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Inhibitory property of the *Piper betel* phenolics against photosensitization-induced biological damages

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Abstract—The *Piper betel* phenolics, allylpyrocatechol (APC) and chavibetol (CHV), were found to protect photosensitization-mediated lipid peroxidation (LPO) of rat liver mitochondria effectively, APC being significantly more potent. The better activity of APC *vis-à-vis* CHV could be attributed to its higher reactivity with $^1O_2$, as revealed from the rate constant values of $^1O_2$ quenching by the respective phenolics. APC also prevented the detrimental effects of the Type II photosensitization-induced toxicity to mouse fibroblast L929 cells. The results suggest that APC may play an important role in protecting biological systems against damage, by eliminating $^1O_2$ generated from certain endogenous photosensitizers.

1. Introduction

Photosensitization is a widely occurring phenomenon in biological systems due to the ubiquitous nature of visible light and a number of endogenous and exogenous compounds which can act as photosensitizers.[1] The potentially damaging event is primarily triggered due to the generation of various radical and non-radical reactive oxygen systems (ROS). Generation of free radicals in skin by solar ultraviolet light accelerates skin cancer, photoaging, and other light-related skin pathologies.[2] Cellular exposure to UV light also leads to iron release resulting in excessive production of ROS and ultimately to pathogenesis.[3] Photosensitization of the skin during drug therapy is a very well-known and frequently reported effect.[4] Several classes of commonly used chemicals are able to react with the UVA/UVB photons yielding stable toxic products or producing free radicals.[5] Therefore, there is a need to develop suitable formulations that can prevent photo-induced biological damages. To this end, exploration of edible herbs/plants that are also credited with various medicinal attributes might be useful from the point of view of low cost and less toxicity.

The *Piper betel* plant is widely growing in the tropical humid climate of South East Asia, and its leaves, with a strong pungent and aromatic flavor, are widely consumed as a mouth freshener. The leaves are credited with wound healing, digestive, and pancreatic lipase stimulant activities in the traditional medicine.[6] During our exploration of non-toxic and affordable herbal medicinal formulations, the *P. betel* leaf extract and its constituent phenolics were found to show impressive gastroprotective,[7] and anti-inflammatory as well as immunomodulatory properties.[8]

Recently, we have also found that the *P. betel* leaf extract can prevent the photosensitization-induced damages to lipids and proteins.[9] Hence, the present work was undertaken with the primary aim to: (i) investigate whether its major antioxidant[10] phenolics, chavibetol (CHV), allylpyrocatechol (APC), are responsible for the activity, (ii) rationalize the differential potency of CHV and APC mechanistically, and (iii) evaluate the protecting efficacy against photosensitization-induced cellular damage. The chemical structures of CHV and APC are shown in Figure 1.

Keywords: Cells; Lipids; Photosensitization; Protection.
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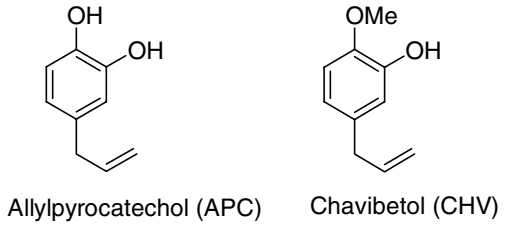

Figure 1. Chemical structures of chavibetol (CHV) and allylpyrocatechol (APC).

2. Results

2.1. Prevention of photosensitization-induced lipid peroxidation by CHV and APC

The individual protective capacity of CHV and APC against photo-induced peroxidation of rat liver mitochondria was studied by assaying the TBARS and LOOH formed during the lipid peroxidation (LPO). Our studies showed that exposure of mitochondria to methylene blue (MB) plus light led to enhanced formation of TBARS as a function of illumination time (data not shown). Exposure of mitochondria to photo-irradiation for 0.5 h led to a significant ($p < 0.001$) increase in the TBARS concentration (12.81 ± 0.22 nmol/mg protein) as against the control value (2.29 ± 0.05 nmol/mg protein) without photo-exposure.

