EXPERIMENTAL
MATERIALS:

Chemicals used for the present studies were obtained from the following sources:

<table>
<thead>
<tr>
<th>CHEMICALS</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Sigma Chemical Co., U.S.A.</td>
</tr>
<tr>
<td>Bathocuproine disulfonate</td>
<td>Sigma Chemical Co., U.S.A.</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>Sigma Chemical Co., U.S.A.</td>
</tr>
<tr>
<td>Catalase</td>
<td>Sigma Chemical Co., U.S.A.</td>
</tr>
<tr>
<td>Deoxyribonucleic acid (calf thymus)</td>
<td>Sigma Chemical Co., U.S.A.</td>
</tr>
<tr>
<td>Diphenylamine</td>
<td>B.D.H., India.</td>
</tr>
<tr>
<td>EcoRI</td>
<td>Banalore Genei Pvt. Ltd., Bangalore, India.</td>
</tr>
<tr>
<td>HaelII</td>
<td>Pharmacia Fine Chemicals, Uppsala, Sweden.</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>Sigma Chemical Co., U.S.A.</td>
</tr>
<tr>
<td>Lambda phage DNA</td>
<td>Isolated and purified according to Maniatis et al., (1982).</td>
</tr>
<tr>
<td>Neocuproine</td>
<td>Sisco Research Laboratories, Pvt. Ltd., Bombay, India.</td>
</tr>
<tr>
<td>Nitroblue tetrazolium</td>
<td>Sisco Research Labs.</td>
</tr>
<tr>
<td>Nutrient Broth</td>
<td>Hi-Media, India</td>
</tr>
<tr>
<td>Nutrient Agar</td>
<td>Hi-Media, India</td>
</tr>
<tr>
<td>S₁-nuclease</td>
<td>Sigma Chemical Co., U.S.A.</td>
</tr>
<tr>
<td>Super oxide dismutase (SOD) (Bovine Erythrocytes)</td>
<td>Sigma Chemical Co., U.S.A.</td>
</tr>
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</table>
Supercoiled pBR322 DNA isolated and purified according to Maniatis et al., (1982).

Smal
Pharmacia Fine Chemicals, Uppsala, Sweden.

Tannic acid
Aldrich Chemical Co., U.S.A.

MICROBIOLOGICAL STRAINS:

<table>
<thead>
<tr>
<th>E. coli strain designation</th>
<th>Relevant Genetic Makers</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>F-, Thi-L, his C4, del (gpt Pro A) 6Z, arg E3, thr-l, len B6, kdg k51, rf6 D1, ara-14, Lac y1, gal k2, xyl-5 mtl-l, tsx-33, sup E44, rps L31, rac. a sup</td>
<td>Dr. Barbara, J. Bachmann E.Coli Genetic Centre, Deptt. of Biology, Yale University, New Haven. CT 06511-7444, USA.</td>
</tr>
<tr>
<td>AB1886</td>
<td>as AB1157 but Uvr A6</td>
<td>-do</td>
</tr>
<tr>
<td>AB2463</td>
<td>as AB1157 but rec. A3</td>
<td>-do</td>
</tr>
<tr>
<td>KL403</td>
<td>F-, Pol A1, thy A34, thi-l, len B6, Pro C32, Lac Z36, ara-14, his F860, mtl-l, xyl-5, rps L109, rps E2015, mal A38.</td>
<td>-do</td>
</tr>
</tbody>
</table>

Bacteriophage \( \lambda_{vir} \) was a generous gift from Prof. R. Thomas, Belgium. All other chemicals used were of analytical grade.
METHODS:

Reaction of Kojic acid/Tannic acid with calf thymus DNA and digestion with S\textsubscript{1}-nuclease:

Reaction mixtures (0.5 ml) contained 10 mM Tris-HCl (pH 7.5), 500 ug calf thymus DNA and varying amounts of Kojic acid/Tannic acid, Ferric chloride/Cupric chloride or other metal ions, Neocuproine, or free radical scavengers were included in some experiments. For anaerobic experiments N\textsubscript{2} was bubbled through the solutions for 2 min. All solutions were sterilized before use. After incubation at room temperature for specified time periods (in case of kojic acid under illumination of 500 lux from a fluorescent lamps) S\textsubscript{1}-nuclease digestion was performed. This assay determines the acid soluble nucleotides released from DNA as a result of enzymatic digestion. The reaction mixture in a total volume of 1.0 ml contained 0.1 M acetate buffer pH 4.5, 1 mM zinc sulphate, water and enzyme. The mixture was incubated for 2 h at 48°C. The reaction was stopped by adding 0.2 ml of bovine serum albumin (10mg/ml) and 1 ml of 14% perchloric acid (ice cold). The tubes were immediately transferred to 0°C for at least 1 h before centrifugation to remove the undigested DNA and precipitated protein. Acid soluble nucleotides were determined in the supernatant using the diphenylamine method of Schneider (1957). To a 1.0 ml aliquot, 2.0 ml diphenyl reagent (freshly prepared by dissolving 1 gm of recrystallized diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of conc. H\textsubscript{2}SO\textsubscript{4})
was added. The tubes were heated in a boiling water bath for 20 minutes. The intensity of blue colour was read at 600 nm.

Treatment of supercoiled plasmid pBR322 DNA with Kojic acid/Tannic acid in presence of Fe(III)/Cu(II):

Reaction mixtures (30 ul) contained 10 mM Tris-HCl (pH 7.5), 0.34 ug of DNA, and other components as indicated in the legends. Incubation at room temperatre was performed for specified time periods. In case of kojic acid, incubation was performed under illumination of 500 lux from a fluorescent lamp. After incubation, 10 ul of a solution containing 40 mM EDTA, 0.05% bromophenol blue tracking dye and 50% (V/V) glycerol was added and the solution was subjected to electrophoresis in submarine 1% agarose gels. The gels were stained with ethidium bromide (0.5 ug/ml) viewed and photographed on a transilluminator. In experiments in which bands were extracted for subsequent studies, a small trough was cut into the gel just ahead of the band to be extracted. Electrophoresis was continued but occasionally interrupted as the stained material (viewed under UV light) entered the trough, which was then emptied with a micropipette. Ethidium bromide was removed from these samples and DNA was processed according to standard methods (Maniatis et al., 1982).

Alkaline gel electrophoresis was carried out as described by Kohen et al., (1986). Alkaline agarose slab gels (15 x 15 x 0.8 cm) contained 0.9% agarose and sodium chloride
The running buffer contained 0.03 M sodium hydroxide and EDTA (0.002 M) and electrophoresis was at 20 V, 40 mA for 14 h. The gel was renatured in 1 M Tris-HCl buffer pH 7.4 containing 0.6 M sodium chloride. The gel was stained for 15 min in 250 ml of water containing (0.3 ug/ml) ethidium bromide. The gel was returned to the renaturation buffer for an additional 30 min and then photographed under ultraviolet illumination.

The photographic negatives were scanned by a GS 300 Transmittance/Reflectance scanning densitometer (Hoffer Scientific Instruments Co., San Francisco, CA) to quantify the relative amounts of DNA in each band.

**Determination of Fe(II) with 1,10-phenanthroline:**

The reaction between Fe(II) and 1,10-phenanthroline to form a red complex serves as a good sensitive method for determining Fe(II) (Sandell, 1959). The complex is stable and the colour intensity does not change appreciably over long periods of time. The intensity of colour is independent of pH in the range of 2.0 to 9.0 and Beer's law is obeyed. The molar absorptivity of the complex \([C_{12}H_8N_2]Fe^{2+}\) is 11,100 at 510 nm. The production of Fe(II) in the presence of kojic acid (50 uM); 1,10-phenanthroline (800 uM) and FeCl\(_3\) (50 uM) was observed and absorption was recorded at 510 nm after the incubation period.
Determination of Cu(I) using neocuproine and bathocuproine:

Cu(I) was determined by employing Cu(I) specific chelating agents neocuproine and bathocuproine. Neocuproine complexes with Cu(I) to form Cu(neocuproine)$_2$ complex which has an absorption peak at 450 nm (Nebesar, 1961), while bathocuproine forms an intense orange complex which absorbs maximally at 480 nm (Jaselow and Dawson, 1951). Production of Cu(I) in a mixture of the drug and Cu(II) was detected both by neocuproine and bathocuproine and absorption was recorded at 450 and 480 nm respectively.

Treatment of λ-DNA with tannic acid and restriction enzyme digestion:

2-3 μg phage DNA was incubated in a total volume of 30 μl in 10 mM Tris-HCl (pH 7.5) with tannic acid which was present at the molarities indicated in the legends. The reaction mixtures were incubated at 37°C for 2 h at the end of which they were dialysed against TE using 0.025 μM pore size Millipore filter to remove unreacted tannic acid. The dialysed solution was digested with the restriction endonucleases. Eco.RI* activity of restriction endonuclease Eco.RI was obtained on using the conditions of low ionic strength, high pH and a high enzyme concentration as described by Polisky et al., (1975). The reaction mixture contained 25 mM Tris-HCl pH 8.5, 5 mM MgCl$_2$, and 100 units of Eco.RI. Other samples were digested with 3-5 units of various restriction endonucleases. The reaction was stopped by adding
1 vol. of a solution containing 0.2% sodium dodecyl sulphate, 20% sucrose and 0.1% bromophenol blue. The mixture was electrophoresed on 1% agarose gels, stained with ethidium bromide and photographed in UV light.

Detection of superoxide anion ($O^{-}_2$):

Superoxide anion was detected by the reduction of nitroblue tetrazollum (NBT) essentially as described by Nakayama, et al., (1983). A typical assay mixture contained, 50 mM potassium phosphate buffer pH 7.8, 0.033 mM NBT, 0.1 mM EDTA and 0.06% triton X-100 in a total volume of 3 ml. The reaction was started by the addition of the drug. After mixing, absorbance was recorded at 560 nm against a blank which did not contain the compound, at different time intervals. To confirm the formation of super oxide anion, superoxide dismutase (SOD) was added into the solution before addition of the compound.

Determination of hydroxyl radical:

This was done by employing aromatic hydroxylation. The assay is based on the ability of hydroxyl radical to hydroxylate aromatic rings at an almost diffusion controlled rates and the measurement of hydroxylated products by a simple colorimetric method using salicylate (2-hydroxy benzoate) as a detector molecule (Richmond et al., 1981). The reaction mixture (2.0 ml) contained the following reagents at the indicated concentrations; 2.0 mM
salicylate, 0.1 mM EDTA, 0.1 mM transition metal ion and 150 mM potassium phosphate buffer, pH 8.0. The reaction was started by adding appropriate amounts of the compound. The mixture was incubated at room temperature for 2 hours. After incubation, the reaction was stopped by adding 80 ul of 11.6 M hydrochloric acid and 0.5 gm of sodium chloride followed by 4.0 ml of chilled diethylether. The contents were mixed by vortexing for 1 min. Next, 3.0 ml of upper ether layer was pipetted out and evaporated to dryness in a boiling tube at 40°C. The tubes were cooled and the residue dissolved in 0.25 ml of cold distilled water to which the following reagents were added in the order stated: (a) 0.125 ml of 10% S/V TCA dissolved in 0.5 M HCl, (b) 0.25 ml of 10% W/V sodium tungstate in water and (c) 0.25 ml of 0.5% W/V sodium nitrite (freshly prepared). After standing for 5 min, 0.5 M potassium hydroxide was added and the absorbance at 510 nm was read exactly after 1 min. To confirm the formation of hydroxyl radical various hydroxyl radical quenchers were added to the reaction mixture before the addition of compound.

