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In vitro analysis of *Calotropis* peroxidase transformed diamine compounds

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Abstract

Benzidine (BZ), O-dianisidine (OD) and Paraphenylenediamine (PPD) compounds are commonly used in hair dyes, textile dying and paper coloring industries. These dyes are reported to have genotoxic property. When such dyes are let it to effluent adds significant carcinogenicity to the rodent and human, removal of such dyes remain essential. Application of *Calotropis gigantea* peroxidase (CgPOD) for biotransformation of these genotoxic dyes found to be one of the effective processes to reduce the toxicity. When BZ and OD were exposed to CgPOD, get transferred from genotoxic to non-genotoxic form, whereas the PPD transformed to the genotoxic form due to the formation of cyclohexa-2,5-dione-1,4-diylidenediamine compound. The structural changes were elucidated using FT-IR & ^{1}H NMR, which confirmed the biotransformed structure of PPD, BZ and OD to cyclohexa-2,5-dione-1,4-diylidenediamine, bis-(4'-amino-biphenyl-4-yl)-diazene and bis-(3,3'-dimethoxy-4'-amino-biphenyl-4-yl)-diazene, respectively. In conclusion, although the BZ and OD are genotoxic and not in the case of PPD when exposed to human systems, the CgPOD mediated metabolites of BZ and OD were non-genotoxic as shown in DNA fragmentation assay and fluorescence assay. Whereas the PPD metabolite induce or activate genotoxicity when exposed to lymphocytes and it shows 13.05% residual fluorescence intensity at 0.15 mg/ml. Hence the CgPOD mediated biotransformation can be effectively used in the treatment of BZ and OD containing dye effluents and should not be used in dyes having PPD.

Keywords: Benzidine, Biotransformation, *Calotropis gigantean*, O-dianisidine, Paraphenylenediamine, Plant peroxidase

1 Introduction

Aromatic amines represent one of the most important classes of industrial and environmental chemicals. Many of them have been reported to be a powerful chemical carcinogens, mutagens and/or hemotoxicants. For example, benzidine (BZ) and O-dianisidine (OD) is recognized as a human carcinogen and rodents [1]. BZ is a major mutagenic moiety of many azo dyes, and Bz or its congeners can be generated from azo dyes through reduction by intestinal and environmental microorganisms [2]. Paraphenylenediamine (1,4-diaminobenzene) (PPD), an arylamine, is one of the most common allergens among patients with allergic contact dermatitis and also it induce myocarditis and myocardial infarction [3]. PPD has several applications, including as an ingredient of the coloring used in hair and fabric dyes, rubber, lacquers, leather, eye shadow, shoe polish and as an intermediate in the manufacture of azo dyes, antioxidants [4].

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Chemical carcinogenesis is a multi-stage process that begins with exposure, to complex mixtures of chemicals that are found in the human environment. Once internalized, these carcinogens frequently compete with compounds involved in metabolic pathways of activation and detoxification. The oxidized forms of aromatic amines can bind with DNA to form DNA adducts and cause DNA damage, which can be a result of normal cellular metabolism, oxidative phosphorylation and oxidative stress due to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), a causative factor for chemically induced cancers [5].

Aromatic amines are oxidized to their N-hydroxy derivatives in the liver by the cytochorme P-450 1A2 isozyme. The N-hydroxy derivatives are esterified by acetyltransferase to the N-acetoxy derivatives or glucuronidated by UDP-glucuronidase to form N-glucuronides, both of which are transported by the blood and urine leading to cancer. The N-acetoxy derivatives and N-glucuronides are converted to aryl nitrenium ions, which can react with DNA to form DNA adducts [6]. The NADPH-cytochrome P-450, various forms of cytochrome P-450, prostaglandin H synt hase and peroxidases such as horseradish peroxidase can also activate aromatic amines to reactive free radical intermediates or produce ROS. The

action of reactive oxygen species generated from benzidine and its analogues contribute to DNA damage such as point mutation(s) [7].

