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Antioxidant Activity of *Piper betel* Leaf Extract and Its Constituents

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The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay of the ethanol extracts of three varieties (Bangla, sweet, and Mysore) of *Piper betel* (pan) revealed the Bangla variety to possess the best antioxidant activity that can be correlated with the total phenolic content and reducing powers of the respective extracts. Column chromatography of the extract of the Bangla variety led to the isolation of chevibetol (CHV), allylpyrocatechol (APC), and their respective glucosides. The HPTLC analyses of the extracts revealed similar chemical profiles in all three *P. betel* varieties, although the concentrations of CHV and APC were significantly less in the sweet and Mysore varieties. Among the isolated compounds, APC showed the best results in all the in vitro experiments. It could prevent Fe(II)-induced lipid peroxidation (LPO) of liposomes and rat brain homogenates as well as γ-ray-induced damage of pBR322 plasmid DNA more efficiently than CHV. The superior anti-LPO and radioprotective activities of APC vis-à-vis those of CHV could not be explained by their respective Fe(II) chelation and •OH radical scavenging capacities. The better ability of APC to scavenge $O_2^{-\bullet}$ radicals and H_2O_2 might account for the results.

KEYWORDS: Antioxidant; active principle; allylpyrocatechol; *Piper betel*

INTRODUCTION

Oxidative stress has been implicated in the etiology of a number of human ailments (*1*−*3*). Compounds, especially from natural sources, capable of protecting against reactive oxygen species (ROS) mediated damage may have potential application in the prevention and/or cure of the disease (*4*−*6*). To this end, the exploration of dietary plants that are also credited with medicinal properties is promising and might provide functional foods. Traditionally, ethnomedicines are extensively used in India and elsewhere due to their low cost, easy accessibility to everyone, and perceived less side effects (*7*). Currently, there is a growing interest in plant-based or herbal medicines even in the Western world. In many respects, the mechanism of action of the herbal drugs differs from that of the synthetic drugs or pure compounds. This can be characterized as a polyvalent action and interpreted as additive or, in some cases, potentiating. However, one or more active principles in the plant-based or herbal preparations are primarily responsible for their health benefits. Consequently, their presence in optimum amounts is essential to obtain the desired effects of these drugs. Because of the lack of proper quality control, this aspect is often neglected, leading to the availability of spurious drugs. 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 and analyze their contents in them.

Ayurveda, the Indian system of medicine, has been an integral part of Indian culture and materia medica. From the rich Indian biodiversity, it has identified various plants/herbs that have been associated with a number of potential therapeutic efficacies (*8*). *Piper betel* (Vedic name: saptasira) is one such widely growing tropical plant. Its leaves possessing a strong pungent and aromatic flavor are widely consumed as a mouth freshener in Eastern Asia and are traditionally credited with wound healing and digestive and pancreatic lipase stimulant activities (*9*). Earlier, the antiulcerogenic property of the leaf extract was attributed to its antioxidant property (*10*, *11*). In addition, its antimicrobial (*12*), antifungal, and anti-inflammatory (*13*), as well as radioprotective, antioxidative, and immunomodulatory properties have been reported by us and others (*14*−*17*). However, these studies were carried out with nonstandardized crude preparations without identifying the active principles. Thus, the primary aim of the investigation was to identify and quantify the potential antioxidant compounds in different varieties of the *P. betel* leaves that are commonly consumed in India.

MATERIALS AND METHODS

Materials. Fresh leaves of three varieties (Bangla, sweet, and Mysore) of *P. betel* were collected from local markets of Mumbai and Kolkata and identified (collection nos. 2610−2612) by taxonomy by the Botanical Survey of India, Indian Botanical Garden, West Bengal.

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The leaves from each variety were collected from several plants and bulked prior to extraction. Ascorbic acid, ferrous ammonium sulfate, 2-thiobarbituric acid (TBA), NADH, nitroblue tetrazolium chloride (NBT), and 2-deoxyribose were obtained from Himedia Laboratory (Mumbai, India). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), phosphatidylcholine, Folin−Ciocalteau's reagent, and α-tocopherol were purchased from Sigma (St. Louis, MO). H_2O_2 (35%) was from Lancaster (Morecambe, UK), while Fe(III) chloride and trichloroacetic acid (TCA) were from Thomas Baker (Mumbai, India). Other materials used were ethylenediaminetetraacetic acid (EDTA) (Sarabhai Chemicals, Baroda, India), phenazine methosulfate (PMS) (Aldrich, Milwaukee, WI), potassium ferricyanide (BDH, Mumbai, India), phenol red (PR) (S.D. Fine-Chem, Mumbai, India), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (both from BDH, England), and horse radish peroxidase (HRPO) and ethidium bromide (both from SRL, Mumbai, India).

Stock solutions of ferrous ammonium sulfate and EDTA were freshly prepared in water. The test samples were dissolved in 10% aqueous ethyl alcohol. Appropriate blanks were used in all the antioxidant assays. A stock solution of 1% (w/v) TBA was prepared in 50 mM NaOH and used within 1 week. All solutions were made with triple distilled water. Stock solutions of compounds (150 mM) and α-tocopherol (100 mM) were prepared in ethanol, while that of mannitol (50 mM) was in water. The concentrations of the reagents and test samples for all the assays indicate final concentrations. Column chromatography was carried out over silica gel (SRL, Mumbai, India) using AR grade solvents. The studies were carried out using the ethanol extracts of the different varieties of *P. betel* and the compounds isolated by us from them.

Instrumentation. The IR spectra were scanned as thin films with a Jasco model A-202 spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AC-200 (200 MHz) instrument in $CDCl_3$. The chemical shift values in δ (ppm) and coupling constants (J) in a Hertz scale are presented. The HPLC analyses were carried out with a Jasco model PU-2080 plus chromatogram using a Hypersil GOLD (250 mm × 4.6 mm, particle size 5 μm, Thermo Electron Corporation) column, acetonitrile/water (1:1, flow rate 1 mL/min) as the eluent, and peak detection at 254 nm. The absorbance spectrophotometry was carried out at 25 °C using a Jasco V-550 UV−vis spectrophotometer. Wavelength scans and absorbance measurements were made in 1 mL quartz cells of 1 cm path length.

