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Photoirradiation of azulene and guaiazulene—Formation of reactive oxygen species and induction of lipid peroxidation☆

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ABSTRACT

Azulene and guaiazulene are popular ingredients in beauty, cosmetic, skin, and body care products. We previously determined that these chemicals are photomutagenic in *Salmonella* and phototoxic, causing DNA damage in human Jurkat T-cells. In this study we report that photoirradiation of azulene and guaiazulene, respectively, by UVA light at 0–70 J/cm^2 in the presence of a lipid, methyl linoleate, resulted in lipid peroxidation in a light dose–responsive manner. When irradiated in the presence of sodium azide or superoxide dismutase, the level of lipid peroxidation decreased, indicating that lipid peroxidation is mediated by free radical and superoxide in particular. In contrast, lipid peroxidation was not enhanced when deuterated methanol was incorporated to the system, which suggests that singlet oxygen is not a predominant photo-induced product. Electron spin resonance (ESR) spin trapping study confirmed that photoirradiation of azulene predominantly generated superoxide and none or very low quantity of singlet oxygen was produced. These results indicate that photoirradiation of azulene and guaiazulene by UVA light generates reactive oxygen species (ROS), and induces lipid peroxidation when irradiation in the presence of a lipid. These results implicate that azulene and guaiazulene are phototoxic when exposed to sunlight.

1. Introduction

Azulene (Fig. 1) is an essential oil derived from the German chamomile plant *Matricaria recutita*. In addition to its wonderful aroma, this oil is an anti-inflammatory, anti-spasmodic, and anti-microbial agent [1,2], and has been used as a folk medicine to treat inflammatory diseases. Azulene is clinically used for the medical treatment of pharyngitis, gastric ulcer, gastritis, conjunctivitis, adenoiditis and stomatitis. In addition, azulene is a popular ingredient for various products for human use [3–5]. Its alkyl derivative guaiazulene, 1,4-dimethyl-7-isopropylazulene (Fig. 1), is a constituent of guaiac wood oil and has been reported to exhibit antioxidative activity [6] and anti-inflammatory effect [7]. Furthermore, guaiazulene has been used as an antiulcer drug.

Azulene was not mutagenic in *Salmonella typhimurium* bacteria strains TA98, TA100, TA1535, and TA1537 with and without an S9 activation enzyme system [1]. In clinical studies, some patients showed allergic reactions to azulene [8].

Both azulene and guaiazulene are popular ingredients in beauty, cosmetic, skin, and body care products. While people using these cosmetic products are unavoidably exposed to sunlight, it is not known whether use of cosmetics containing azulene and guaiazulene with concomitant exposure to sunlight results in any deleterious effects. It had been reported that azulene and its derivatives exhibit photochemical reactivity [9]. We previously reported that concomitantly exposed to UVA/visible light, azulene and guaiazulene were photomutagenic in *Salmonella* TA102 and in human skin Jurkat T-cells [3]. Azulene can cause DNA strand cleavage in T-cell nucleus or pure ΦX174 plasmid DNA in solution [4]. In this paper, we report that photoirradiation of azulene and guaiazulene by UVA light produces reactive oxygen species (ROS) and induces lipid peroxidation.

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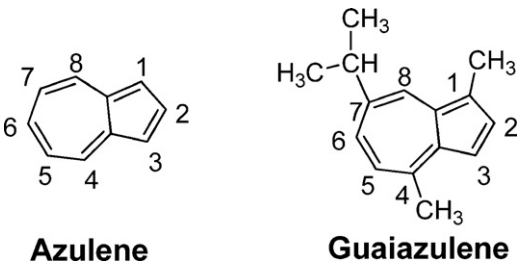

Fig. 1. Chemical structures of azulene and guaiazulene.

2. Materials and methods

2.1. Materials

Azulene, guaiazulene, methyl linoleate, sodium azide (NaN_3), superoxide dismutase (SOD), diethylene-triaminepentaacetic acid (DTPA), and 2,2,6,6-tetramethyl-piperidine (TEMP) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). The nitrone spin trap, 5-*tert*-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO), was a gift from Professor Kalyanaraman (Medical College of Wisconsin). All other reagents were obtained through commercial sources. All solvents were HPLC grade.

