Cancer chemopreventive effects of Pinus Maritima bark extract on ultraviolet radiation and ultraviolet radiation-7,12, dimethylbenz(a) anthracene induced skin carcinogenesis of hairless mice

Maria Kyriazi*, Dido Yova, Michail Rallis, A. Lima

School of Electrical and Computer Engineering, National Technical University of Athens, Iroon Polytechniou 9, Zografou Campus, 157 80 Athens, Greece

Pharmaceutical School, National University of Athens, Zografou Campus, 157 71 Athens, Greece

University Hospital of Skin Diseases A. Syggros, Ilissia, Athens, Greece

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Abstract

The bark extract of Pinus Maritima (PBE), a rich in phenolic acids, polyphenols and in particular flavonoids mixture, was examined for skin cancer preventive action that was evaluated in two different experimental animal tumor models induced by ultraviolet radiation (UVR) and combination of UVR with 7,12-dimethylbenz[a]anthracene. Significant decrease in the number of animals bearing tumors and the number of tumors per animal was observed in the PBE treated animals. In the same time significant increase in the viability of these animals was also observed. Furthermore, PBE delayed the appearance of tumors. These results provide strong evidence about the preventive anticancer activity of this extract on non-melanoma skin cancer and its protective effect not only from UVR, but also from more potent carcinogenic agents.

Keywords: Chemoprevention; Skin carcinogenesis; Pine bark extract; Hairless mice

1. Introduction

Skin cancer is by far the most common type of cancer, with tremendous impact on health and morbidity. The three main types of skin carcinomas are basal cell carcinomas (BCCs), squamous cell carcinomas (SCCs) and cutaneous melanomas (CMs). Non-melanoma skin cancers (NMSCs) are the most frequently diagnosed malignancies in the USA, accounting for approximately 40% of all cancer cases. It is estimated that more than 1,000,000 new cases of NMSCs diagnosed in the USA in 2003 resulted in 2200 deaths [1].

UV radiation is considered to be the major carcinogenic factor for all types of skin cancers. However, many other factors contribute to the initiation and promotion of skin carcinogenesis.
For instance, occupational exposures to chemical pollutants (e.g. polycyclic aromatic hydrocarbons), volatile organic compounds (e.g. benzene), and heavy metals (e.g. arsenic, cadmium and lead) considered very potent genotoxic factors for some population groups such as steel, agriculture, petrochemical, textile and pesticide industry workers [2,3].

UVR and chemical agents can initiate damages to biomolecules either by direct photochemical reactions or/and via oxidative mechanisms generated by reactive oxygen species (ROS) [4–8]. Skin spontaneously responds to increased ROS levels, induced by ultraviolet radiation (UVR) or chemical agents, by detoxifying enzymes such as superoxide dismutase, catalase, thioredoxin reductase and low-molecular mass antioxidant molecules such as glutathione, a-tocopherol and ascorbic acid. However, this response may not be sufficient to prevent the oxidative damage of cutaneous cells after excessive or repetitive exposure to carcinogenic agents [9]. Thus, ROS may oxidize lipids, proteins and DNA leading to formation of oxidized products such as lipid hydroperoxides, protein carbonyls and 8-oxo-gouanosine [5]. If these alterations occur to genes involved in normal homeostatic mechanisms that control proliferation and cell death, significant abnormalities are observed in the cell cycle, leading to the first cancer stage, initiation [10,11].

Chemoprevention represents a relatively new and promising strategy which can slow, reverse or completely halt the process of carcinogenesis by the use of natural or synthetic antioxidants. These chemopreventive compounds are known to be antimutagenic, anticarcinogenic, non-toxic and to have the ability to exert striking inhibitory effects on diverse cellular events associated with multistage carcinogenesis [9,12]. Such antioxidants are carotenoids, vitamins E and C and botanical antioxidants as tea polyphenols, proanthocyanines, circumcin, silymarin, apigenin and resveratrol [12–15].

The pine bark extract (PBE) obtained from the French Maritime Pine, Pinus Maritima, owning to the chemical structure of its components, presents remarkable antioxidant activity. A chromatographic profile of PBE has revealed that this mixture contains phenolic, procyanidin/proanthocyanidin, and flavonoid compounds existing as monomers, dimers and oligomers of 5–7 units [16,17]. In addition, PBE lacks toxicity and non-mutagenic [18]. This extract is now widely used as a nutritional supplement and phytochemical remedy for various diseases associated with prooxidant pathology such as atherosclerosis, diabetes, ischemia, and ageing process [16,17].