When the mitochondria were irradiated for 0.5 h in the presence of various concentrations (30, 60, and 180 μM) of APC or CHV, significant protection was observed. Even at a very low concentration (30 mM), APC showed measurable (~32%, $p < 0.01$) prevention in TBARS formation, which increased in a concentration-dependent manner. The protections offered by 60 and 180 μM of APC were ~48% ($p < 0.01$) and 86% ($p < 0.001$), respectively. The protective capacity of CHV also showed a similar trend, although it was less effective than APC at all the test concentrations. For example, CHV (30, 60, and 180 μM) could prevent ~22% ($p < 0.05$), 40% ($p < 0.01$), and 72% ($p < 0.001$) of photo-induced LPO. Under similar conditions, the protections offered by the positive controls, sodium azide (10 mM) and vanillin (2 mM), were 73% and 34%, respectively. The results with CHV and APC are summarized in Figure 2a. The protections offered (for a 30 min photo-exposure) by some other known antioxidants under similar conditions are shown in Table 1.

Photo-irradiation of the rat liver mitochondria expectedly led to the increased formation of LOOH, which, however, was prevented by CHV and APC. Following photo-irradiation for 0.5 h, the LOOH concentration increased more than 2-fold ($p < 0.001$). This was revealed from the increase in the absorbance at 560 nm from 0.032 ± 0.003 (unirradiated control) to 0.078 ± 0.009 (after photo-irradiation). Addition of APC, CHV (each 180 μM) or sodium azide (10 mM) to the mitochondria prior to photo-irradiation reduced the absorbance at 560 nm to 0.010 ± 0.003, 0.030 ± 0.005, and 0.039 ± 0.006, respectively. These corresponded to

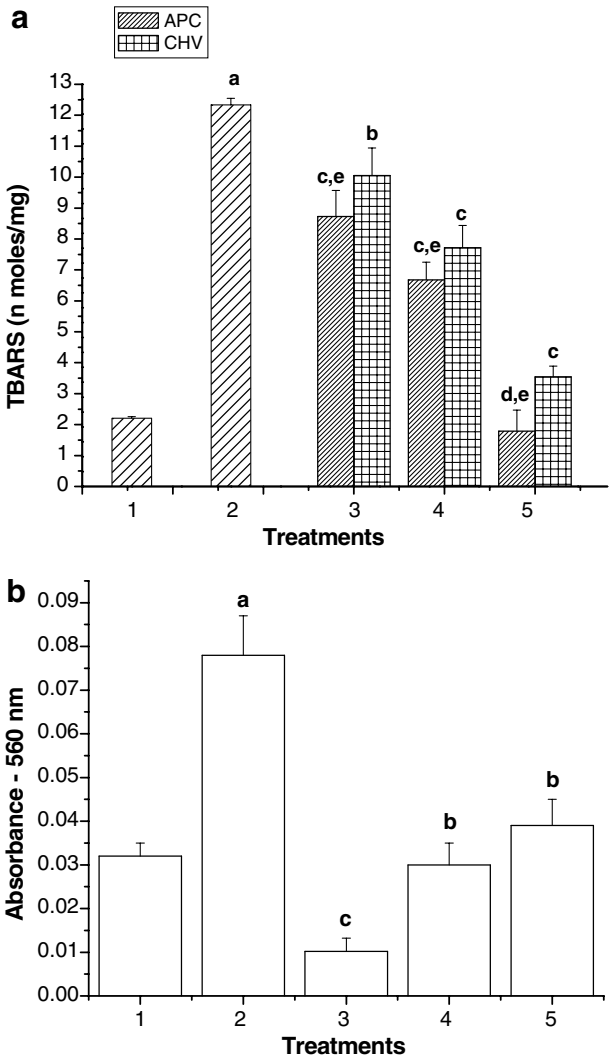

Figure 2. (a) Photosensitization-induced formation of TBARS in rat liver mitochondria and prevention by CHV and APC. A mixture of mitochondrial protein (0.5 mg/mL), methylene blue (50 μg/mL) without and with different concentrations of CHV or APC was incubated for 30 min and the amounts of TBARS were estimated. The values are means ± SEM ($n = 5$). 1, control mitochondria; 2, mitochondria + MB + hν; and 3–5, mitochondria + MB + hν in the presence of CHV or APC (30, 60, and 180 μM, respectively). [a]$p < 0.001$ compared to normal; [b]$p < 0.05$, [c]$p < 0.01$, [d]$p < 0.001$ compared to photo-irradiated experimental control; [e]$p < 0.05$ compared to CHV treatment. (b) Photosensitization-induced formation of LOOH in rat liver mitochondria, as revealed from the absorbance at 560 nm and its prevention by APC, CHV (each 180 μM) and sodium azide (10 mM). A mixture of mitochondrial protein (0.5 mg/mL), methylene blue (50 μg/mL) without and with the test samples was incubated for 30 min and the amounts of LOOH were estimated. The values are means ± SEM ($n = 5$). 1, control mitochondria; 2, mitochondria + MB + hν; and 3–5, mitochondria + MB + hν in the presence of APC, CHV or NaN_3, respectively. [a]$p < 0.001$ compared to normal; [b]$p < 0.01$, [c]$p < 0.001$ compared to photo-irradiated experimental control.