2.2. Light sources

The UVA light box was custom made using 4 UVA lamps (National Biologics, Twinsburg, OH) [10]. The irradiance of the light box was determined using an Optronics OL754 Spectroradiometer (Optronics Laboratories, Orlando, FL), and the light dose was routinely measured using a Solar Light PMA-2110 UVA detector (Solar Light Inc., Philadelphia, PA). The maximum emission of the UVA light box was determined to be between 340 and 355 nm. The light intensities at wavelengths below 320 nm (UVB light) and above 400 nm (visible light) are approximately two orders of magnitude lower than the maximum in the 340–355 nm spectral region.

In this study, the UVA-irradiation dose was from 7 to 70 J/cm^2, approximately 23–230 min exposure at the dose rate of 5 mW/cm^2. 10 J/cm^2 of UVA, equates to about 2 h exposure at the noontime of sunny days during the summer around world, based upon observations of UVA intensity of 2.1 mW/cm^2 in Okayama, Japan in September [11], 3.6 mW/cm^2 in Jackson, MS, USA in August [12], 5.4 mW/cm^2 in Paris, France in July [13], 6.6 mW/cm^2 in Coimbatore, India in July [14].

2.3. Peroxidation of methyl linoleate initiated by photoirradiation of azulene and guaiazulene

Experiments were conducted using a solution of 100 mM methyl linoleate and 1.0 mM azulene in methanol. Samples were placed in a UV-transparent cuvette and irradiated with 0, 14, 35, 56 or 70 J/cm^2 of UVA light. After irradiation, the levels of lipid peroxidation were expressed as the amount of methyl linoleate hydroperoxides determined by HPLC peak area by monitoring the elution at 235 nm [10,15]. Methyl linoleate hydroperoxides and the recovered substrate (azulene or guaiazulene) were separated by HPLC using a Prodigy 5 μm ODS column (4.6 mm × 250 mm, Phenomenex, Torrance, CA) eluted isocratically with 10% water in methanol (v/v) at 1 mL/min.

2.4. Peroxidation of methyl linoleate initiated by photoirradiation of azulene and guaiazulene in the presence of a free radical scavenger or enhancer

Experiments were conducted using a solution of 100 mM methyl linoleate and 1.0 mM azulene in methanol in the presence and absence of NaN_3 or SOD. All experiments were carried out as described in the previous paragraph. The concentration of SOD was 200 U/mL and NaN_3 was 20 mM.

It has been established that the lifetime of singlet oxygen is longer in deuterated solvent, such as water or methanol, than in protic solvent [16]. The effect on the levels of lipid peroxide formation induced from UVA photoirradiation of azulene and guaiazulene with CH_3OH and CH_3OD was conducted similarly.

2.5. ESR spectral measurements

The UVA light photoirradiation of azulene dissolved in 60% (for super oxide anion) and 90% (for singlet oxygen) CH_3CN in water was performed *in situ* at room temperature. A 50 μL quartz capillary tube was used. The UVA light was provided by a Schoeffel 500 W Xenon lamp coupled with a Schoeffel broad band (300–360 nm) photochromator. All experiments were performed in duplicate. The data were obtained with error of less than 10%.

Conventional ESR spectra were obtained with a Bruker EMX spectrometer (Bruker Instruments Inc., MA, USA). ESR signals were recorded at 20 mW incident microwave and 100 kHz field modulation of 1 G. The scan width was 100 G for both BMPO and TEMP experiments. All measurements were performed at room temperature.

2.5.1. Generation of singlet oxygen

Singlet oxygen generated from the photoirradiation of azulene with UVA light was detected by the ERS spin trapping method using the spin trap TEMP as described by Rinalducci et al. [17]. Samples were mixtures of ethanol (15 μL ethanol), 5 mg/mL azulene in ethanol (80 μL) and 100 mM TEMP in water (5 μL). The ESR spectra were recorded at room temperature at different times after exposure to UVA light (320 nm).

