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Research article

Photoprotection of Fractions Obtained from *Phyllanthus Orbicularis* Extract against UVB -Induced DNA Damage

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Abstract

The use of plants as sources of photochemopreventive agents has been increased in the last years. The assessment of photoprotective properties of plant extracts improves the development of pharmaceutical and cosmetic products. In the present work, we evaluate the effect of *Phyllanthus orbicularis* extracts against UVB-induced damage to DNA. The total aqueous extract of this plant (POE) and chemical fractions obtained from it: aqueous (AF), chloroformic (CF) and dichloromethane (DF) were assessed. For this, plasmid DNA solutions were exposed to UVB radiation in presence of plant extracts. The DNA repair enzymes Formamidopyrimidine-DNA glycosylase (Fpg) and T4 bacteriophage endonuclease V (T4-endo V) were employed to distinguish oxidized DNA and cyclobutane pyrimidine dimer (CPD) lesions. The supercoiled and relaxed forms of DNA were separated through electrophoretic migration in agarose gels. Additionally, the UVB wavelength transmittances of total extract and chemical fractions of *P.orbicularis* were measured. The results showed that POE and AF protect DNA against the damage induced by UVB light. This protective effect is related to the antioxidants and absorptive properties of some flavonoids present in the extracts.

Keywords

Absorptive Properties; Antioxidant; Flavonoids; Plasmid DNA; Ultraviolet Radiation

Introduction

During the past decade, the use of vegetal compounds against diseases induced by ultraviolet (UV) light, such as photoaging and photo carcinogenesis, became available. This activity of plant extracts is related of their physical and bioactive properties. Nowadays, the development of sun protection approaches includes compounds with the ability of absorbing UV-radiation in combination with compounds able to decrease the oxidative stress induced by UV. In this sense, numerous phytocompounds have been evaluated as potential photochemopreventive agents due to their absorptive and antioxidative properties [1-4].

The *Phyllanthus orbicularis* Kunth species is an endemic Cuban plant, whose antigenotoxicity against UV radiation has been demonstrated. The photoprotective activity of its total aqueous extract in plasmidic DNA and bacterial cells has been related to its absorptive and antioxidant properties [5,6]. In human cell lines the UVB-bio antimutagenic effect of POE has been relat-

ed to the extract ability to modulate the NER system [7] One important approach to identify compounds responsible for the observed protective effect can be reached through the study of chemical fractions obtained from the plant extract.

The UV of sunlight that reach the surface of the Earth is composed of proximately 95% UVA and 5% UVB. Despite this, the UVB is the most mutagenic component of terrestrial UV, as it is directly absorbed by the DNA molecule. This results in the formation of DNA photoproducts, such as cyclobutane pyrimidine dimers (CPDs) and 6-pyrimidine-4-pyrimidone photoproducts (6-4 PPs), which are, probably, the main causes for the carcinogenic effect of solar exposure. Also, UVB light can produce reactive oxygen species (ROS) that acts as powerful mutagens also causing DNA damage [8-10].

Accordingly, the aim of the present work is to evaluate the antigenotoxicity of chemical fractions obtained from *P. orbicularis* plant extract against UVB artificial light in plasmid DNA.

Materials and Methods

Phyllanthus orbicularis total aqueous extract and chemical fractions

The specimens of *P. orbicularis* plants were collected at Cajálbana, Pinar del Río, Cuba, in March 2015. These were authenticated by Dr. C. Rosalina Berazain, Havana University, Cuba. A voucher specimen (No.7/220 HAJB) is deposited at the herbarium of the National Botanical Garden, Havana City, Cuba.

The total aqueous extract of *P. orbicularis* (POE) was obtained according to methodology described by Del Barrio and Parra (2000) [11]. The chemical fractions were obtained as previously described by Sánchez-Lamar and collaborators, 2015 [12]. The plant lyophilized extract was dissolved in methanol for 48 hours with gentle shaking. The solvent was removed under reduced pressure (Fraction A) and the insoluble fraction was discharged. The complete Fraction A was dissolved in water and extracted 3 times with chloroform (3:1), obtaining an organic fraction, the chloroform fraction (CF) and aqueous fraction. Both fractions were dried and aqueous fraction was extracted 3 times with a water/dichloromethane mix (3:1), rendering an aqueous (AF) and an organic fraction (DF).