reduction in LOOH formation by 87% ($p < 0.001$), 61.5% ($p < 0.01$), and 50% ($p < 0.01$), respectively, compared to the photo-irradiated sample. The formation of LPO by photosensitization of rat liver mitochondria, and its prevention by APC, CHV, and sodium azide are shown in Figure 2b.

Table 1. Protective activities[a] of some known antioxidants against photosenstitization-induced oxidation of rat liver mitochondria

Antioxidant (concn)	% Protection[b] against lipid peroxidation
Mannitol (100 mM)	20.1 ± 2.12
Glutathione (10 mM)	35.6 ± 4.44
Ascorbic acid (10 mM)	25.7 ± 3.26
SOD (1000 U/assay)	8.4 ± 0.60

[a] The values are for photo-exposure for 30 min.
[b] The values are means ± SEM ($n = 5$).

2.2. Singlet oxygen scavenging activity of APC and CHV

The luminescence of singlet oxygen at 1270 nm decreased progressively in the presence of increasing concentrations of CHV and APC. A plot of the inverse of the lifetime of singlet oxygen against the concentrations of the test compounds showed excellent linearity (Fig. 3a and b). The rate constant for total (physical and chemical) $^1O_2$ quenching (k_Q) by a quencher Q (CHV, APC or NaN_3) was determined by the Stern–Volmer equation: $k_{obs} = k_d + k_Q$ [Q], where k_{obs} and k_d are the decay rate constants of $^1O_2$ phosphorescence, measured in the presence and absence of CHV and APC, respectively. Under similar conditions, the rate constants for CHV, APC, and the positive control, NaN_3 were 5.36×10^6, 8.42×10^6, and 2.41×10^8 M^{-1} s^{-1}, respectively.

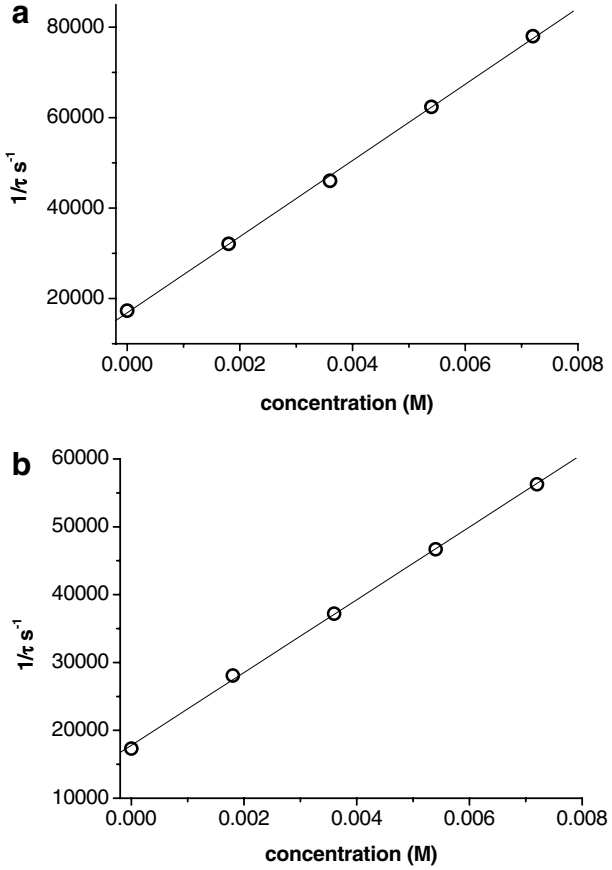

Figure 3. Stern–Volmer plot for the reaction $^1O_2$ with (a) APC and (b) CHV in acetonitrile.