Nowadays, enzyme based treatment processes have received a great deal of interest for the hazardous compounds detoxification [8-11]. Numerous reports were available on detoxification of aromatic amine using peroxidase and laccase *in vitro*. Though they are from bacterial, fungal and plant sources [12-14], enzymes from medicinal plants are not yet employed in detoxification processes. During our preliminary investigation stated that higher peroxidase activity in the latex of *Calotropis gigantea*, found to be equivalent to Horseradish peroxidase. The shrub *Calotropis gigantea* found mainly in tropical regions and to a lesser extent in sub-tropical areas. The plant is well known for its great capacity of producing latex which exudates from damaged parts. Wide ranges of proteins are found in latex fluids, such as carbohydrate binding proteins (lectins) and N-acetyl-β-D-glucosaminidases. Proteolytic enzymes, mainly serine and cysteine types, are more abundant proteinases found in latex fluids [15]. Evidences have supported the possible involvement of laticifer proteins (LP) in the plant defense mechanisms [16]. *Calotropis gigantea* is extensively used in Ayurvedic medicine for treatment of diabetes mellitus, bronchial asthma, rheumatoid arthritis and nervous disorders [17] and also in traditional folkloric medicine for the treatment of asthma [18]. However, additional information about its occurrence, biological activities and structure of latex proteins is still required to confirm this hypothesis. The peroxidase activity of latex obtained from *Calotropis gigantea* has not been reported so far. Hence, the present investigation was made to identify the biotransformation ability of partially purified peroxidase from *Calotropis gigantea* latex against PPD, BZ and OD.

2 Materials and methods

2.1 Chemicals

Paraphenylenediamine, Benzidine, O-Dianisidine, DMSO, Carboxy methyl Cellulose, Ethidium bromide (EtBr), EDTA, and heparine were obtained from Sigma-Aldrich, USA. All other chemicals were analytical grade.

2.2 Extraction and partial purification of CgPOD

The *Calotropis gigantea* latex was collected from non-cultivated healthy plants and placed in distilled water under room temperature until laboratory analysis was undertaken. A gentle agitation was made to collect the natural coagulant. The samples were centrifuged at 5000x*g* for 10 min at 4°C and the supernatant, still containing non-precipitated rubber, was exhaustively dialyzed against distilled water. Again the supernatant was subjected to centrifugation as described above. Then clean supernatant was stored until use and this fraction was denominated as laticifer proteins. The dialysis step successfully precipitated 100% of the remaining rubber, and washed out small metabolites soluble in water.

Partial purification of CgPOD was achieved by ion exchange chromatography. Ion exchange resin Carboxy methyl Cellulose was packed in 2x12cm column and pre-equilibrated with 10 bed volume of 20mM sodium acetate buffer (pH 5.5). The dialysate was loaded onto the column and washed with 10 bed volume of equilibration buffer (20mM of sodium acetate buffer). The bound proteins were eluted with gradient of 0 -0.35M NaCl in equilibration buffer. The active fractions were pooled and dialyzed against 20mM Tris buffer (pH 8.8) for further experiment.

2.3 Peroxidase assay

The peroxidase activity with O-Dianisidine dihydrochloride (OD) as reducing substrate was determined using modified protocol of Rompel et al. [19] in brief, 1ml reaction mixture containing aliquot of partially purified enzyme, 20mM sodium acetate buffer (pH 4.5), 0.4mM OD and 1mM hydrogen peroxide. The reduction of OD was observed in terms of increase in absorbance at 460nm (ε_{460} =11.3x10^{3}mM cm^{-1}). One unit of the peroxidase activity is defined as the amount of enzyme that reduces 1mmole of substrate per min at 25°C.

2.4 Biotransformation experiment

The biotransformation of BZ, OD and PPD was achieved using CgPOD. The transformation reactions were performed in 1ml reaction mixture containing 20mM sodium acetate buffer (pH: OD-4.1, BZ-4.5, PPD-5.5), 0.2U mL^{-1} of partially purified CgPOD, 0.4mM substrate (PPD/BZ/OD) and 0.6mM H_2O_2 for 10min at room temperature. Then the reaction mixture was subjected to centrifugation at 5000x*g* for 10 min. The pellets were allowed to air dry at room temperature and resuspended in dimethyl sulfoxide (DMSO).

2.5 FT-IR and ^{1}H NMR analysis

The analysis of structural elucidation and functional group of non-transformed compound and biotransformed metabolites were performed using FT-IR and ^{1}H NMR. For FT-IR, a solid about 0.5-1 mg is intimately mixed with about 100mg of dry and powdered KBr. The mixture is pressed with special disc, under a pressure of 10,000–15,000 pounds per square inch, to give a transparent disc. An FT-IR spectrum was recorded on Shimadzu FT-IR 8201 (PC) spectrometer using KBr pellets and absorption frequencies were expressed in reciprocal centimeters (cm^{-1}). For ^{1}H NMR, samples were prepared in 100% d6-DMSO under dry nitrogen purge. About 25µg of the sample was dissolved in 1ml of DMSO and transferred into high quality 5mm tubes and capped under nitrogen. ^{1}H NMR spectra were acquired on a varion Inova-500 instrument with proton resonance frequency at 499.97 MH2 with an INVERSE probe. ^{1}H NMR spectra were recorded on JEOL GSX 400 (MHZ) spectrophotometer using tetramethylsilane (TMS) as an internal standard. The chemical shifts are expressed in parts per million (ppm).

