergistically (Yoshida et al., 2011).

3. Pharmacokinetics of PBEs

PBEs exhibit complex pharmacokinetics and the existing literature is limited. As stated above, proanthocyanidins are the primary active constituents of PBEs, and studies on pharmacokinetics, discussed below, have mainly focused on proanthocyanidins.

3.1. Absorption

The so-called 'rule of five' can be used to describe the physicochemical properties on which the oral bioavailability of molecules with drug-like properties depends (Lipinski et al., 2001). If a compound has less than five hydrogen bond donors, less than 10 hydrogen acceptors, a relative molecular mass below 500, or a log P smaller than five, it is likely to be readily absorbed. All monomeric polyphenols detected in plasmas in the studies discussed in this review comply with this designation (Grimm et al., 2006).

The absorption of proanthocyanidins through the gut barrier is likely to be limited by

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their polymeric nature and high molecular weight (Fernandes et al., 2012); lower molecular weight components and metabolites are more easily absorbed (Fernandes et al., 2012; Kosin´ska and Andlauer, 2012). Most *in vivo* studies suggest that procyanidin dimers can be absorbed (Baba et al., 2002; Prasain et al., 2009; Shoji et al., 2006; Tsang et al., 2005). The gut is the predominant location for absorption, and a large percentage of the parent compound may be transformed by the gut microflora, and the resulting metabolites may constitute the predominant form of absorption (Stoupi et al., 2010).

3.2. Metabolism

More than seven different constituents or metabolites were detected in plasma following oral administration of Pycnogenol, and five (catechin, caffeic acid, ferulic acid, taxifolin and M1 (δ-(3,4-dihydroxy-phenyl)-γ-valerolactone) were derived directly from Pycnogenol. Maximum concentrations (Cmax) were as follows: catechin = 107 ng/ml, caffeic acid = 17 ng/ml, ferulic acid = 15 ng/ml, taxifolin = 33 ng/ml, and the metabolite M1 = 4 ng/ml. The analysis of steady state plasma samples showed that compounds were present as conjugates of sulfate and/or glucuronic acid, indicating phase II metabolism in the liver (Grimm et al., 2006).

3.3. Distribution

Proanthocyanidins undergo structural modifications during phase-II metabolism that may affect protein-binding properties and tissue distribution (Fernández-Murga et al., 2011). With procyanidin-rich simple food matrices, the highest metabolite accumulation was observed in liver, and methyl catechin–glucuronide was the only procyanidin phase-II

metabolite detected in this organ (Serra et al., 2013).

For procyanidin-rich complex food matrices, kidney was the major site of metabolite accumulation (Serra et al., 2013), and glucuronidated and methylated forms of epicatechin and catechin were the predominant phase-II procyanidin metabolites detected in this organ (Baba et al., 2002; Natsume et al., 2003; Tsang et al., 2005). All major procyanidin metabolites detected in plasma were present in lung tissue, which contained the highest concentrations of glucuronide and methyl glucuronide epicatechin metabolites (Serra et al., 2013).

No phase-II procyanidin metabolites were identified in the heart with procyanidin-rich simple or complex food matrices. However after ingestion of a procyanidin-rich complex food matrix, three phenolic acid compounds were detected in heart tissue, with phenylacetic acid predominating. Increased phenolic acids also were also observed in thymus, testicles and spleen (Serra et al., 2013), although the increase was only slight for vanillic acid (Serra et al., 2013).

3.4. Excretion

After intravenous administration of procyanidin B2, 76% was excreted in the urine, reflecting extensive renal clearance (Stoupi et al., 2010). Several procyanidin metabolites were present in the kidney, which further indicated that urine was the main procyanidin excretion pathway in rat (Serra et al., 2011). Furthermore, Düweler and Rohdewald (2000) identified two further metabolites of PYC in the urine of human volunteers.

In male rats, extensive enterohepatic cycling of flavonoids and their conjugated metabolites has been reported (Coldham and Sauer, 2000; Silberberg et al., 2006), and 28%

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of intravenously administered procyanidin B2 was excreted in feces, which is consistent with biliary excretion and enterohepatic recycling (Stoupi et al., 2010).

4. Immunomodulatory effects of PBEs

4.1. Antioxidant function and free radical scavenging

Free radicals are strong oxidizing agents that cause considerable damage to proteins, lipids and DNA that leads to chronic cardiovascular, cerebral, and metabolic diseases, as well as senescence. Oxidative stress occurs when free radical levels exceed the cellular antioxidant capacity (Flohé et al., 1997). PYC, Enzogenol, and PMBE are rich in proanthocyanidins, which are strong quenchers of reactive oxygen species (ROS) and hence potent antioxidants (Afaq and Mukhtar, 2006). These extracts differ in their exact physiological effects, as discussed below.

4.1.1. Oxidative stress-reducing activity

PYC and Enzogenol protect biomacromolecules from oxidative stress. Specifically, PYC inhibits lipid peroxidation, regenerates the ascorbyl radical, and further protects endogenous vitamin E and glutathione from oxidative damage (Grimm et al., 2004). Additional protective effects arise from their ability to inhibit the generation of thiobarbituric acid reactive products, and oxidative hemolysis by peroxide hydrogen (Andrea, 2010).

For defense against photoaging and UV damage, an oral antioxidant treatment would be a highly desirable option. Orally administered vitamins or botanical polyphenols demonstrated UV-protective properties in the skin (Stahl and Sies, 2007), and PYC can help to reduce UV skin damage and may protect against photoaging. A significant reduction in

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age pigment was demonstrated using skin color measurements, and clinical trials demonstrated that PYC protects skin from UV damage (Furumura et al., 2012). The efficacy and safety of these treatments have been validated.

Aside from directly protecting against UV, PYC has been shown to prevent ROS-induced skin damage (Yoshida et al., 2011). Research on hairless mice showed that PYC has the ability to reduce ROS-mediated oxidative stress in maxillofacial region circulation due to its ROS scavenging activity. This indicates that PYC may be useful as a prophylactic antioxidant for the prevention of ROS-induced skin diseases. Furthermore, PYC is also effective in preventing potassium dichromate-induced oxidation-mediated nephrotoxicity, but additional studies are needed to explore the potential of PYC as a nephroprotective agent (Parveen et al., 2009).

Enzogenol has marked superoxide radical scavenging activity *in vitro*, and is a more effective antioxidant than vitamin C, trolox, catechin, and grape seed extracts (Wood et al., 2002). Enzogenol in combination with vitamin C can significantly reduce the oxidation of proteins and DNA (Senthilmohan et al., 2003), and a clinical trial of this combination successfully reduced systemic plasma protein oxidation to significantly lower levels than vitamin C alone (Young et al., 2006).

