JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Comparative Evaluation of Antimutagenicity of Commonly Consumed Fruits and Activity-Guided Identification of Bioactive Principles from the Most Potent Fruit, Java Plum (*Syzygium cumini*)

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Supporting Information

ABSTRACT: Commonly consumed fruits showed remarkable variation in antimutagenicity when assayed by *E. coli* rifampicin resistance assay, and Java plum (*Syzygium cumini*) was found to be one of the most potent fruits. Its anthocyanins contributed maximally to the observed antimutagenicity and resolved into three distinct bands in HPTLC. Although these bands displayed similar antioxidant capacity, the band at R_f 0.22 was the most antimutagenic and resolved into two peaks in HPLC. The second peak (t_R 3.8 min) displayed a strong and broad spectrum antimutagenicity and was identified as petunidin-3,5-diglucoside by analysis of its molecular ion and fragmentation pattern by ESI-MS/MS. The presence of glucose moiety was confirmed by TLC analysis of acid-hydrolyzed products. This purified anthocyanin was found to suppress mutagenic SOS DNA repair process in *E. coli* and thus indicated suppression of the error-prone DNA repair pathway as one of the major mechanisms of antimutagenicity of this fruit.

KEYWORDS: *fruits, Syzygium cumini, antimutagenicity, antioxidant, anthocyanins, petunidin-3,5-diglucoside*

INTRODUCTION

Mutations are known to be involved in various diseases including neoplastic inductions.[1,2] Mutagenesis in a cell can be induced by cosmic radiation, chemical mutagens from industrial effluents, and also certain secondary metabolites such as mycotoxins.[3] The role of natural dietary components in the prevention of cardiovascular, neurodegenerative diseases and cancer has been investigated as a disease prevention strategy.[4,5] Identification of antimutagenic compounds from health protective fruits could be helpful in the development of strategies to prevent the onset of neoplastic inductions or other mutation-associated diseases.[6] In a recent study, the health-promoting anthocyanins from snapdragon *Antirrhinum majus* when expressed in transgenic tomato were found to impart a substantial protective effect against cancer progression in susceptible mice.[7] Recent studies from this laboratory with commonly consumed vegetables as well as honey showed their broad spectrum of antimutagenicity.[8,9] In general fruits including *S. cumini* are known to be a rich source of dietary flavonoids that also confer different colors to the fruits.[10,11] *Syzygium cumini* [Java plum, Jamun (Hindi)] belongs to the family *Myrtaceae* and is believed to have originated in the Indian subcontinent. Its fruits are small (2−3 cm long) in size and oblong in shape (Supplementary Figure S1). The ripe fruit has varied skin color such as purple-red to black and has a fleshy pink pulp.[12] In a recent study *S. cumini* fruit extract has been found to possess selective antiproliferative effects against cancer cells. [13]

Mechanism of mutation is quite common for different mutagens irrespective of the biological system. A bacterial system, due to its comparatively shorter generation time and ease of handling and manipulation, has been preferred for scientific studies for analysis of mutagenicity/antimutagenicity. Here too, evaluation of antimutagenicity of different fruits upon mutagenic challenge was performed using *E. coli* RNA polymerase β (*rpoB*)-based rifampicin resistance ($Rif^S \rightarrow Rif^R$) assay, where mutation(s) in *rpoB* gene abolished its interaction with rifampicin and confered rifampicin-resistant phenotype (Rif^R) to *E. coli* cells.[14,15] It provided an easily selectable phenotypic marker. The *rpoB* gene in the *E. coli* genome has many hotspots of mutations, but due to its stringent regulation the level of spontaneous mutation frequency is very low.[16] These properties make this assay highly authentic, reliable, and reproducible. Further study was aimed to evaluate the antimutagenicity of most potent *S. cumini* fruits and to isolate and characterize the bioactive principle(s) responsible and explain its chemopreventive potential.

MATERIALS AND METHODS

Fruit Samples and Chemicals. A number of fresh and mature fruits including *S. cumini* (Java plum, Indian blackberry, Jamun) were procured from a local market in Mumbai, India (Table 1). A total of 6 kg of fruits were procured in three independent sets of 2 kg each. The fruits were washed with potable water. Clear juice was obtained by homogenization of whole fruits after removal of the seeds, followed by filtration using double-layered muslin cloth. In case of mango, muskmelon, pineapple, and pomello, the fruits were peeled before homogenization.

Chemicals. Acetonitrile, ethyl methanesulfonate (EMS), methanol, and sodium chloride were procured from Sigma Chemical Co. (St. Louis, MO, USA). Luria−Bertani (LB) medium, nalidixic acid, and rifampicin were procured from Hi Media (India). Magnesium sulfate was obtained from Qualigens (India). Glacial acetic acid and *n*-butyl alcohol were obtained from Thomas Baker (India). Formic acid and

Received: March 12, 2013
Revised: September 27, 2013
Accepted: September 30, 2013
Published: September 30, 2013

dx.doi.org/10.1021/jf403005x | J. Agric. Food Chem. 2013, 61, 10033−10042

Table 1. Antimutagenicity of Juice Extract of Different Fruits against EMS[a]

fruit	% reduction in mutation frequency
apple (*Malus domestica* cv. Granny Smith)	75 ± 2b
sapota (*Manilkara zapota*)	25 ± 2e
guava (*Psidium guajava*)	70 ± 2c
Java plum (*Syzygium cumini*)	**85 ± 4a**
jujube (*Zizyphus mauritiana*)	55 ± 2d
mango (*Mangifera indica* cv. Alphonso)	85 ± 2a
mango (*Mangifera indica* cv. Badami)	10 ± 2f
muskmelon (*Cucumis melo*)	60 ± 3d
pineapple (*Ananas comosus*)	28 ± 2e
pomello (*Citrus maxima*)	30 ± 4e
plum (*Prunus persica*)	25 ± 2e

[a]Values with different letters (a−f) in the column are significantly different at $p < 0.05$.

hydrochloric acid were procured from SD Fine Chemical (India). All solvents were HPLC grade. Milli-Q water (conductivity 5.5 μS/m) was obtained using a Millipore system.

Bacterial Strains. *Escherichia coli* strains MG1655 (*F- λ- ilvG-rfb-50 rph-1*) and SG104 (MG1655 H-19B Δ*N::KanRcro-tnp*^{R}*168ΔOP::Amp*^{R}*galK::resC-Tet*^{R}*-resC*) were gifted by Prof. M. Z. Humayun, University of Medicine and Dentistry, NJ, USA.