Preparation of the Plant Extracts and Their HPTLC Analyses. The air-dried leaves of the three varieties of *P. betel* (each 250 g) were chopped into fine pieces and soaked in 95% ethanol (1 L) for 2 days, and the supernatant was decanted. The entire process was repeated three times, and the combined extracts were filtered through a nylon mesh and evaporated in vacuo. Each of the extracts (∼8.0 g) was dissolved in methanol (50 mL) and treated with activated charcoal (0.2 g), and the mixture was warmed at ∼60 °C. After filtration, the extracts were concentrated in vacuum and finally lyophilized to obtain chlorophyll-free amorphous yellowish brown solids that were stored in a vacuum desiccator. These were designated as *P. betel* Bangla, *P. betel* sweet, and *P. betel* Mysore ethanol extracts (PEB, PES, and PEM), respectively, and were obtained in 1.23, 0.68, and 0.96% w/w yields.

The chemical constituents of PEB, PES, and PEM were analyzed by HPTLC (Camag instrument) using silica gel plates ($60F_{254}$, E. Merck, Germany). Given that the constituents were of different polarities, their HPTLC analyses were carried out using different mobile phases. The best resolution for chevibetol (CHV) and allylpyrocatechol (APC) was found with 2:8 ethyl acetate/hexane, while for the glucosides, 3:3:4 toluene/acetone/formic acid was used. The compounds were detected under UV light (254 nm).

Isolation of Chemical Constituents. PEB (4.7 g) was subjected to column chromatography over silica gel using a gradient elution. The column was eluted with 0, 2, 5, and 10% EtOAc/hexane followed by 0, 5, 10, 15, 50, and 100% methanol/chloroform (250 mL each), and 50 mL fractions were collected. The fraction eluting with 2% ethyl acetate/hexane furnished a mixture (0.005% w/w with respect to PEB) of two compounds with very close R_f values. The fractions eluting with 5 and 10% ethyl acetate/hexane, respectively, furnished CHV and APC, which were characterized using IR, ^{1}H and ^{13}C NMR, and mass spectroscopic data. The fraction eluting with 10 and 50% methanol/chloroform furnished two viscous sticky glycosides **1** (0.21%) and **2** (0.45%) as revealed by the Molisch test. The apolar minor fraction and the glycosides did not show any DPPH radical scavenging activity. Hence, these were not fully identified. However, hydrolysis of an aliquot of each of the glycosides (20 mg) with trifluoroacetic acid (5 mL) in methylene chloride (10 mL) at ∼80 °C for 4 h furnished CHV and APC, respectively. The sugar part obtained in the aqueous extract of the previous reactions was analyzed as glucose. Thus, compounds **1** and **2** were the glucosides of CHV and APC. The structures of the isolated compounds are shown in **Figure 1**. The yields reported for all the compounds are with respect to the weight of PEB.

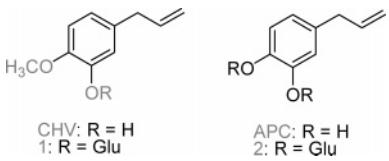

Figure 1. Chemical structures of CHV and APC.

3-Hydroxy-4-methoxyallylbenzene (CHV). Yield, 0.16%; oil; HPLC ret. time: 6.9 min (single peak); IR (film): 3509, 3070, 3003, 1638, 1509, 1350, 1236, 1175, 1129, 995, 865, 760 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 3.28 (d, J = 6.5 Hz, 2H, $ArCH_2$), 3.85 (s, 3H, OCH_3), 5.01−5.09 (m, 2H, olefin), 5.58 (broad, 1H, Ar-OH), 5.82−5.99 (m, 1H, olefin), 6.63−6.79 (m, 3H, ArH); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 38.1, 55.7, 114.8, 118.2, 122.4, 130.4, 132.2, 141.4, 147.5; MS (*m*/*z*, rel. int.): 56 (57), 66 (39), 78 (67), 92 (100), 103 (43), 105 (40), 121 (31), 132 (25), 150 (26), 165 (M + 1$^+$, 37).

3,4-Dihydroxyallylbenzene (APC). Yield, 0.9%; oil; HPLC ret. time: 5 min (single peak); IR (film): 3398, 1606, 1444, 1282, 1195, 1114, 964, 863, 789 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 3.28 (d, J = 6.5 Hz, 2H, $ArCH_2$), 4.99−5.08 (m, 2H, olefin), 5.36 (broad, 2H, 2 × Ar-OH), 5.80−6.01 (m, 1H, olefin), 6.59−6.79 (m, 3H, ArH); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 38.2, 114.6, 118.2, 122.4, 131.2, 132.5, 139.7, 145.1; MS (*m*/*z*, rel. int.): 56 (38), 66 (82), 78 (100), 91 (51), 103 (100), 111 (30), 123 (100), 132 (100), 150 (M$^+$, 100).

DPPH Scavenging Assay. An ethanolic solution of DPPH (100 μM) was incubated with an ethanolic solution of different concentrations of each of the test samples, and the absorbance was monitored spectrophotometrically at 517 nm. The percentage of reduction of the DPPH absorbance gave the DPPH scavenging activity of the test samples and was used as a marker of their antioxidant activities. The concentrations (IC_{50}) of the test samples that induced a 50% decrease of the DPPH absorbance during a 30 min observation were calculated by carrying out concentration-dependent studies (*18*). An ethanol solution of the respective test samples was used as the blank, while α-tocopherol was the positive control. The time-dependent studies were carried out for 0.5−4 min using a fixed concentration (10 μM) of CHV, APC, and α-tocopherol.

Assay of Reducing Powers. The reducing powers of PEB, PES, and PEM were quantified by a known method (*19*). Briefly, a reaction mixture (total volume 1 mL) containing each of the extracts in phosphate buffer (0.2 M, pH 6.6) was incubated with potassium ferricyanide (1% w/v) at 50 °C for 20 min. The reaction was terminated by adding a TCA solution (10% w/v), and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was mixed with distilled water and Fe(III) chloride (0.1% w/v) solution, and the absorbance at 700 nm was measured. An increase in the absorbance of the reaction mixture was indicative of increased reducing power. BHT was used as the standard.