4.1.2. Influence on oxidative enzymes

Recent studies suggested that the antioxidant properties of PMBEs may be associated with effects on antioxidant enzymes. PMBE protects normal human liver L-02 cells from hydrogen peroxide-induced damage. Malondialdehyde (MDA) is an indicator of lipid peroxidation, and levels of glutathione peroxidase (GSH-Px), glutathione (GSH), catalase

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(CAT), and superoxide dismutase (SOD) reflect the degree of cell oxidative damage. Hydrogen peroxide directly causes permanently damage to cells, and also increases MDA and decreases GSH and CAT. PMBE treatment decreases MDA and increases GSH, CAT, GSH-Px, and SOD, and alleviates damage induced by carbon tetrachloride (Wang et al., 2010). Despite the different physiological properties of the various bark extracts, they are likely to exert their effects via similar molecular mechanisms.

4.2. Anti-inflammatory activity

Park *et al*. (2011) evaluated the immunomodulatory effects of proanthocyanidin-rich *P. radiata* extracts (PAEs) in specific pathogen free (SPF) white leghorn chickens. PAEs enhanced the proliferation of immune cells such as peripheral blood mononuclear cells (PBMC), splenocytes and bursal cells. PAEs also induced production of the Th1 cytokine IFN-γ, suppressed the production of the Th2 cytokine IL-6, and increased IL-18 mRNA (Park et al., 2011). Other researchers have reported on the anti-inflammatory properties of PAEs (Torras et al., 2005).

PYC possesses anti-inflammatory properties, and inhibits anti-dinitrophenyl (DNP) IgE-mediated passive cutaneous anaphylaxis in rats when administered orally. *In vitro*, PYC reduces histamine release from rat peritoneal mast cells (RPMC) triggered by anti-DNP IgE in a dose-dependent manner, and inhibits protein expression and secretion of tumor necrosis factor (TNF)-α and IL-6. Moreover, PYC decreases anti-DNP IgE-induced calcium uptake in RPMCs, and suppresses the activation of nuclear factor-kappa B (NF-kB) (Choi and Yan, 2009), a transcription factor controlling the expression of genes involved in inflammation. Together, these results have inspired the clinical use of PYC in mast

cell-mediated immediate-type allergic diseases.

PYC treatment simultaneously downregulates cyclooxygenase (COX)-2 and 5-lipoxygenase (LOX) gene expression, and reduces leukotriene biosynthesis in human polymorphonuclear leukocytes upon pro-inflammatory stimulation *ex vivo* (Choi and Yan, 2009). Furthermore, phospholipase A2 activity is also inhibited. Downregulation of COX-2 is partially compensated by upregulation of COX-1. Downregulation of 5-LOX consequently decreases leukotriene levels in asthmatic patients (Canali et al., 2009), and the anti-inflammatory activity of PYC may be best utilized in combination with inhalation corticosteroids (ICS), enabling reduced dosage and frequency of ICS administration, and potentially reducing the need for other asthma medications (Belcaro et al., 2011). Anti-inflammatory properties include alleviating pain and stiffness in arthritis and osteoarthritis patients (Belcaro et al., 2008[a]), and PYC is a promising anti-arthritic drug candidate.

PMBE can be used to treat autoimmune diseases since it can block inflammation while normal human horn (HaCaT) cells remain unaffected during short treatment periods. For example, when the action time is less than 12 h, PMBE has little effect on HaCaT cells at all concentrations of PMBE tested. However during prolonged action time, PMBE can induce apoptosis in a dose-dependent manner. PMBE does not induce cell cycle arrest in HaCaT cells, since the number of surviving cells in every phase decreased after treatment. PMBE is therefore alleged to involve less side reactions if the action time is carefully controlled (Wu et al., 2008).

In conclusion, PBEs have strong anti-inflammatory properties and immunomodulatory

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effects, and are promising candidates for treating type I allergies, asthma and arthritis. Although a number of animal trials have been reported, more clinical trials are needed.

4.3. Anti-infection activity

PBEs also possess anti-infection properties. Epstein-Barr virus (EBV) is associated with numerous diseases including nasopharyngeal carcinoma, and this pathogen undergoes two distinct life cycle stages: the lytic cycle, and the lytic productive cycle (Xu et al., 2012). The EBV immediate early genes Zta and Rta have an important function in the lytic cycle, and EA-D expression is also involved. PMBE inhibits the expression of Zta and Rta, which results in the failure to transcribe the EA-D gene. PMBE also inhibits transfection of plasmid pRluc (BRLF1 promoter) and pZluc (BZLF1 promoter), suggesting PMBE simultaneously inhibits transcription of immediate early genes and the lytic cycle. PMBE acts on EBV in a dose-dependent manner, and inhibiting EBV entry into the lytic cycle requires a low concentration, while completely blocking entry needs a higher concentration (Xu et al., 2012).

PYC inhibits the binding of human immunodeficiency virus type-1 (HIV-1) to host cells, and also inhibits viral replication and T-cell interaction in cell culture experiments (Feng et al., 2008). This activity may be due to up-regulation of the intracellular antioxidant protein Mn-SOD, and/or inhibition of phosphorylation of the ribosomal S6 protein. Additionally, the ectopic expression of Mn-SOD also inhibits HIV-1 replication, providing another route by which PYC acts as an anti-HIV-1 agent (Feng et al., 2008). PYC also inhibits the growth of *Helicobacter pylori*, albeit to a limited extent, and perturbs also their adherence to gastric cells (Rohdewald and Beil, 2008). Enzogenol has also been shown to be an effective

antibacterial and antiviral agent.

4.4. Anti-cancer activity

Proanthocyanidins may regulate carcinogenesis and the development and migration of tumors, and also possess antimutation properties (Senthilmohan et al., 2003). The anti-cancer activity of PMBE has received the most attention.

4.4.1. Selective induction of apoptosis

PMBE induces apoptosis in human cervical cancer HeLa cells, human hepatoma $HepG_2$ cells (Ma et al., 2010), and murine sarcoma S180 cells (Zhang et al., 2012) in a dose-dependent manner, but has very little apoptosis-inducing activity in normal cells (Ma et al., 2010), making PMBE a potential anti-cancer therapeutic lead. PMBE induces apoptosis via the death receptor and mitochondrial pathways (Fig. 1). It is not known whether PMBE acts via the endoplasmic reticulum pathway. PMBE treatment up-regulates Bax expression in HeLa cells but not in $HepG_2$ cells (Ma et al., 2010), and further studies are needed to understand the mechanisms involved.

