(h) ABSTRACT OF THESIS:

Bisphenol-A (BPA) is a well-known environmental pollutant and established endocrine disruptor. In spite of this fact, it is widely used in the manufacture of plastic bags, toys, bottles, recycled paper, electronics, dental sealants, coated tins, eyeglass lenses, sports safety equipment, medical equipment, tubing, etc. (Rubin 2011; Fenichel et al. 2013). BPA leaches off from baby bottles, polycarbonate plastics, dental products and food cans leading to its environmental contamination and exposure to the general population (Vandenberg et al. 2007; Rathee et al. 2012; Michałowicz 2014). The toxic effects of BPA have been investigated in several studies both *in vivo* and *in vitro* (Michałowicz 2014).

In 2008, National Toxicology Program (NTP), expressed concern regarding its adverse effects on the health and development of fetuses, infant, and children (Shelby 2008). Canada in 2010 was the first to ban the use of BPA in baby bottles (Government of Canada 2010). After that, European Commission also restricted the usage of BPA in infant feeding bottles (European Parliament 2011). In 2012, FDA banned the use of BPA in baby bottles and sippy cups (FDA 2012), and in 2013 restricted the use of BPA in the inner surface coating of infant formula packaging (FDA 2013). This restriction on BPA use led to its replacement by other bisphenols such as bisphenol-S (BPS), bisphenol-B (BPB), bisphenol-F (BPF), and bisphenol-AF (BPAF).

Bisphenol-B (2,2-bis(4-hydroxyphenyl)butane) is one of the most widely used BPA analogues involved in manufacturing epoxy resins (Cunha and Fernandes 2010). It has an ethyl group on the central carbon atom instead of a methyl group found in BPA. Similar to BPA, it leaches into canned food items, which is evident from the reports of its detection in numerous products such as beverages, seafood, peeled tomatoes, powdered infant formula, commercial milk and a variety of other food items (Grumetto et al. 2008, 2013; Cunha et al. 2011, 2012; Alabi et al. 2014). BPB has also been shown to contaminate various environmental samples such as wastewaters, sediments and indoor dust (Liao et al. 2012a, b; Tao et al. 2015; Česen et al. 2018; Usman et al. 2019). Moreover, it has also been detected in human urine, saliva and sera affirming its human exposure (Cobellis et al. 2009; Cunha and Fernandes 2010; Song et al. 2017; Russo et al. 2018a).

With regards to its toxic potential, there are some reports suggesting BPB to possess endocrine disrupting properties. In line with BPA, it also behaves as estrogenic and anti-androgenic chemical, and also acts as an agonist to the human Pregnane-X receptor (Hashimoto et al. 2001; Yoshihara et al. 2004; Kitamura et al. 2005; Sui et al. 2012). Unfortunately, BPB has also been reported to have more acute toxicity than BPA (Chen et al. 2002). Moreover, is more resistant to aerobic and anaerobic biodegradation than BPA hence has a greater tendency for bio-accumulation (Usman and Ahmad 2016).

Considering the above-mentioned facts and its structural similarity with BPA, there is a high probability that BPB may also have adverse effects on environment and human health. Therefore, BPB was selected to be thoroughly studied for its various toxic effects.

In the first chapter an attempt was made to study the endocrine disrupting potential of BPB using *in silico* techniques. A webserver (endocrine disruptome) capable of performing molecular docking with multiple hormone nuclear receptors was employed to study affinity of BPB towards them. Thereafter, target nuclear receptors of BPB were selected for re-docking with Autodock to get a detailed insight into the binding of BPB with these selected nuclear receptors. Results of endocrine disruptome suggested a relatively high affinity of BPB with five human nuclear receptors, namely antagonist conformation of human androgen receptor (hAR), and agonist conformation of human estrogen receptor-α (hER-α), human glucocorticoid receptor (hGR), human thyroid receptor-α (hTR-α), and human thyroid receptor-β (hTR-β). The binding energies of BPB with hAR an, hER-α, hGR, hTR-α, and hTR-β obtained via endocrine disruptome were -8.3, -8.3, -7.8, -8.2 and -8.9 kcal/mol, respectively. Re-docking of selected nuclear receptors further confirmed strong affinity and binding of BPB towards hAR, hER-α, hGR, hTR-α, and hTR-β. Moreover, the docked structure of BPB with nuclear hormone receptors (BPB-nuclear receptor complex) were also superimposed with the docked structures of respective nuclear receptors with their well-known ligands. The superimposed structures showed that BPB binds to the ligand binding domain (LBD) of human nuclear hormone receptors just like their well-known ligands namely; flutamide (antagonist of AR), estradiol (agonist of ERs), dexamethasone (agonist of GR), thyroxine (agonist of TRs). Moreover, we also found that the hydrophobic interactions were mainly responsible for the binding of BPB to the ligand binding domains of these human nuclear receptors. In view of the above, we concluded that, BPB has a potential to act as an endocrine disruptor as it can lead to unwanted activation/inactivation of various nuclear hormone receptors. This undesired activation or inactivation of hormone signaling can have serious adverse effects on health, especially on endocrine balance.