Assessment of Antimutagenicity. Antimutagenic activity was determined in *E. coli* with the change of Rif^S (Rifampicin-sensitive) phenotype to Rif^R (Rifampicin-resistant) phenotype upon mutagenic challenge.[14,15] In this assay, control (non-mutagen-exposed) *E. coli* cells are not able to grow on rifampicin-selective plates as rifampicin inhibits DNA-dependent RNA polymerase (RNAP) through a simple steric-occlusion mechanism and therefore physically prevents the synthesis of RNA.[16] *E. coli* RNAP consists of five ($\alpha_2\beta\beta'\omega$) subunits. Mutations in the *rpoB* gene reduce the affinity of rifampicin binding to RNAP and thus confer rifampicin resistance (Rif^R) to *E. coli* cells.[16] Therefore, induced mutagenesis leads to acquisition of rifampicin resistance by these cells, which are thus able to grow on rifampicin-selective plates. The *rpoB* gene encodes the β-subunit of RNAP and is vulnerable to mutagens due to the presence of several hotspots for mutations.[14] The advantage of this assay system is its simplicity, sensitivity, accuracy, and very low level of spontaneous mutation frequency.

Ethyl Methanesulfonate (EMS) Induced Mutagenesis. The assay was performed as per the method of Cupples and Miller.[14] Overnight-grown *E. coli* (MG1655) culture was diluted (1.50) in fresh LB medium (25 mL) and grown at 37 °C at 125 rpm up to mid-log phase (∼3 h). It was incubated on ice for 15 min and centrifuged (8000*g* for 10 min). The pellet was washed with LB broth (50 mL) and resuspended in 25 mL of the same. Antimutagenicity was assayed by incubating cell suspension (5 mL) with EMS (133 mM) in the absence (control) as well as presence of fruit juice (effective concentration 50%) or purified anthocyanin, at 37 °C in a rotary shaker (75 rpm). EMS is a directly acting mutagen and induces mutagenesis by alkylating nitrogenous bases (especially guanine) in DNA by a nucleophilic substitution reaction predominantly leading to GC→AT transitions.[17]

Similarly, the effect of preincubation with purified anthocyanin was evaluated by mixing cell suspension with anthocyanin 1 h prior to EMS treatment. For evaluating the post-treatment effect of anthocyanin, the cell suspension was treated with EMS followed by washing with fresh LB twice and then treating with anthocyanin. The cells were then centrifuged, and the pellet was washed twice with LB (2 mL) and resuspended in 5 mL of the same. At the end of the treatments an aliquot (50 μL) of cell suspensions was inoculated in LB broth (1 mL) in different replicates and incubated overnight on a rotary shaker (125 rpm) at 37 °C. Proper dilutions of the cultures were spread plated on LB-agar-rifampicin (100 μg/mL) plates for scoring Rif^R (rifampicin-resistant) mutants and on LB agar plates for enumerating viable cells and incubated at 37 °C for 24 h. Mutation frequency was calculated as the ratio of total number of Rif^R mutants per milliliter to the total number of viable cells per milliliter. Spontaneous mutation frequency was determined by incubating the cell suspension in the absence of mutagen.

Ultraviolet (UV) Induced Mutagenesis. Overnight LB grown *E. coli* cells were subcultured in 25 mL of LB (1:50 dilution) and grown to mid-log phase (∼3 h). The cells were pelleted (8000*g* for 10 min), resuspended in sterile $MgSO_4$ (12.5 mL, 0.2 M), and placed on ice for 10 min. Under test conditions, HPLC-purified anthocyanin was added, thoroughly mixed, and incubated for 2 h. As a control, an equal volume of sterile Milli-Q water was added. The cell suspension was washed twice, and a 50-μL aliquot was exposed to UV (254 nm; lamp power, 8 W; intensity, 220 μW/cm^2) for 15 s in dark (to prevent photoreactivation) and inoculated (50 μL) in LB (1 mL) and incubated overnight. On the following day, these cultures were spread plated as described above. UV produces mutations due to formation of DNA lesions such as pyrimidine dimer and pyrimidine-pyrimidone photoproducts.[18]

Nalidixic Acid Induced Mutagenesis. Nalidixic acid (10 μg/mL) treatment was carried out as described above for EMS except the treatment was performed for 3 h. Nalidixic acid is an antibacterial quinolone that induces formation and stabilization of gyrase cleavage complex resulting in DNA breaks and mutagenesis.[19]

Antioxidant Assays. The 2,2-diphenyl-1-picrylhydrazyl (DPPH°) radical scavenging potential was evaluated by spraying DPPH° solution (2.54 mM) on the HPTLC bands. Development of a yellow color on a purple background indicates a positive reaction.[20] The 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) ($ABTS^+$) radical scavenging activity was assessed by adding purified anthocyanin (0.1 mg/mL) to $ABTS^+$ solution and measuring the decrease in absorbance at 734 nm (UV-2450 spectrophotometer, Shimadzu, Japan). [21]

Extraction and Purification of Anthocyanins and Non-anthocyanin Phenolics. The extraction of anthocyanins was carried out as described by Faria.[12] Acidified (0.3% conc HCl) methanol was added to the homogenized *S. cumini* fruit pulp (72 g), and the suspension was kept at 4 °C for 16 h with intermittent vortexing. The residue was repeatedly extracted with acidified methanol (75 mL × 10) until the filtrate obtained was nearly colorless. Later, the colored solution was filtered (Whatman paper no. 1) and vacuum dried (Eppendorf Concentrator 5301, Hamburg, Germany). This served as the crude anthocyanin extract. For the other experiments, the non-anthocyanin phenolics were extracted from the homogenized pulp (50 g) using methanol/water (8:2, 250 mL × 2) and vacuum dried. These extracts were subjected to solid-phase extraction (SPE) using a Sep-Pak C-18 plus cartridge (Waters Corporation, MA, USA).[22] The column was preconditioned with methanol followed by acidified Milli-Q water (0.01% conc HCl). Both of these extracts (dissolved in acidified Milli-Q water) were separately loaded on two different columns followed by washing with acidified Milli-Q water to remove sugars, acids, and other water-soluble compounds. In set I non-anthocyanin phenolics were eluted from the total phenolic extract using ethyl acetate.[22−24] In set II the C-18 column loaded with crude anthocyanin phenolics was first treated with ethyl acetate to remove non-anthocyanin phenolics and later eluted using acidified methanol (0.01% conc HCl) to obtain the anthocyanin-enriched fraction.[24] These fractions (non-anthocyanin phenolics from set I and anthocyanin phenolics from set II) were dried under vacuum at ambient temperature and later dissolved in acidified (0.01% conc HCl) Milli-Q water (for anthocyanins) and normal Milli-Q water (for non-anthocyanin phenolics), filtered (0.45 μm), and tested for antimutagenic activity at neutral pH.