Plasmid

Plasmid pCMUT (1762 bp) (C – chloramphenicol resistance, and MUT – sup^F, mutation target gene) derived from pAC189 plasmid was used. Purification of DNA samples was prepared by using Qia gen Plasmid Maxi Kit (Valencia, CA) with freshly transformed *E. coli* strain DH10b and stored in TE buffer (10 mM Tris-HCl [pH 8.0], 1M Methylenediaminetetracetic acid) at -20°C [13].

Evaluation of P. orbicularis extract effects on DNA and the enzyme used in the experiments

In order to test an eventual damage effect of the plant extract on plasmid DNA used in the experiments, 10 µl of plasmid DNA solution (20 ng/µl diluted in TE) was mixed with 10 µl of POE at different concentrations (10, 100 and 1000 µg/ml), and incubated at 37°C, for 2, 3 and 4 h. Then, the induction of DNA breaks was evaluated.

In order to test an eventual inhibition of the enzymatic activities of Fpg protein and T4- endo V by the vegetal extract in study, 0.8 U of Fpg protein (from New England Biolabs, Ipswich, USA) and 70 ng of T4-endo V (produced in this laboratory) were first incubated with POE during 1 h, and then their enzymatic activities were assayed in irradiated plasmid DNA. The detection of form II DNA (relaxed circle) bands, after electrophoresis in agarose gel, was taken as a measure of enzymatic activities.

Evaluation of *P. orbicularis* extracts on UVB-irradiated DNA

In the photoprotection experiments, the *P. orbicularis* total extract (POE) and chemical fractions (AF, CF, DF) were tested. They were mixed with plasmid DNA, 1:1 proportion (v/v). The extracts concentrations evaluated were 1000, 500, 10 and 6.5 µg/mL for POE, AF, CF and DF, respectively.

The samples DNA-extracts were UVB irradiated with Vilber Loumart T15M 15 W lamp. The dose rates of the UVB lamps were measured with a UV radiometer VLX 3W (Vilber Lourmat,Torcy, France), whereby values obtained was 4.8 $Jm^{-2}s^{-1}$.

After irradiation, 200 ng of DNA samples were incubated with 0.8 U of Formamidopyrimidine-DNA glycosylase (Fpg protein, from New England Biolabs, Ipswich, USA), 70 ng of T4 bacteriophage endonuclease V (T4-endo V, produced in this laboratory) to discriminate the different types of DNA lesions. These samples were incubated at 37°C, the incubation conditions were previously described by Schuch and 2009 [13].

Quantification of DNA-CPD and oxidative lesions

To determine the average number of DNA lesions generated by UVB irradiation, the amounts of supercoiled (FI) and circle (FII) plasmid DNA forms were determined as described before. For DNA-CPD and oxidative damage specific quantification, the number of single strand breaks corresponding to T4 endo-V and Fpg enzyme sensitive sites respectively were calculated for each treatment. The number of enzyme-sensitive sites per Kbp of plasmid DNA was calculated, assuming a Poisson distribution adapted to this technique, by the following equation:

$$X = -\ln (1.4\times FI/1.4\times FI+FII) / 1.8$$

where FI represents the intensity of fluorescence measured in the supercoiled DNA bands, FII represents the intensity in the open-circular relaxed DNA bands, 1.4 is a correction factor to account for the increased fluorescence of ethidium bromide when this compound is bound to relaxed DNA compared to supercoiled DNA, and 1.8 is pCMUT vector size in Kbp [13].

Evaluation of *P. orbicularis* extracts transmittance

To evaluate the physical capacity of the total extract and chemical fractions to absorb UV radiations, the transmittance was determined for 1000, 500, 10 and 6.5 µg/mL of POE, AF, CF and DF, respectively. The absorbance was measured at 280-320 nm wavelength, by Ultrospec 2100 spectrophotometer (Amersham Biosciences. Cambridge CB4. England).

