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Interaction of anthraquinones of *Cassia occidentalis* seeds with DNA and Glutathione

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ABSTRACT

Consumption of *Cassia occidentalis* (CO) seeds has been associated with the hepatomyoencephalopathy (HME) in children. Recently, we have characterized the toxic anthraquinones (AQs) such as Emodin, Rhein, Aloe-emodin, Chrysophanol and Physcion in CO seeds and detected these moieties in the bio fluids of CO poisoning cases. As AQs were detected in the serum of HME patients, their interaction with key biomolecules including protein, DNA and glutathione (GSH) is imperative. In this regard, we have previously reported the interaction of these AQs with serum albumin protein and their subsequent biological effects. However, the interaction of these AQs with DNA and GSH remained unexplored. In the present work, we have studied the binding of these AQs of CO seeds with DNA and GSH by fluorescence spectroscopy, UV–vis spectral analysis, molecular docking, and biochemical studies. Results indicated a higher binding affinity for Emodin ($K_a = 3.854 \times 10^4\,L\,mol^{-1}\,S^{-1}$), Aloe-emodin ($K_a = 0.961 \times 10^4\,L\,mol^{-1}\,S^{-1}$) and Rhein ($K_a = 0.034 \times 10^4\,L\,mol^{-1}\,S^{-1}$) towards calf thymus DNA may be associated with their higher cytotoxicity. Alternatively, Physcion and Chrysophanol which showed less cytotoxicity in our earlier studies exhibited very low DNA binding. The binding pattern of all these AQs is consistent with the *in-silico* data. Absorption spectroscopy studies indicated the possible formation of GSH conjugate with Aloe-emodin and Physcion. Further biochemical measurement of GSH and GSSG (Glutathione disulfide) following incubation with AQs indicated that Aloe-emodin (28%) and Rhein (30%) oxidizes GSH to GSSG more as compared to other AQs. Taken together, these results suggest that the higher cytotoxicity of Rhein, Emodin and Aloe-emodin may be attributed to their potent DNA and GSH binding affinity.

1. Introduction

In recent past, accidental poisoning of *Cassia occidentalis* seeds has been known to be the causative factor for children death in several parts of India [1–3]. Our previous studies have established the association of children death with CO poisoning [1] and have identified the toxic anthraquinones (AQs) including Aloe-emodin, Chrysophanol, Emodin, Physcion and Rhein in CO seeds [4]. All these AQs were further detected in the serum of CO seeds exposed patients as well as in the experimental rats, linking their role to the CO toxicity [4]. In another study, we have reported that CO seeds treatment to rats modulate an array of transcripts including oxidative stress and xenobiotic metabolism [5]. Impairment of xenobiotic metabolism due to CO seeds exposure may lead to the accumulation of active ingredients including AQs inside the body that may cause toxic manifestations.

Although the toxicity of some of these AQs is known, the mechanism (s) of toxicity is not fully understood [6,7]. The AQs group of compounds falls under a large group of bioactive Quinones. Quinones primarily exhibit toxicity in two ways; firstly, forming conjugates with cellular macromolecules (Protein, DNA and Glutathione), secondly by generation of semi Quinone radical by one electron transfer leading to oxidative stress [8]. In one of our earlier studies we have examined the binding affinity of AQs of CO seeds including Aloe-emodin, Chrysophanol, Emodin, Physcion and Rhein with bovine serum albumin pro-

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tein and its association with the cytotoxicity in hepatic cells [9]. However, the interaction of these AQs with DNA and GSH remain unexplored.

Earlier, through the hepatic transcriptional analysis of CO seeds exposed rat, we have demonstrated that the toxic ingredients of CO seeds induce the DNA damage and apoptotic pathways in rat liver [5]. In addition, recently we have seen that Rhein, a toxic anthraquinones in CO seeds causes DNA damage and induce apoptosis in rat primary hepatocytes [10]. Further, we have shown that CO seeds or the anthraquinones therein decreases the free-SH or GSH both *in vitro* and *in vivo* [1,10]. Therefore, it is essential to explore the AQs-DNA and AQs-GSH interaction in detail. In this study we have investigated the binding affinity and interactions of AQs with DNA and GSH in a context to their toxicity.

2. Material and methods

2.1. Chemicals and reagents

Calf thymus DNA (ctDNA), Emodin, Rhein, Aloe-emodin, Chrysophanol, Physcion, Glutathione (GSH), oxidized Glutathione (GSSG), Ethidium bromide (EtBr) Orthopthaldehyde (OPT) and N-ethylmaleimide (NEM) were purchased from Sigma Aldrich Co. (St. Louis, MO). All other chemicals used were of the highest purity available from commercial sources.

2.2. DNA stock preparation and quantification

The stock solution of calf thymus DNA was prepared in Tris-EDTA buffer (1 M Tris and 0.5 M EDTA, pH 8.0) as described earlier [11]. DNA solution with UV absorbance ratio at 260 and 280 nm (A_{260}/A_{280}) greater than 1.9 showed that the DNA was free of protein. DNA concentration was determined by Spectrophotometer ND1000 (NanoDrop Technologies Inc., USA) and expressed as ng/μl. Further the concentration was converted to micromolar equivalent using A_{260} unit of double stranded DNA corresponding to 50 μg/ml, which is equivalent to 0.15 mM. Absorption coefficient of 6600 M^{-1} cm^{-1} for DNA was used for quantification.