Estimation of the Total Phenolic Contents. The method (*20*) downscaled to 1 mL final volume was followed to determine the amounts of total phenolics in the test samples. The test samples (each 100 μL) were mixed with 500 μL of 1:10 Folin−Ciocalteau's reagent followed by the addition of Na_2CO_3 (400 μL, 7.5%). After incubating the reaction mixture at 24 °C for 2 h, the absorbance at 765 nm was recorded. Gallic acid monohydrate was used as the standard. The total phenolic contents of PEB, PES, and PEM are expressed as gallic acid equivalents (μg of GAE)/mg dry weight of the samples.

Lipid Peroxidation (LPO) Assay. Lipid peroxidation of liposomes was carried out as reported earlier (*21*) with minor modifications.

Briefly, small unilammelar liposomes were prepared from phosphatidylcholine at 10% concentration in 10 mM Hepes pH 7.4 buffer. In the reaction mixture (0.5 mL) containing potassium phosphate buffer pH 7.4 (50 mM), to the liposome (1.5× from the 10× stock) with or without test samples was added Fe(II) (50 μM) and ascorbic acid (500 μM), and the mixture was incubated at 37 °C for 30 min. The TCA-TBA-HCl (15% TCA, 0.375% TBA, 0.25 M HCl) solution was added, and the mixture was heated for 10 min on a boiling water bath. After cooling, the precipitate formed was removed by centrifugation at 1000*g* for 10 min, and the absorbance of the supernatant at 532 nm was read.

In a separate set of experiments, brain homogenate was prepared from male Wistar rats. Briefly, the brain tissues were removed immediately from the freshly sacrificed rats and put into chilled Krebs buffer pH 7.4. The brain tissues were washed three times with cold Krebs buffer pH 7.4 and homogenized at 4000 rpm using a ceramic glass homogenizer (three cycles 30 s each) in the same buffer to obtain a 10% homogenate. This was centrifuged at 2000 rpm for 15 min, the pellet was thrown, and the supernatant preserved at 4 °C was used as the brain homogenate. The Fe(II)-induced LPO of the brain homogenate was carried out as stated previously (*21*), and the concentration-dependent anti-LPO activities of CHV and APC were assessed.

Lipid Hydroperoxide Assay. This assay was carried out essentially according to the reported method (*22*). Briefly, in the reaction mixture (0.5 mL) containing Tris buffer pH 7.4 (125 mM), KH_2PO_4 (1 mM), and the liposome (1.5X from the 10X stock) with or without APC, was added Fe(II) (200 μM) and ascorbic acid (200 μM), and the mixture was incubated at 37 °C for 30 min. At the end of the incubation, a 50 μL aliquot was taken to which 0.5 mL of FOX2 reagent was added. The tubes were vortexed, and the absorbance at 560 nm was read after 30 min.

Iron Chelation by CHV and APC. The iron chelation study was carried out by recording the UV/vis spectra (190−800 nm) of a solution of CHV or APC (10 μM) in water as such and after the addition of aliquots of ferrous ammonium sulfate (5−80 μM) solution. The chelating capacities of CHV and APC were evaluated from the change and/or shift of the absorbance (*23*).

Protective Activities of CHV and APC against γ-Ray-Induced Strand Breaks in Plasmid DNA. The assay was carried out as described earlier (*22*). The samples were prepared in a final volume (14 μL) and irradiated for 2.45 min at 25 °C up to a dose of 12.25 Gy using a ^{60}Co source (dose rate: 5 Gy/min). In all experiments, the concentration of the supercoiled pBR322 DNA was 200 ng in a 10 mM potassium phosphate buffer pH 7.4. CHV or APC was added as an aqueous solution to achieve the final concentration. After irradiation, the resulting forms of the plasmid, form I (supercoiled) and form II (open circular), were separated by agarose gel electrophoresis, (72 V, 1 h) stained with ethidium bromide, and visualized under UV light. The relative intensities of the bands were determined with a Kodak Gel Logic 200 imaging system.

Hydrogen Peroxide Scavenging Assay. The procedure employed was essentially the same as described earlier (*24*). The buffered phenol red solution (PRS) used in all the assays contained the following reagents in the final concentration stated: sodium chloride (140 mM), dextrose (5.5 mM), phenol red (0.28 mM), and HRPO (8.5 U/mL) in 10 mM potassium phosphate buffer pH 7.0. Phenol red and HRPO were added to the buffer briefly before carrying out the experiment. Different concentrations of CHV, APC, and H_2O_2 (final concentration 60 μM) were incubated at 25 °C for 15 min, and the concentration of H_2O_2 remaining in each case was assayed by adding PRS (190 μL) followed by NaOH (10 μL, 1 N) and measuring the absorbance at 610 nm against an appropriate blank. The concentration of the H_2O_2 stock solution was calculated from its absorbance at 230 nm, using the extinction coefficient as 81 mol^{-1} cm^2. α-Tocopherol was used as a reference inhibitor.

2-Deoxyribose Assay. The reaction mixture (1 mL) contained 2-deoxyribose (2.8 mM), Fe(III) chloride (20 μM), EDTA (100 μM) [EDTA and Fe(III) chloride were mixed prior to the addition of 2-deoxyribose], and H_2O_2 (200 μM) without or with CHV, APC, or mannitol (1.0 mM) in a 10 mM potassium phosphate buffer pH 7.4. The reaction was triggered by adding ascorbic acid (300 μM) and subsequent incubation of the mixture for 1 h at 37 °C. Solutions of Fe(III) chloride, ascorbic acid, and H_2O_2 were prepared in deaerated water just prior to use. A solution of TBA in 50 mM NaOH (1 mL, 1% w/v) and TCA (1 mL, 2.8% w/v aqueous solution) was added, the mixture was heated for 15 min in a boiling water bath, and the amount of chromogen produced was spectrophotometrically measured (*25*) at 532 nm.

Superoxide Radical Scavenging Assay. The superoxide scavenging ability of CHV and APC was assessed by a reported method (*26*). The reaction mixture contained the following reagents in the final concentration stated: CHV or APC (50 μg/mL), PMS (30 μM), NADH (338 μM), and NBT (72 μM) in a phosphate buffer (0.1 M, pH 7.4) was incubated at room temperature for 5 min, and the absorbance of the solution at 560 nm was measured against an appropriate blank that did not contain any NADH. BHA was used as a reference inhibitor.

