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Total inhibition of $^1O_2$-induced oxidative damage to guanine bases of DNA/RNA by turmeric extracts

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ABSTRACT

The guanine base of nucleic acids is known to be very reactive towards degradation by $^1O_2$-induced oxidative stress. Oxidative reactions of DNA are linked to many human diseases including cancer. Among the various forms of reactive O_2 species ($^{\cdot}OH$, $^1O_2$ or $O_2^{-\cdot}$), the oxidative stress caused by $^1O_2$ is of particular physiologic importance because of its selectively long life in aqueous medium and its ability to diffuse through a cell membrane. In this study we investigated the degradation of a model compound guanosine (Guo) by $^1O_2$, which was generated by riboflavin-induced photosensitization and by molybdate ion catalyzed disproportionation of H_2O_2. We observed the remarkable ability of an aqueous and alcoholic extracts of Turmeric (*Curcuma longa*) as an extraordinary scavenger of $^1O_2$ to completely inhibit the degradation of Guo. The alcoholic extracts were more effective in their antioxidant activity than the corresponding water extract. This naturally occurring antioxidant offers a most economical supplement to protect biologically significant molecules from the oxidative stress induced by $^1O_2$.

1. Introduction

Oxidative reactions of DNA are known to be involved in mutagenesis, carcinogenesis, aging and other chronic diseases [1–7]. The chemical reactions involved in oxidative stress include base modifications, strand breaks, interstrand cross-linking, adduct formation by primarily involving reactive oxygen species (ROS) [8–10]. Out of the various forms of ROS, singlet oxygen ($^1O_2$) is of considerable interest as it is known to specifically target the guanine base of nucleic acids by a reaction mechanism known as photodynamic action [11,12]. In photosensitization reactions, synergistic action of a photosensitizer, light and O_2 leads to the formation of $^1O_2$ that results in cellular destruction [13,14]. With the realization that $^1O_2$ has a relatively long lifetime (from microseconds to milliseconds) and that it can diffuse through cell membranes, its role in biological system becomes more apparent [15]. Photodynamic reactions also have remarkable success in killing microorganisms and treatment of cancer [13,16,17]. Although, $^1O_2$ can be generated by a number of methods, the photosensitization and chemical reactions are two most commonly employed methods for its formation in the laboratory [18,19].

For the present study, we used both photosensitization and chemical methods for generating $^1O_2$. Riboflavin (RF, 7,8-dimethyl-10-ribitylisoalloxazine or Vitamin B_2) was used as a photosensitizer to generate $^1O_2$ under ultraviolet light (UVB, 290–320 nm) [20,21]. Selection of RF is of special interest because in addition to a photosensitizer, it is also an essential nutritional factor of our physiological system [22]. Earlier studies revealed RF as a potential chromophore for inducing genetic damage when phototherapeutic illumination is used in the presence of multivitamins to treat neonatal hyperbilirubinemia for enhancing the photodecomposition of bilirubin [23,24]. Illuminated RF formed adducts with DNA and poly(dA)-poly(dT) and also caused DNA modifications [23–25]. Our initial studies have shown that RF, upon exposure to UV radiation degrades DNA and RNA bases [21]. Out of the five heterocyclic bases of DNA and RNA (A, G, C, U and T), the guanine (G) base was found to be the most reactive to the synergistic action of UV radiation and RF. In a follow up study, we demonstrated that certain naturally occurring antioxidants like ascorbic acid, glutathione, glycolic acid and quercetin could inhibit the photosensitized degradation of these bases [26]. In this study, we demonstrate a remarkable ability of the extracts of turmeric (primarily curcumin) as an extraordinary scavenger of $^1O_2$ to completely inhibit the degradation of Guo. Curcumin, the yellow pigment in turmeric, is well known for its antioxidant,

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anti-inflammatory, antimicrobial and anticarcinogenic properties in model reactions [27–29].