2.5.2. Detection of superoxide radical anion generation

The ESR-spin trapping method for superoxide radical detection using the spin trap BMPO is essentially the same as described by us previously [18], with the exception that all samples were dissolved in an ethanol/PBS buffer (70/30, v/v). For detection of superoxide radical anion formed from photoirradiation of azulene with UVA light, samples contained BMPO and azulene at final concentrations of 100 mM and 0.35 mg/mL, respectively.

3. Results

3.1. Photoirradiation of azulene and guaiazulene in the presence of methyl linoleate

Photoirradiation of azulene in methanol with UVA light in the presence of methyl linoleate was studied to determine whether photoirradiation of azulene can initiate lipid peroxidation. Photoirradiation of azulene, methyl linoleate, and a mixture of methyl linoleate and azulene with 0, 14, 35, 56, and 70 J/cm^2 of UVA light were conducted in parallel. The extent of lipid peroxide formation following irradiation was measured by calculation of the amount of methyl linoleate hydroperoxides based on the HPLC peak area detected at 235 nm (Fig. 2) [15]. As expected, without light photoirradiation, azulene did not produce any methyl linoleate hydroperoxides (Fig. 2). When irradiated at 14 J/cm^2 in the presence methyl linoleate, photoirradiation of azulene at concentrations of 0.002, 0.02, and 0.2 mM did not generate lipid

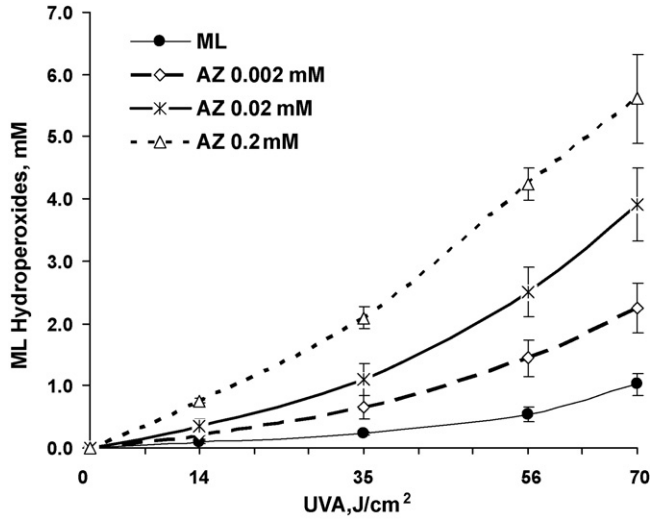

Fig. 2. Lipid peroxidation induced by photoirradiation of azulene (AZ) in methanol in the presence of methyl linoleate (ML) with UVA light at a light dose of 0, 14, 35, 56, and 70 J/cm^2, respectively. The levels of peroxidation were measured by HPLC analysis monitoring the elution at 235 nm.

peroxidation (linoleate hydroperoxides) significantly. At light dose of 35 J/cm^2, azulene at 0.02 and 0.2 mM both induced lipid peroxidation ($P < 0.05$). When light doses at 56 J/cm^2 or higher were used, photoirradiation of azulene at different concentrations all produced lipid peroxidation significantly. When azulene at 0.2 mM was used, lipid peroxidation generated significantly ($P < 0.05$) even irradiated at 7 J/cm^2 (data not shown). As shown in Fig. 2, lipid peroxidation was generated in a dose response manner.

Photoirradiation of guaiazulene by UVA in the presence of methyl linoleate was similarly conducted. Lipid peroxidation also increased in a dose dependent manner (Fig. 3). It was determined that the levels of lipid peroxidation generated by photoirradiation of guaiazulene are closely similar to those of azulene at different concentrations and at different light doses. However, it was found that at 1 mM in methanol with light doses at 0, 35, and 70 J/cm^2, respectively, azulene induced lipid peroxidation at a higher level than guaiazulene (data not shown). These overall results indicate that both azulene and guaiazulene can generate lipid peroxidation (methyl linoleate hydroperoxides) upon photoirradiation with UVA light.