2.6 DNA adduct formation and fluorescence assay

DNA was isolated from human peripheral blood by previously described method [20]. The purity of DNA was checked by the ratio of A260/A280 and the DNA was quantified at A260.To the samples (containing 0.01 - 0.04mg of BZ and its product; 0.015, 0.03, 0.45, 0.060, 0.075, 0.090mg of OD and its product; 0.2mg of PPD and 0.03, 0.05, 0.07, 0.09, 0.11, 0.13mg of PPD metabolite), human peripheral blood DNA (2µg/25µl) and 4mM citrate phosphate buffer (pH 5.6) were added individually and

incubated for 30min at room temperature. A control DNA was prepared with the same composition excluding the compound. The reaction mixture was loaded on 0.8% agarose gel with control DNA to analyze the genotoxic effect.

DNA intercalation was determined by the displacement of ethidium bromide (EtBr) from DNA using modified method of Sakore [21]. The 25µl reaction mixture consists of human peripheral blood DNA (2µg), 4mM citrate phosphate buffer (pH 5.6). The control was maintained without any compounds. After 30min incubation, the measurements were made by excitation at 518nm and emission at 605nm, in the absence and presence of the compound using Fluorescence spectrophotometer (Hitachi F2000). The fluorescence intensity of EtBr-DNA was converted and expressed as percentage.

2.7 Effect of BZ, OD and PPD on Lymphocyte

Human lymphocytes were isolated according to Sierens et al. [22] method with slight modification. Blood withdrawn from a male donor (healthy non-smoker) was collected into Ficol-Hypaque. The samples were centrifuged at 2000rpm at 25°C for 20min. The lymphocyte forming a layer was directly above the Ficol-Hypaque in the vacutainer. Lymphocytes were then removed and washed with PBS (50mM, pH 7.4). Then it was centrifuged for 5min at 1500rpm and the cell pellets were resuspended in 50mM phosphate buffer saline solution. The cells were diluted prior to use at a concentration of $2.5x10^5$cells mL^{-1}. Cells were then incubated at 37°C for 2 h with chemicals being tested at previously standardized doses.

2.8 Cytotoxicity analysis

The cytotoxicity of test chemical was analyzed following Yen et al. [23] method. A volume of 0.49ml of cell suspension was treated separately with standard concentration of each tested chemicals. After 5min, lµl of 0.4% Trypan blue solution was added to determine the cell viability. The cells were analyzed through microscopic observation to determine the cell viability.

3 Results and discussion

3.1 Genotoxicity of BZ and its biotransformant on human DNA

The BZ and its congeners are bases for commercially used dyes, which are metabolized and released as free aminobiphenyls in the human systems. BZ is a strong carcinogen causing cancer in bladder, liver, mammary gland and also developing carcinoma in thyroid gland of animals [24]. BZ and its biotransformed product were treated with human peripheral blood DNA. With the increase in BZ concentration (0.01, to 0.04mg) the DNA intensity get decreased, it is proportionally showing that the EtBr displacement of BZ. On the other hand, the 0.01 to 0.04mg concentration of BZ biotransformant treated DNA samples did not show any difference when compared with the EtBr-DNA fluorescence of control DNA (Fig.1). But the action of ROS generated from BZ and its analogues contribute to DNA damage such as point mutation(s) [9]. All well-known free radical scavengers exerted their inhibitory effects on the levels of DNA damage caused by BZ. This indicates that the action of ROS generated from the BZ would contribute to DNA breakage [25].

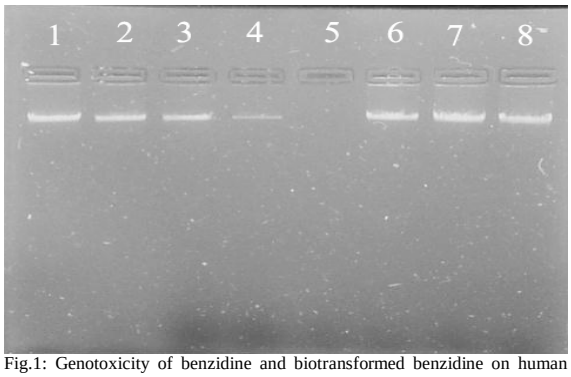


Fig.1: Genotoxicity of benzidine and biotransformed benzidine on human DNA. Agarose gel electrophoresis of high molecular weight DNA from human blood (~2 µg per lane) exposed to reaction medium (4mM citrate phosphate buffer pH5.6, 0.1 mg/ml DNA) for 30 min at room temperature: 1) DNA; 2 - 5) increasing concentration of BZ, system without POD (0.01mg (2); 0.02mg (3); 0.03mg (4); 0.04mg(5); and 6 - 8) increasing concentrations of BZ metabolite (0.01mg (6); 0.03mg (7); 0.04mg (8).