Study of Acute Toxicity of APC on Mice. The acute toxicity of APC on mice was studied by oral intubation of APC (25 mg/kg of body wt) and observing the animals for 1 month. At the end of the observation period, the animals were sacrificed, and the histology of the liver was assessed.

Statistics. Data are presented as mean ± SEM of five experiments. The data presented for protection against γ-ray-induced plasmid DNA damage are the mean of two experiments.

RESULTS AND DISCUSSION

Besides oxygen metabolism, various reactive oxygen species (ROS) can be formed in cells by transition metal (especially Fe(II)) mediated reactions (*27*) and radiation exposure (*28*) leading to the deleterious effects on membrane lipids and DNA. For the present work, the antioxidant activity of the ethanol extracts (PEB, PES, and PEM) of three varieties of *P. betel* leaves was screened by the DPPH radical scavenging and reducing power assays. Using DPPH assay results as an indicator, the antioxidant principles were isolated from the most active extract PEB. The activities of the extracts were correlated with the relative concentrations of the active principles revealed by a HPTLC analyses and phenolics content in them. Subsequently, the protective activity of the active constituents against iron mediated lipid peroxidation and γ-ray-induced DNA damage was studied. In addition, the scavenging activities of the active constituents for various biologically relevant ROS were also assessed. Of the chosen *P. betel* varieties, the leaves of the Bangla variety (name derived from its cultivation in West Bengal, India) are moderately green, most pungent, and consumed widely in India. The leaves of the sweet variety, commonly known as meetha pan are dark green and most expensive and valued for their least pungency (explaining its sweet name, meetha (Hindi or Urdu for sweet)) and exotic flavor. The leaves of the Mysore variety are light green and moderately pungent with a rather unusual flavor liked by the people of southern India.

Composition Profiles of PEB, PES, and PEM and Isolation of the Chemical Constituents. Preliminary TLC analyses of PEB, PES, and PEM showed similar profiles of the constituent chemicals. Subsequently, the constituents in the extracts were quantified by HPTLC analyses that were standardized using various mobile phases. All the extracts contained four major compounds, although in different concentrations (**Table 1**). A detailed phytochemical analysis of PEB by conventional chromatography furnished CHV and APC (*29*), as well as their glucosides, **1** and **2** (**Figure 1**). The glucosides were earlier isolated from the rhizomes of smaller glalnga (*30*). PEB was found to be most enriched with CHV and APC. We did not quantify the concentrations of the glucosides in PES and PEM, as these did not show any DPPH radical scavenging activity. However, visual inspection of the TLC plates revealed them to be the major constituents of PES and PEM. The lesser

Table 1. Chemical Composition of Different Varieties of *P. betel* Leaf Ethanol Extracts

compound (R_f)	concn (%)[a,b] PEB	PES	PEM
CHV (0.55)	0.21	0.10	0.13
APC (0.24)	1.23	0.14	0.37
chevibetol glycoside **1** (0.24)	0.26		
allylpyrocatechol glycoside **2** (0.19)	0.52		

[a] Based on the weight of *P. betel* leaves. The values are the mean of two experiments. [b] Concentrations of the glycosides were not determined as these did not show any radical scavenging activity.

concentrations of the free phenolics in them might account for less pungency.

DPPH Radical Scavenging Activities and Reducing Powers of Different *P. betel* Extracts/Constituents. The DPPH scavenging ability and reducing power assays provide two simple methods for preliminary assessment of the antioxidant activity of a test compound. The DPPH assay results are indicative of the hydrogen-donating propensity of a test compound (*31*). Likewise, the antioxidant activity of plant extracts is also correlated with their reducing powers, which are generally associated with the presence of reductones (*32*). These exert antioxidant action by breaking the free radical chains as well as reacting with certain precursors of peroxide and preventing peroxide formation (*33*).

All the extracts showed a concentration-dependent scavenging of the DPPH radicals. Among these, the best sample, PEB, was active even at a very low concentration (3 μg/mL). It showed 56.53 and 71.24% scavenging activity at 6 and 7.5 μg/mL concentrations, respectively. PES and PEM were effective only at higher concentrations (>20 μg/mL). Their respective scavenging activities were 33 and 16% at the highest test concentration (50 μg/mL). Among the constituents of PEB, CHV and APC showed concentration-dependent activity, while glucosides **1** and **2** were inactive. The DPPH scavenging activities of APC and α-tocopherol were similar and significantly higher than that of CHV. The DPPH assay results are summarized in **Table 2**, which also contains the respective IC_{50} values of PEB, CHV, APC, and the positive control, α-tocopherol. The IC_{50} values PES and PEM were not evaluated.

We have also carried out the time-dependent DPPH radical scavenging studies with CHV and APC and α-tocopherol, each at a final concentration of 10 μM. It was found that the reaction was initially very fast with APC and α-tocopherol, while the reactivity of CHV was insignificant even up to 4 min. Within 1 min, APC and α-tocopherol could scavenge 32 ± 1 and 19 ± 1% of the radical. Thereafter, this increased to 37 ± 0.3 and 19 ± 2%, respectively, in 4 min. Thus, APC was found to react faster with DPPH as compared to the other test samples including α-tocopherol.

The relative reducing powers of the extracts also corroborated with those obtained with their DPPH scavenging activities. The increase in the absorbance at 700 nm of the reaction mixture, caused by the extracts, is indicative of their increased reducing power, and the comparative results are presented in **Table 3**. PEB showed significantly higher reducing potential than PES, PEM, and BHT used at 10-fold more concentrations. The reducing power of BHT was better than those of PES and PEM over the entire and identical concentration range tested. This factor is likely to contribute significantly toward the observed antioxidant effects of PEB ≫ PEM > PES.