High Performance Thin Layer Chromatography (HPTLC). SPE-purified anthocyanin extract was further purified using HPTLC (CAMAG, Switzerland) equipped with winCATS 4 software. The sample (250 μg/mL) was spotted on silica gel $60F_{254}$ TLC plates (0.5 mm thick, Merck, Darmstadt, Germany) by Linomat 5 (CAMAG) sample applicator. The plate was developed in butanol/acetic acid/water (4:1:5), air-dried, and photographed. The bands were selectively scraped off the plate, suspended in acidified methanol, ultrasonicated,

and thoroughly vortexed for efficient liberation of anthocyanin from the silica. The eluted suspension was centrifuged (10,000*g* for 10 min), filtered (0.22 μm), vacuum dried, and dissolved in acidified methanol for further HPLC analysis.

High Performance Liquid Chromatography (HPLC). HPLC was performed using a reverse-phase HPLC system (UltiMate 3000; C-18 column, Acclaim 120; size, 4.6 × 250 mm; particle size, 5 μm; Detector VWD-3000; Software, Chromeleon Version 6.8 SR8; Dionex Corporation, Sunnyvale, CA, USA). Separation was carried out using an isocratic mobile phase of acetic acid/formic acid/water (A, 2:5:93) and acetic acid/acetonitrile/water (B, 2:30:68) in the ratio 50:50 A:B at the flow rate 1 mL/min. The chromatograms were monitored at 280 nm.[25] Individual peaks were collected, vacuum dried, dissolved in Milli-Q water, and subjected to antimutagenicity assays against EMS, UV, and nalidixic acid as described above.

Quantification of Total Phenolics and Anthocyanins. Total phenolic content was determined with the Folin-Ciocalteau reagent using gallic acid as standard.[26] Total phenolic content was expressed in milligrams of gallic acid equivalent (mg GAE)/100 g sample (dry weight).

Total monomeric anthocyanin (TMA) content was determined using the pH differential method.[27] The sample was diluted with potassium chloride buffer (0.025 M, pH 1.0) until the absorbance at 520 nm was within the linear range (<1.2). The sample was similarly diluted with sodium acetate buffer (0.4 M, pH 4.5), and the dilution factor (DF) was considered during quantification. These dilutions were allowed to equilibrate (15 min) before the absorbance was measured. TMA content was equated as $(A \times MW_{Cy} \times DF)/(\varepsilon_{Cy})$, where A is $[(A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5}]$, with respect to cyanidin-3-glucoside (MW 449.2; ε = 26,900 L/cm/mol) and expressed as mg Cy-3-glu/100 g sample (dry weight).

Glycosidic Analysis of Anthocyanin. HPLC-purified anthocyanin (~0.5 mg) dissolved in acidified methanol (2 mL, 2 N HCl) was subjected to acid hydrolysis for 1 h at 100 °C. The hydrolysate was cooled and extracted with amyl alcohol (2 mL × 3), and the upper colored portion was separated off. The remaining aqueous phase, containing hydrolyzed sugar, was vacuum dried, dissolved in Milli-Q water, and analyzed using TLC on silica plates with standard hexoses (glucose, galactose, mannose, rhamnose, xylose, fructose) using butanol/acetic acid/diethyl ether/water (9:6:3:1). The plate was air-dried, and sugars were detected by spraying sulphuric acid solution (10% H_2SO_4 in ethanol) and placing the plates in a hot air oven (100 °C) for 15 min for spot development.

Characterization of Purified Anthocyanin. Electrospray Mass Spectrometry. HPLC-purified peaks were vacuum dried, dissolved in acidified methanol (0.01% conc HCl), and analyzed by mass spectrometry (API-3200 triple quadrupole mass spectrometer, Applied Biosystem-MDS Sciex, Toronto, Canada) employing the turbo electro spray ionization (ESI) technique. The sample was introduced into the ESI (+)-MS by infusion. The mass spectrometer parameters were ion spray voltage, 5500 V; turbo gas flow, 7 L min^{-1}; and temperature, 350 °C. Collision-activated dissociation during MS-MS was performed in a LINAC Q2 collision device, using N_2 (7 mTorr). Declustering potential and collision energy was automatically optimized. The collision cell exit potential was 25 V. The instrument was operated in positive ion mode, and the data were processed by the software Analyst 1.4.

Mechanism of Antimutagenicity. Effect of *S. cumini* Anthocyanin on Mutagenic DNA Repair Pathway. Mutagenic exposure leads to induction of an error-prone DNA repair pathway such as SOS (Save Our Soul) repair in *E. coli*, which is one of the important routes to hypermutagenesis.[28] This mutagenic DNA repair pathway is regulated by more than 40 genes, which are under a common repressor LexA.[29,30] Upon DNA damage RecA serves as a DNA damage sensor protein and binds to single-strand regions of DNA. RecA-ssDNA nucleoprotein complex mediates autocleavage of LexA repressor protein leading to expression of SOS regulated genes including *umuCD*, which encodes polymerase V ($UmuD'_{(2)}C$), a low fidelity error-prone polymerase and inserts nucleotides opposite various DNA lesions in a process termed translesion DNA synthesis (TLS) leading to mutagenesis.[30] However, polymerase V is strictly regulated

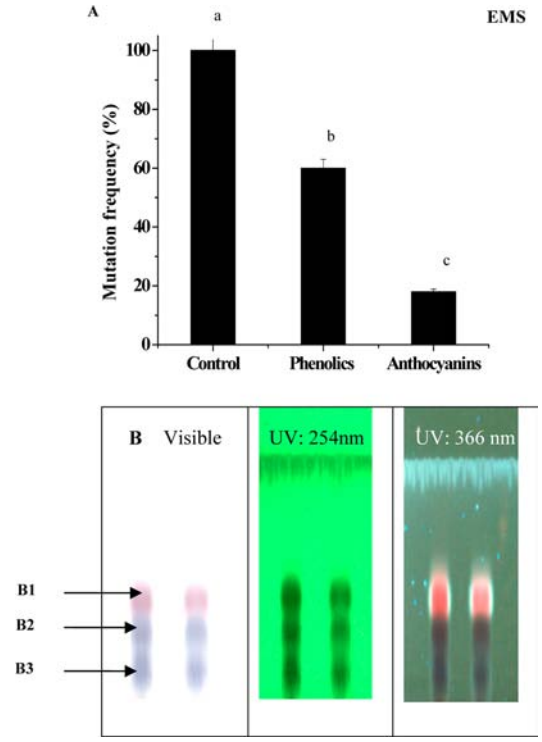

Figure 1. (A) Comparative antimutagenic potential of non-anthocyanin and anthocyanin phenolics (1 mg/mL) against EMS. (B) HPTLC chromatogram of column purified anthocyanin extract analyzed in normal as well as under UV (254 and 366 nm). Values with different letters (a–c) on the bar are significantly different at $p < 0.05$.

in the cell to avoid genomic mutation overload. SulA is another gene under the SOS regulon that is inhibitory for septum formation during the normal course of cell division.[31] SulA binds with FtsZ protein and makes it unavailable for cell septation.[31] Upon SOS induction, septa are not formed in *E. coli* cells, leading to cell filamentation due to activation of *sulA*. Besides, certain *E. coli* cells contain lysogenic prophage in their genome. However, extensive DNA damage resulting in SOS induction leads to switching from lysogenic to lytic mode. This has been analyzed using a reporter system called Selectable *In-Vivo* Expression Technology (SIVET) Assay where induction of the defective lambdoid prophage does not lead to cell lysis but instead makes *E. coli* cells chloramphenicol-resistant.[32] In the *E. coli* SIVET strain SG104 the genes required for lambda phage lytic cycles (N, O, P) have been inactivated by replacement with antibiotic-resistant genes.