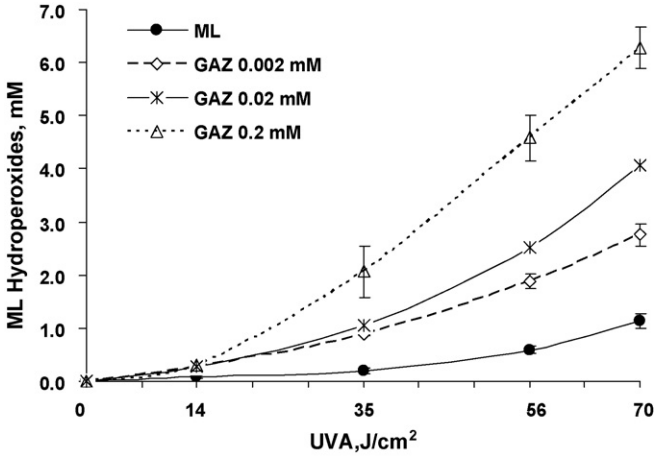

Fig. 3. Lipid peroxidation induced by photoirradiation of guaiazulene (GAZ) in methanol in the presence of methyl linoleate (ML) with UVA light at a light dose of 0, 14, 35, 56, and 70 J/cm^2, respectively. The levels of peroxidation were measured by HPLC analysis monitoring the elution at 235 nm.

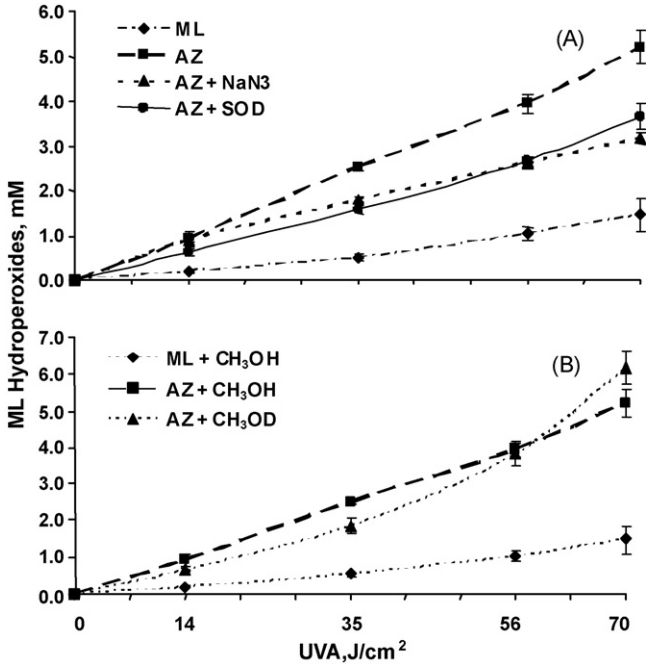

Fig. 4. Effects of NaN_3, SOD, and CH_3OD on peroxidation of methyl linoleate (ML) initiated by 1 mM azulene (AZ) in CH_3OH under UVA irradiation. The levels of peroxidation were measured by HPLC analysis monitored at 235 nm.

3.2. Peroxidation of methyl linoleate initiated by photoirradiation of azulene and guaiazulene in the presence of a free radical scavenger or CH_3OD

The involvement of free radical intermediates in the peroxidation of methyl linoleate initiated by photoirradiation of azulene and guaiazulene was examined. The free radical scavenger NaN_3 [3,4,10,18] and the superoxide free radical scavenger superoxide dismutase (SOD) [3,4,10,18] were employed for this study. As the results shown in Fig. 4A, lipid peroxidation was inhibited by NaN_3 and SOD at 35 J/cm^2 and higher ($P < 0.05$). These results suggest that peroxidation of methyl linoleate initiated by photoirradiation of azulene is mediated by free radicals and that lipid peroxidation is partly induced by superoxide.