2.3. Steady-state photolysis of APC

The steady-state photolysis of APC in the presence of visible light + MB, but not with visible light alone, led to its degradation as revealed from the HPTLC analyses. The extent of degradation increased in a time-dependent manner. As shown in a typical HPTLC profile (Fig. 4) the photolysis of APC led to the formation of three major products together with several minor ones. Two of these products were identified as **1** and **2** and their structures are shown in Figure 5. The photosensitized degradation was not noticed under an aerobic condition and was markedly suppressed by sodium azide (data not shown).

2.4. Cytotoxicity of APC against L929 cells

The cytotoxicity of APC (5, 10, and 20 μM) against the L929 cells following incubation of the cells for 20 h was evaluated by the MTT method.[11] The preincubation time (20 h) of the L929 cells was chosen based on a time-dependent trial study (data not shown). APC did not show any cellular toxicity at 5 and 10 μM concentrations. However, at a concentration of 20 μM, it showed ~25% cell killing. For carrying out the photocytotoxicity experiments, the cytotoxicity of MB (9 and 18 μM) against the L929 cells was also determined. It was found that MB (9 μM) was non-toxic to the cells, while its toxicity even at a higher concentration (18 μM) was also

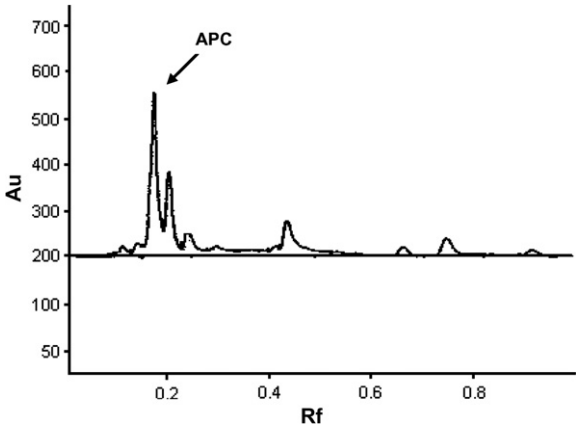

Figure 4. A typical HPTLC chromatogram of the products, obtained after photolysis of APC for 4.5 h in the presence of MB. The HPTLC analysis was carried out using silica gel plates using 20% EtOAc/hexane as the solvent. The products were detected at 310 nm.

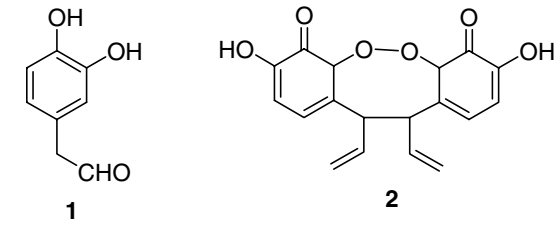

Figure 5. Chemical structures of two of the products formed by the reaction of $^1O_2$ with APC.

Table 2. Evaluation of cytotoxicity of different concentrations of APC and MB

Treatment	% Survival[a]
Control L929 cells	100.2 ± 3.2
APC (5 μM)-treated L929 cells	96.2 ± 2.8
APC (10 μM)-treated L929 cells	96.5 ± 5.4
APC (20 μM)-treated L929 cells	75.7 ± 3.5[b]
MB (9 μM)-treated L929 cells	102.2 ± 2.6
MB (18 μM)-treated L929 cells	95.3 ± 4.3

[a] The L929 cells were treated with different concentrations of APC or MB and the % survival was determined by the MTT assay. The values are means ± SEM ($n = 5$).
[b] $p < 0.05$.

marginal (5%). Hence these were chosen for the further experiments. The results are summarized in Table 2.