Table 2. Comparative DPPH Radical Scavenging Activities of *P. betel* (Bangla) Leaf Ethanol Extract, Its Constituent Phenolics, and α-Tocopherol

test sample	concentration	% scavenging activity	IC_{50} value[a]
PEB	3 μg/mL	36.95 ± 1.54	52.43 ± 1.24 μg/mL
	4.5 μg/mL	46.14 ± 1.97	
	6.0 μg/mL	56.53 ± 2.29	
	7.5 μg/mL	71.24 ± 2.64	
PES	20 μg/mL	14.78 ± 0.87	
	30 μg/mL	18.44 ± 1.13	
	40 μg/mL	25.18 ± 1.24	
	50 μg/mL	33.01 ± 1.39	
PEM	20 μg/mL	7.2 ± 0.43	
	30 μg/mL	9.17 ± 0.30	
	40 μg/mL	12.44 ± 0.39	
	50 μg/mL	16.56 ± 0.14	
CHV	50 μM	41.63 ± 1.45	78.12 ± 2.78 μM
	100 μM	59.63 ± 1.31	
	150 μM	70.45 ± 0.51	
APC	10 μM	26.08 ± 0.98	20.17 ± 0.07 μM
	20 μM	49.35 ± 0.67	
	30 μM	73.49 ± 0.18	
α-tocopherol	10 μM	21.62 ± 0.27	20.73 ± 0.13 μM
	20 μM	48.47 ± 0.28	
	30 μM	74.19 ± 0.52	

[a] Values are mean ± SEM ($n = 5$). Means were compared statistically by one-way ANOVA at $p < 0.05$. For PEB, CHV, and APC, the mean values for IC_{50} were significantly different. The values of CHV and α-tocopherol were significantly different, while those of APC and α-tocopherol were not significantly different from each other.

Table 3. Comparative Reducing Powers of Different Varieties of *P. betel* Leaf Extracts and BHT

sample	concentration (μg/mL)	absorbance at 700 nm[a]
PEB	0.1	1.08 ± 0.03
	0.25	1.13 ± 0.04
	0.50	1.23 ± 0.04
	1.0	1.37 ± 0.06
PES	1.0	1.06 ± 0.02
	2.0	1.10 ± 0.02
	4.0	1.16 ± 0.02
	6.0	1.20 ± 0.02
PEM	1.0	1.08 ± 0.01
	2.0	1.14 ± 0.02
	4.0	1.21 ± 0.02
	6.0	1.26 ± 0.04
BHT	1.0	1.05 ± 0.02
	2.0	1.27 ± 0.03
	4.0	1.67 ± 0.05
	6.0	1.93 ± 0.08

[a] Values are mean ± SEM ($n = 5$). Means were compared statistically by one-way ANOVA at $p < 0.05$. The values of PES and PEM were significantly different from that of BHT at 4 and 6 μg/mL but not at 1 and 2 μg/mL. The value of PEB at 1 μg/mL was significantly different from those of PES, PEM, and BHT.

Total Phenolics in Different *Piper* Extracts. It is well-known that plant phenolics, in general, are highly effective free radical scavengers and antioxidants. Consequently, the antioxidant activities of plant/herb extracts are often explained with their total phenolics content with good correlation. We also observed similar correlations in the present study. The total phenolics in the extracts (PEB, PES, and PEM) were determined spectrophotometrically by the Folin−Ciocalteu method and expressed as μg of GAE/mg of the test samples (**Table 4**). As revealed by the data, the total phenolic content of PEB (820.53 ± 10.8 μg of GAE/mg) was significantly higher than those of both PES and PEM (72.25 ± 1.42 and 106.72 ± 3.42 μg of

Table 4. Comparative Total Phenolic Contents of Different Varieties of *P. betel* Leaf Extracts

test sample	total phenolic activity (μg of GAE/mg)[a]
PEB	820.53 ± 10.8
PES	72.25 ± 1.42
PEM	106.72 ± 3.42

[a] Values are mean ± SEM ($n = 5$). Means were compared statistically by one-way ANOVA at $p < 0.05$. The value of PEB was significantly different from that of PES and PEM.

GAE/mg). Overall, the order of the phenolic contents of the test samples was PEB > PEM > PES that matched with their respective DPPH radical scavenging abilities and reducing powers.

Thus, the previous results clearly established that among the extracts of the three varieties of *P. betel*, PEB was the best antioxidant due to its higher reducing power as well as higher content of phenolics, especially CHV and APC. All the subsequent studies were carried out with these compounds using a series of in vitro methods.

Antilipid Peroxidation (LPO) Activities of CHV and APC. Owing to the high levels of unsaturation, lipids are susceptible to oxidative damage. Lipid peroxidation (LPO) can inactivate cellular components that play a major role in oxidative stress in biological systems. Further, several toxic byproducts of the peroxidation can damage other biomolecules including DNA away from the site of their generation (*34*, *35*). Therefore, compounds possessing the anti-LPO activity are extremely important for health benefits and food preservation. Hence, the anti-LPO activities of CHV and APC were studied by carrying out Fe(II)−ascorbic acid mediated peroxidation of liposomes in the presence and absence of the test compounds. The extent of LPO was assessed by measuring the end products in terms of the thiobarbituric acid reactive substrates (TBARS) as well as lipid hydroperoxide (LOOH) formed.

In unstimulated experiments, the amount of TBARS was marginal, the absorption at 532 nm (A_{532}) being only 0.116 ± 0.009 ($n = 5$). In control experiments, lipid peroxidation in liposome was stimulated by the addition of Fe(II) (50 μM) and ascorbic acid (500 μM), and the A_{532} value increased to 0.665 ± 0.012 ($n = 5$). CHV, APC, and the positive control, α-tocopherol, inhibited the LPO in a concentration-dependent manner (**Figure 2**). APC was a very strong antioxidant and prevented the LPO by 45.5% even at a very low concentrations (2 μM). Overall, CHV, APC, and α-tocopherol prevented LPO with IC_{50} values of 25 ± 2, 2 ± 0.3, and 10 ± 0.2 μM, respectively. The data correlated well with their respective DPPH scavenging activities. APC was the best candidate, its activity being significantly better than that of α-tocopherol.

The anti-LPO activities of CHV and APC were also assessed using rat brain homogenate as the lipid source and Fe(II)−ascorbic acid as the oxidant. In this case also, CHV and APC showed dose-dependent anti-LPO activities as shown in **Figure 3**. Under these conditions, both CHV and APC showed better efficacy with the IC_{50} values of 20 ± 2 and 0.44 ± 0.1 μM, respectively. The superior efficacy of CHV and APC in this system might be due to their recycling by various reducing agents available in rat brain homogenate.

Considering that the TBA assay is fairly nonspecific, the anti-LPO activity of APC (2 μM) was also investigated by measuring the LOOH formed in the Fenton mediated liposomal peroxidation. APC (2 μM) inhibited the iron-stimulated LOOH formation in liposomes, showing 54 ± 3% ($n = 5$) protection.