2.3. Interaction of anthraquinones with DNA-ethidium bromide complex

The fluorescence emission spectra of all the AQs were recorded separately and compared with the emission spectra of DNA, EtBr and DNA-EtBr complex. Since, Emodin emission spectra showed no overlapping with DNA-EtBr, Emodin was further studied by this method as described earlier [12]. In brief, Perkin Elmer Luminescence spectrometer (Waltham, MA) with a quartz cell of 1 cm path length was used to measure the fluorescence. The excitation wavelength was set at 350 nm while the emission spectrum was scanned from 500 to 750 nm. The slit width of the excitation and emission was set at 10 nm. The fluorescence spectra of different concentrations of Emodin (5–50 μM) in a fixed concentration of DNA-EtBr complex (75 μM DNA and 6.3 μM EtBr) were measured in 0.4 M Britton-Robinson buffer (0.04 M acetic acid, 0.04 M boric acid and 0.04 M orthophosphoric acid at pH 7.4). The quenching constants of Emodin to DNA-EtBr complex was quantitatively calculated from the Stern Volmer [13] and modified Stern Volmer plot [14] as described in the results section.

2.4. Interaction of DNA with the emission spectra of anthraquinones

The fluorescence emission spectra of all the AQs (20 μM) were recorded. Subsequently, fluorometric titration was carried out by adding ctDNA (10–100 μM). Among all the AQs, only the emission spectra of Rhein and Aloe-emodin were efficiently quenched by the addition of ctDNA. Hence, the binding of these two AQs with DNA was carried out following the method described earlier [15]. In brief, fluorescence measurements were carried out on a Perkin Elmer Luminescence spectrometer (Waltham, MA) by progressive addition of ctDNA (0–1.5 mM) to a fixed concentration (50 μM) of Aloe-emodin or Rhein in BR buffer. The excitation wavelength was set at 400 nm and the emission spectra were recorded from 450 nm to 750 nm. The intrinsic fluorescence of Aloe-emodin and Rhein was obtained at 582 nm when excited at 400 nm. The quantitative analysis of the potential interaction of Aloe-emodin and Rhein with DNA was performed based on the fluorometric titration. The dynamic quenching constant, quenching rate constant and effective quenching constant were calculated using Stern-Volmer equation (SVE) and modified Stern-Volmer equation (MSVE).

2.5. Molecular docking study of anthraquinones with ctDNA

Docking of AQs with DNA were carried out using Discovery Studio version 4.0 (Accelrys, San Diego). The crystal structure of Calf thymus DNA (ctDNA) was obtained from Protein Data Bank (PDBID: 453D) [16]; [17]. In order to prepare the structure for molecular docking, we removed water molecules and added gasteiger charges on the crystal structure. The structures of five anthraquinones (AQs) i.e. Rhein (Pubchem CID:10168), Emodin (Pubchem CID:3220), Aloe-emodin (Pubchem CID:10207), Chrysophanol (Pubchem CID:10208) and Physcion (Pubchem CID:10639) were retrieved from pubchem [18]. The minimum energy conformation of ligand molecules was generated using “Generate Conformation” protocol in Discovery Studio using CHARMm (Chemistry at HARvard Macromolecular Mechanics) force field [19,20]. Molecular docking studies were performed using the CDOCKER module implemented in Discovery Studio. A set of 10 random orientations of each ligand molecule were produced. In order to achieve docking poses with high accuracy and to measure the amelioration of a docking study, CDOCKER score was used as a standard [21]. To further validate our *in-silico* results, molecular docking studies were also performed using Autodock 4.2 program (version 1.5.6), using the same receptor and ligand set. The detailed methodology for molecular docking using Autodock version 4.2 program has been described in supplementary material.

2.6. Absorption spectroscopy studies of anthraquinones-GSH interaction

To investigate the interaction of AQs with GSH, UV–vis absorption spectroscopy was employed following the method described earlier [22]. Initially, the UV–vis absorption spectra of all the five AQs (Aloe-emodin, Chrysophanol, Emodin, Physcion, and Rhein) at a maximum concentration of 50 μM were recorded in Shimadzu UV 2550 double beam spectrophotometer (Tokyo, Japan). The AQ-GSH conjugate was studied by mixing the AQ (25 μM) with GSH (25 and 75 μM) in 0.1 M phosphate buffer (pH 7.4) followed by incubation of 15 min at 37 °C. Equal amount of compound was added to the reference and sample cuvettes and the difference spectra of various AQs (350–500 nm for Aloe-emodin, Chrysophanol, Physcion and Rhein; 375–600 nm for Emodin) was recorded.

2.7. Measurement of GSH and GSSG in GSH-anthraquinone incubation mixture

The content of GSH and GSSG was assayed in GSH-AQ incubation mixture using OPT as a fluorescent probe, which binds GSH or GSSG to form highly fluorescent derivative [23]. For GSH assay, the reaction mixture in a final volume of 3.5 mL contained 3.25 mL sodium phosphate-EDTA buffer (0.1 M, pH 8.0), 0.25 mL OPT (1 mg/ml methanol). The reaction was initiated by the addition of AQ (50 μM) in a cuvette containing GSH (100 μM) at room temperature. After 15 min, the formation of fluorescent GSH-OPT adduct was detected and read on the spectrofluorometer at an excitation and emission wavelengths of 350 and 450 nm (slit widths 5 & 10 nm), respectively. For GSSG assay, NEM was used to prevent oxidation of GSH to GSSG during the assay. The