2.5. Prevention of photocytotoxicity of L929 cells by APC

For this, the effect of the light plus MB combination on the proliferation of L929 cells in the presence of APC was investigated by the MTT assay. The experiments were carried out at three photo-exposure periods (5, 10, and 15 min) using two different concentrations of APC (5 and 10 μM) and MB (9 and 18 μM). It was found that with 9 μM MB, the damage was less (14–21%) even for a photo-exposure period of 15 min. The protection offered by APC under this condition was measurable (6–14%), but not very significant. However, survival of the L929 cells reduced progressively on increasing the photo-exposure time in the presence of MB (18 μM). For example, the percentages of cell survival were ~67.6, 64.4 ($p < 0.05$), and 48.9 ($p < 0.01$), respectively, for the photo-exposure of 5, 10, and 15. Preincubation of the cells with APC (5 and 10 μM) improved the cell survival over the entire photo-exposure period significantly, better protection being observed with 10 μM of APC. The survival of the L929 cells incubated with APC (10 μM) ~94.2%, 92.2%, and 82.9% during photo-exposure of 5, 10, and 15 min. The corresponding figures for the APC (5 μM)-pretreatment were ~89.1%, 88.3%, and 85.4%, respectively. The results (Fig. 6) revealed that compared to the photo-irradiated, untreated cells, those treated with APC had significantly better viability for photo-exposure up to 10 ($p < 0.05$) and 15 min ($p < 0.001$). However, when the cells were pretreated with a higher concentration APC (20 μM), the percentages of cellular survival were less (data not shown). This may be attributed to the cytotoxicity of APC at the higher concentration. Under the same condition, vanillin (50 μM) increased the cell viability by only 12.5% for a photo-exposure up to 15 min.

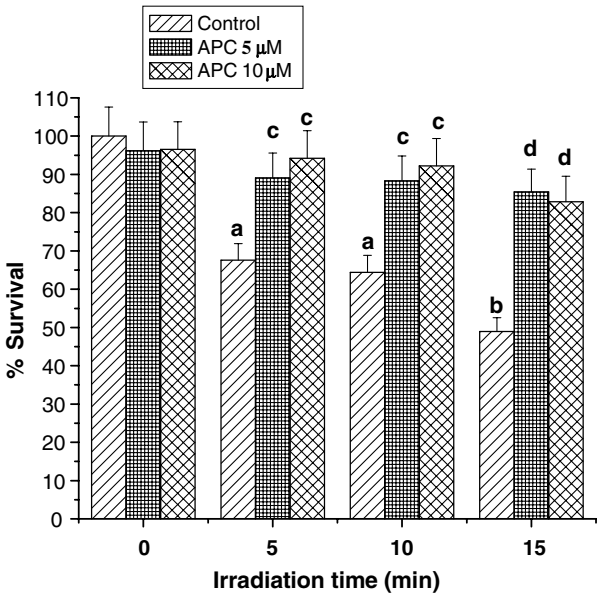

Figure 6. Time-dependent photosensitization-induced killing of L929 cells and its prevention by APC. The L929 cells (2×10^4 cells per well) in serum free RPMI solution containing APC (0–10 μM) were irradiated for 0–15 min with visible light in the presence of MB (18 μM). Following incubation in FCS for 20 h, the cell viability was determined. The values are means ± SEM ($n = 5$). [a]$p < 0.05$, [b]$p < 0.01$ with respect to unirradiated cells; [c]$p < 0.05$, [d]$p < 0.001$ with respect to the respective irradiated, but non-treated, cells.

3. Discussion

The photosensitization generates several reactive oxygen species (ROS) via type I (superoxide radicals) and predominantly type II (singlet oxygen, $^1O_2$)[12] processes leading to damages to various sub-cellular structures and molecules.[13,14] Among these, singlet oxygen, produced under various normal and pathophysiological conditions,[15] causes maximum damage to membrane lipids, DNA, etc.,[16] due to its relatively long half-life in the cellular milieu. The cellular membrane can play an important role in the photosensitization. In particular the peroxidation of polyunsaturated fatty acids, which are extremely sensitive to ROS, has been suggested as the primary event that, through chain reactions, triggers metabolic pathways leading to inflammatory response and to cell death.[14,17,18] Products of lipid oxidation can activate membrane phospholipases[19,20] and can induce alterations of the membrane lipid packing, favoring the penetration of water molecules inside the bilayer.[21] The induced alterations in membrane permeability, transport systems, loss of membrane bound enzymes, etc., can eventually lead to cell lysis and death under specific conditions.[22] Adverse effect of photosensitization to specific carriers for succinate, citrate, and oxalacetate has been reported.[23]