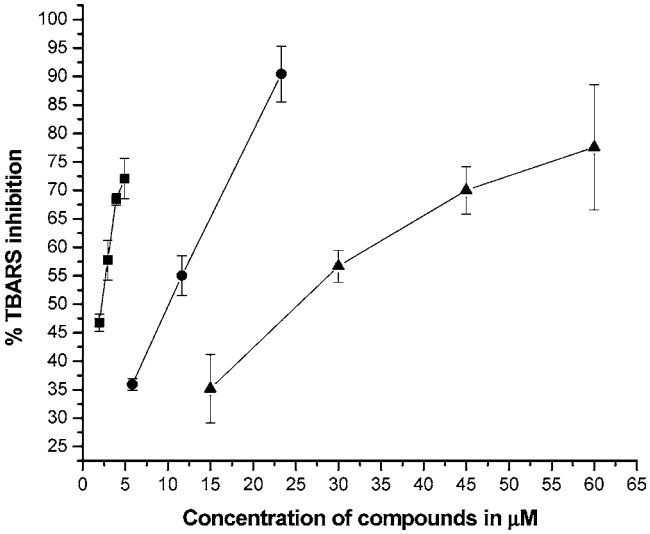

Figure 2. Concentration-dependent protective activities of CHV, APC, and α-tocopherol against Fe(II) (50 μM)–ascorbic acid (500 μM) mediated lipid peroxidation of liposome, measured in terms of TBARS formed. ▲: CHV; ■: APC; and ●: α-tocopherol. The values are mean ± SEM ($n = 5$). The means of the IC_{50} values were compared statistically by one-way ANOVA at $p < 0.05$. The individual values of CHV, APC, and α-tocopherol were significantly different.

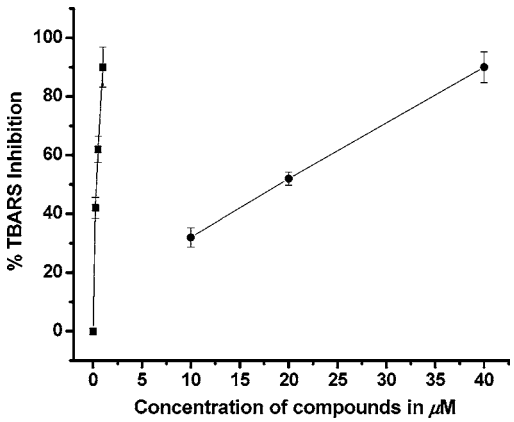

Figure 3. Concentration-dependent protective activities of CHV and APC against Fe(II) (50 μM)–ascorbic acid (500 μM) mediated lipid peroxidation of rat brain homogenate, measured in terms of TBARS formed. ●: CHV and ■: APC. The values are mean ± SEM ($n = 5$). The means of the IC_{50} values were compared statistically by one-way ANOVA at $p < 0.05$. The individual values of CHV and APC were significantly different.

Under similar conditions, the positive control, α-tocopherol (10 μM), showed 35 ± 2% ($n = 5$) prevention of LOOH formation. Thus, the results obtained with other LPO parameters also confirmed the superior anti-LPO activity of APC as compared to α-tocopherol.

The extraordinary anti-LPO activity of APC in comparison to CHV and even α-tocopherol might be due to its better radical scavenging and/or iron chelating abilities. Consequently, for a better understanding of the operative mechanism, the iron chelation capacity of CHV and APC was also assessed.

Iron Chelation by CHV and APC. CHV and APC showed strong complexation with Fe(II) since the addition of Fe(II) in increasing concentrations (5−80 μM) to a fixed concentration of CHV or APC (10 μM) led to a gradual reduction in the intensities of their absorption bands (204 and 280 nm) due to

the free compounds. However, the hypochromicity shown by both compounds was similar. This indicated that while complexation with Fe(II) might contribute to their anti-LPO activity, the vastly enhanced activity of APC was due to its better radical scavenging potential arising out of the constituent unprotected catechol moiety. It is well-known that the presence of a substituent with a higher electron donating capacity lowers the O−H bond dissociation enthalpy of a phenol and increases the rate of H-atom transfer to peroxyl radicals (*36*). The electron donating effect of a substituent depends on its nature and the position with respect to the phenol moiety. Thus, the activity of a strong electron donating group, such as the hydroxyl group, at the ortho and para positions is much higher than that at the meta position, explaining the relative order of the antioxidant potencies of catechols, quinols, and resorcinols. Between CHV and APC, the latter contains a free catechol moiety. Hence, its antioxidant capacity is anticipated to be better as reported with various other catechols (*37*).

Radioprotection of pBR322 DNA by CHV and APC. It is well-known that radiation causes mutation and carcinogenesis, the main factor of these events being attributed to scission of DNA molecules (*28*, *35*). Because of the high concentration of water in metabolizing cells, radiation exposure of biological systems primarily leads to its radiolysis furnishing e^-_{aq}, •OH, and H•. These radicals react with DNA due to the presence of various reactive sites (base and sugar) in them. For a variety of tissues, the pathophysiological importance of ROS mediated oxidative injury caused by the exposure to radiation is widely appreciated. Hence, the protective capacity of the CHV and APC against γ-ray-induced DNA single strand breaks (SSB) was also assessed.

During radiation exposure, a single strand break (ssb) of the supercoiled (SC) DNA generates an open-circular (OC) form, which migrates slower than the SC form in the agarose gel electrophoresis. Exposure of the pBR322 plasmid DNA to γ-radiation, as a function of dose, resulted in a significant increase in the ssbs of DNA. A linear increase in ssbs, assessed as average strand breaks per DNA molecule, was observed up to a dose of 12.25 Gy (data not shown), which was sufficient to convert the entire supercoiled DNA to an open circular form. Hence, this dose was chosen for the studies, and the results are shown in **Figure 4a**,**b**.