NaN_3 is also an effective singlet oxygen ($^1O_2$) and hydroxyl radical ($^{\bullet}OH$) scavenger [10,18]. As a result, NaN_3 alone cannot be relied upon to determine whether singlet oxygen is involved in peroxidation of methyl linoleate by photoirradiation of azulene or guaiazulene. Since singlet oxygen has a longer half-life in deuterated methanol (CH_3OD) than in methanol [10,16], use of both NaN_3 and CH_3OD should provide a reliable approach for determining whether singlet oxygen is involved in peroxidation. Subsequently, the effect of deuterated methanol on lipid peroxidation induced by photoirradiation of azulene was determined. As shown in Fig. 4B, deuterated methanol did not significantly enhance lipid peroxidation. The results of NaN_3 and of deuterated methanol indicate that singlet oxygen is not involved in lipid peroxidation induced by photoirradiation of azulene at concentration of 1 mM.

The involvement of free radicals on peroxidation of methyl linoleate initiated by photoirradiation of guaiazulene was similarly studied. The results are shown in Fig. 5A indicate that both NaN_3 and SOD significantly inhibited lipid peroxidation initiated by guaiazulene. Lipid peroxidation was not significant enhanced by CH_3OD irradiated 14, 35, or 56 J/cm^2, but was enhanced at 70 J/cm^2 ($P < 0.05$) (Fig. 5B). The overall results indicate that UVA photoirradiation of guaiazulene induced lipid peroxidation mediated mainly by superoxide.

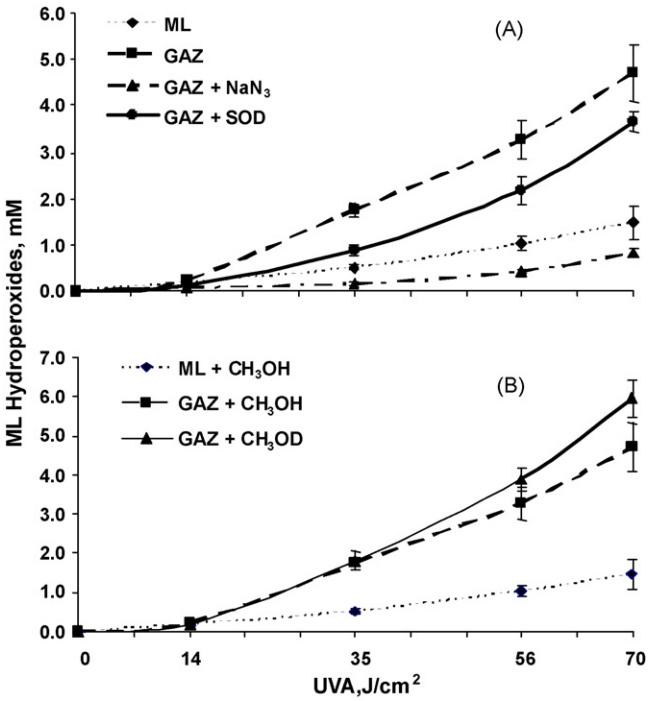

Fig. 5. Effects of NaN_3, SOD, and CH_3OD on peroxidation of methyl linoleate (ML) initiated by 1 mM guaiazulene (GAZ) in CH_3OH under UVA irradiation. The levels of peroxidation were measured by HPLC analysis monitored at 235 nm.

3.3. Photodecomposition of azulene and guaiazulene by UVA light

To determine the extent of photodecomposition of azulene, photoirradiation of 1 mM of azulene in ethanol irradiated with 0, 14, 35, 56, and 70 J/cm^2 of UVA light was conducted. As determined by calculation of the amount of azulene based on the HPLC peak area detected at 272 nm, the percentages of azulene recovered after 0, 14, 35, 56, and 70 J/cm^2 of UVA light irradiation were 100, 85, 28, 9, and 6%, respectively. As illustration, the HPLC profiles of azulene recovery upon irradiation with 0, 35, and 70 J/cm^2 of UVA light are shown in Fig. 6.

Photoirradiation of 1 mM of guaiazulene in ethanol by UVA light irradiation was similarly conducted. The percentages of guaiazulene recovered after 0, 14, 35, 56, and 70 J/cm^2 of UVA light irradiation were 100, 89, 32, 12, and 9%, respectively (data not shown).