Cell Filamentation Assay. The *E. coli* cell filamentation assay was performed as described by Gottesman et al.[33] Overnight-grown *E. coli* culture was subcultured in 1 mL of LB (1:100 dilution), grown to mid-log phase, and centrifuged (8000*g* for 10 min). The cell pellet was resuspended in saline (0.85% NaCl). Under test conditions, filter-sterilized HPLC peak 2 fraction (B2 f2, 100 μg/mL) was added, while in the control an equal volume of sterile Milli-Q water was added. An aliquot (1 mL) was exposed to γ radiation (100 Gy) (Gamma Cell-220, AECL Canada, dose rate 5 Gy/min). The suspension was inoculated into LB (10 mL), grown for 2 h, and centrifuged at 2000*g* for 1 min. The pellet was suspended in 100 μL of saline and smeared on a glass slide. The smear was heat fixed, stained with crystal violet, and visualized for filamentation under a compound microscope (Carl Zeiss, GmbH, Germany) with an oil immersion objective. Measurement of the filament length was performed using software Axio Vision AC4.1. Cells having length ≥3.0 μm were recorded to be filamentous. Filamentation frequency was calculated as percentage of filamented cells with respect to total cell population.

SIVET Assay. SIVET assay was performed using the methods previously reported.[32,34,35] A single colony of *E. coli* SG104 strain was grown overnight in LB (10 mL) supplemented with tetracycline

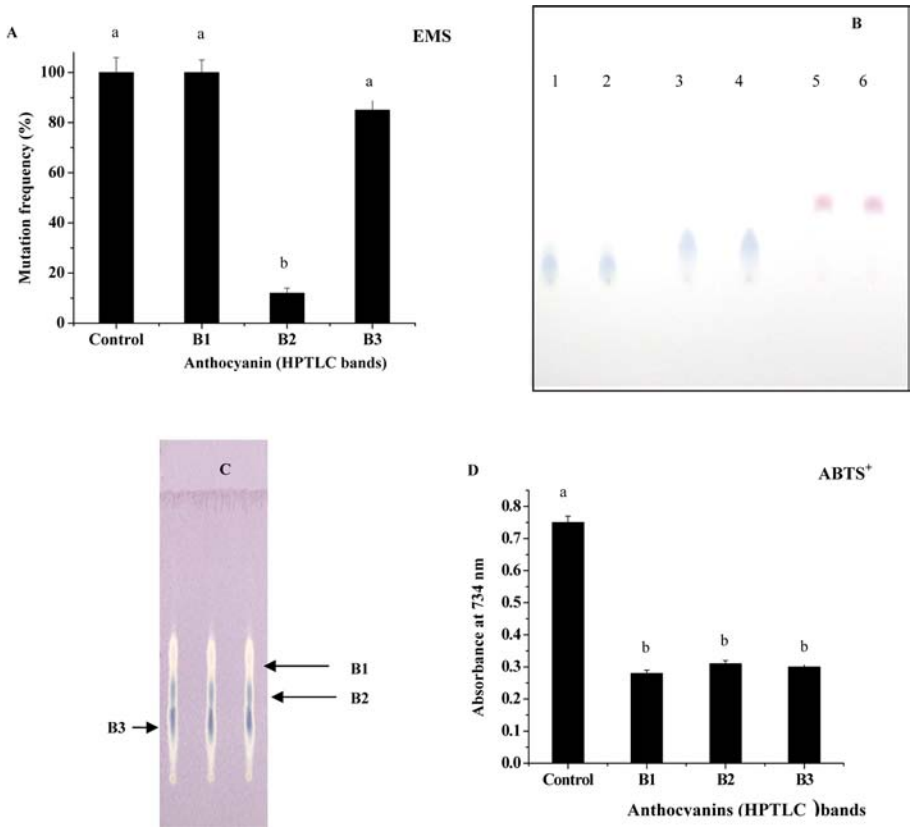

Figure 2. (A) Comparative antimutagenicity of HPTLC purified bands (B1, B2, B3) against EMS. (B) Chromatogram showing stability of B1, B2, and B3 at pH 5 and 7 [tracks l, 3, 5: B1, B2, B3 at pH 5; tracks 2, 4, 6: B1, B2, B3 at pH 7]. (C) Comparative antioxidant activity: HPTLC plates sprayed with DPPH° solution and visualized. (D) $ABTS^+$ scavenging activity. Values with different letters (a,b) on the bar are significantly different at $p < 0.05$.

(5 μg/mL), kanamycin (20 μg/mL), and ampicillin (30 μg/mL). The culture was subcultured in LB (50 mL; 1:50 dilution) containing the above antibiotics and grown to mid-log phase to a density of ~2×10^8 cfu/mL. The cells were centrifuged, suspended in saline, and exposed to γ radiation (100 Gy) in the presence of HPLC-purified peak 2 (100 μg/mL). The cell suspension was washed twice in LB. Later an aliquot (50 μL) was inoculated in LB broth (1 mL) and grown for 18 h without any antibiotic. Appropriate dilutions of these cultures were spread plated on LB agar having ampicillin (30 μg/mL), kanamycin (20 μg/mL), and chloramphenicol (10 μg/mL) for estimating SIVET-induced population and on ampicillin and kanamycin to select all SIVET lysogens and incubated for 48 h at 37 °C. SIVET induction frequency was measured as ratio of SIVET-induced cells (Amp^R Kan^R Cm^R) to total viable cells (Amp^R Kan^R).

Statistical Analysis. Statistical analysis was performed using BioStat 2009 Version Professional 5.8.0.0 (AnalystSoft Inc., Canada). Antimutagenicity was performed for 5 replicates in three independent sets. Means and standard deviations were calculated. The analysis of variance (one-way ANOVA) was applied to establish significant differences between the values obtained. A probability value of $p < 0.05$ was adopted as the criterion for significant differences. Chromatographic analysis was performed in three independent sets.