Very recently, we have identified[10] CHV and APC as the major antioxidants of the *P. betel* leaf extract, which also showed protecting activity against photosensitization.[9] However, this does not guarantee protective efficacy of CHV and APC against photosensitization, since different ROS are involved in the latter process. Consequently, in the present study, the capacity of CHV and APC in preventing photosensitization-induced LPO in rat liver mitochondria and scavenging $^1O_2$ was assessed. Subsequently, the study was extended to cellular system using the most active compound, APC. Using methylene blue as the sensitizer, the extent of oxidation of lipids by photosensitization was investigated by measuring the amounts of TBARS formed in

the absence and presence of CHV and APC. Methylene blue (MB) is an ideal photosensitizer for the investigation as it does not interfere with spectrophotometric measurement of microsomal lipid peroxidation. The combination of MB, light, and oxygen mainly generates $^1O_2$ and can induce oxidative damage in membrane.[22] It was found that while both CHV and APC inhibited the TBARS formation in a concentration-dependent manner, the efficacy of APC was significantly better ($p < 0.05$). Given that the TBA assay is fairly non-specific, the lipid-protecting capacities offered by a fixed concentration (180 μM) of the test compounds were also assayed by measuring LOOH, the relatively unstable product of LPO. Addition of the compounds to the mitochondria prior to photo-irradiation could inhibit the formation of LOOH very effectively. Lipid peroxidation is a complex multistep process wherein the initially formed lipid radicals get converted to TBARS via the intermediacy of LOOH and other products. CHV and APC could efficiently prevent the photosensitization-induced LPO at its various stages, as is revealed from our results.

Given that sodium azide a specific $^1O_2$ scavenger could prevent the photosensitization-induced LPO, the involvement of $^1O_2$ in the process was apparent. Our studies on the $^1O_2$ scavenging activity of CHV and APC also confirmed this. Kinetic measurements revealed that APC was ~1.5-fold more efficient than CHV in scavenging $^1O_2$. Earlier, APC was found to scavenge the superoxide radicals more effectively than CHV.[10] These factors, taken together, might account for their relative efficacy in preventing the photosensitization-induced LPO.

The steady-state photolysis experiments showed that irradiation of APC with visible light in the presence of MB led to its time-dependent depletion. The process required an aerobic condition and was markedly suppressed by sodium azide (data not shown). All these data confirmed that the photosensitized degradation of APC is mediated by $^1O_2$. Formation of a number of degradation products in the reaction (as revealed by the HPTLC analysis) suggested that the degradation proceeds through a complex process. One of the degradation products, **1**, is produced by an expected oxidative cleavage of the olefinic function of APC, while **2** is formed by dimerization of two APC molecules at the allylic position, followed by oxygenation of the dimeric product at the *ortho*-position of the phenolic moieties.

The chemical reaction with $^1O_2$ appears to contribute a large share to the process of $^1O_2$ quenching by APC. For the present, however, there is no evidence against a possible involvement of physical quenching of $^1O_2$ in the APC–$^1O_2$ interaction. Thus, the measured rate constant may actually be for the total reaction, which includes the physical quenching, if any, in addition to the chemical quenching.

Subsequently, we extended the work to cellular system wherein the protective activity of the best candidate, APC, against the photosensitization-induced damage to L929 cells was assessed. As such APC was non-toxic to the mouse fibroblasts L929 cells up to a concentration of 10 μM, although it showed toxicity at 20 μM concentration. The photosensitization-mediated cell killing could be prevented very efficiently by APC at very low concentrations, while another naturally occurring phenolic, vanillin, was much less effective. Interestingly, the protective effect of APC was more pronounced at a higher concentration of MB. UV screening by APC has apparently no connection with its protective effect against photosensitization since the investigations were conducted using light devoid of UVB (APC absorbs only in the UVB region, 204 and 280 nm). There can be little doubt that APC quenches $^1O_2$ with a high reactivity and thus removes the ROS efficiently from biological systems, protecting them under photosensitization conditions.

It is worth mentioning that the fungicidal and nematocidal activity of *P. betel* and allergic reaction of eugenol has been reported earlier.[24] Also, reactive oxygen species (ROS) are crucial for hydroxychavicol toxicity toward κB epithelial cells.[25] Possibly, the toxicity of APC depends on the cell types explaining our results. Earlier, we have found APC to be strong scavenger of various ROS in cell free systems.[10] Our results are consistent with previous reports where catechols were found to produce less ROS compared to other benzene diols.[26] In our previous studies, we did not observe any side effect of the whole *P. betel* extract or its constituents (APC and CHV) to rats up to a dose of 0.5 g/kg body weight.[7,10,27]