3.4. Electron spin resonance (ESR) spin trapping of reactive oxygen species formed from photoirradiation of azulene with UVA Light

3.4.1. Detection of singlet oxygen ($^1O_2$) formation

The spin trap TEMP has been commonly used as a specific probe for singlet oxygen formation [18]. Upon reaction of singlet oxygen with TEMP, the resulting TEMPO, a stable nitroxide, can be detected by ESR spectroscopy [19,20]. UVA irradiation of TEMP (in 90% CH_3CN and 10% water) for 10 min did not result in an ESR signal (Fig. 7A, panel *a*). Reaction of TEMP and azulene in the absence of UVA light also did not result in an ESR signal (Fig. 7A, panel b). With concomitant exposure of 3 mM azulene and TEMP to UVA light for 10 min, singlet oxygen was still not generated (Fig. 7A, panel c). When 10 mM azulene was used for irradiation by UVA light for 10 min, a very weak ESR spectral profile, which is typical of TEMPO [18], was detected (Fig. 7A, panel d). Because the intensity of these ESR signals is extremely weak, it indicates that singlet oxygen formation is not the main pathway.

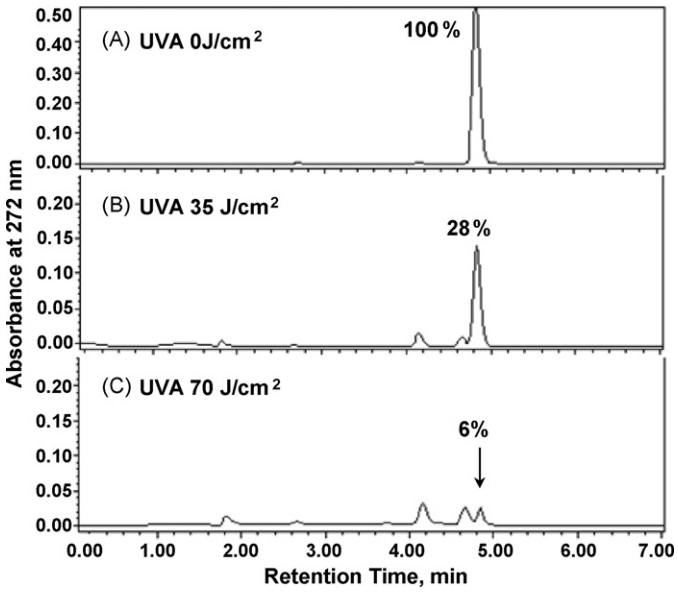

Fig. 6. Reversed-phased HPLC profile of the photodecomposition of guaiazulene (1 mM in ethanol) after irradiation with (A) 0 J/cm^2, (B) 35 J/cm^2, and (C)70 J/cm^2 of UVA light. HPLC analysis was conducted on a Prodigy 5 μm ODS column (4.6 mm × 250 mm) eluted isocratically with 10% water in methanol (v/v) at 1 mL/min.

3.4.2. Detection of superoxide radical anion ($O_2^{\bullet -}$) formation

To determine whether photoirradiation of azulene with UVA light generates superoxide radical anion, BMPO, a trapping agent that efficiently traps superoxide radical anion [21], was employed. Photoirradiation of BMPO with UVA light, in the absence of azulene, did not result in an ESR signal (data not shown). In addition, no ESR signal was observed when a solution of azulene was mixed with BMPO in the absence of UVA light (data not shown). However, an ESR signal was observed after photoirradiation of azulene (1 mM) and BMPO (20 mM) with UVA light and the intensity of the signal increased with longer irradiation time (Fig. 7B, curve a). The ESR spectral profile is identical to that produced from the reaction of xanthine with xanthine oxidase in the presence of BMPO reported previously by Xia et al. [18], indicating that BMPO-$^{\bullet}$OOH adducts were formed and detected by ESR-spin trapping methods [21]. The ESR signal intensity also correlates with concentration of azulene as shown in Fig. 7B, curve b and Fig. 7B curve c using 2 and 3 mM of azulene, respectively. These results indicate that superoxide radical anion was generated from photoirradiation of azulene with UVA light.