RESULTS

Comparative Evaluation of the Antimutagenic Potential of Common Indian Fruits. The antimutagenic potential of 11 commonly consumed fruits in India was analyzed. Among these, Java plum (*S. cumini*), mango (cv. *Alphonso*), apple (cv. Granny Smith), and guava were found to display very high antimutagenicity (Table 1), and the percentage reduction in mutagenic frequency was found to be 70% or more. Mango (cv. *Badami*), plum, sapota, pineapple, and pomello were found to display less antimutagenicity reducing the mutation frequency by less than 30%. Muskmelon and jujube were found to be moderately antimutagenic with the reduction in mutation frequency in the range of 50−60%. Mango (cv. *Alphonso*) and Java plum displayed highest antimutagenicity among the fruits tested. As Java plum is traditionally known to have therapeutic and prophylactic properties in complementary medicine, it was further selected for characterization of its bioactive compound(s).[36]

Purification of Bioactive Compound from *S. cumini* Fruit. Anthocyanins and non-anthocyanin phenolics were extracted from the *S. cumini* fruit pulp and evaluated for antimutagenicity. The total phenolic content was 809 mg GAE/100 g, and the anthocyanin content was 400 mg Cy-3-glu/100 g on dry weight basis. The SPE-purified anthocyanin extract consisted of 96.2% total monomeric anthocyanins (TMA), whereas the non-anthocyanin phenolic fraction was almost devoid of TMA (<0.13%). In comparison with the non-anthocyanin phenolic fraction, the anthocyanin fraction displayed ~3-fold higher antimutagenic potential (Figure 1A). Further separation of the anthocyanin fraction was performed using HPTLC and HPLC to identify the specific anthocyanin(s) associated with antimutagenicity. During HPTLC, efficient separation of anthocyanins was observed in a solvent system composed of butanol/acetic acid/water (4:1:5) resulting in three distinct bands under visible light: an upper pinkish red band (B1, R_f 0.44), a middle blue band (B2, R_f 0.22), and a lower blue band (B3, R_f 0.16) (Figure 1B). No fluorescent bands were detected under UV

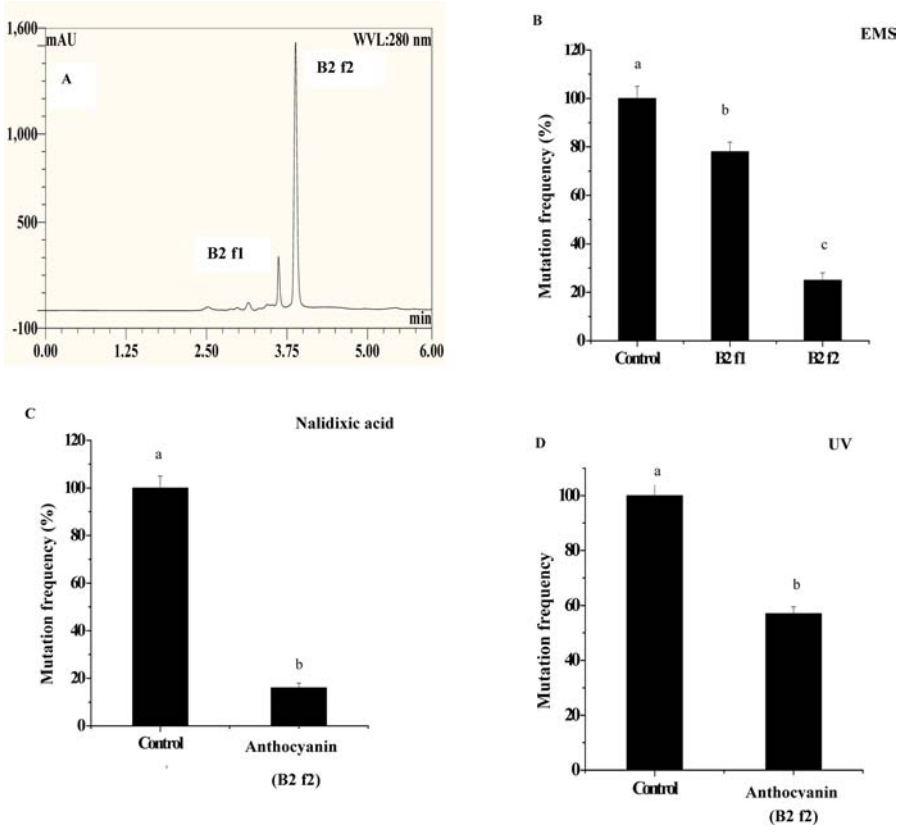

Figure 3. (A) HPLC profile of HPTLC purified middle band (B2); antimutagenicity evaluation of B2 f1 and B2 f2 using *E. coli rpoB* based Rif^S Rif^R assay system against (B) ethyl methanesulfonate, (C) nalidixic acid, and (D) UV. Values with different letters (a–c) on the bar are significantly different at $p < 0.05$.

(254 nm) (Figure 1B). However, B1 was found to fluoresce at 366 nm (Figure 1B).

Among these, B2 displayed significantly higher and prominent antimutagenicity against EMS as compared with the other two bands at equivalent concentration of 500 μg/mL (Figure 2A). B1 did not show any antimutagenicity, whereas B3 showed marginal antimutagenic activity. In order to rule out the possibility that the differential antimutagenicity shown by B1, B2, and B3 was not related to their stability or lability under the assay conditions (pH 7.0), the HPTLC eluted anthocyanin bands (B1, B2, B3) were subjected to two different pH conditions (Milli-Q water adjusted to pH 5 and pH 7) and assessed for their stability through TLC. The anthocyanin bands were not found to degrade even after 20 h incubation at 37 °C, and remained stable at the two pH values (Figure 2B). The three bands displayed similar radical scavenging activity in DPPH° (~95% scavenging by densitometry measurements) as well as $ABTS^+$ (~57% scavenging) assays (Figure 2C,D).

HPLC-Based Purification of Antimutagenic Anthocyanins. HPTLC band B2 when subjected to HPLC analysis was resolved into two peaks at t_R 3.66 and 3.88 min (Figure 3A). The peak fractions were collected, vacuum dried, and evaluated for antimutagenicity against EMS. B2 fraction 2 (B2 f2) displayed >70% antimutagenicity as compared to B2 fraction 1 (B2 f1) when analyzed at equivalent concentrations of 250 μg/mL (Figure 3B). Also, purified anthocyanin (B2 f2) was comparatively more effective as compared to a mixture (1:1) of anthocyanins B2 f1 (125 μg/mL) and B2 f2 (125 μg/mL) making a final concentration of 250 μg/mL at equivalent concentrations (data not shown). In pre and post incubation experiments, B2 f2 displayed ~40% and ~33% antimutagenicity, respectively (data not shown).

To check whether B2 f2 possessed a broad-spectrum antimutagenic property, it was further assayed against UV and nalidixic acid. In the presence of B2 f2, the UV-induced mutation frequency was reduced to half and that of nalidixic acid to one-fifth of the control treated cells (Figure 3C,D).