Overall, the present studies revealed its high potency as an in vitro antioxidant against photosensitization-induced biological damages. All these results suggested its possible use as a natural, non-toxic protector against photosensitization-induced oxidative biological damages. Currently, there is a growing interest of the plant-based or herbal medicines all over the World. However, lack of proper quality control of these often leads to spurious drugs. Variation in the profile/concentrations of the active principles in the plant-based or herbal preparations primarily contributes to this. It is well established that factors such as habitation, time of collection, maturity of the plants, etc., affect the concentrations of their bioactive chemical constituents. Hence, for a proper quality control of the plant-based drugs, it is essential to identify the bioactive constituents. From this perspective, the identification of APC and CHV as the active principles for the preventive property of *P. betel* leaf extract against photosensitization is extremely important. The results suggested that to promote *P. betel* as an effective antioxidant, it is essential to assay the content of the CHV and especially APC in the samples.

4. Experimental

4.1. Materials

Following our reported procedure APC and CHV were isolated from the methanol extract of *P. betel* leaves and

fully characterized.[10] Ascorbic acid and 2-thiobarbituric acid (TBA) (both from Himedia Lab. Pvt. Ltd, India), ethylenediamine tetraacetic acid (EDTA) (E. Merck, India), trichloroacetic acid (TCA) (Thomas Baker, India), and potassium phosphate, sodium azide, KOH, and HCl (all from SRL, India) were used. Tetraethoxypropane, guanidine hydrochloride, glutathione, α-tocopherol, Superoxide dismutase (SOD) (type I, from bovine liver, sp. Act. 26,000 U/mg protein), methylene blue (MB), hematoporphyrin, RPMI 1640, thizolyl blue tetrazolium (MTT), fetal calf serum (FCS), and penicillin were obtained from Sigma Chemicals, USA. All other reagents were of analytical grade. Mouse fibroblasts L929 cells were obtained from National Centre for Cell Science (NCCS), Pune, India.

Stock solutions of ascorbic acid and EDTA were prepared in deaerated water just prior to use. The test sample was used as an aqueous solution containing 0.1% ethanol. All solutions were made with triply distilled water.

4.2. Animals and preparation of mitochondria

The rats were bred in the BARC Laboratory Animal House Facility and procured after obtaining clearance from the BARC Animal Ethics Committee. All the experiments were conducted with strict adherence to the ethical guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India on the use of animals in scientific research. Mitochondria were isolated from male Wistar rats weighing 250 ± 20 g as described earlier.[27]

4.3. Photosensitization-induced lipid peroxidation assay

The system for exposing mitochondria to photosensitization was simple, physiologically relevant, and similar to that described earlier.[9] In brief, the reaction mixture (final volume 1.0 mL) containing mitochondrial protein (final concentration 0.5 mg/mL), MB (50 μg/mL), and APC or CHV (0–180 μM, final concentration) in KH_2PO_4–KOH buffer (pH 7.4) was kept in a trap maintained at 37 °C and irradiated for 30 min with a 100 W tungsten lamp. The distance from the light source and the trap was 15 cm. The light intensity of the system, as measured by a Lux meter, was calculated to be 32.45 W m^{-2}.

The light-induced lipid peroxidation of the rat liver mitochondria was assayed by the TBA method described elsewhere.[28] For estimating the thiobarbituric acid reactive substrates (TBARS), the reactants were heated for 15 min on a boiling water bath with the TBA reagent (0.5% TBA/10% TCA/6 mM EDTA/0.63 M HCl). After cooling, the precipitate formed was removed by centrifugation at 1000*g* for 10 min. The absorbance of the sample was determined at 532 nm against a blank that contained all the reagents except the mitochondria. Malonaldehyde standard was prepared by the acidic hydrolysis of tetraethoxypropane. For correction of endogenous TBARS, fresh samples were boiled without photo-irradiation, and the values were subtracted. Various antioxidants were used as the positive controls. The lipid hydroperoxide (LOOH) was assayed as described earlier.[27]

4.4. Assay of singlet oxygen scavenging by CHV and APC

Singlet oxygen was produced by photosensitization of hematoporphyrin (120 μM, OD at 532 nm 0.521) in acetonitrile, and its luminescence at 1270 nm in the absence and presence of various concentrations of CHV and APC was detected with a transient luminescence spectrometer model TL 900 (Edinburgh Instr., UK). Hematoporphyrin was excited using the pump beam from an Nd-YAG-pumped optical parametric oscillator laser (5 ns full-width half-maximum pulse), and the signals were passed through a cutoff filter.