As compared to the unirradiated DNA (control, lane 1), exposure of DNA to γ-radiation (12.25 Gy) led to an extensive conversion of the supercoiled form to the open circular form (lane 2) (**Figure 4a**,**b**). The addition of CHV and APC in increasing concentrations to the DNA, prior to irradiation, progressively reduced the intensity of the band due to the open circular form. Quantification of the DNA bands in **Figure 4b** revealed that APC at 0.5 (lane 3), 1.0 (lane 4), 2.5 (lane 5), and 5.0 (lane 6) μM concentrations offered 31, 45, 64, and 76% protection to the radiation-induced ssb formation. In comparison, the protecting efficiency of CHV even at higher concentrations (1, 2.5, 5.0, and 10 μM) was significantly less (26, 41, 58, and 72%, respectively). None of the compounds themselves caused any DNA scission as even a 2 h incubation of DNA with them did not increase the open circular form. The differential radioprotection of DNA by CHV and APC may be attributable to the respective scavenging ability of various ROS generated during radiation exposure to biological systems.

Besides the •OH radicals, radiation exposure of aerobic cells can also generate the superoxide ($O_2^{-\bullet}$) radicals from e^-_{aq}, produced by radiolysis of cellular water. Further, $O_2^{-\bullet}$ radicals can disproportionate to produce H_2O_2. All these reactive oxygen species (ROS) are well-known to cause biological damage induced by the low linear energy transfer (LET) radiation (*28*, *38*). Hence, for a better understanding of the radioprotective mechanism of CHV and APC, their scavenging ability against these ROS was also assayed.

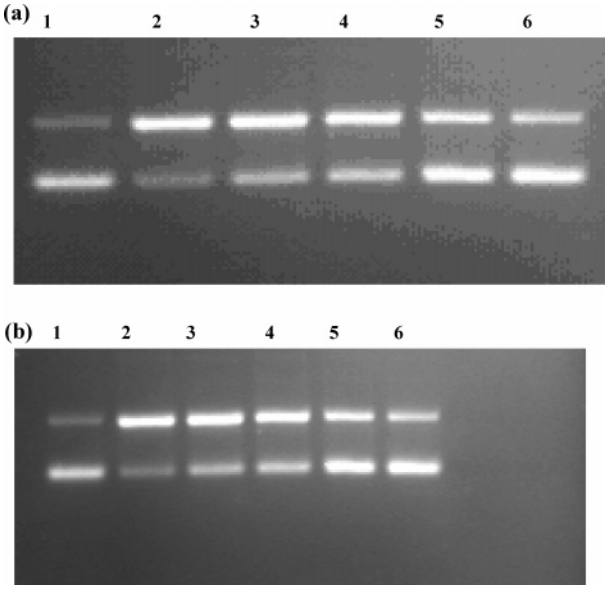

Figure 4. Gel electrophoresis pattern of pBR 322 plasmid DNA showing the open-circular (OC) and super-coiled (SC) forms after exposure to γ-rays in the presence and absence of different concentrations of CHV and APC. Plasmid DNA (200 ng) samples in 10 mM potassium phosphate buffer, pH 7.4, in a 14 μL volume were irradiated at 25 °C up to a dose of 12.25 Gy using a ^{60}Co source (dose rate: 5 Gy/min). (**a**) Lane 1: DNA sample without irradiation; lane 2: DNA sample after γ-irradiation; and lanes 3–6: DNA samples after irradiation in the presence of 1.0, 2.5, 5.0, and 10.0 μg/mL of CHV, respectively. (**b**) Lane 1; DNA sample without irradiation; lane 2: DNA sample after γ-irradiation; and lanes 3–6: DNA samples after irradiation in the presence of 0.5, 1.0, 2.5, and 5.0 μg/mL of APC, respectively.

Scavenging of •OH Radical by CHV and APC. The highly reactive •OH radicals are believed to be contributing maximum in DNA cleavage. In the 2-deoxyribose oxidation assay, both CHV and APC showed a high affinity for the hydroxyl radicals generated by the Fenton reaction.

In a reaction mixture containing Fe(III)−EDTA−H_2O_2 and 2-deoxyribose, very little TBARS was formed (A_{532} = 0.076 ± 0.010/h, n = 5), while the addition of ascorbic acid triggered production of TBARS (A_{532} = 1.692 ± 0.0351/h, n = 5). As shown in **Figure 5**, CHV and APC inhibited the degradation of 2-deoxyribose in a concentration-dependent manner. However, the assay revealed an almost equal scavenging efficiency of the CHV and APC (IC_{50} 67 ± 2 and 67 ± 1 μM). Under similar conditions, mannitol (1.0 mM) showed 19 ± 1% scavenging for the hydroxyl radicals. Given the high reactivity of the hydroxyl radicals, the results with the phenols were not surprising and could not explain the better anti-LPO and radioprotecting activities of APC as compared to CHV. Consequently, their efficacy in mitigating the other relevant ROS, $O_2^{-\bullet}$, and H_2O_2 was also investigated.

Scavenging of $O_2^{-\bullet}$ Radical and H_2O_2 by CHV and APC. Despite its involvement in many pathological processes, the superoxide radical by itself is not very reactive but can generate a hydroxyl radical via a superoxide-driven Fenton process. It has also been implicated in ischemeia-reperfusion injury and

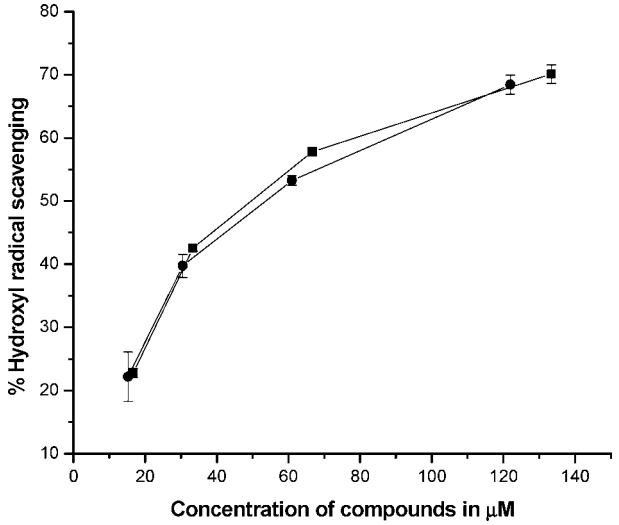

Figure 5. Concentration-dependent hydroxyl radical scavenging activities of CHV and APC. The assays were carried out by measuring the TBARS formed by the oxidation of 2-deoxyribose (2.8 μM) with Fe(III) (20 μM), EDTA (100 μM), ascorbic acid (300 μM), and H_2O_2 (200 μM). ●: CHV and ■: APC. The values are mean ± SEM ($n = 5$). Means were compared statistically by one-way ANOVA at $p < 0.05$. The values for CHV and APC were not significantly different at all the test concentrations.