Mass Spectrometry Analyses. The HPLC fractions were subjected to ESI-MS/MS analyses. For B2 f2, a molecular ion of m/z 641 was detected. Upon fragmentation, this resulted in two characteristic daughter product ions with m/z 479 and 317 (Figure 4A). These correspond to daughter ions formed due to loss of one and two glucosyl moieties, respectively, from m/z 641. For the anthocyanins, the product ion m/z 317 is characteristic of petunidin aglycone.[24] Upon further fragmentation, the ion with m/z 479 resulted in a single ion at m/z 317, thus confirming the inference that m/z 479 is a petunidin glucoside that was formed due to loss of 1 glucosyl residue from m/z 641 (Supplementary Figure S2). On the basis of the m/z value of the molecular ion and the characteristic fragmentation pattern, the identity of anthocyanin B2 f2 was confirmed to be petunidin-3,5-diglucoside.

HPLC B2 f1 corresponded to a molecular ion having an m/z value of 611. This resulted in two daughter ions of m/z values 287 and 449, respectively (Figure 5A,B), from the loss of two glucosyl moieties, i.e., $[M]^+ - 2 \times 162$ (mass of one glucosyl moiety). For anthocyanin, the m/z value of 287 is characteristic for cyanidin aglycone.[24] Thus B2 f1 was identified as cyanidin-3,5-diglucoside.

dx.doi.org/10.1021/jf403005x | J. Agric. Food Chem. 2013, 61, 10033–10042

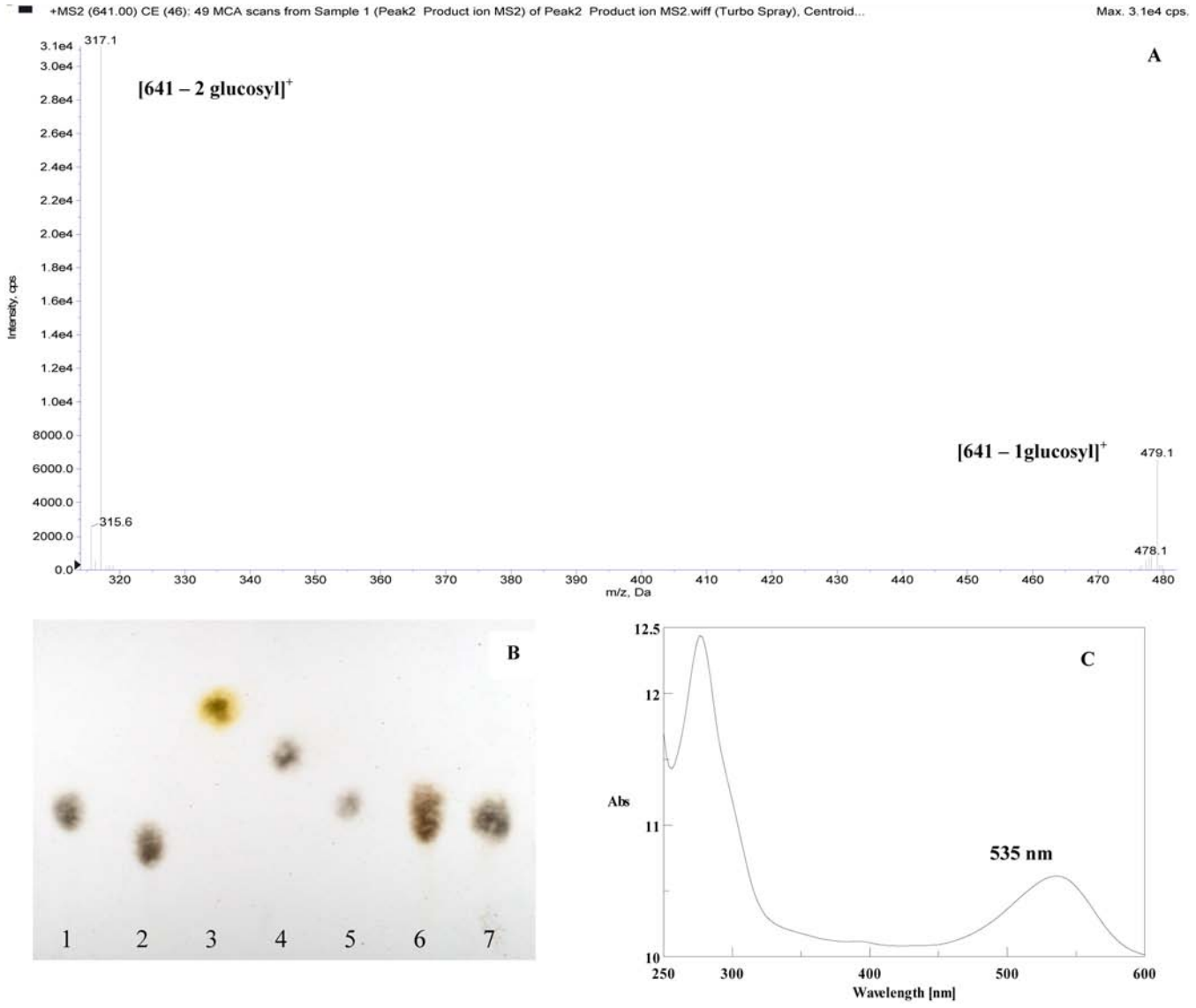

Figure 4. (A) Characteristic fragmentation pattern of anthocyanin (B2 f2) showing daughter ions of m/z 479 and 317 (Petunidin aglycone). (B) TLC chromatogram of acid hydrolyzed B2 f1 and standard sugars [1, glucose; 2, galactose; 3, rhamnose; 4, xylose; 5, sample; 6, mannose; 7, fructose]. (C) UV–vis spectrum of anthocyanin (B2 f2).

In reverse-phase HPLC, the elution order of anthocyanidins is reported to be delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin.[37] Our HPLC data are consistent since cyanidin was eluted first followed by petunidin.

For all of these anthocyanins, the hexose was assigned as glucose considering that glucose was the only monosaccharide previously found after acid hydrolysis of anthocyanin extract obtained from fruits of *S. cumini*.[38] Moreover, the presence of 3,5-diglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin in *S. cumini* was recently confirmed by nuclear magnetic resonance (NMR).[39] Our observations are consistent with those reported in earlier studies with *S. cumini* fruits, where the 3,5-diglucoside of petunidin is the major anthocyanin present.[38] The presence of glucose released from B2 f2 was also confirmed with the qualitative TLC analysis of sugars (Figure 4B).

B1 and B3 bands contained predominantly malvidin 3,5-diglucoside (m/z 655; product ions m/z 493.1 and 331.1) and delphinidin 3,5-diglucoside (m/z 627; product ions m/z 465.1 and 303.1), respectively, as indicated by ESI-MS/MS analyses (Supplementary Figures S3, S4A,B). Besides, B1 was found to fluoresce at 366 nm, which is indicative of malvidin 3,5 diglucoside.[40]

Glycosidic Analysis. The aqueous hydrolysate obtained by acid hydrolysis of anthocyanin (B2 f2) was evaluated by qualitative TLC analysis. The R_f values of standard hexose were glucose, 0.49; galactose, 0.42; rhamnose, 0.69; xylose, 0.62; mannose, 0.46; and fructose, 0.45. The R_f value of the sample was found to be 0.49, thus confirming the identity of the hexose moiety as glucose (Figure 4B).