4. Discussion

Photoirradiation of azulene or guaiazulene in the presence of a lipid, methyl linoleate, induced lipid peroxidation, suggesting that reactive oxygen species (ROS) are formed. In the present study, this is first determined by incorporation of a free radical scavenger, sodium azide or superoxide dismutase, to the reaction system. Then we employ an ESR-spin trap technique to provide direct evidence as to whether photoirradiation of azulene by UVA light produces ROS. BMPO is a specific probe for superoxide. When photoirradiation of azulene was conducted in the presence of BMPO, ESR signal profiles specifically for BMPO-$^{\bullet}$OOH [18] were obtained. These results unambiguously confirmed the formation of superoxide radical anion. Additional studies with reduction of lipid peroxidation formation by addition of superoxide dismutases on the UVA photoirradiation of azulene (Fig. 4A). On the other hand, photoirradiation of azulene by UVA in the presence of TEMP, a spe-

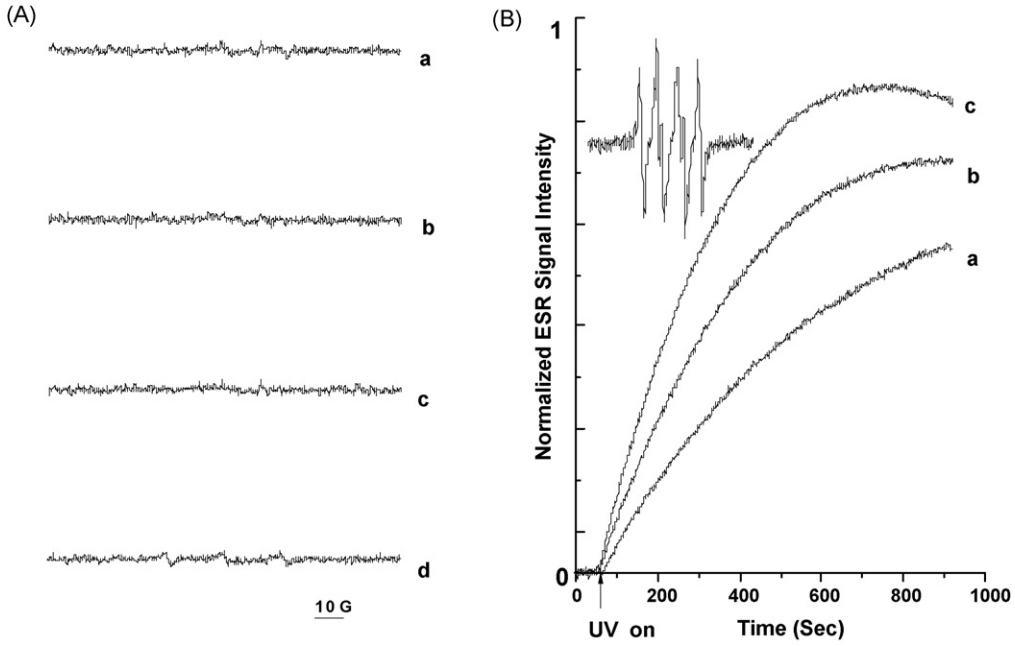

Fig. 7. Panel A: Signet oxygen formation from photoexcited azulene (300–360 nm). A sample containing a mixture of 5 mM TEMP (90% CH_3CN in water) with: (a) no azulene with light for 10 min; (b) 3 mM azulene without light for 10 min; (c) 3 mM azulene with light for 10 min; and (d) 10 mM of azulene with light for 10 min. Panel B: Generation of superoxide anion from photoirradiation of azulene under UVA light in a time and azulene concentration dependent manner. ESR spectra were recorded at room temperature. Samples containing 20 mM BMPO and (a) 1 mM, (b) 2 mM, and (c) 3 mM of azulene were irradiated with UVA light at 340 nm. The insert is the CW ESR spectrum of BMPO-OOH.

cific probe for singlet oxygen, resulted in the formation of TEMPO at an extremely low level only when azulene at a very high concentration (10 mM) was used.

The ease of superoxide formation can be attributed to the fact that azulene and guaiazulene are both extremely unstable photochemically. This can contribute to the formation of superoxide rapidly under UVA irradiation as compared to the formation singlet oxygen. Both compounds can absorb UV and visible light. Upon light absorption, the molecule is excited to upper energy states. The excited state molecules can often undergo a series of reactions leading to cellular damages that are usually not possible at ground state [22].