Although many strong antioxidant substances have been studied for their preventive-anticancer activity, no sufficient data exist yet for PBE. In the present work, the PBE anticancer activity was examined on non-melanoma skin carcinomas in hairless mice without melanin (SKH-HR1) and with melanin (SKH-HR2). The tumors were induced by UVR and combination of UVR and 7, 12, dimethylbenz[a]anthracene (DMBA), for simulating the UVR carcinogenic action and the genotoxic effects of UVR with chemical agents combination.

2. Materials and methods

2.1. Chemicals

DMBA and acetone (with purity >95%) were purchased from Sigma Aldrich Chem. Co. The PBE was of the highest available purity kindly offered by Vilco S.A. The concentration of PBE solution was 0.02 w/v%. Fresh PBE solutions were prepared twice per week.

2.2. Animals

Animal care was performed according to the guidelines established by the European Council Directive 86/909/EEC and the Greek Committee for Experimental Animals. In order to evaluate the preventive-anticancer activity of PBE, both female and male hairless mice SKH-HR1 (albino) and SKH-HR2 (with melanin) were used. They entered the experiments at ages varying between 8 and 10 weeks. All mice originated from the breading stock of the Pharmaceutical Technology Laboratory of National University of Athens. The animals were housed in cages of five depending on their sex and experimental group. The animals room was kept at 25 ± 1 °C and illuminated by yellow fluorescent tubes in a 12-h cycle of light and dark (switched on at 8:00 and off at 20:00). These lamps do not emit any measurable UV
radiation. The mice had unrestricted continuous access to solid pellets and water.

2.3. UV radiation

Solar simulated UV radiation (280–400 nm) was obtained from a Xenon lamp 1000 W (6269) placed in an Arc Lamp Housing (66020-M) connected with a Universal Power Supply (68820) of Oriel Instruments. The light was filtered through appropriate for these wavelengths filters. The irradiance was measured before every experiment by a Goldilux Smart Meter (70239) of Oriel Instruments.

2.4. DMBA-UVR induced skin carcinogenesis

The procedure followed for the DMBA-initiation and UVR-promotion of two-stage mouse skin carcinogenesis was essentially as that reported by Husain et al. [19] with minor modifications. Five topical treatments were applied on the back of each animal, once every 5 days, with 0.2 ml of DMBA solution (0.015% in acetone). Two weeks after the last DMBA application, the backs of the animals were exposed to UVA (8.5 mW/cm²) and UVB (8 mW/cm²) radiation for 5 weeks, five times per week. During the first week, the exposure time was equal to a median erythemal dose MED 32 mJ/cm² [19] and was increased 25% each week to avoid skin thickening. The animals were divided into two experimental groups, the control and the test group. Each group consisted of five female and five male animals from each strain. The PBE treatment of the test group started 2 weeks before the first DMBA application and continued up to the end of the experiment. PBE was diluted in the drinking water of the animals. Test and control animals consumed about 10 ml/day of either water or PBE solution.

2.5. UVR induced skin carcinogenesis

The procedure followed for the UVR initiation and promotion of mouse skin carcinogenesis was essentially as that reported by Husain et al. [19] with minor modifications. The backs of the animals have been exposed to UVA (8.5 mW/cm²) and UVB (8 mW/cm²) radiation for 16 weeks, five times per week. The UVR dose which the animals received during the first week was equal to MED 32 mJ/cm² and was increased by 25% each week, until the 10th week. After the 10th week, the irradiation time remained equal to 7 MED. The animals were divided into two experimental groups, the control and the test group. Each group consisted of five female and five male animals from each strain. The PBE treatment of the test group started 2 weeks before the first UV treatment and continued up to the end of the experiment. PBE was diluted in the animal drinking water. The consumption of water and PBE solution per animal was similar with that of DMBA-UVR treated groups.