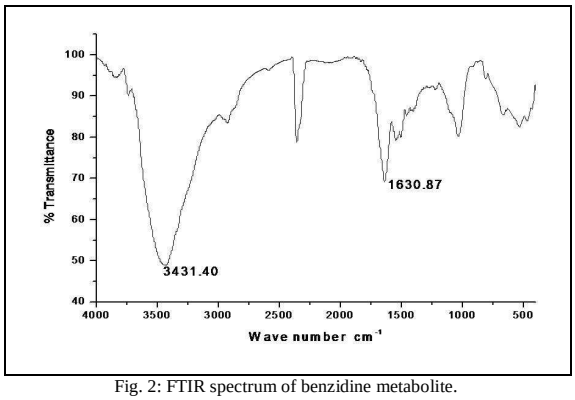
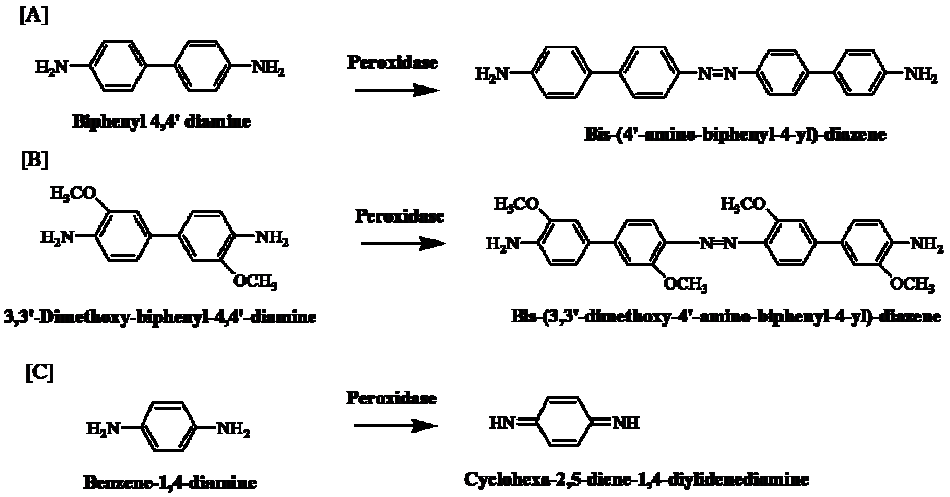


Fig. 2: FTIR spectrum of benzidine metabolite.

The non-genotoxicity of biotransformed BZ was further analyzed through the structural changes using FT-IR and ^{1}H NMR spectrum. From the FT-IR spectrum of BZ biotransformant (Fig.2) the stretching vibrations at 3505cm^{-1} and 3431.40cm^{-1} correspond to amine group while the band at 1637cm^{-1} responsible for the azo (N=N) group. The ^{1}H NMR spectrum of BZ biotransformant with a broad singlet at δ 5.10ppm was due to NH_2 (Fig.3). A sixteen-proton multiplet at δ 7.90–8.00ppm was due to aromatic protons. Hence we can confirm that, with the presence of an enzyme there is a reduction in the primary amino group and this reduction leads to the formation of azo compound according to the equation shown in Scheme 1a.



Scheme - 1: Calotropis peroxidase mediated Biotransformation of aromatic amines. [A] Benzidine [B] O-Dianisidine [C] Paraphenylene diamine

The CgPOD mediated biotransformed product (Bis-(4'-amino-biphenyl-4-yl)-diazene) of BZ may vary from other reported products, which have an action ambiguous to that of monoacetylated BZ. In earlier report the metabolite (i.e., N-acetylbenzidine) of monoacetylated benzidine was more toxic and predominant in DNA adduct formation than benzidine itself [26]. The genotoxicity of monoacetylated benzidine metabolite varied between the isoenzymes of N-acetyl transferase (NAT1 and NAT2) in humans [27]. These results indicate that an initial N-acetylation is involved in activation of benzidine, while acetylation of the second amino group is involved in a part of a detoxification pathway [28]. Hence the non-genotoxicity of benzidine biotransformant was due to the deamination of both amines by CgPOD.