4.4.2. Promotion of cell cycle arrest

PMBE treatment affects cell cycle distribution in four types of human cancer cells: it induces cell cycle arrest at S and G_2/M in a dose-dependent manner, and increased treatment time increased the proportion of HeLa cells in G_2/M, but the opposite was true in $HepG_2$ cells (Ma et al., 2010). PMBE also arrests the cell cycle of BEL-7402 (Cui et al., 2005[a]) and LoVo cells (Cui et al., 2007) at G1 and S phase, respectively, resulting in apoptosis. These properties are also dose- and time-dependent, and tissue specificity may explain the different effects on different cell types.

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4.4.3. Inhibition of HeLa cell migration

PMBE has no significant effect on the adhesion capability of HeLa cells. However, the scratch migration assay indicated a strong inhibition of the migration capability of HeLa cells, and the molecular mechanism awaits further investigation (Wu et al., 2011).

4.4.4. Immune enhancement

PMBE also induces peripheral leukocyte and lymphocyte proliferation, and may inhibit the growth of cancer cells via activation of the immune system (Zhang et al., 2012). In experiments on tumor-bearing mice, PMBE (at a concentration above 200 mg/kg) raises the thymus index and spleen index, which reflects the immunological function of mice (Zheng et al., 2007).

4.4.5. Reduction of the side-effects of radiotherapy and chemotherapy

The ability of PYC to markedly reduce side-effects in patients receiving radiotherapy and chemotherapy has received widespread attention. A semi-quantitative evaluation showed that symptom severity was less than half that of control groups. Furthermore, symptoms such as cognitive impairment, cardiotoxicity and neutropenia were improved. The most apparent improvements in the acute side-effects in radiotherapy patients were decreased soreness and ulceration in the mouth and throat, and mitigation of dryness in the mouth and eyes. For chemotherapy patients, nausea, vomiting, diarrhea, and weight loss were all alleviated. PYC also effectively reduces the side-effects of drugs through its endothelial protective, anti-inflammatory and anti-edema activities (Belcaro et al., 2008[b]).

4.4.6. Other anti-cancer activities

Enzogenol shares similar physiological properties with PMBE, and studies on the

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potential anti-cancer effects of Enzogenol are few. ROS scavenging and protecting DNA from damage by peroxides are possible mechanisms underlying the anti-cancer properties of PBE. Finally, PYC appears to protect skin against squamous cell carcinomas (Pavlou et al., 2009), but the mechanisms are unknown at present.

5. Organ protective effects of PBE

5.1. Cardiovascular protection

Proanthocyanidins are flavonoids which are known to relax vascular smooth muscle to help tissues absorb nutrients and excrete metabolites. PYC can noticeably reduce many of the risk factors associated with cardiovascular disease (CVD), such as systolic blood pressure, glycosylated hemoglobin, hyperlipidemia and platelet aggregation (Parveen et al., 2009). PYC also improves endothelial function in patients with CAD by reducing oxidative stress (Enseleit et al., 2012). Enzogenol has been shown to block early atherogenesis, and may therefore be useful for treating atherosclerosis (Kim et al., 2010). In combination with vitamin C, Enzogenol shows potential benefits for endothelial function, systolic blood pressure maintenance, plasma protein carbonyl balance, and leukocyte DNA protection (Abd El Mohsen et al., 2002; Young et al., 2006).

Hypertension is an important factor of CVD that has received considerable attention, and treatments for hypertension have been developed (Fitzpatrick et al., 1998). PYC possesses a potent endothelium-dependent vasorelaxant activity on rat aortic rings *in vitro*, and vasorelaxation is suppressed by pretreatment with a non-selective NO synthase (NOS) inhibitor (Fitzpatrick et al., 1998), suggesting that PYC-induced endothelium-dependent

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vasorelaxation is associated with NOS. Indeed, a report in 2009 demonstrated that PYC-induced endothelium-dependent vasorelaxation was mediated by the eNOS-NO-soluble guanylate cyclase signaling system (Kwak et al., 2009). Other studies involving hypertensive patients showed that dietary PYC supplementation decreases plasma endothelin-1 concentrations, whereas NO metabolite levels in plasma tend to increase, suggesting PYC has beneficial effects on endothelial function in hypertensive patients (Liu et al., 2004). The antihypertensive effects of PYC are therefore attributed to both antioxidant-mediated protective effects against endothelial dysfunction, and to endothelium-dependent vasorelaxation mediated by endothelial NOS activation (Kwak et al., 2009). Additionally, at levels of 100 mg/day, PYC reduced the dosage of the calcium channel blocker nifedipine required to maintain normal blood pressure levels (Liu et al., 2004).

Patients with type 2 diabetes mellitus (DM) are at considerable risk of excessive morbidity and mortality from CVD (Parveen et al., 2010). PYC exhibits antidiabetic effects in a type 2 DM rat model by stimulating the antioxidant defense system (Zibadi et al., 2008). Other research showed that PYC improves diabetes control, lowers CVD risk factors, and reduces the required dosage of antihypertensive medicines (Parveen et al., 2010).

Research on a type 1 diabetes model involving PYC administered alone or in combination with an anti-platelet therapy showed that PYC lowers platelet hyperactivity and exerts antithrombotic effects. However, the anti-thrombotic effects of long-term PYC treatment in diabetes patients is associated with enhanced thromboxane synthesis, which may lead to severe vascular complications (Nocun et al., 2008).

PYC has also been shown to be useful for treating and controlling chronic venous

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insufficiency (CVI), and may be combined with compression stockings to good effect; combined PYC and compression decreased ankle swelling, resting flux, transcutaneous oxygen pressure, and other clinical symptoms (Cesarone et al., 2010). Clinical studies have shown that PYC can reduce leg edema in CVI, reduce the incidence of deep vein thrombosis during long-haul flights, and enhance the healing of venous ulcers and hemorrhoidal episodes by topical application and/or oral administration (Gulati, 2014).

Although PYC is essentially a dietary supplement and not a replacement for pharmaceutical treatments, it is cost-effective and efficient at negating many of the risks associated with CVD, especially when applied in early heart disease patients prior to drug therapy. Additive or substitutive use of PYC may help to reduce the dosage and cost of existing treatments, and may improve their effectiveness (Chowdhury et al., 2011).

5.2. Neuroprotective effects

Free radicals may be involved in the pathogenesis of particular brain disorders. In an aging brain, oxidative damage can be observed, and antioxidants may protect the brain from ROS damage. In one study, PBE in combination with vitamin C showed potential as a migraine treatment (Chayasirisobhon, 2013). Enzogenol displays a positive influence on cognitive performance, specifically on working memory, a type of short-term memory. In combination with vitamin C, Enzogenol improved the response speed of spatial working memory and immediate recognition tasks (Pipingas et al., 2008). In patients with 3–12 months post-mild traumatic brain injury, self-perceived cognitive failures were reduced (Theadom et al., 2013). Similarly, PYC prevents the accumulation of oxidatively damaged proteins, and protects nerve cells against β-amyloid in a glutamate-induced toxicity model

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(Gulati, 2005), suggesting it may reduce the risks of neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's disease (Voss et al., 2006).