Spectral Analysis. Anthocyanins typically show an absorption band in the 490–550 nm region of the visible spectrum. B2 f2 when analyzed in the range of 250–600 nm displayed maximum absorption at 535 nm (Figure 4C). This matches with the absorption maximum of petunidin-3,5-diglucoside.[10]

Effect of Anthocyanin (B2 f2) on Radiation-Induced Cell Filamentation As Marker of SOS Response. The normal *E. coli* cell measures 1–2.5 μm in length. Its morphology upon γ radiation exposure changed drastically to long cell filaments even as long as 30 μm (Figure 6A). The filamentation frequency was found to be ~60% in a radiation-exposed

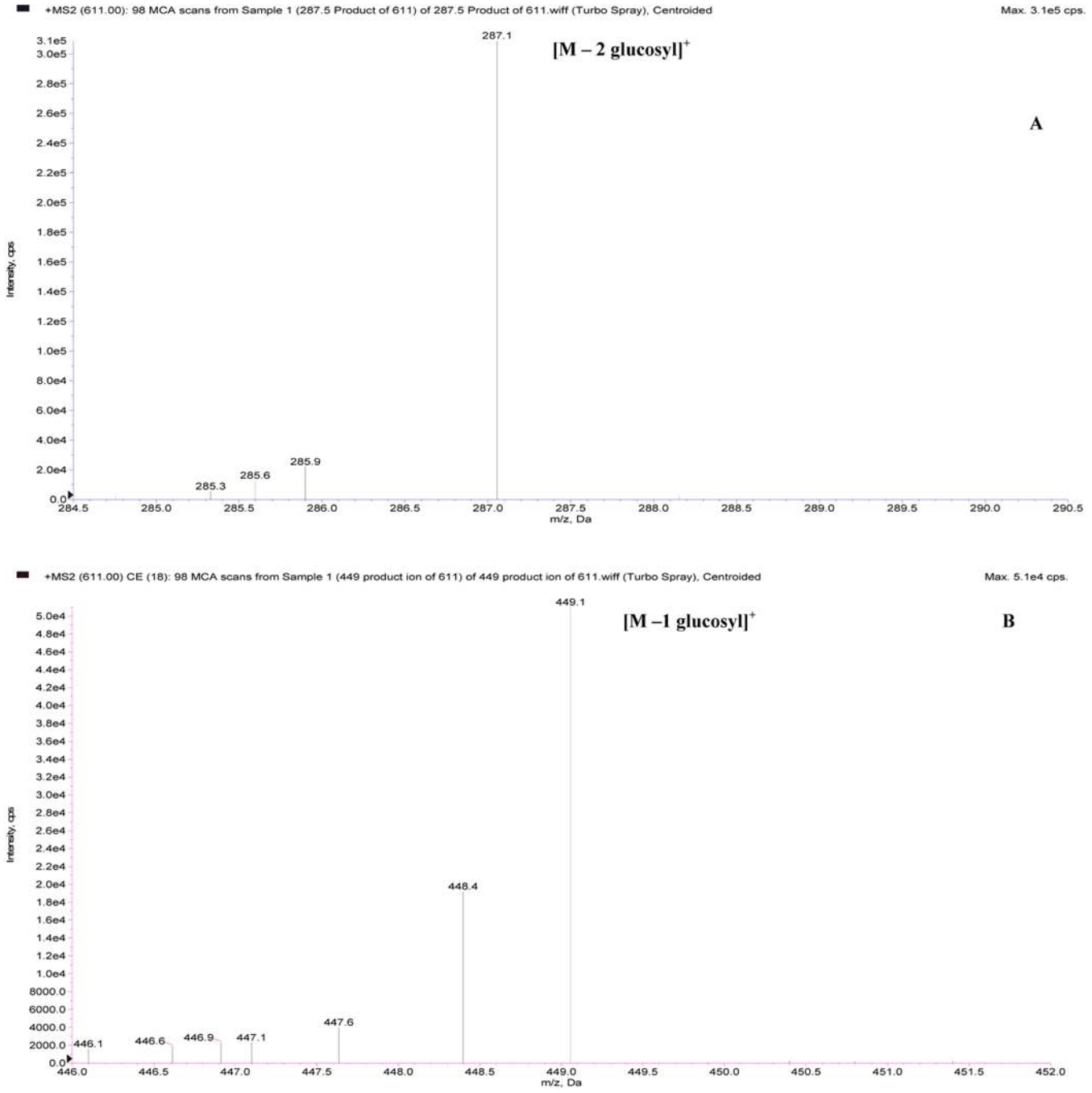

Figure 5. (A) Mass spectrometry of anthocyanin (B2 f1) showing loss of two glucosyl moieties to yield product ion of m/z 287 (Cyanidin aglycone). (B) Loss of one glucosyl moiety to yield a product ion of m/z 449.

population, which in the presence of anthocyanin (B2 f2) was significantly reduced by ~87% (Figure 6C).

Effect of Anthocyanin (B2 f2) on Radiation-Induced Defective Lambdoid Prophage Induction Using SIVET Assay. SIVET induction frequency in radiation-exposed *E. coli* (SG104) cells and its inhibition by anthocyanin (B2 f2) is shown in Figure 6D. The SIVET induction frequency in γ radiation-treated cells was found to be ~90%, which reduced to ~43% in the presence of anthocyanin (B2 f2).

DISCUSSION

Most of the fruits are known to have a number of health-protective phytochemicals including flavonoids. Among the flavonoids, anthocyanins are a relatively small group of water-soluble vacuolar pigments. The flavylium cation (anthocyanidin) is glycosylated, and the glycosides are often acylated, which creates multiple potential structural and functional variants. There are about 17 naturally occurring anthocyanidins, of which only 6 anthocyanidins are ubiquitous in nature, namely, pelargonidin (Pgn, m/z 271), cyanidin (Cyd, m/z 287), peonidin (Pnd, m/z 301), petunidin (Ptd, m/z 317), malvidin (Mvd, m/z 331), and delphinidin (Dpd, m/z 303).[41] ESI-MS/MS analysis served as a powerful and rapid technique for characterization of anthocyanins.[24] Although numerous studies have shown that anthocyanins display a wide range of biological activities, including cancer chemopreventive attributes, there