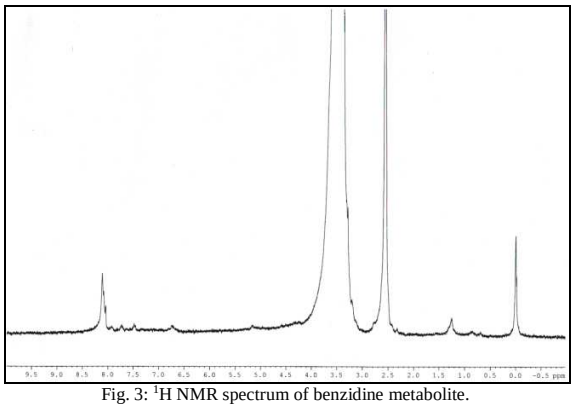


Fig. 3: ^{1}H NMR spectrum of benzidine metabolite.

3.2 Genotoxicity of OD and its biotransformant on human DNA

The genotoxicity of OD on human DNA was observed (Fig.4). The OD (0.015 and 0.030mg) was incubated with peripheral blood DNA shown in lane 2 and 3 were similar to control DNA (lane 1). However, the gradual decrease in EtBr-DNA fluorescent intensity was observed at 0.05mg of OD (lane 4) whereas the lanes 5-7 were shown (0.060, 0.075 and 0.090mg of OD, respectively) drastic decreases in EtBr-DNA fluorescence intensity. This result indicates that the higher concentration (>0.030mg) of OD might effectively form adduct(s) with DNA and hence decreases in fluorescence intensity of DNA was observed. The genotoxic effect of biotransformed OD was shown in lane 8 & 9, there was no change observed when compared with control DNA, even at 0.015 and 0.110mg of biotransformed OD.

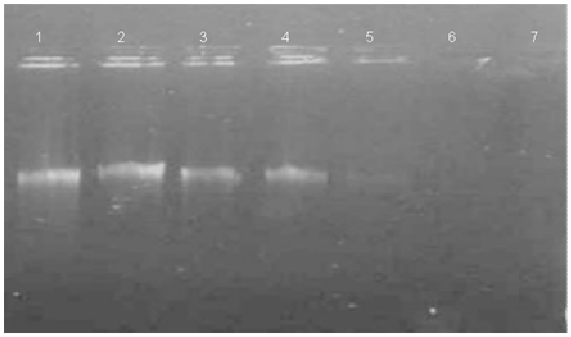


Fig.4: Genotoxicity of O-dianisidine on human DNA. Agarose gel electrophoresis of high molecular weight DNA from human blood (~2 µg per lane) exposed to reaction medium (4mM citrate phosphate buffer pH 5.6, 0.1 mg/ml DNA) for 30 min at room temperature: 1) DNA; 2&3) increasing concentrations of OD metabolite (0.015mg; 0.110mg, respectively), 4-7) increasing concentration of OD, system without POD (0.015mg; 0.030mg; 0.050mg; 0.062mg, respectively).

In the FT-IR spectrum of OD biotransformant (Fig.5) the strong NH stretching band at 3434cm^{-1} and 3340cm^{-1} corresponds to the amine group and the band at 1626cm^{-1} is for azo (N=N) group. A stretching vibration at 1226cm^{-1} corresponds to O-CH_3. The ^{1}H NMR spectra (Fig.6) revealed that two singlet at δ 3.95 and 4.00ppm each for three-proton integration were due to two O-CH_3.

Twelve aromatic proton multiplets at δ 8.11 to 8.32ppm were due to aromatic protons. A broad singlet at δ 5.10ppm was due to NH_2. From these spectral studies we can suggest the reaction (Scheme 1b) for the reduction of primary

amino group from OD into an azo group of its respective biotransformants.

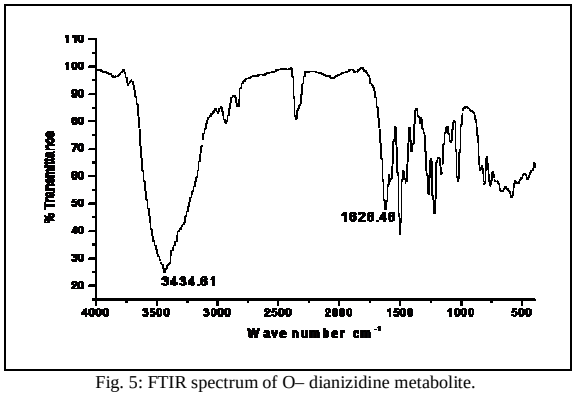


Fig. 5: FTIR spectrum of O– dianizidine metabolite.