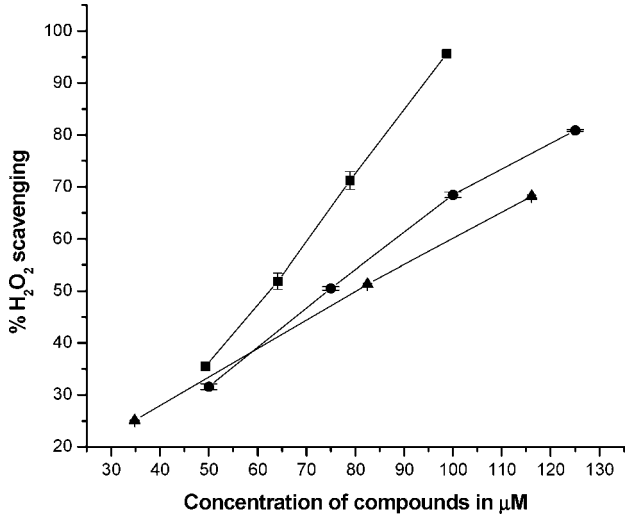

Figure 6. Concentration-dependent hydrogen peroxide scavenging activity of CHV, APC, and α-tocopherol. The assays were carried out by measuring the decrease in absorbance of hydrogen peroxide (60 μM) at A_{610} nm, after addition of the test samples. ●: CHV; ■: APC; and ▲: α-tocopherol. The values are mean ± SEM ($n = 5$). The means of the IC_{50} values were compared statistically by one-way ANOVA at $p < 0.05$. The individual values of CHV, APC, and α-tocopherol were significantly different.

promoting human gout (*39*). In the present study, we assessed the potential of a fixed concentration (each 50 μg/mL) of CHV and APC to scavenge the $O_2^{-\bullet}$ radical, generated by a PMS/NADH system. APC was found to scavenge the superoxide radicals more effectively (26 ± 1%) than CHV (6 ± 1%). Under the same conditions, the positive control, BHA (100 μg/mL), showed 22 ± 1% scavenging of the superoxide radicals. Thus, APC was more effective than both CHV and BHA.

Although not a radical species, H_2O_2 is an important ROS contributing to oxidative stress. The generation of even low levels of H_2O_2 in biological systems may be important. Naturally occurring iron complexes are believed to react with H_2O_2 in vivo to generate the highly reactive hydroxyl radicals via the Fenton reaction (*40*). CHV and APC depleted H_2O_2 in a concentration-dependent manner (**Figure 6**) with IC_{50} values of 76 ± 0.5 and 62 ± 0.2 μM, respectively. In comparison, the positive control α-tocopherol was marginally less effective (IC_{50} value 81 ± 0.5 μM) in scavenging H_2O_2.

Thus, the superior radioprotecting activity of APC as compared to that of CHV can be attributed to its better ability to scavenge $O_2^{-\bullet}$ radicals and H_2O_2. The excellent anti-LPO activity of APC as compared to CHV and α-tocopherol might be due to a combination of factors such as Fe (II) chelation and scavenging H_2O_2 and lipid (L•) as well as lipid peroxide (LOO•) radicals.

Many of the therapeutic properties of *P. betel* are attributed to its antioxidant action. However, the antioxidant principles have so far not been established. This would be important for developing plant-based drugs since chemical constituents of plants are dependent on various factors. This has also been reported with the leaves of *P. betel* (*41*). Earlier, phytochemical investigations of *P. betel* by different groups led to the isolation of chavicol, CHV, and APC along with their acetates (*29*), triterpenes, and β-sitosterol (*42*) as well as several neolignan piperbetols and piperols (*43*). In contrast, the extracts used by us were found to contain only CHV and APC as the antioxidant principles. Our studies using the liposome, rat brain homogenate, and plasmid DNA as in vitro models showed that the antioxidant and radioprotective activities of the *P. betel* ethanol extract could be attributed to one of its constituent phenolics, APC, while the contribution of the other partially methylated phenol CHV is insignificant. The studies also revealed that among the three most widely used varieties (Bangla, sweet, and Mysore) of *P. betel*, the Bangla variety is rich in phenolics, especially APC, and, hence, is the most effective antioxidant. Thus, to promote *P. betel* as an effective antioxidant, it is essential to assay the content of the total phenolics and CHV and APC in the samples.

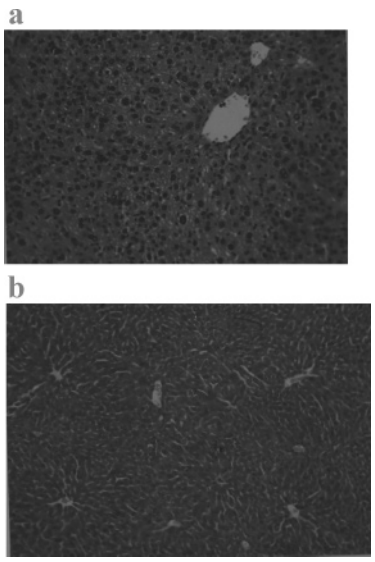

Figure 7. Histological section of untreated and APC-treated mouse liver. (**a**) Normal mouse and (**b**) mouse treated with APC (25 mg/kg of body wt).

Our own evaluation of the acute toxicity of APC (up to a dose of 25 mg/kg of body wt) on mice did not reveal any

observable physical sign change, and the animals had normal food and water as well as stool during the experimental period. After 1 month of APC administration, there were no signs of abnormality in the liver of the mice as shown in the comparative hisotological photographs of the livers of normal and the APC-treated mice (**Figure 7a**,**b**). It is worth mentioning that in spite of the reported (*29*) fungicidal and nematocidal activity of *P. betel* and allergic reaction of eugenol, we did not observe any side effect of even the whole extract (PEB) in our previous studies (*15*). All these findings suggested that APC, given at the current dose, does not have any potential side effect in the animals. This was expected considering that it is the major constituent of *P. betel* leaves that is freely consumed in India and Southeast Asian countries. In the light of the previous statements, APC appears to be a promising candidate for further in vivo bioevaluation.

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