The positive effects of Enzogenol on cognitive performance may operate through antioxidant action, improvements of cerebral blood supply and circulation, influences on neuronal signal transduction, and modification of cell contents following neurological signaling (Abd El Mohsen et al., 2002).

The brain is incredibly complex, and the exact mechanisms of neuroprotection are yet to be determined, but PBEs show promise as potential drugs for treating neurodegenerative diseases.

5.3. Endocrine and metabolic effects

5.3.1. Improvement in perimenopausal symptoms

Menopausal women receiving a low daily dose of PYC for a month showed significant improvement in the symptoms of menopausal syndrome, especially vasomotor and insomnia/sleep problems (Kohama and Negami, 2012). These results indicated that PYC is particularly correlated with climacteric symptoms. However a placebo also had a degree of effect, which is a reminder of the importance of psychological factors (Kohama and Negami, 2012).

5.3.2. Suppression of excessive lipid accumulation

Fatty liver formation is markedly reduced in mice with adipose differentiation-related protein (ADRP) expression abrogated by gene knockout or antisense oligonucleotides (Imai et al., 2007). ADRP abrogation also prevents atherosclerosis development (Paul et al., 2008) and diet-induced insulin resistance (Varela et al., 2008). These findings strongly implicate

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ADRP as a promising target for preventing excessive lipid accumulation, which can induce the production of oleic acid (OA) if intracellular. PYC has been shown to reduce OA-induced ADRP expression and to suppress the formation of lipid droplets (Fan et al., 2009). Fan and co-workers (2009) demonstrated that PYC achieved these effects by enhancing ADRP mRNA degradation. The intracellular lipid accumulation suppressing activity of PYC indicates a potential use in treating fatty liver disease (Fan et al., 2009).

Lipopolysaccharide (LPS) upregulates ADRP transcription by first inducing the expression of interleukin (IL)-6, IL-1α and interferon (IFN)-β that subsequently stimulate ADRP expression (Gu et al., 2008). PYC suppresses the expression of ADRP and the aforementioned cytokines, and may therefore be useful for the prevention of atherosclerosis.

5.4. Properties of other pine extracts

Extracts from numerous other types of pine trees have been shown to have beneficial effects, and bark extracts are a particularly rich source of physiologically and potentially pharmacologically active compounds (Table 1).

6. Toxicological effects

PBE is generally regarded as safe by independent toxicology experts based on the results of 70 clinical trials that include healthy subjects and patients with particular disease histories (n = 5723). The frequency rate of adverse effects which include gastrointestinal discomfort, dizziness, headache and nausea is 2.4% and 0.19% in patients and healthy subjects, respectively. No alarming side effects, even at high dosages, have been reported to date. Therefore PBE at a dosage of 20–100 mg per day for long periods (months) and

100–300 mg for shorter periods are considered nontoxic (Heather, 2010).

With Enzogenol, adverse events including emesis and diarrhea occurred in un-fed dogs given the highest doses (Frevel et al., 2012). However in human trials no safety issues have arisen (Frevel et al., 2012; Theadom et al., 2013; Drieling et al., 2010).

7. Human exposure

Enzogenol, derived from the bark of *Pinus radiata*, is a commercially available proanthocyanidin-rich flavonoid extract used widely in functional foods. Its recommended dosage ranges from 480-960 mg/day, and treatment durations have ranged from five weeks to 6=six months in human clinical trials. A typical study on protein oxidation and DNA damage in older human subjects involved a dosage of 480 mg/day and treatment duration of 12 weeks (Senthilmohan et al., 2003).

Pycnogenol is another common functional food ingredient that has been used for a long time. A typical dosage of 150 mg/day for six months can improve health risk factors in subjects with metabolic syndrome and may also be effective in changing some behavioral patterns that contribute to persistent undesirable traits in patients with metabolic syndrome (Belcaro et al., 2013). Mood and attention changed favorably upon Pycnogenol treatment in a study involving student volunteers (Luzzi et al., 2011). Based on data from recent reports, and given the safe dosage range discussed above, we speculate that a dosage from 100 to 200 mg/day could be safely recommended.

Most reports on PMBE have focused at the cellular level, and clinic data for recommending appropriate dosage is lacking. Functional foods containing PMBE are

relatively few in number, but SongZhen capsules (Zhongjianxing Group Co., China) contain >5g/100g of procyanidins and 400-600 mg of flavone.

8. Summary

To date, PYC has received more attention than other PBEs, and many beneficial effects have been reported (as discussed above). PYC is already commercially available as a herbal dietary supplement that may be taken to ameliorate various degenerative disorders (Rohdewald, 2002) and is a promising candidate for treating endothelial dysfunction and as a prophylactic for protecting against vascular diseases (Gulati, 2005). PYC also has beneficial effects on chronic disorders, but further investigations are required before it can be recommended for this use (Sivoňová et al., 2004). As a dietary supplement or health care product, PYC decreases the risk of many common diseases and/or reduces the required dosage of many conventional drugs. However, for more extensive pharmaceutical use of PYC, full clinical trials would be needed. The biologically active components of PYC are polyphenolic compounds, and other PBEs containing high levels of polyphenolics that are potential alternatives to PYC in dietary supplements, pharmaceuticals, cosmetic products, and foods/beverages (Iravani et al., 2011).

PBEs have undergone numerous animal experiments and clinic trials, and almost all reports found them to be safe and well-tolerated with few side-effects. Frevel *et al*. (2012) found that Enzogenol exhibited no adverse effects on liver or kidney function in animals and humans (Frevel et al., 2012), confirming the safety of this extract as a food supplement.

PMBE reportedly inhibits HeLa cell migration, but the underlying mechanism is currently

unknown (Wu et al., 2011). Although PMBE has not been thoroughly investigated, it is a potential chemotherapy drug that can effectively inhibit the growth of cancer cells and protect normal cells. Chemotherapeutic agents with fewer side-effects are highly desirable. The time- and dose-dependent action of PMBE results in a failure to completely kill cancer cells, therefore PMBE must be used in combination with other chemotherapies. The mechanism of the actions of PMBE on cancer cells has not been completely elucidated, and studying this mechanism may reveal valuable information on the potential uses of traditional Chinese medicines in combination with modern Western medicines for chemotherapy.