In chapter 2, our objective was to evaluate the cytotoxic and genotoxic potential of BPB under *in vitro* conditions using human peripheral blood mononuclear cells (PBMCs) as a study model. PBMCs with a cell density of 1.56 x 10^6 cells/ml and viability around 90% were used in this study. The PBMCs were incubated with varying concentrations of BPB (200-800 μM) for 3 hrs. Thereafter, the cell viability was estimated using MTT assay. Intracellular ROS generation was also assessed using DCFH-DA staining and NBT assay. Moreover, lipid peroxidation and glutathione levels were determined in BPB treated PBMCs. Side by side scanning electron microscopy was employed to study cell morphology and integrity. Furthermore, the genotoxic potential of BPB was also evaluated by comet assay. It was found that BPB was able to induce a dose-dependent decline in cell viability. Moreover, BPB was also able to cause significant ROS generation in treated PBMCs as suggested by NBT assay, and microscopic examination of DCFH-DA stained PBMCs. Scanning electron microscopic observations suggested significant morphological alterations such as membrane distortion, cell membrane blebbing along with loss of cell integrity and shape. Moreover, a substantial increase in tail length and tail moment was also observed in BPB treated cells suggesting the genotoxic potential of BPB. In summary of the findings obtained in this chapter, we can say that BPB is capable of inducing cytotoxicity, oxidative stress, and genotoxicity under *in vitro* conditions.

Taking lead from the *in vitro* work, we proceeded to *in vivo* evaluation of BPB induced toxicity as presented in chapter 3 of this thesis. Determination of intraperitoneal (IP) LD_{50} of BPB in Swiss albino male mice using Dixon's up and down method was the first objective of this study. It was estimated to be 250 mg/kg bw. Thereafter, acute and sub-acute exposure of BPB was given to adolescent male mice. Weekly IP doses of 5, 10, and 15% of calculated LD_{50} was given to the adolescent male mice. After sacrifice on 16th (acute group) and 30th day (sub-acute group), serum testosterone levels were determined. Body and testes weights were also recorded to determine gonadosomatic index (GSI). Moreover, enzymatic and non-enzymatic oxidative stress markers levels in liver, kidney, and testes tissues were evaluated to understand the effect of BPB exposure on oxidant-antioxidant balance. Comet assay was performed to determine the possible genotoxic effects of acute and sub-acute BPB treatment. Furthermore, histopathological studies were also conducted on liver, kidney and testes tissues. There was significant decline in blood testosterone levels in both acute and sub-acute exposure groups. It was found that BPB exposure led to a reduction in testosterone levels and GSI in adolescent male mice. Moreover, BPB exposure to the experimental mice induced significant alterations in SOD, CAT, GST, GR activities as well as MDA and GSH levels in liver, kidney, and testes tissues suggesting overproduction of ROS as a consequence of BPB exposures. BPB was also found to induce DNA damage in liver, kidney and testes tissues at higher doses following acute and sub-acute exposures. We also found clear-cut morphological changes in the said organs of adolescent mice subjected to both acute and sub-acute exposures of BPB. Therefore, we conclude that acute and sub-acute exposure of BPB could induce oxidative stress resulting in genotoxic outcomes along with damage to tissue architecture. A decline in testosterone levels and GSI index could be an indicator of possible reproductive toxicity of BPB exposure.

We further explored the adverse effects of acute and sub-acute BPB exposure on sperms of treated mice in the next chapter (chapter 4). Sperm cells from BPB exposed mice were isolated from cauda epididymis isolated from previously sacrificed adolescent male mice. Both enzymatic and non-enzymatic oxidative stress markers were determined in sperm cells. Comet assay was also performed on sperm cells to evaluate the genotoxic effects of BPB exposure on sperm cells. Moreover, sperm count, motility of sperm cells, along with number of sperm cells having various morphological defects were determined. We also studied sperm cells movement using computer-assisted sperm analysis (CASA) tool. A significant change in the activity of SOD, CAT, GST, and GR were observed in both acute and sub-acute BPB exposure groups. Moreover, a decline in GSH content and increased MDA level were recorded suggesting overproduction of ROS leading to oxidative stress and lipid peroxidation. Micrographs of DCFH-DA stained sperm cells also revealed the presence of ROS. Results obtained in comet assay and micrographs of DAPI and acridine orange stained sperm cells confirm DNA damage in sperm samples on acute and sub-acute exposure which is a consequence of oxidative stress. Furthermore, BPB exposure led to a marked decline in sperm count and increase in the number of sperm cells having defective head, tail, and midpiece morphology. Moreover, an increase in the number of sperm cells having immature nucleus was observed in both groups. CASA revealed a significant decrease in sperm quality and compromised CASA parameters, i.e. curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity of curvilinear path (LIN), wobble

coefficient (WOB), straightness coefficient (STR), amplitude of head lateral displacement (ALH), beat cross frequency (BCF), and progressive motility. Thus, both the acute and sub-acute exposures of adolescent male mice to BPB led to adverse effects on sperms' quality and quantity. It is clear from the results presented in this chapter that acute or sub-acute exposure of BPB during the age of sexual development could have an adverse effect, especially on sperm cells.