Statistical analysis

Median values and the corresponding standard deviation were calculated for each treatment. Normality, variance homogeneity and one-way analysis of variance (ANOVA) tests were also undertaken. Median values were obtained from three independent experiments. The median of treatment were compared with the control using Dunnett test ($p<0.05$). The results were analyzed using the computational program GradhPad (GraphPad v4 Software, Inc, USA).

Results

P. orbicularis extracts´ transmittance against UVB radiation

The transmittance of POE and its chemical fractions were evaluated in UVB wavelength range. The order of transmittance of extracts was DF~CF>AF>POE. The DF and CF had almost the same transmittance physical behavior, and no absorbance of UVB light (Figure1). The AF blocked around 60% of the UVB radiation and POE, at the concentration used, completely blocked UVB.

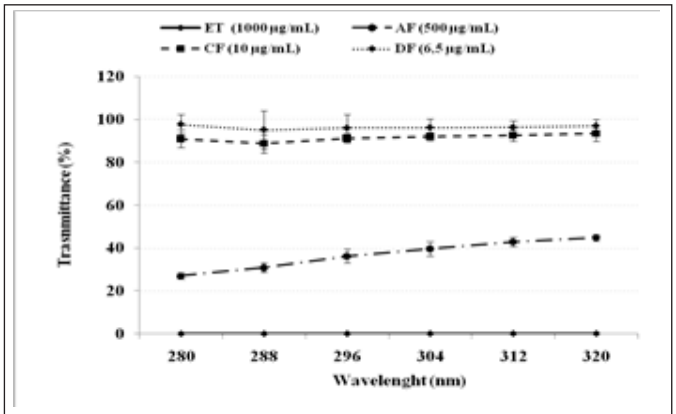

Figure 1: Transmittance of *P.orbicularis* aqueous total extract and its chemical fractions for UVB wavelength range (280-320 nm). Mean curves of three independent experiments are presented.

Genotoxicity of POE and its effect on T4-endo V and Fpg endonuclease activities

To exclude a possible direct effect of phytocompounds of *P. orbicularis* on DNA, we evaluated the supercoiled conformation of plasmid DNA pCMUT after direct incubation with POE. Up to 4 h of incubation, there was no change of plasmid conformation, at any concentration tested, thus compounds present in *P. orbicularis* did not induce damage in DNA. The single strands breaks were not generated after the treatment with the extract (Figure 2). On the other hand, the POE didn't affect the enzymatic activities of T4-endo V and Fpg enzymes used in this study. Both enzymes were able to recognize and nick the respective specific damaged sites, with independent on the presence of the extract (Figure 3).

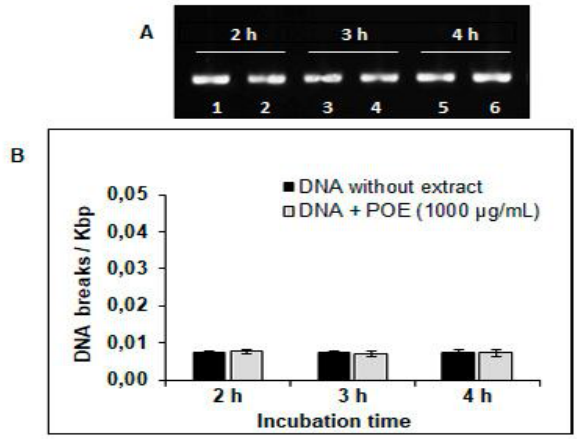

Figure 2: Genotoxicity of *P. orbicularis* extract on plasmid DNA conformation A

Agarose electrophoresis of plasmid DNA. Positions 1, 3 and 5: DNA controls; 2, 4 and 6: DNA + POE (1000 µg/mL) after incubation during the indicated times. B) DNA strand break quantification. Means ± SD of three independent experiments are presented. No significant differences were detected, Tukey test, $p<0.05$.