In summary, PBEs are rich in proanthocyanidins, and include Pycnogenol, Enzogenol, and PMBE. These extracts have been widely tested in animal and clinical studies as dietary supplements due to their potent antioxidant, anti-inflammatory, anti-cancer, cardio- and neuroprotective activities. They are safe and exhibit few undesirable side-effects. Following suitable clinical trials, PBEs may be used in functional foods or pharmaceuticals to treat numerous diseases. The efficacy of PBEs may be improved by addition of supplementary ingredients, determining optimum dosage, or changing the administration route. Finally, the molecular mechanisms of the various biological activities of PBEs are yet to be determined, and should be studied if the true medicinal potential of these extracts is to be realized.

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Authorship Contributions

Participated in research design: YY Cui, YY Li, J Feng and XL Zhang

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References

Abd E. Mohsen, M.M., Kuhnle, G., Rechner, A.R., Schroeter, H., Rose, S., Jenner, P., Rice-Evans, C.A. (2002) Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. *Free Radical Bio Med* **33**:1693-1702

Afaq, F., Mukhtar, H. (2006) Botanical antioxidants in the prevention of photocarcinogenesis and photoaging. *Exp Dermatol* **15**: 678-684

Andrea, G.D. (2010) Pycnogenol: A blend of procyanidins with multifaceted therapeutic applications? *Fitoterapia* **81**:724-736

Apetrei, C.L., Tuchilus, C., Aprotosoaie, A.C., Oprea, A., Malterud, K.E., Miron, A.(2011) Chemical, antioxidant and antimicrobial investigations of Pinus cembra L. bark and needles. *Molecules* **16**:7773-7788

Baba, S., Osakabe, N., Natsume, M., Terao, J. (2002) Absorption and urinary excretion of procyanidin B2 [epicatechin-(4β-8)-epicatechin] in rats. *Free Radic Biol Med* **33**:142-148

Belcaro, G. , Cornelli, U., Luzzi, R., Cesarone, M. R., Dugall, M., Feragalli, B., Errichi, S., Ippolito, E., Grossi, M. G., Hosoi, M., Cornelli, M., Gizzi, G. (2013) Pycnogenol® Supplementation Improves Health Risk Factors in Subjects with Metabolic Syndrome. *Phytother Res* **27**:1572-1578

Belcaro, G., Cesarone, M.R., Errichi, S., Zulli, C., Errichi, B.M., Vinciguerra, G., Ledda, A., Di Renzo, A., Stuard, S., Dugall, M., Pellegrini, L., Errichi, S., Gizzi, G., Ippolito, E., Ricci, A., Cacchio, M., Cipollone, G., Ruffini, I., Fano, F., Hosoi, M., Rohdewald, P. (2008[a]) Treatment of osteoarthritis with Pycnogenol®. The SVOS (San Valentino

osteo-arthrosis study). Evaluation of signs, symptoms, physical performance and vascular aspects. *Phytother Res* **22**:518-523

Belcaro, G., Cesarone, M.R., Genovesi, D., Ledda, A., Vinciguerra, G., Ricci, A., Pellegrini, L., Gizzi, G., Ippolito, E., Dugall, M., Cacchio, M., Di Renzo, A., Stuard, S. (2008[b]) Pycnogenol may alleviate adverse effects in oncologic treatment. *Panminerva Med* **50**:227-234

Belcaro, G., Luzzi, R., Cesinaro, D.R.P., Cesarone, M.R., Dugall, M., Feragalli, B., Errichi, B.M., Ippolito, E., Grossi, M.G., Hosoi, M., Errichi, S., Cornelli, U., Ledda, A., Gizzi, G. (2011) Pycnogenol® improvements in asthma management. *Panminerva Med* **53**:57-64

Canali, R., Comitato, R., Schonlau, F., Virgili, F. (2009) The anti-inflammatory pharmacology of Pycnogenol® in humans involves COX-2 and 5-LOX mRNA expression in leukocytes. *Int Immunopharmacol* **9**:1145-1149

Cesarone, M.R., Belcaro, G., Rohdewald, P., Pellegrini, L., Ledda, A., Vinciguerra, G., Ricci, A., Ippolito, E., Fano, F., Dugall, M., Cacchio, M., Di Renzo, A., Hosoi, M., Stuard, S., Corsi, M. (2010) Improvement of signs and symptoms of chronic venous insufficiency and microangiopathy with Pycnogenol®: A prospective, controlled study. *Phytomedicine* **17**:835-839

Chayasirisobhon, S. (2013) Efficacy of Pinus Radiata Bark Extract and Vitamin C Combination Product as a Prophylactic Therapy for Recalcitrant Migraine and Long-term Results. *Acta Neurol Taiwan* **22**:13-21

Choi, Y.H., Yan, G.H. (2009) Pycnogenol® inhibits immunoglobulin E-mediated allergic

response in mast cells. *Phytother Res* **23**:1691-1695

Chowdhury, Z.T., Schonlau, F., Zibadi, S., Watson, R.R.(2011) Pycnogenol Supplementation and Cardiovascular Health: Treatment and Cost-Benefit Analysis, Nutrients, in *Dietary Supplements, and Nutriceuticals :Cost Analysis Versus Clinical Benefits* (Watson, R. R., Gerald, J. K. , Preedy, V. R. Eds) pp. 467- 475, Humana Press, London

Coldham N.G., Sauer M.J. (2000) Pharmacokinetics of [(14)C]Genistein in the rat: gender-related differences, potential mechanisms of biological action, and implications for human health. *Toxicol Appl Pharmacol.* **164**:206-215

Cui, Y.Y., Wang, H.B., Xie, H., Wu, C.L., Peng, Q.Q., Zheng, G.Y., Wang, J.F. (2007) Primary exploration of mechanism of Pinus massoniana bark extract inhibiting growth of human colorectal carcinoma cells in vitro. *J Chin Agric Univ* **12**:7-14 (in Chinese)

Cui, Y.Y., Xie, H., Qi, K.B., He, Y.M., Wang, J.F.(2005[a]) Effects of Pinus massoniana bark extract on cell proliferation and apoptosis of human hepatoma BEL-7402 cells. *World J Gastroenterol* **11**:5277-5282

Cui, Y.Y., Xie, H., Wang, J.F.(2005[b]) Potential Biomedical Properties of Pinus massoniana Bark extract. *Phytother Res* **19**:34-38

Drieling, R.L., Gardner, C.D., Ma, J., Ahn, D.K., Stafford, R.S.(2010) No Beneficial Effects of Pine Bark Extract on Cardiovascular Disease Risk Factors. *Arch Intern Med* **170**:1541-1547

Drozd, N.N., Kuznetsova, S.A., Lapikova, E.S., Davydova, A.I., Makarov, V.A., Kuznetsov, B.N., Butylkina, A.I., Vasil'eva, NIu., Skvortsova, G.P.(2008) Anticoagulant activity of arabinogalactane sulfate and cedar bark extract studied in vitro. *Eksperimental'naia i*

klinicheskaia farmakologiia **71**:30-34

Düweler, K.G., Rohdewald, P.(2000) Urinary metabolites of French maritime pine bark extract in humans. *Pharmazie* **55**:364-368