2.6. Data evaluation

During 1 year, the animals were examined weekly, for the appearance of pigmented lesions, papillomas, basal and squamous carcinomas, and the effect of PBE on tumor prevention was evaluated. The lesions were examined histopathologically after routine staining with eosin haematoxylin. The weight of the animals was recorded weekly, too. Tumor appearance and growth was recorded for each mouse for tumors of diameter between 1 mm and 1 cm. Mice with tumor diameter greater than 1 cm were euthanatized for ethical reasons. The diameter of the tumors was recorded every 15 days up to the end of animals lives by a digital imaging system. The digital imaging system that was developed consists of a CCD camera (Cool Snap, RS Photometrics) connected to a computer. The software programs used for image acquisition and processing are Image-Pro® Plus and Adobe Photoshop®. Statistical analysis of all these data were also performed using the Student’s t-test.

3. Results

3.1. Inhibition of experimental carcinogenesis by PBE

The results of the examinations showed that administration of PBE exhibited a chemopreventive effect on the experimental carcinogenesis induced by
either UV or combination of DMBA-UV in mice. The preventive effect was evident as a significant reduction in tumor incidence, multiplicity and delay in tumor latency period. In both cancer models, the administration of PBE through drinking water did not exhibit any toxic effect in the animals as monitored by their weight profile. In addition, no noticeable difference in the weight of animals was observed between the control and experimental groups, and the mean water consumptions per animal between the test and control group were equal.

3.2. Effect of PBE on DMBA-UV induced mouse skin carcinogenesis

Tumor appearance was consistent with previously described studies in hairless mice, so tumors first appeared as benign papillomas, progressing toward more malignant states, and finally squamous cell carcinomas [20]. Figs. 1 and 2 show the effect of PBE on the DMBA-UV induced skin papillomas in SKH-HR1 and SKH-HR2 male and female mice. As shown in Figs. 1 and 2 when data were analyzed for

![Graph](image1.png)

Fig. 1. Delay-inhibition of first DMBA-UV induced papilloma appearance in SKH-HR1 and SKH-HR2 male mice by oral administration of PBE.

![Graph](image2.png)

Fig. 2. Delay-inhibition of first DMBA-UV induced papillomas appearance in SKH-HR1 and SKH-HR2 female mice by oral administration of PBE.
papillomas incidence, oral administration of PBE significantly delayed the incidence of skin papillomas. Compared with the control group of mice the time of appearance of the first papilloma was delayed 9 weeks for SKH-HR1 male mice, 8 weeks for SKH-HR2 male mice, 17 weeks for SKH-HR2 female mice. It must also be highlighted that administration of PBE completely inhibited the appearance of skin papillomas and tumors in SKH-HR1 female mice. At the end of the experiment (Fig. 3), 100% of SKH-HR1 and 100% of SKH-HR2 male mice bore skin tumors in the control group, comparing with 60% of SKH-HR1 and 60% of SKH-HR2 male mice in the test group, accounting for 40% reduction–inhibition of the tumor incidence. Similarly, 80% of SKH-HR1 and 80% SKH-HR2 female mice bore skin tumors in the control group at the end of the experiment, comparing with 0% of SKH-HR1 and 40% of SKH-HR2 female mice bearing skin tumors in the test group, accounting for 100 and 50%, respectively, reduction–inhibition of tumor incidence. Fig. 4 shows that administration of PBE decreased the tumor multiplicity (cumulative number of tumors per group or number of tumors per mouse) from 2.8 to 0.8 in male SKH-HR1 mice, from 4.5 to 1.5 in male SKH-HR2 mice, from 1.4 to 0 in female SKH-HR1 mice and from 2.8 to 0.4 in female SKH-HR2 mice. In addition, oral administration of PBE prolonged the average SKH-HR1 male mouse life for 7.6 weeks (23%) compared with the test group and the average SKH-HR2 male mouse for 8.4 (25%) weeks (Fig. 5). The respective prolongation of the average SKH-HR1 female mouse life was 6.6 weeks (18%) and of the SKH-HR2 female life was 7.2 weeks (20%) (Fig. 5). There was no difference in the weight profiles between the two different groups.