. Xenobiotics like BPB can also interact with vital biological macromolecules such as DNA and serum albumin. Therefore, in order to understand their mechanism of interaction with BPB, firstly we studied the interaction of BPB with ctDNA (chapter 5). Absorption spectra of BPB titrated with increasing concentrations of ctDNA, and vice versa were taken. Moreover, fluorescence spectra of BPB in the presence of ctDNA at 298 and 308 K were also recorded to study the thermodynamics of the interaction. To unravel the mode of binding, the dye displacement assay was also performed using EtBr and Hoechst dyes as markers of intercalatory and groove binding modes of interaction, respectively. To further confirm the results of dye displacement assay, KI quenching experiment was conducted. Moreover, DNA melting assay and CD spectroscopy were also done to verify the binding mode of BPB with ctDNA. Finally, molecular docking studies were performed to get a detailed insight into the interaction and binding of BPB with ctDNA. Results of UV-Visible spectral analysis suggested an appreciable amount of interaction of BPB with ctDNA. Fluorescence spectral analysis of ctDNA in the presence of BPB also revealed significant interaction of BPB with ctDNA. Moreover, temperature-dependent fluorescence studies further strengthened our contention with an additional input regarding the nature of binding. The values of binding constant, ΔG, ΔH, and ΔS at 298 K were calculated to be 1.15×10^4 M^{-1}, -40.44 kcal/mol, -5.50 kcal/mol, and -0.06 kcal/mol, respectively. The Ksv and thermodynamic parameters suggested that the interaction between BPB and ctDNA was spontaneous and favorable and mainly mediated by van der Waals forces. The Ksv value determined in competitive dye displacement assay of BPB with EtBr and Hoechst directed us to suggest groove binding mode of interaction. Similarly, KI quenching studies also supported our proposed mechanism of binding. Circular dichroism spectroscopy, DNA melting profiles further affirmed our proposal regarding the exact mechanism of interaction. Molecular docking studies also proved BPB to be DNA minor groove binder. Moreover, the binding free energy of the interaction calculated using molecular docking also corroborated with the binding free energy obtained in temperature-dependent fluorescence studies. This study provides a detailed insight into the binding and interaction of BPB, a potential endocrine disruptor with ctDNA, a macromolecule of great biological significance.

In the final chapter of this thesis, we carried out the interaction studies of BPB with BSA, a vital carrier protein (chapter 6). Xenobiotics interacts with serum albumins and some of them may also induce temporary or permanent conformational changes in the protein structure affecting protein's normal functioning and transport of critical endogenous substances. Several techniques namely, steady-state, time-resolved, and synchronous fluorescence, circular dichroism, and molecular docking studies were used to study the interaction of BPA and BPB with BSA. Results obtained in steady-state fluorescence suggested that both bisphenols bind to BSA with a binding constant within the optimum range 10^4-10^6 M^{-1}, because of which BSA can easily transport and distribute BPA and BPB. Data obtained in time-resolved fluorescence

predicted the mechanism of quenching of BSA by test bisphenols was dynamic in nature. There was also a high probability of energy transfer between bisphenols and BSA as suggested by FRET results. Moreover, BPB was able to reduce the α-helical content in BSA more significantly than BPA resulting in significant conformational changes in BSA as compared to BPA. Similarly, SDS refolding studies also predicted BPB to induce more intense and irreversible conformational alternations in native structure of BSA than BPA. Interaction energies and docked poses obtained in molecular docking suggested that the typical binding mode of BPA and BPB with BSA could be hydrophobic in nature. Moreover, BPB binds with -6.57 kcal/mol of binding energy whereas, BPA binds with -5.62 kcal/mol of binding energy. BPA occupied a position in between the subdomain IIA and IIB at the FA6 binding site, while BPB binds in the subdomain IB at the FA1 binding site. This difference in the binding site of bisphenols within BSA may be the underlying reason behind the difference in the binding energies of BPA and BPB with BSA. Therefore, we concluded that BPB binds to BSA with higher affinity than BPA and induces more severe irreversible conformational changes in the native structure of BSA.

In conclusion, the data obtained in this thesis makes us suggest that the test bisphenol i.e. BPB to be a potential endocrine disruptor as it has high affinity towards important nuclear hormone receptors. Moreover, it can induce oxidative stress and genotoxicity leading to cytotoxicity under *in vitro* conditions in PBMCs. Acute and sub-acute exposures of BPB can lead to induction of oxidative stress resulting in DNA damage and alterations in normal tissue architecture in liver, kidney and testes tissues in adolescent male mice. Apart from this, acute as well as sub-acute exposure of BPB during sexual development age lead to a decline in testosterone levels, compromised gonad development and is able to induce adverse effects on the quality, maturation and morphology of sperm cells. Moreover, BPB was also found to interact and bind in the minor grooves of DNA which might lead to some alteration in normal DNA functions. Besides, it also binds to serum albumin resulting in irreversible conformational changes in protein structure which may also lead to compromised transportation of endogenous and exogenous important substances. Therefore, we conclude that use of BPB as a safe replacement of BPA in various industries and consumer products should be questioned.