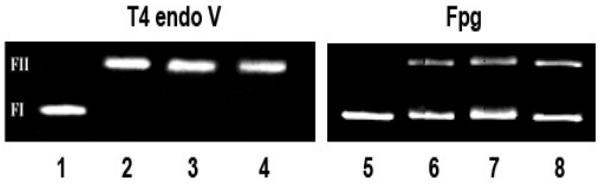

Figure 3: Toxicity of *P. orbicularis* extract in T4 endo V and Fpg endonuclease activity on irradiated plasmid DNA (UVB 10 kJ/m2).

Agarose electrophoresis of plasmid DNA. Positions 1: DNA control; 2: T4 endo V without preincubation; 3: T4 endo V pre-incubated (1h) only in buffer reaction; 4: T4 endo V pre-incubated with 1000 µg/mL of POE (1h). column 5: DNA control 6: Fpg without pre-incubation; 7: Fpg pre-incubated (1h) only in buffer reaction; 8: Fpg pre-incubated with 1000 µg/mL of POE.

Effect of *P. orbicularis* extracts on UVB radiation induced CPDs and oxidized DNA bases

The results related to photoprotective action showed that POE and AF extracts inhibited significantly the CPD formation on DNA by UVB light. For oxidatively generated damage, this protective effect was only demonstrated for POE. The CF and DF did not result in any reduction of DNA-photodamage induced (Figure 4).

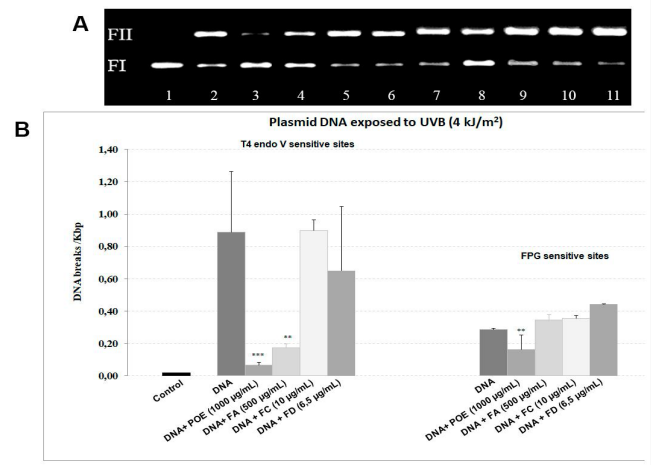

Figure 4: Plasmid DNA exposed to UVB artificial radiation in presence of plant extracts (POE, AF, CF, DF).

Agarose electrophoresis of plasmid DNA digested with the T4 endo V and Fpg endonucleases. Column 1: Control DNA; column 2: T4 endo V positive control (DNA irradiated and digested with T4 endo V without extract); columns 3-6: DNA irradiated in presence of extracts (POE, AF, CF, DF) and digested with T4 endo V column 7: Fpg positive control (DNA irradiated and digested with Fpg without extract); columns 8-11: DNA irradiated in presence of extracts (POE, AF, CF, DF) and digested with T4 endo V column B) Quantification of CPDs and oxidized lesions in DNA exposed to UVB light. Means ± SD of three experiments, are shown. Significant differences were found, Dunnett test, $p<0.05$

Discussion

UV radiation from sunlight, particularly UVB, alters DNA by promoting the formation of pyrimidine dimers, and oxidized bases due to the generation of ROS. UVB exposure directly and through ROS causes skin cancer, including cutaneous malignant melanoma and the non-melanoma skin cancer. The skin cancer incidences have been increasing dramatically over the past few years, so, skin protection against UV exposure is a fundamental part of cancer prevention. In this sense, the inhibition of UVB-induced CPDs or ROS formation could be an important approach for the prevention of UVB- skin neoplasic process [2,3,9,14,15].