Singlet oxygen monitoring:

Formation of singlet oxygen ($^{1}O_2$) was determined in aqueous solution by the method of Kralijic and Mohsni (1978); p-nitrosodimethylaniline (pRNO) solution was prepared in 0.01 M phosphate buffer, pH 7.8. Histidine (33.3 ug/ml) was added to the pRNO solution as a selective acceptor of $^{1}O_2$. Irradiation by fluorescent
light of the reaction mixture was performed for varying time periods of incubation. Singlet oxygen \(^{1}\text{O}_2\) formed a transannular peroxide intermediate complex with histidine leading to the bleaching of pRNO which was then measured spectrophotometrically at 440 nm. The generation of singlet oxygen \(^{1}\text{O}_2\) in the reaction system was further established by carrying out quenching studies with sodium azide (Joshi, 1985).

Assay of hydrogen peroxide (H\(_2\text{O}_2\)) production:

The production of H\(_2\text{O}_2\) was assayed by the method of Nakayama et al., (1983) with modifications. Titanium sulphate solution was prepared from titanium dioxide (Snell and Snell, 1949) and diluted so that the final concentration was 1% (W/V) Ti(SO\(_4\)) in 1.25 M H\(_2\text{SO}_4\). A 2 ml sample containing different amounts of tannic acid in water was mixed with 2 ml 50 mM sodium phosphate buffer, pH 7.2 and incubated at 37°C for 1 h. An aliquot of the mixture was added to 2 ml of a Ti(SO\(_4\))\(_2\) solution. A blank sample was also prepared which did not contain Ti(SO\(_4\))\(_2\). Absorbance was measured at 410 nm. In order to confirm that the colour change was due to the generation of H\(_2\text{O}_2\), in a separate reaction, 0.4 ml of catalase solution (1 mg/ml) was added to the reaction mixture before incubation at 37°C.

Microbiological Methods:

Exponentially growing bacterial cultures were raised in
nutrient broth containing (per litre): peptone, 5 g; beef extract, 1.5 g; yeast extract, 1.5 g; NaCl, 5 g. The bacterial strains of E. coli were plated on nutrient broth containing 1.5% (W/V) agar, whereas the soft agar used in the overlay technique contained 0.7% (W/V) agar. Phage was propagated and titered using E. coli strain AB1157 as host. For assay of phage inactivation, Kojic acid/Tannic acid in water and a stock of aqueous FeCl₃/CuCl₂, previously sterilised by filtration were added to a suspension (0.1 ml) of phage in Tris/Mg²⁺ (0.01 M, pH 8.0). FeCl₃/CuCl₂ was added 10 min after the addition of compound and reaction mixture was incubated at room temperature for specified time periods during which it was vortexed at 5 min intervals. In some experiments deferoxamine/neocuproine or oxygen free radical quenchers were added before the addition of FeCl₃/CuCl₂. After incubation treated phage was diluted with 0.01 M MgSO₄ and 0.1 ml of diluted phage was added to a 0.3 ml suspension of E. coli host strains. λ-E. coli complexes were vortexted for 1 min and incubated for 20 min at 37°C. After incubation, 3.0 ml of soft agar (40°C) was added to the treated phage and immediately poured on the nutrient agar plates. Plates were incubated at 37°C for 5-6 hours followed by counting of plaque forming units (PFU).

For experiments in which the indicator bacteria were pre-treated with UV light, cells were harvested, resuspended and washed three times by centrifugation in 0.15 M NaCl, 0.015 M potassium phosphate buffer, pH 7.5 and the final suspension (10⁸
cells/ml) was poured into a petridish to a depth of 3 mm. The dish was exposed to an uncalibrated bactericidal UV lamp for different periods of time. For experimental assays, the viability of the cells used for assay was determined by dilution and plating on nutrient media.