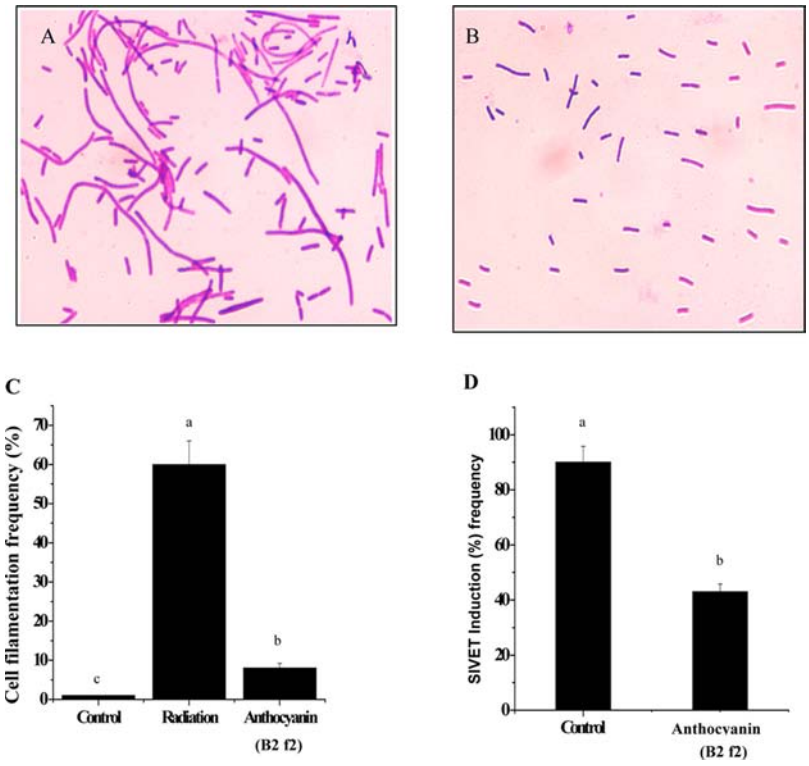

Figure 6. (A) Filamentation in radiation-exposed *E. coli* cells. (B) Inhibition of cell filamentation by anthocyanin (B2 f2). (C) Increase in cell filamentation upon radiation exposure and its inhibition by anthocyanin (B2 f2). (D) Increase in SIVET induction upon radiation exposed *E. coli* SG 104 cells and its inhibition by anthocyanin (B2 f2). Values with different letters (a−c) on the bar are significantly different at $p < 0.05$.

are only a few reports on antimutagenicity of anthocyanins in general.[41,42] In the current study *S. cumini* fruit juice was found to significantly reduce the extent of chemical (EMS)-induced mutagenesis when evaluated using an *E. coli* based forward mutation assay. Bacteria are relatively easy to handle and have a higher multiplication rate (shorter doubling time) that makes them suitable to screen mutagenesis/antimutagenesis events. For this reason the most widely and routinely used assay is the Ames test, which is employed by various national and international regulatory agencies.[43] Due to practical limitations, the findings in bacterial systems are extrapolated to higher systems because fundamental biomolecules (viz., DNA), their response to various damaging agents, and to a large extent the process of their rectification (DNA repair) are quite similar across the life forms from prokaryotes to eukaryotes and humans.[44,45]

Our findings also indicated lack of correlation between the antioxidant and antimutagenic activities of anthocyanins. Although significant difference in antimutagenicity was observed among different anthocyanins (HPTLC bands B1, B2, B3), their antioxidant activities were similar. Pedreschi and Cisneros-Zevallios[46] have made similar observations for purple corn anthocyanins, and Bandyopadhyay and colleagues for vegetables.[8] The current results indicate the possibility of a structure-based modulation of antimutagenic property but not antioxidant capacity for the anthocyanins. In contrast to antimutagenicity, a high antioxidant capacity may not be a good indicator of high anticancer activity.[47] It is plausible that substitutions (hydroxylation and methoxylation) on the B-ring of different anthocyanins are important in affecting the antimutagenicity.[48] These substitutions may affect the polarity of anthocyanins and their cellular diffusion characteristics.[49] However, the exact mechanism explaining their structure− activity relationship remains to be elucidated. Petunidin 3,5-diglucoside (B2 f2) containing a methoxy substitution at the 3′ position of the B-ring displayed significantly higher antimutagenicity compared to that of cyanidin-3,5-diglucoside (B2 f1). As mentioned earlier, B3 also displayed marginal antimutagencity. It is apparent that a single methoxy substitution at the 3′ position is critical for the observed antimutagenicity because double methoxy substitutions (at both the 3′ and 5′ positions of the B-ring) present in malvidin 3,5-diglucoside (predominant in B1) drastically reduced its antimutagenic potential.

Gamma radiation results in expression of a resolvase gene (in the SIVET strain) that excises the insertion sequence located within the *cat* (chloramphenicol acetyl transferase) gene that is subsequently functionally expressed, rendering the cells chloramphenicol-resistant [(Tet^R tetracycline-resistant) Cm^S (chloramphenicol-sensitive) to a Tet^S (tetracycline-sensitive) Cm^R (chloramphenicol-resistant)] phenotype. The frequency of Cm^R cells serves as a measure of prophage induction, which in turn parallels SOS induction. Upon γ radiation exposure very strong SIVET induction was observed, but this significantly reduced in the presence of anthocyanin (B2 f2).

Relatively little is known about how anthocyanins enter the body, distribute in tissues, and exert beneficial health effects. Most animal and human studies have found that anthocyanins are absorbed mainly as their intact glycosidic form and rapidly reach the circulatory system. The apparent bioavailability is consistently very low across all studies with often less than 0.1% of the ingested dose appearing in the urine.[50] However, the observed health protective effects of anthocyanins in both *in vitro* and *in vivo* studies indicate that these phenolics have very high bioefficacy. Even their low concentrations seem to provide good health protective effects by protecting against

oxidative stress, induction of apoptosis and growth inhibition, and changes in inflammatory response.[51]

In conclusion, significant variation was observed in the antimutagenic potential of commonly consumed fruits in India. Among these *Syzygium cumini* fruits displayed remarkable antimutagenicity with an anthocyanin being the major contributing factor. Chromatographic purification and mass spectrometry based characterization of the antimutagenic anthocyanin showed that it was petunidin-3,5-diglucoside. This anthocyanin exhibited broad spectrum antimutagenicity against various physical (UV) and chemical (EMS and nalidixic acid) mutagens. High antioxidant activity of an anthocyanin does not predict its high antimutagenicity. Further, our study indicated that the inhibitory potential of anthocyanin petunidin-3,5-diglucoside against the error-prone DNA repair pathway (SOS) in *E. coli* is one of the possible mechanisms of its antimutagenicity. Thus, the current findings provide credible evidence of the health protective effects of *S. cumini* fruit.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors wish to thank Prof. M. Z. Humayun, UMDNJ, USA, for providing *E. coli* SG104 and MG 1655 strains. The authors also acknowledge the technical assistance of Sanjeev Kumar, Sunil Shukla, Pritam Patil, and Bhumika Kulai during mass spectrometry and antimutagenicity assays.

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