In this work, the *P. orbicularis* whole aqueous extract and its chemical fractions were evaluated as photoprotectors against UVB light. Before that, the toxic effect of compounds of *P. orbicularis* on DNA and restriction enzymes was studied. For this, only the total extract of *P orbicularis* was evaluated as representative of the all possible compounds present in the plant. Given the characteristics of the model and methodology experimental of this work, the extract can interact directly with the DNA molecule. The observed effect is the result of direct chemical interactions of POE phytocomponents with plasmid. There was not DNA strand break of pCMUT generated by POE's compounds. Related to the effect of extract on restriction enzymes previous works had demonstrated POE's capacity to inhibit restriction enzymes such as: *BamHI*,*EcoRI* and *PvuII*, impeding their ability to cut DNA specific sequences [16]. In our experimental conditions, restriction activity of T4 endo V and *Fpg* enzymes were not inhibited by components of this extract.

For the photoprotection experiments, the levels of CPDs and oxidized bases in DNA treated and not treated with POE, AF, CF and DF were assessed. The results demonstrated that POE is able to decrease the CPDs and oxidated bases levels after UVB-irradiation. The AF also reduced the direct photodamage generated by UVB.

The diminished photodamage in DNA could be related of the absorptive properties of phytocompounds present in POE and AF. The transmittance of these extracts showed that they are able to absorb UVB light. In presence of POE, the UVB light was effectively blocked, for AF the approximately 60% of UVB radiation is absorbed. The remaining UVB transmission (40%) in the presence AF could explain the lack of protection of this fraction for UVB-ROS generation. However, the presence of POE significantly protected DNA molecules from CPD formation and UVB-ROS generation. For this extract free oxygen radical scavenging activity, was previously demonstrated, which contributes to UVB-photooxidative protective effect [5,17-20]. According to the UVB transmittance of CF and DF, none of these fractions protected DNA against the damages induced by UVB light. In fact, the phytocompounds separated in these fractions are not chromophores elements, and also they did not act as antioxidative agents. Previous works have demonstrated the antioxidative properties of CF and DF extracts in cellular experimental system [12], but not in plasmid DNA model. Maybe, these fractions could be operating as UV-protector agents in the cells context. Future studies, using cellular assays, could clarify the bioactivity of these fractions against UV light.

The fractioning vegetal process is an important approach to identify the compounds responsible for the researched activity in vegetal extracts. In this sense, our results demonstrated that less or non-polar compounds separated in chloroform and dichloromethane fractions (CF, DF) did not act as photoprotector agents. The protection observed is related of UVB-filter and/or antioxidant properties of POE and AF. In these extracts, polyphenols with media and high polarity are present. Among these, flavones, flavanols, flavanols and flavanonols had been studied for their absorptive properties since they are able to block the pass of UVB light. The quercetin and kaempferol show UV absorption peak in the vicinities of 250-280 nm. The catechins and epicatechins may act as UVB filters too. Similarly, the maximum UV absorption of silymarin and gallic acid derivatives occurs at this

wavelength. Besides the absorptive properties, these compounds scavenge oxygen free radical [4,20-23]. The mentioned compounds above have been identified in POE [12,24]. Likewise, due to its polarity and the fractioning process employed, some of that phytocompounds should be present in AF and be responsible of the effect obtained against UVB light.

In our experimental conditions, among the extracts of *P. orbicularis* (POE, AF, CF, DF) evaluated as sunscreen, POE resulted to be the best. The whole herbal extracts contain of numerous compounds that together could provide a better bioactivity. In previous studies, the UV- desmutagenic effect of POE has been demonstrated against UV light [5,6]. In these scientific works, we confirm the photoprotective effect of POE, related to its absorptive and antioxidative properties. Additionally, through the evaluation of fractions obtained from this plant, we demonstrated that some polar flavonoids contained in aqueous fraction are the responsible for the UV- protection.

Currently a successful approach for reducing the harmful effects of UV radiation on the skin is the use of phytocompounds with UV-blockers, DNA repair and antioxidants properties as photoprotective agents in the sunscreen. According to this, our results highlight the relevance of this plant extract as valuable source of compounds useful in cosmetic and medical practices.

Acknowledgment

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Conflict of interest

The authors report no conflicts of interest.

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