2. Materials and methods

2.1. Chemicals

The chemicals used for the study were obtained as follows: catalase, curcumin, G, deoxyguanosine (dGuo), 1,4-diaza-[2.2.2]-bicyclo-octane (DABCO), guanosine 5′-phosphate (GMP), Guo, histidine (HIS), hydrogen peroxide (H_2O_2), mannitol, N,N-dimethyl-p-nitrosoaniline (RNO), RF, sodium azide (NaN_3), sodium molybdate (Na_2MoO_4), sodium phosphate (NaH_2PO_4), superoxide dismutase and trifluoroacetic acid (TFA) were purchased from Sigma/Aldrich Chemical Company. Acetonitrile (CH_3CN) and sodium hydroxide were purchased from Mallinckrodt. Turmeric samples were procured in powder form (New Delhi, India), dry roots (Reliance™Select) and fresh roots (Amazon.com).

2.2. Generation of $^1O_2$

$^1O_2$ was generated by either UV radiation-induced photosensitization of RF or by the disproportionation of H_2O_2 catalyzed by Na_2MoO_4 [20,30]. For the photosensitization reaction, a 2.5 μg/mL RF solution was prepared in a 0.01 carbonate buffer (pH 10). Samples (10 mL each) were transferred into a Petri dish and irradiated under UVB (290–320 nm) for different dose ranging from 0 to 12 J. The UVB irradiation system used for this study comprised a horizontal planar array of three 4-feet medium wave UVB-emitting (T-40M) fluorescent tubes manufactured by Vilber Lourmat, Marne La Valle, France. The irradiance of the emitted light was measured by a VLX-3W UVR-probe, which was equipped with UVB (SX-12 nm) detector manufactured by Vilber Lourmat. The UVR dose is expressed in Joule (J = W/cm^2 × s). $^1O_2$ was measured in an aqueous solution by the method proposed by Kraljic and El Mohsni [31]. $^1O_2$ forms a trans-annular peroxide intermediate with histidine leading to the bleaching of RNO, which is measured spectrophotometrically at 440 mm. $^1O_2$ was generated chemically by following the method of Aubry and Cazin [30]. This method is based upon the formation of $^1O_2$ in the disproportionation of H_2O_2, which is catalyzed by mineral compounds in an alkaline solution. A solution of Na_2MoO_4 (20 mM) was prepared in NaOH (10 mM) and H_2O_2 (10 mM) was added to the solution prior to the reaction.

$$H_2O_2 + Na_2MoO_4 \rightarrow {}^1O_2 + {}^3O_2$$

2.3. Extraction of turmeric

Powered dry root (5 g), dried whole root (5 g) or fresh whole root (25 g) were extracted with 100 mL each of deionized H_2O, 60% C_2H_5OH in H_2O, 60% CH_3OH in H_2O and 30% C_2H_5OH + 30% CH_3OH in H_2O. The fresh whole roots were peeled, rinsed with distilled deionized H_2O and then minced in a Cuisinart Mini-Prep food processor. The dried whole roots were soaked overnight in 40 mL H_2O for softening and then minced. After refluxing and cooling to room temperature, each extract was filtered and stored in the refrigerator. Each extracts were prepared for studies in the following manner: five samples of various volumes (2–10 mL, Table 1) of the extract were dried at room temperature in Petri dishes. Deionized H_2O (4 mL) was added to the extracts and the mixture was transferred to a glass vial (40 mL). Freshly prepared $^1O_2$ reagent (5 mL each) was added to the five turmeric extract samples along with 1 mL of 10 mM Guo (total volume 10 mL). The sixth sample consisted of 5 mL $^1O_2$ reagent with 1 mL of 10 mM Guo diluted to 10 mL with H_2O was used as a control. The final sample consisting of 10 mM Guo (1 mL), which was diluted to 10 mL, with H_2O. Samples were reacted at room temperature for 24 h. 0.1 mL aliquots of each sample were diluted to 1.0 mL with H_2O and filtered with Alltima 0.45 μ nylon syringe filter prior to analysis using high pressure liquid chromatography (HPLC).

Table 1
A dose dependent effect of different turmeric extracts on $^1O_2$-induced guanosine degradation. Each extract (2–10 mL) were air dried before use.