This reaction also carried out with a loss of proton from one end of amino group which leads to the formation of nitrenium ion as an intermediate and the product is a derivative of Hydrazine.

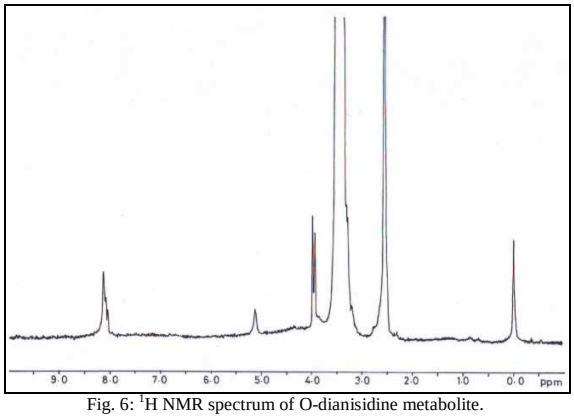


Fig. 6: ^{1}H NMR spectrum of O-dianisidine metabolite.

Adris and Chung et al. [29] reported that human opportunistic pathogenic bacteria may also contribute to the bioactivation of these aromatic amines (BZ and OD). Rodgers et al. [30] have shown that OD is rapidly metabolized in the rat via N-acetylation, hydroxylation, O-demethylation, and glucuronidation, and that of the eight metabolites identified in urine and bile, N-acetyl-OD is the most potent bacterial mutagen. As per Martelli et al. [31] report, OD induces a dose-dependent degree of DNA fragmentation in primary cultures of rat hepatocytes. This is similar with our report that OD has shown genotoxicity with human peripheral blood DNA and DNA fragmentation in lymphocytes. These results are consistent with the carcinogenic activity of OD in the rat liver [31]. The active primary amine group in OD was modified to its product [Bis-(3,3'-dimethoxy-4'-amino-biphenyl-4-yl)-diazene] by the peroxidation reaction and it was confirmed from the FT-IR and ^{1}H NMR spectrum. OD oxidation catalyzed by peroxidases obviously includes several steps and formation of intermediate radicals, which undergo dimerization finally yielding the strongly colored derivative *bis*-(3,3'-dimethoxy-4-amino) azodiphenyl.

3.3 Genotoxicity of PPD and its biotransformant on human DNA

The commonality among many carcinogens is that they exhibit a chemical reactivity towards cellular macromolecules such as DNA [32]. Therefore, PPD and its biotransformant were treated with human peripheral blood DNA. A high concentration of PPD (0.20mg) treated DNA sample did not show any damaging effect when compared to the control DNA. The same experiment with PPD biotransformant at various concentrations (0.03-0.13mg) showed a decrease in EtBr-DNA fluorescence conferring that DNA adduct formation has occurred with increasing concentration of PPD product (Fig.7). It was reported that the binding of a carcinogen to DNA fragment can affect the subsequent binding of a second compound and this effect is dependent upon both the chemical nature of the carcinogen and the neighbors of the target quinine bases [33]. The DNA adduct forming potency differs between the compounds and it has been shown to correlate with carcinogenic potency [34].

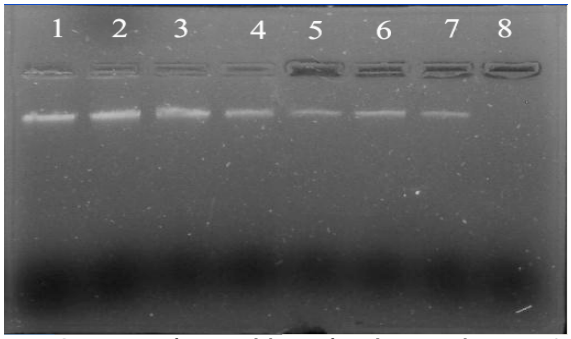


Fig.7: Genotoxicity of PPD and biotransformed PPD on human DNA. Agarose gel electrophoresis of high molecular weight DNA from human blood (~2 µg per lane) exposed to reaction medium (4mM citrate phosphate buffer pH 5.6, 0.1 mg/ml DNA) for 30 min at room temperature: 1) DNA; 2) 0.2mg PPD, system without POD; 3 - 8) increasing concentrations of PPD metabolite (0.03mg (3); 0.05mg (4); 0.08mg (5); 0.10mg (6)); 0.12mg (7); 0.15mg (8).