Enseleit, F., Sudano, I., Périat, D., Winnik, S., Wolfrum, M., Flammer, A.J., Fröhlich, G.M., Kaiser, P., Hirt, A., Haile, S.R., Krasniqi, N., Matter, C.M., Uhlenhut, K., Högger, P., Neidhart, M., Lüscher, T.F., Ruschitzka, F., Noll, G.(2012) Effects of Pycnogenol on endothelial function in patients with stable coronary artery disease: a double-blind, randomized, placebo-controlled, cross-over study. *Eur Heart J* **33**:1589-1597

Fan, B., Ikuyama, S., Gu, J. Q., Wei, P., Oyama, J., Inoguchi, T., Nishimura, J. (2009) Oleic acid-induced ADRP expression requires both AP-1 and PPAR response elements, and is reduced by Pycnogenol through mRNA degradation in NMuLi liver cells. *Am J Physiol-Endoc M* **297**:E112-E123

Feng, W.Y., Tanaka, R., Inagaki, Y., Saitoh, Y., Chang, M.O., Amet, T., Yamamoto, N., Yamaoka, S., Yoshinaka, Y. (2008) Pycnogenol, a procyanidin-rich extract from French maritime pine, inhibits intracellular replication of HIV-1 as well as its binding to host cells. *Jpn J Infect Dis* **61**: 279-285

Fernandes, I., Nave, F., Gonçalves, R., De Freitas, V., Mateus, N.(2012) On the bioavailability of flavanols and anthocyanins: Flavanol–anthocyanin dimers. *Food Chem* **135**: 812- 818

Fernández-Murga, L., Tarín, J.J., García-Perez, M.A., Cano, A.(2011) The impact of chocolate on cardiovascular health. *Maturitas* **69**:312-321

Fitzpatrick, D.F., Bing, B., Rohdewald, P. (1998) Endothelium-dependent, vascular effects of

Pycnogenol. *Cardiovasc Pharm* **32**:509-515

Flohé, L., Brigelius-Flohé, R., Saliou, C., Traber, M.G., Packer, L. (1997) Redox regulation of kappa B activation. *Free Radical Bio Med* **22**:1115-1126

Frevel, M.A., Pipingas, A., Grigsby, W.J., Frampton, C.M., Gilchrist, N.L. (2012) Production, composition and toxicology studies of Enzogenol Pinus radiata bark extract. *Food Chem Toxicol* **50**: 4316- 4324

Fuentes, J.L., Vernhe, M., Cuetara, E.B., Sánchez-Lamar, A., Santana, J.L., Llagostera, M. (2006) Tannins from barks of Pinus caribaea protect Escherichia coli cells against DNA damage induced by γ-rays. *Fitoterapia* **77**:116 -120

Furumura, M., Sato, N., Kusaba, N., Takagaki, K., Nakayama, J.(2012) Oral administration of French maritime pine bark extract (Flavangenol®) improves clinical symptoms in photoaged facial skin. *Clin Interv Aging* **7**:275-286

Grimm, T., Skrabala, R., Chovanová, Z., Muchová, J., Sumegová, K., Liptáková, A., Duracková, Z., Högger, P. (2006) Single and multiple dose pharmacokinetics of maritime pine bark extract (pycnogenol) after oral administration to healthy volunteers. *BMC Clin Pharmacol* doi:10.1186/1472-6904-6-4

Grimm, T.,. Schäfer, A, Högger, P. (2004) Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (pycnogenol). *Free Radical Biol Med* **36**:811-822

Gu, J.Q., Ikuyama, S., Wei, P., Fan, B., Oyama, J., Inoguchi, T., Nishimura, J.(2008) Pycnogenol, an extract from French maritime pine, suppresses Toll-like receptor 4-mediated expression of adipose differentiation-related protein in macrophages. *Am J*

Physiol-Endoc M **295**: E1390-E1400

Gulati, O. P. (2005) The nutraceutical Pycnogenol: its role in cardiovascular health and blood glucose control. *Biomed Rev* **16**:49-57

Gulati, O.P.(2014) Pycnogenol® in Chronic Venous Insufficiency and Related Venous Disorders. *Phytother Res* **28**:348-362

Heather, O. (2010) Scientific and Clinical Monograph for PYCNOGENOL. American Botanical Council, Austin, Tex, USA (See:http://abc.herbalgram.org/site/DocServer/Pycnog_FullMono120809_LOW.pdf?docID=1741, July 14, 2014.)

Hsu, T.Y., Sheu, S.C., Liaw, E.T., Wang, T.C., Lin, C.C.(2005) Anti-oxidant activity and effect of Pinus morrisonicola Hay. on the survival of leukemia cell line U937. *Phytomedicine***12**:663- 669

Imai, Y., Varela, G. M., Jackson, M. B., Graham, M.J., Crooke, R.M., Ahima, R.S.(2007) Reduction of hepatosteatosis and lipid levels by an adipose differentiation-related protein antisense oligonucleotide. *Gastroenterology* **132**:1947-1954

Ince, I., Yesil-Celiktas, O.,Karabay-Yavasoglu, N.U., Elgin, G.(2009) Effects of Pinus brutia bark extract and Pycnogenol in a rat model of carrageenan induced inflammation. *Phytomedicine* **16**:1101-1104

Iravani, S., Zolfaghari, B. (2011) Pharmaceutical and nutraceutical effects of Pinus pinaster bark extract. *Res Pharm Sci* **6**:1-11

Jerez, M., Selga, A., Sineiro J., Torres, J.L., Núñez, M.J. (2007) A comparison between bark extracts from Pinus pinaster and Pinus radiata: Antioxidant activity and procyanidin

composition. *Food Chem* **100**:439- 444

Kaushik, D., Kumar, A., Kaushik, P., Rana, A.C.(2012[a]) Analgesic and Anti-Inflammatory Activity of Pinus roxburghii Sarg. *Adv Pharmacol Sci* doi: 10.1155/2012/245431

Kaushik, D., Kumar, A., Kaushik, P., Rana, A.C.(2012[b]) Anticonvulsant activity of alcoholic extract of bark of Pinus roxburghii Sarg. *Chin J Integr Med* **10**:1056-1060

Kim, D.S., Kim, M.S., Kang, S.W., Sung, H.Y., Kang, Y.H. (2010) Pine bark extract Enzogenol attenuated tumor necrosis factor-α-induced endothelial cell adhesion and monocyte transmigration. *J Agric Food Chem* **58**:7088-7095

Kohama, T., Negami, M.(2012) Effect of low-dose French maritime pine bark extract on climacteric syndrome in 170 perimenopausal women: a randomized, double-blind, placebo-controlled trial. *J Reprod Med* **58**:39- 46