Extraction reagents	% inhibition of Guo degradation from dry extracts				
	2 mL	4 mL	6 mL	8 mL	10 mL
Turmeric powder					
Water extract	2.42	09.3	15.2	17.7	21.2
Ethanol extract	47.6	55.0	63.5	71.6	75.8
Methanol extract	18.2	25.0	30.6	70.4	77.4
Ethanol + methanol + water extract	33.3	63.6	80.5	82.4	89.5
Turmeric roots (fresh)					
Water extract	8.00	11.5	16.7	23.3	30.4
Ethanol extract	25.4	70.9	100		
Methanol extract	15.6	36.2	78.0	89.6	100
Ethanol + methanol + water extract	57.8	89.2	100		
Turmeric roots (dry)					
Water extract	3.80	11.1	14.2	19.0	32.7
Ethanol extract	67.3	91.5	100		
Methanol extract	55.6	85.4	97.3	100	
Ethanol + methanol + water extract	68.4	100			

2.4. Determination of guanosine

HPLC analysis was performed by using a Hitachi L-6200A intelligent pump system, which was equipped with a Hitachi L-4000 UV detector operating at 260 nm. The analyses of Guo and dGuo were performed on a reverse phase Alltima C-18, 5 μ (4.6 mm × 250 mm) column (Grace) using a gradient of 0.02 M NaH_2PO_4 with 0.2% TFA (pH 2.5) and 30% CH_3CN in water with 0.2% TFA (pH 2.5). Percent degradation of Guo and dGuo was estimated by comparison of average peak area (ChromJet Integrator, Spectra Physics) of control samples with $^1O_2$-reagent treated samples in the presence or absence of turmeric extracts determined from three independent measurements.

3. Results

3.1. Production of $^1O_2$

Fig. 1 demonstrates a steady state increase in the decomposition of RNO by RF upon exposure to UVB radiation. $^1O_2$ production was measured by recording a decrease in the absorption of RNO at 440 nm. This test is specific for $^1O_2$ detection as the bleaching of RNO takes place only in the presence of HIS (10 mM), which forms a trans-annular peroxide intermediate with $^1O_2$. Other well recognized free radicals and ROS like $^{\cdot}OH$, H_2O_2 or $O_2^{\cdot-}$, which can also be generated under oxidative stress, do not react with RNO. Addition of well recognized scavengers of $^1O_2$ like NaN_3 (10 mM) or DABCO (25 mM) to the RNO + HIS + RF reaction mixture, resulted in 90–100% inhibition of RNO bleaching (or indirectly $^1O_2$ production). We have already reported that RF generates $^1O_2$ comparable to equimolar amounts of Rose Bengal [21] or hematoporphyrin derivatives [32]. Both Rose Bengal and hematoporphyrin are well-recognized photodynamic agents exhibiting photobiological reactions by $^1O_2$ generation [33,34].

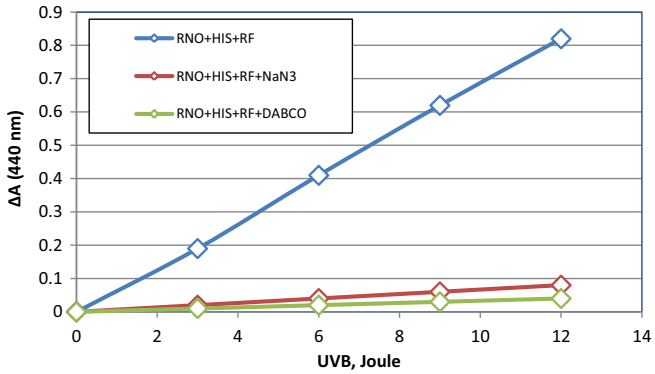

Fig. 1. Production of singlet oxygen ($^1O_2$) with riboflavin (RF) plotted as a function of UVB (290–320 nm) exposure dose. The formation of $^1O_2$ was ascertained by evaluating the bleaching of p-nitrosodimethylaniline (RNO) at 440 nm in the presence of histidine (HIS) as a selective acceptor of $^1O_2$. Reaction system: RNO ($3.5–3.0 \times 10^{-5}$M) + HIS (1×10^{-2}M) + RF (2.5 μg/mL). The effect of quenchers was determined by adding NaN_3 (10 mM) or DABCO (25 mM) to the reaction mixture.

3.2. Photosensitized degradation of guanosine and deoxyguanosine

The UVB sensitized reactions of RF led to almost a similar level of degradation to Guo and dGuo (Fig. 2). A more or less complete inhibition in the degradation of Guo was observed when reactions were carried in the presence of 25 mM DABCO. Another well recognized $^1O_2$ quencher, NaN_3, produced over 90% inhibition of Guo degradation. These observations clearly suggested that $^1O_2$ was largely responsible for the photodegradation of the G bases. Other antioxidants like superoxide dismutase ($O_2^{\cdot-}$ scavenger), catalase (H_2O_2 scavenger) and mannitol ($^{\cdot}OH$ scavenger) did not show any influence in preventing the degradation of Guo under similar conditions.