4.5. Assay of photosensitized degradation of APC

A solution of APC (0.13 M) and MB (100 mM) in water (containing 0.1% methanol) was exposed to visible light as above. Aliquots were taken from the samples at different intervals of irradiation and subjected to high-performance thin layer chromatography (HPTLC) on a silica gel plate C18 analytical column (YMC, 25034.6 mm id) using 20% EtOAc/hexane as the solvent. The extent of APC degradation was measured by a decrease in the area of APC peak measured at 280 nm.

The preparative scale photolysis was carried out for 24 h in 4 sets each containing APC (10 mg), the other conditions remaining same. The reaction mixture was extracted with $CHCl_3$ (3× 5 mL), the combined organic extracts were dried and concentrated in vacuo. The residue was subjected to preparative thin layer chromatography (silica gel, 10% EtOAc/hexane) to isolate **1** and **2**.

4.5.1. 2-(3,4-Dihydroxyphenyl)ethanal (1). IR: 2720, 1720 cm^{-1}; ^1H NMR: δ 4.20 (d, J = 2.0 Hz, 2H), 5.35 (br s, 2H), 7.26–7.36 (m, 1H), 7.51–7.56 (m, 1H), 7.69–7.78 (m, 1H), 9.98 (t, J = 2.0 Hz, 1H); MS (*m*/*z*, rel. int.): 152 (M^+, 48), 125 (100).

4.5.2. 3,4-Bis[4-hydroxy-5-oxo-6-peroxocyclohexa-1,3-dienyl]hexa-1,5-diene (2). IR: 3443, 1698 cm^{-1}; ^1H NMR: δ 3.23–3.32 (m, 2H), 3.77 and 3.82 (two s, 2H), 4.96–5.11 (4H), 5.81–5.92 (m, 2H), 6.43–6.62 (m, 2H), 6.77–6.85 (m, 2H); MS (*m*/*z*, rel. int.): 327 ($M+1^+$, 16), 295 (18), 162 (100).

4.6. Cell line maintenance and evaluation of cytotoxicity of APC

The L929 cells were routinely seeded at a density of $0.1–3 \times 10^6$ cells/mL and grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. Cells were passaged every 3–4 d so as to maintain the cell density below 0.4×10^6 cells/mL. The cell density and viability were determined by the Trypan blue dye exclusion test. Subcultures were obtained by trypsinization (0.25% trypsin in PBS). The cells were discarded after 3 weeks of subculture.

The cytotoxic effect was evaluated by the conventional MTT dye reduction assay.[11] The L929 cells (2×10^4 cells per well) were grown in 96-well microtiter plates for 2 h in serum free RPMI solution (180 μL) containing APC (0, 5, 10, and 20 μM). Fetal calf serum (20 μL) was added to the cells and incubated for another 20 h. Subsequently, the medium was removed, and the cells were washed once with PBS. The cell viability was determined. The cytotoxicity of MB (9 and 18 μM) was also evaluated to decide its concentration to be used for the photosensitization experiment.

4.7. Prevention of photocytotoxicity by APC

The L929 cells (2×10^4 cells per well) grown as above in 96-well microtiter plates in the absence or presence of APC (5 and 10 μM) were treated with MB (9 or 18 μM) in PBS (200 μL) and incubated for 2 h. After removal of MB solution, the cells were washed once with PBS (200 μL), and 100 μL PBS was added. The microplate was covered with a glass plate and irradiated with a 100 W tungsten lamp at room temperature for different times (0, 5, 10, and 15 min). Subsequently, PBS solution was removed and the cells were incubated with freshly added RPMI solution containing 10% FCS (200 μL) for 72 h. To determine cell toxicity, the medium was removed by aspiration and 100 μL of PBS containing MTT (0.5 mg/mL) was added to each well. After 4 h, 100 μL of SDS solution (10% SDS in 0.01 M HCl) was added to the cells, kept at 37 °C for another 12 h and the absorbance at 550 nm was read using a spectrophotometric plate reader (Bio-Tek, USA).[11]

5. Statistical analysis

The data were analyzed using a paired *t*-test for the paired data or one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparisons post test. In addition, Bonferroni correction was also carried out for knowing the simultaneous statistical inference among the groups under investigations. The IC_{50} values of the test samples were estimated using the Probit analysis and the significance level of the analyses was also investigated by the chi-square test. A probability value of $p < 0.05$ was considered significant.

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