Kosin´ska, A., Andlauer, W.(2012) Cocoa polyphenols are absorbed in Caco-2 cell model of intestinal epithelium. *Food Chem* **135**: 999-1005

Kwak, C.J., Kubo, E., Fujii, K., Nishimura, Y., Kobuchi, S., Ohkita, M., Yoshimura, M., Kiso, Y., Matsumura, Y.(2009) Antihypertensive effect of French maritime pine bark extract (Flavangenol): possible involvement of endothelial nitric, oxide-dependent vasorelaxation. *Hypertension* **27**:92-101

Li, K., Li, Q., Li, J., Zhang, T., Han, Z., Gao, D., Zheng, F.(2007) Antitumor activity of the procyanidins from Pinus koraiensis bark on mice bearing U14 cervical cancer. *J Yakugaku Zasshi* **127**:1145-1151

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J.(2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and

development settings. *Adv Drug Deliv Rev* **46**:3-26

Liu, X.M., Wei, J.P., Tan, F.S., Zhou, S.M., Würthwein, G., Rohdewald, P.(2004) Pycnogenol®, a French maritime pine bark extract, improves endothelial function of hypertensive patients. *Life Sci* **74**:855-862

Luzzi R, Belcaro G, Zulli C, Cesarone, M.R., Cornelli, U., Dugall, M., Hosoi, M., Feragalli, B.(2011) Pycnogenol® supplementation improves cognitive function, attention and mental performance in students. *Panminerva Med* **53**:75-82

Ma, H.L., Liu, B., Feng, D.R., Xie, H., Li, R.D., Yuchi, Y.X., Wang, H., Wang, J.(2010) Pinus massoniana bark extract selectively induces apoptosis in human hepatoma cells, possibly through caspase-dependent pathways. *Int J Mol Med* **25**: 751-759

Natsume, M., Osakabe, N., Oyama, M., Sasaki, M., Baba, S., Nakamura, Y., Osawa, T., Terao, J. (2003) Structures of (−)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (−)-epicatechin: differences between human and rat. *Free Radic Biol Med* **34**:840-849

Nocun, M., Ulicna, O., Muchova, J., Durackova, Z., Watala, C.(2008) French maritime pine bark extract (Pycnogenol®) reduces thromboxane generation in blood from diabetic male rats. *Biomed Pharmacother* **62**:168-172

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

Park, I.J., Cha, S.Y., Kang, M., So, Y.S., Go, H.G., Mun, S.P., Ryu, K.S., Jang, H.K.(2011) Effect of proanthocyanidin-rich extract from Pinus radiata bark on immune response of

specific-pathogen-free White Leghorn chickens. *Poultry Sci* **90**:977- 982

Parveen, K., Khan, M.R., Mujeeb, M., Siddiqui, W.A. (2010) Protective effects of Pycnogenol® on hyperglycemia-induced oxidative damage in the liver of type 2 diabetic rat. *Chem-biol Interact* **186**:219-227

Parveen, K., Khan, M.R., Siddiqui, W.A. (2009) Pycnogenol® prevents potassium dichromate (K2Cr2O7)-induced oxidative damage and nephrotoxicity in rats. *Chem-Bio Interact* **181**:343-350

Paul, A., Chang, B.H., Li, L., Yechoor, V.K., Chan, L.(2008) Deficiency of adipose differentiation-related protein impairs foam cell formation and protects against atherosclerosis. *Circ Res* **102**:1492-1501

Pavlou, P., Rallis, M., Deliconstantinos, G., Papaioannou, G., Grando, S.A.(2009) In-vivo data on the influence of tobacco smoke and UV light on murine skin. *Toxicol Ind Health* **25**:231-239

Pipingas, A., Silberstein, R.B., Vitetta, L., Rooy, C.V., Harris, E.V., Young, J.M., Frampton, C.M., Sali, A., Nastasi, J. (2008) Improved cognitive performance after dietary supplementation with a Pinus radiata bark extract formulation. *Phytother Res* **22**:1168-1174

Prasain, J.K., Peng, N., Dai, Y., Moore, R., Arabshahi, A., Wilson, L., Barnes, S., Wyss, J.M., Kim, H., Watts, R.L. (2009). Liquid chromatography tandem mass spectrometry identification of proanthocyanidins in rat plasma after oral administration of grape seed extract. *Phytomedicine* **16**: 233-243

Rohdewald, P. (2002) A review of the French maritime pine bark extract (Pycnogenol), a

herbal medication with a diverse clinical pharmacology. *Int J Clin Pharmacol Ther* **40**:158-168

Rohdewald, P., Beil, W.(2008) In vitro inhibition of Helicobacter pylori growth and adherence to gastric mucosal cells by Pycnogenol® . *Phytother Res* **22**:685-688

Senthilmohan, S.T., Zhang, J., Stanley, R.A. (2003) Effects of flavonoid extract Enzogenol with vitamin C on protein oxidation and DNA damage in older human subjects. *Nutr Res* **23**:1199-1210

Serra, A., Macià, A., Romero, M.P., Anglès, N., Morelló, J.R., Motilva, M.J. (2011) Distribution of procyanidins and their metabolites in rat plasma and tissues after an acute intake of hazelnut extract. *Food Funct* **2**:562-568

Serra, A., Macià, A., Rubió, L., Anglès, N., Nàdia Ortega, N., Morelló, J.R., Romero, M.P., Motilva, M.J. (2013) Distribution of procyanidins and their metabolites in rat plasma and tissues in relation to ingestion of procyanidin-enriched or procyanidin-rich cocoa creams. *Eur J Nutr* **52**:1029-1038

Shoji, T., Masumoto, S., Moriichi, N., Akiyama, H., Kanda, T., Ohtake, Y., Goda, Y. (2006). Apple procyanidin oligomers absorption in rats after oral administration: Analysis of procyanidins in plasma using the porter method and highperformance liquid chromatography/tandem mass spectrometry. *J Agric Food Chem* **54**: 884-892

Silberberg M., Besson C., Manach C., Remesy C., Morand C.(2006) Influence of dietary antioxidants on polyphenol intestinal absorption and metabolism in rats. *J Agric Food Chem.* **54**:3541-3546

Sivoňová, M., Waczulikova, I., Kilanczyk, E., Hrnciarová, M., Bryszewska, M., Klajnert, B.,

Duracková, Z.(2004) The effect of Pycnogenol on the erythrocyte membrane fluidity. *Gen Physiol Biophys* **23**:39-51

Stahl, W., Sies, H.(2007) Carotenoids and flavonoids contribute to nutritional protection against skin damage from sunlight. *Mol Biotechnol* **37**:26-30