3.3. Effect of curcumin in inhibiting the $^1O_2$-induced photodegradation of Guo

Guo was allowed to degrade to near-completion (97.5%) by UVB-induced sensitization of RF (Fig. 3). Addition of curcumin (4–20 μg/mL), an active ingredient of turmeric (*Curcuma longa*), to the reaction mixture resulted in a dose dependent inhibition of the Guo degradation (Fig. 3). Interestingly, curcumin in spite of exhibiting strong absorption in the UV/visible spectral range (resembling RF) did not generate $^1O_2$ by its own UVB-induced photosensitization. The scavenging study with curcumin could not be extended beyond 20-μg/mL due to its strong absorption in the UVB spectral range and also its lack of solubility in H_2O. Therefore,

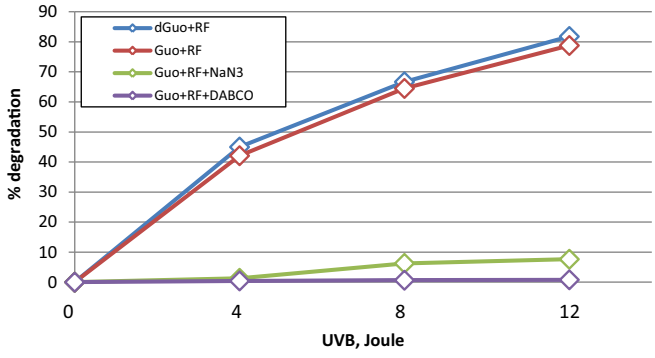

Fig. 2. UVB sensitized degradation of deoxyguanosine (dGuo) and guanosine (Guo) in a 0.01 M carbonate buffer (pH 10) by 2.5 μg/mL riboflavin (RF) and it's quenching by sodium azide (10 mM) and DABCO (25 mM).

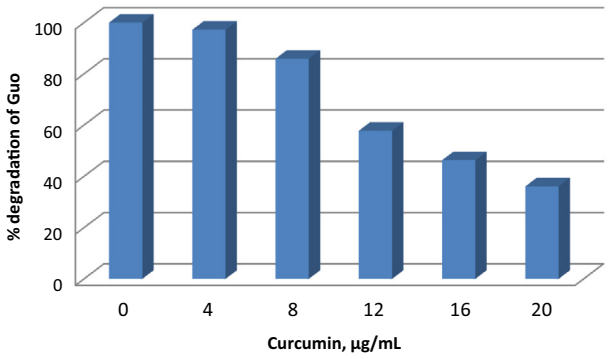

Fig. 3. A dose dependent inhibition of $^1O_2$-induced degradation of guanosine (Guo) by varying amounts of curcumin.

for the studies involving effect of turmeric extracts in Guo degradation, reactions were carried out on a chemically generated $^1O_2$ system [30]. In this system, both dGuo and Guo were degraded to completion (100%) in an alkaline solution of 20 mM Na_2MoO_4 and 10 mM H_2O_2 in 24 h at room temperature. When the reagent was diluted to 50% with water, it produced ~84.7% degradation of Guo under similar conditions. Further dilution of the reagent to 40%, 30%, 20% and 10% led to 81.1%, 70.1%, 59.6% and 38.1% degradation of Guo degradation, respectively. The Guo degradation by chemically generated $^1O_2$ was also inhibited by ~90% when NaN_3 (10 mM) was added to each of the diluted reaction mixture.