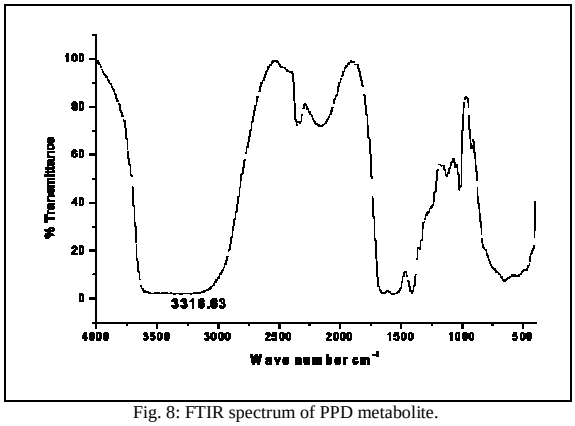


Fig. 8: FTIR spectrum of PPD metabolite.

The FT-IR spectrum of PPD biotransformant (Fig.8) showed a strong band around 3316cm^{-1} which corresponds to the NH stretching band of secondary amine. A strong stretching band at 1620cm^{-1} was due to C=N. The ^{1}H NMR spectrum (Fig.9) of PPD biotransformant showed a four proton doublet at δ 8.35ppm due to CH=CH.

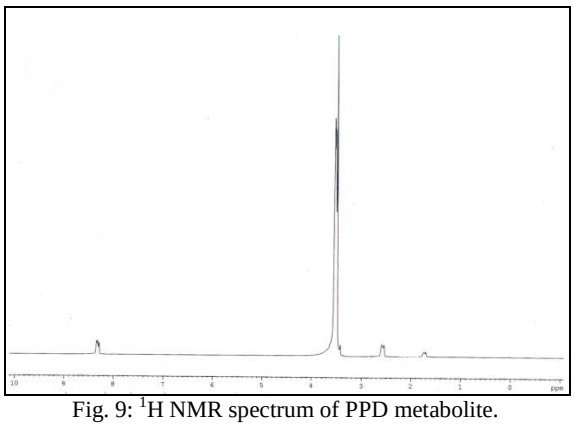


Fig. 9: ^{1}H NMR spectrum of PPD metabolite.

A two proton broad singlet at δ 3.60ppm was due to –C=NH. Hence, from the FT-IR and ^{1}H NMR studies we can infer that the primary amine of PPD can be converted into a secondary amine in the presence of CgPOD and yields a diylidene diamine compound. In this conversion there is a reduction in the amino group with a loss of proton and the same is given in Scheme 1c. Krasteva et al. [35] reported that PPD may be oxidized to benzoquinone diimine, which in turn may form the trinuclear dye N,N 9-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine called Bandrowski's base (BB) and which is involved in contact dermitis in human. Merk et al. [36] reported that PPD need to form reactive metabolites to induce allergy. Similarly the CgPOD biotransformant (Cyclohexa-2,5-diene-1,4-diylidenediamine) of PPD induced genotoxicity on human peripheral blood DNA *in vitro*; which could be substantiated with the FT-IR and ^{1}H NMR spectra. Our results are in harmony with earlier reports [37,38].

3.4 Genotoxicity assay using fluorimetry

To substantiate the effect of BZ, OD and PPD biotransformants on EtBr-DNA fluorescence intensity, the same experiment was done in fluorescence spectrophotometer (Table 1). The fluorescence intensity of the human peripheral blood DNA-EtBr complex was decreased when exposed to 0.15mg of PPD biotransformant as 13.05% and the PPD biotransformant unexposed DNA was assumed as control (100%). The test compounds such as BZ and OD have shown reduced fluorescence intensity at the concentrations of 0.05 and 0.09mg as 07.51 and 10.10% respectively. It seems that the test compounds have higher efficiency in competing with ethidium bromide binding to DNA than biotransformants, which substantiates our previous results.

3.5. Genotoxicity of untreated and biotransformed products on human lymphocytic DNA

Human lymphocytes provide a useful model to study the potential of a chemical to bind to human DNA, the types of DNA adducts formed in human cells and inter-individual variations in binding capacity. The isolated lymphocytes are pre-incubated with the previously determined concentrations of the test compounds and their biotransformants.