It is worthy to note that none of the radical inhibitors used in the investigation were able to completely suppress the photosensitized peroxidation of methyl linoleate in the presence of azulene or guaiazulene. These results clearly indicate that, beside the generation of superoxide and single oxygen, other free radicals, such as carbon-centered radicals and peroxyl radicals, are also formed and capable of inducing lipid peroxidation. Indeed, upon UVA irradiation in the presence of molecular oxygen, carbon-centered radicals and peroxyl radicals can be generated from methyl linoleate, azulene, and guaiazulene. We previously reported that UVA photoirradiation of methyl linoleate resulted in the formation of methyl linoleate 9-hydroperoxides and methyl linoleate 13-hydroperoxides mediated by methyl linoleate carbon-centered radicals and peroxy radicals [23]. In this present article, we determined that both azulene and guaiazulene were photodecomposed upon UVA light irradiation. As shown in Fig. 6, there are several HPLC peaks formed from UVA irradiation of azulene, indicating that azulene was photodecomposed into photodecomposition products. The formation of these photodecomposition products is probably mediated by azulene-derived peroxyl radicals. Selco et al. [24] reported that photoirradiation of azulene generated polyoxygenated compounds. Fiori et al. [25] studied the photostability of guaiazulene under UVA irradiation and determined that oligomeric polyoxygenated compounds were formed. On the basis of their findings, the photodecomposition products formed from UVA irradiation of azulene and guaiazulene in our study should be oligomeric polyoxygenated compounds as well.

A mechanism of UVA photoirradiation of azulene leading to formation of free radicals and induction of lipid peroxidation is proposed (Fig. 8). For photosensitization through ROS formation, photoexcited azulene (AZ*) serves as the initiating species and is found in both the excited singlet state (^{1}AZ*) and excited triplet state (^{3}AZ*). Based on the experimental results, the excited singlet and/or triplet state of azulene transfer an electron to molecular oxygen to generate superoxide radical anion (Type I mechanism), and itself becomes a radical cation. Under the experimental conditions employed in our study, although both superoxide radical anion and singlet oxygen can initiate lipid peroxidation, the pathway involving the transfer of energy from the excited azulene to molecular oxygen to generate singlet oxygen ($^1O_2$) (Type II mechanism) is not a favored process in comparison to the electron-transfer initiated superoxide formation [26–28].

In general, lipid peroxidation generates free radicals, which can cause DNA damage, form DNA adducts, and are tumorigenic in experimental animals [10,29–32]. Lipid peroxidation in human

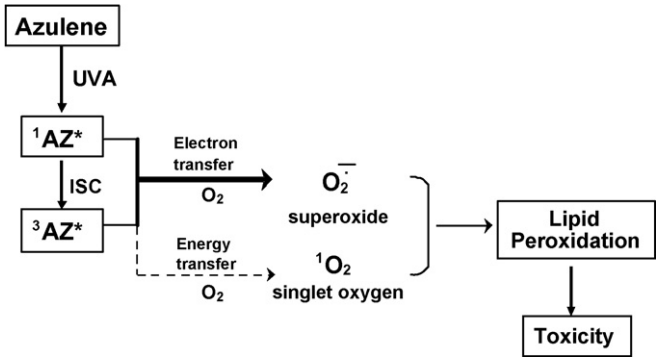

Fig. 8. Proposed mechanism of azulene-induced lipid peroxidation and associated reactions when irradiated by UVA.

has also been associated with many diseases including cancer, athereosclerosis, ischemia, inflammation, liver injury, aging, etc. [5,33–37]. Conceivably, human concomitantly exposed to azulene (or guaiazulene) and UV light may initiate lipid peroxidation and thus, result in significant biological consequences.

On the basis of our present results, people use cosmetics containing azulene and guaiazulene with concomitant exposure to sunlight may result in deleterious effects if the level of these ingredients is high and if sunlight exposure is long. To ensure safe use of cosmetics containing azulene or guaiazulene, more tests on animals and ultimately on humans are warranted.

Acknowledgments

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