3.3. Effect of PBE on UV induced mouse skin carcinogenesis

In the UV treated group, the first induced papilloma on skin of SKH-HR1 and SKH-HR2 male mouse appeared as early as the 14th and 15th week, respectively, after the beginning of the
experiment. Oral administration of PBE resulted in 6-week for SKH-HR1 and 3-week delay for SKH-HR2 male mice. It must be underlined that administration of PBE completely inhibited the appearance of skin papillomas and tumors in SKH-HR1 and SKH-HR2 female mice. At the end of the experiment (Fig. 6), 100% of SKH-HR1 and 100% of SKH-HR2 male mice bore skin tumors in the control group, comparing with 60% of SKH-HR1 and 20% of SKH-HR2 male mice in the test group, accounting for 40 and 80%, respectively, reduction—inhibition of the tumor incidence. Similarly, 60% of SKH-HR1 and SKH-HR2 female mice born skin tumors in the control group, with 0% of SKH-HR1 and SKH-HR2 female mice bearing skin tumors in the test group, accounting for 100% reduction—inhibition of tumor incidence. Fig. 7 shows that the administration of PBE decreased the tumor multiplicity (cumulative number of tumors per group or number of tumors per mouse) from 3 to 1 in male SKH-HR1 mice, from 1.2 to 0.2 in male SKH-HR2 mice, from 1.8 to 0 in female SKH-HR1 mice and from 0.6 to 0 in female SKH-HR2 mice. Moreover, oral administration of PBE prolonged the average SKH-HR1 male mouse life for 7.4 weeks (22%) compared with the test group and the average SKH-HR2 male mouse life for 7.4 (21%) weeks (Fig. 8). The respective prolongation of the average SKH-HR1 female mouse life was 6.4 weeks (17%) and of the SKH-HR2 female life was 7.2 weeks (19%) (Fig. 8).

4. Discussion

ROS can be frequently generated in biological systems either by normal metabolic pathways or as a consequence of exposure to physical and chemical agents. ROS are extensively studied and it is known that contribute to mutagenesis, carcinogenesis, tumor promotion and many diseases. Many tumor initiators and promotors have been shown to exert their action by production of ROS [21,22]. Since tumor promotion is closely linked to oxidative stress, a compound that exhibits antioxidative properties is expected to present
anticancer activity. Thus, a large number of naturally occurring compounds possessing antioxidant activities including curcumin, tea, garlic and grapes extracts have been tested by other researchers for their potential to inhibit the process of carcinogenesis [9,12–15].

Among them PBE, a mixture of phenols, polyphenols, broadly divided into monomers (e.g. catechin, epicatechin and taxifolin), dimers (e.g. procyanidin B1, B2, B3 and B7) trimers (e.g. procyanidin C1 and C2) and oligomers up to heptamers and phenolic acids (e.g. caffeic, ferulic and p-hydroxy-benzoic acid), has shown remarkable antioxidant activity [23,24]. Previous studies have shown that long-term oral PBE supplementation significantly raised the MED in fair-skinned people [25]. This observation is consistent with other in vivo studies in rats and albino mice which have shown that PBE can protect from inflammation and edema induced by repetitive exposure to solar simulated UV radiation or croton-oil [17,26]. There is evidence that this mechanism is consistent with a protective activity of the skin antioxidant system as PBE has been shown to protect endogenous low molecular weight antioxidants levels such as a-tocopherol and ascorbic radical [23,27].

The present study demonstrates the long-term protective action of PBE on skin carcinogenesis. This statement is supported by the above-mentioned results, which are compatible with those observed by other researchers [26] indicating that oral administration of PBE indeed possesses significant cancer chemopreventive potential. Concerning the anti-tumor effects of PBE, it is important to note the delay in the first papilloma/tumor appearance as well as the significant reduction of tumors per animal. Furthermore, it must be underlined the prolongation of animals life for about 20%, the high tumor inhibition rates (about 40%) for male mice and the almost complete inhibition of skin tumors in female mice.

The results are in agreement with those of other researchers studies which examine the chemopreventive/anti-tumor effect of other phenolic and polyphenolic mixtures such as grapes [28,29], black and green tea extracts [30,31]. Although the mechanism by which these extracts exhibit chemopreventive action is not yet revealed exactly, it has been shown that these mixtures: (i) protect endogenous low molecular weight antioxidants [23,27], (ii) increase glutathione and glutathione-S-transferase levels in cells [28,30] and (iii) inhibit tumor promotion biomarkers activities such as ornithine decarboxylase and myeloperoxidase [28,29].

In summary, there is strong evidence that PBE exhibits potent NMSC preventive, anticancer activity. This chemopreventive action was demonstrated in the late appearance of first skin papillomas/tumors, the reduced number of skin tumors per animal, the reduced percentage of animals bearing skin tumors and the prolongation of animal viability.

References