3.4. Inhibition of Guo degradation by turmeric extracts

Different amounts of turmeric extracts which were isolated from 4 different extraction procedures, when added to the mixture of chemical $^1O_2$ generation reagents in the presence of Guo, showed remarkable inhibition of Guo degradation (Fig. 4, Table 1). HPLC analysis provided an efficient analytical tool for monitoring the scavenging of turmeric extracts within 98% accuracy. The retention times of the reagent (Fig. 4A) and Guo (Fig. 4B) were separated by a wide margin to facilitate analysis. Although, turmeric extracts were resolved into multiple peaks in HPLC (Fig. 4D), the interference caused by a few minor peaks at retention time coinciding with the Guo (~19-min) was always below 2%. This was ensured by computerized integration of the HPLC peaks of the extracts with or without Guo. The reagent alone (alkaline

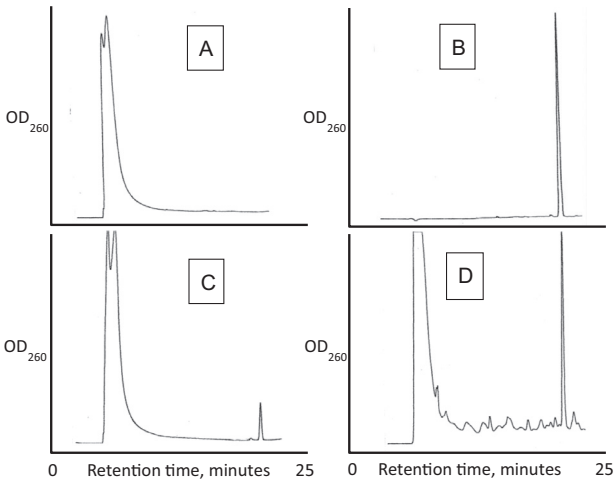

Fig. 4. HPLC analysis of chemically generating $^1O_2$ system consisting of alkaline $Na_2MoO_4 + H_2O_2$ (A), Guo alone (B), Guo after 24 h reaction with the reagent (C) and protection of Guo with a Turmeric extract (D).

$Na_2MoO_4 + H_2O_2$) was eluted between 6–8 min (Fig. 4A). Guo was degraded by about 83% in a 1:1 ratio of reagent with H_2O (Fig. 4C). The combined ethanol + methanol extracts of turmeric provided 89.5–100% protection of Guo degradation in all the samples tested (Table 1).

4. Discussion

Three significant aspects of oxidative stress are addressed in this study: (i) sensitivity of the G bases of DNA/RNA to oxidative damage (ii) involvement of a nutrient (Vitamin B_2) in oxidative stress and (iii) a common ingredient of Asian spices (turmeric) showing remarkable ability to protect G bases against $^1O_2$-induced damage. Turmeric is a rhizomatous herbaceous perennial plant of ginger family, Zingiberaceae [35]. Its active ingredient curcumin has been linked to antioxidant, anti-inflammatory, antimicrobial and anticarcinogenic properties [36]. It also inhibits $^1O_2$-induced damage in plasmid DNA against single-strand breaks [37]. We focused our attention on protecting the G bases of DNA/RNA using Turmeric extracts, in view of its high reactivity towards $^1O_2$ [8,9,38]. In a recent study, we investigated the $^1O_2$-induced photodegradation of G derivatives (G, Guo and guanosine 5′-phosphate) and observed the following order of reactivity: base > nucleoside > nucleotide [21]. Therefore, Guo was selected as a model compound due to its intermediate level of reactivity to photosensitization action, although both Guo and dGuo were almost equally reactive to $^1O_2$ (Fig. 2). Generation of $^1O_2$ by RF is of special significance because this vitamin supplement is an endogenous chromophore with a potential to enhance the harmful effects of solar radiations by photodynamic action. The water extract of turmeric was not as rich antioxidant as were the methanolic, ethanolic or their mixtures. Visible observation of the extracts revealed yellow pigment of Turmeric (curcumin) was predominantly extracted in alcoholic solvents. The most remarkable outcome of this study was that the alcoholic extracts of fresh and dry turmeric roots caused total prevention of $^1O_2$-induced Guo degradation. Commercial sample of curcumin was also effective in partially inhibiting the Guo degradation. Curcumin show potential for being used as an antioxidant supplement because various animal studies and human subjects have shown it to be a safe compound at high doses [35]. Regular intake of Turmeric as a dietary supplement may reduce adverse effects of endogenous and exogenous compounds capable of forming free radicals and ROS in sunlight or indoor lights. Controlled application of turmeric in photodynamic therapy can also prevent DNA damage in healthy cells. In conclusion, our results demonstrate a potentially unique way of protecting the vulnerable base constituents of DNA and RNA from oxidative stress with alcoholic extracts of turmeric.

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