Table 1: Fluorimetric assay of DNA residual fluorescence intensity

Concentration (mg/ml)	Fluorescence intensity at 605nm	Residual fluorescence intensity (%)
PPD metabolite		
0.00	32.27	100.00
0.03	21.78	67.49
0.05	15.02	46.54
0.09	14.53	45.03
0.11	11.34	35.14
0.13	07.19	22.18
0.15	04.21	13.05
Benzidine		
0.00	32.21	100.00
0.01	25.37	75.66
0.02	16.09	49.95
0.03	11.14	34.59
0.04	02.42	07.51
O-dianisidine		
0.000	32.19	100.00
0.015	27.52	85.49
0.030	21.99	68.31
0.045	17.46	54.24
0.060	12.24	38.02
0.075	09.10	28.27
0.090	03.25	10.10

Human DNA (6µg) on incubation with ethidium bromide in 4mM citrate phosphate buffer (pH5.5) gave optimum fluorescent intensity at 605nm, which is considered as control (100%). Similarly the pre incubation of DNA with PPD metabolite (prepared as given under materials and methods) for30 minutes at room temperature, at different concentrations gave decreasing fluorescent intensity as given in the table.

The test compounds and biotransformants exposed lymphocyte DNA shows ladder banding patterns (Fig. 10). The biotransformants of BZ, OD and test compound-PPD, were not fragmented and mimics the control (Lane-1). But, the number of fragments appeared in the lanes were 2, 3 and 4 of PPD biotransformants, test compound BZ and OD respectively. The lifetime of these metabolite is very short (few milliseconds) by reason of their high reactivity and fast conversion into stable hydroxylated derivatives. In other cases, slow decomposition of metabolite favors their entry into the nucleus, mitochondria, and other cell organelles. A small portion of aromatic amine frequently biotransformed to electrophiles, which can bind covalently to DNA and other macromolecules to form adducts.

All the tested compounds showed a distinct disparity in their respective DNA-damaging effects when lymphocytes were exposed to these chemicals for 2h [9]. Among all the tested chemicals, the biotransformant of BZ and OD did not show effects on lymphocyte DNA, whereas PPD biotransformant was found to be the most active aromatic amine, which causes damage on lymphocyte DNA. It is similar to the results of the genotoxicity of benzidine and its analogues determined by the Ames test [39], this study also reveals the genotoxic effects on lymphocytes. Josephy [25] depicted that the free radical oxidation pathway of benzidine metabolism resulted in the formation of reactive electrophoilic species and the attack on DNA to form DNA adducts. The possibility that DNA damage induced by

benzidine in lymphocytes resulted from the generation of free radicals was envisaged.

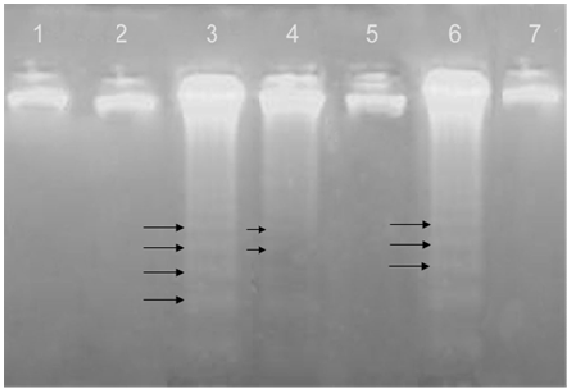


Fig.10: Genotoxicity of untreated and peroxidase treated products on human lymphocytic DNA under *in vivo*. Agarose gel electrophoresis of high molecular weight DNA from human lymphocyte (~2 µg per lane) exposed to reaction medium (4mM citrate phosphate buffer pH5.6, 0.1 mg/ml DNA) for 30min at room temperature: 1) DNA; 2) 0.20mg PPD, system without POD; 3) 0.15mg PPD metabolite; 4) 0.04mg BZ, system without POD; 5) 0.04mg BZ metabolite; 6) 0.06mg OD, system without POD; 7) 0.011mg OD metabolite. The fragment of human DNA shown ( ).

4 Conclusions

In conclusion, although the BZ and OD are genotoxic and not in the case of PPD when exposed to human systems, the CgPOD mediated metabolites of BZ and OD were non-genotoxic as shown in DNA fragmentation assay and fluorescence assay. But, the PPD metabolite exposed lymphocytes has shown genotoxicity. These results has gave clue that the Calotropis peroxidase posses the ability to transform the genotoxic to non-genotoxic form of BZ and OD. Similarly it transforms the non-genotoxic into genotoxic forms of PPD than other peroxidases. The FT-IR and ^{1}H NMR results elucidate and substantiate the hypothesis for genotoxic and non-genotoxic nature of aromatic amines used in this study.

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