Stoupi, S., Williamson, G., Viton, F., Barron, D., King, L.J., Brown, J.E., Clifford, M.N.(2010) In Vivo Bioavailability, Absorption, Excretion, and Pharmacokinetics of [^{14}C]Procyanidin B2 in Male Rats. *Drug Metab Dispos* **38**:287-291

Theadom, A., Mahon, S., Barker-Collo, S., McPherson, K., Rush, E., Vandal, A.C., Feigin, V.L.(2013) Enzogenol for cognitive functioning in traumatic brain injury: a pilot placebo-controlled RCT. *Eur J Neurol* **20**:1135-1144

Torras, M.A.C., Faura, C.A., Schönlau, F., Rohdewald, P.(2005) Antimicrobial activity of Pycnogenol. *Phytother Res* **19**:647- 648

Tsang, C., Auger, C., Mullen, W., Bornet, A., Rouanet, J.M., Crozier, A., Teissedre, P.L. (2005) The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *Br J Nutr* **94**: 170-181

Varela, G. M., Antwi, D. A., Dhir, R., Yin, X., Singhal, N.S., Graham, M.J., Crooke, R.M., Ahima, R.S.(2008) Inhibition of ADRP prevents diet-induced insulin resistance. *Am J Physiol-Gastr L* **295**:G621-G628

Voss, P., Horakova, L., Jakstadt, M., Kiekebusch, D., Grune, T.(2006) Ferritin oxidation and proteasomal degradation: protection by antioxidants. *Free Radical Res* **40**:673- 683

Wang, M., Ma, H.L., Liu, B., Wang, H.B., Xie, H., Li, R.D, Wang, J.F.(2010) Pinus massoniana bark extract protects against oxidative damage in L-02 hepatic cells and

mice. *Am J Chin Med* **38**:909-919

Wood, J.E., Senthilmohan, S.T., Peskin, A.V.(2002) Antioxidant activity of procyanidin-containing plant extracts at different pHs. *Food Chem* **77**:155-161

Wu, C.L, Wang, H.B, Xie H, Wang, JF.(2008) Cytotoxity study of Pinus Massoniana Bark Extract (PMBE) on Human Horn HaCaT. *Mod Tradit Chin Med* **28**:41-44 (in Chinese)

Wu, D.C., Li, S., Yang, D.Q., Cui, Y.Y.(2011) Effects of Pinus massoniana bark extract on the adhesion and migration capabilities of HeLa cells. *Fitoterapia* **82**: 1202-1205

Xu, S.X., Zhang, S.M., Wang, X.D., Gao, Y.Q., Qin, X., Wu, K.(2012) Effect of Pinus massoniana Lamb. bark extract on lytic cycle of Epstein-Barr virus. *Indian J Exp Biol* **50**:708-713

Yoshida, A., Yoshino, F., Tsubata, M., Ikeguchi, M., Nakamura, T., Lee, M.C.(2011) Direct assessment by electron spin resonance spectroscopy of the antioxidant effects of French maritime pine bark extract in the maxillofacial region of hairless mice. *J Clin Biochem Nutr* **49**:79-86

Young, J.M., Shand, B.I., McGregor P.M., Scott, R.S., Frampton, C.M.(2006) Comparative effects of Enzogenol and vitamin C supplementation versus vitamin C alone on endothelial function and biochemical markers of oxidative stress and inflammation in chronic smokers. *Free Radical Res* **40**:85- 94

Zhang, J.H., Feng. D.R., Ma. H.L., Liu, B., Wang, H.B., Xie, H., Li, R.D., Wang, J.F.(2012) Antitumor Effects of Pinus massoniana Bark Extract in Murine Sarcoma S180 Both in Vitro and in Vivo. *Am J Chin Med* **40**:861- 875

Zheng, G.Y., Li, R.D., Wang, C.Z., Xie, H., Ye, J.Z., Gao, C.X.(2007) Study on Anti-tumor of

Extract from Masson's Pine Bark. *Chem Ind Forest Prod* **27**:16-20 (in Chinese)

Zibadi, S., Rohdewald, P.J., Park, D., Watson, R.R.(2008) Reduction of cardiovascular risk factors in subjects with type 2 diabetes by Pycnogenol supplementation. *Nutr Res* **28**:315-320

Footnotes

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Figure legend

Figure 1. Mechanism of PMBE induction of apoptosis in cancer cells.

PMBE induces apoptosis via two pathways: the death receptor and the mitochondrial pathways. In the death receptor pathway, PMBE binds to and stabilizes the trimerization of the death receptor, leading to procaspase-8 autoactivation (it is not known whether PMBE interacts with the death receptor directly). Caspase-8 promotes pro-apoptosis factor Bid truncation into tBid and activates caspases-3, 6, and 7. In the mitochondrial pathway, PMBE increases the expression of the pro-apoptotic protein Bax, and decreases the expression of the anti-apoptotic protein Bcl-2. This results in the translocation of cytochrome C from the mitochondria to the cytoplasm, further leading to apoptosis. In addition, PMBE also facilitates cell apoptosis and increases the expression of p53, which promotes DNA damage repair. If repair is successful, cells can recover and avoid apoptosis.

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Table 1. Other pine bark extracts, components and biological activities.

Pinus bark extract	Main / effective component	Laboratory model	Functions
Pinus roxburghii sarg bark extract	flavonoids	In vivo experiment Animal model: Wistar rats and Swiss albino mice; Wistar and albino rats.	Diuretic, laxative, Antispasmodic, anti-hypertensive, Analgesic and anti-inflammatory (Kaushik et al., 2012[a]; Annegowda et al., 2010; Kaushik et al., 2012[b]).
Pinus siberian cedar crust bark extract	arabinogalactan sulphate	In vitro experiment	Anticoagulate (Drozd et al., 2008).
Pinus brutia bark extract	taxifolin	In vitro experiment	Anti-inflammatory likely to PYC (Ince et al., 2009).
Pinus caribaea Morelet bark extract	tannins	In vivo experiment Cell line: E.coli	Antioxidant, anti-lipid peroxidation and antigenotoxic (Fuentes et al., 2006).
Pinus morrisonicola Hay. bark extract	various flavonoids	In vivo experiment Cell line: u937	Free radical scavenging and anti-cancer (Hsu et al., 2005).
Pinus koraiensis bark procyanidins extract	procyanidine	In vivo experiment Animal model: mice	Antioxidant, anti-cancer (Li et al., 2007).
Pinus cembra L.	phenolics,flavonoids, proanthocyanidins	In vitro experiment	Antioxidant and antimicrobial (Apetrei et al., 2011).

Other pine bark extracts have important biological activities, and like Pycnogenol, Enzogenol, and PMBE, are also rich in flavonoids and may function as ROS scavengers and have anti-cancer properties.