**Investigate the dopaminergic neurotoxicity profile of designer drugs
(Piperazine derivatives)**

by

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Abstract

Different botanical derived, or synthetic addictive substances have been "misused" and/or "abused" for centuries around the world. To overcome the abuse by these substances, strict legal laws were constituted globally. However, novel and drugs with chemical structures similar to illegal psychoactive drugs substances (with a slight structural change) were manufactured in undercover laboratories to have the same or augmented psychostimulatory effects. Currently, the major classes of designer drugs are piperazines, cathinones, synthetic cannabinoids, synthetic opioids, tryptamines, and phenethylamines. These classes of designer drugs have shown to elicit significant psychostimulatory effect by a different mechanism of action. There are very few reports on the dopaminergic neurotoxicity of piperazine derivatives. However, they have shown to affect various monoaminergic neurotransmission. In the current study, we have synthesized and elucidated the neurotoxic mechanisms of new piperazines.

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List of Abbreviations

5-HT	Serotonin
ACTH	Adrenocorticotropin
AVP	Arginine Vasopressin
BZP	N-benzylpiperazine
CAT	Catalase
CNS	Central Nervous System
CYP	Cytochrome P
DA	Dopamine
DCF-DA	2`, 7-Dichlorofluorescindiacetate
DMSO	Dimethylsulfoxide
FBS	Fetal Bovine Serum
GC/MS	Gas Chromatography/Mass Spectrometry
GSH	Glutathione
GSH-Px	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxide
LC/MS	Liquid Chromatography/Mass Spectrometry
MAO	Monoamine Oxidase
mCPP	1-(3-chlorophenyl) piperazine
MDA	Malondialdehyde
MDBP	1-(3,4-methylenedioxybenzyl) piperazine
MDMA	Methylenedioxymethamphetamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NA	Noradrenaline
NADH	Nicotinamide Adenine Dinucleotide
NO	Nitric Oxide

OPA   O-Phthalaldehyde

PBS   Phosphate Buffer Saline

PNS   Peripheral Nervous System

ROS   Reactive Oxygen Species

SCN   Suprachiasmatic Nucleus

TBA   Thiobarbituric Acid

TBARS   Thiobarbituric Acid-Reactive Substances

TCA   Trichloroacetic Acid

TFMPP   Trifluoromethylphenylpiperazine

TFMBzPP Trifluoro-Methyl-Benzyl-Phenylpiperazine

BzPP   Benzyl-Phenylpiperazine

1. literature Review

1.1. Introduction-History and Current Scenario of Drugs of Abuse

Drug addiction rates and deaths resulting from drug abuse has become a huge problem worldwide. In the United States, which is one of the largest countries in terms of percentage mortality rate due to substances of abuse, 1 of every 20 deaths connects to addiction (Report, 1997; World Health Organization, 2012). Despondently this addiction epidemic is also found Europe, Asia, Australia and Africa. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) discovers a new legal high drugs-numbers of novel drugs have raisin from 14 in 2005 to 300 in 2014 (Hill and Thomas, 2011; Musselman and Hampton, 2014; Liechti, 2015). Historically, designer drugs or Novel psychoactive substances began to be used in the late 1960s as substitutes for banned control substances. Designer drugs are substances manufactured with a slight change in chemical structures that similar to illegal psychoactive drugs, for the purpose of marketing and avoid interdiction from authorities. Interestingly, designer drugs were synthesized by pharmaceutical companies with the ultimate goal of therapeutic interventions for the various central nervous system and peripheral disorders, but abuse liability proved as the collateral. Therefore, the United State authorities in 1970 founded the Controlled Substances Act, which is a legal system to identify and organize abuse substances, depending on the medical value, abuse possibility, and physiological physical effects. The Controlled Substances Analogue Enforcement defined the designer drugs as "a substance other than a controlled substance that has a chemical structure

substantially similar to that of a controlled substance in schedule I or II or that was specifically designed to produce an effect substantially similar to that of a controlled substance in schedule I or II." The Controlled Substances Act divide the Substances of Abuse into 5 classes, in 1 to 5 scales, where Class-I is having a great risk of abuse, and 5 is having the minimal risk of abuse. First termed designer drugs were in 1988 on a compound called "China White" which is a synthetic opioid (Kram et al., 1981). In the recent times, trading and embracing of these drugs have increased because of the Internet (the main marketer of such drugs of various kinds). Designer drugs industry depends on two main sources; plant, where the raw materials are taken and then hidden laboratories that synthesize the final product (Henderson, 1998). The final product is often marketed as unfit for human consumption, also attempting to cheat for distribution purpose as scientific laboratory materials or plant supplements (Musselman and Hampton, 2014). Addictive drugs are known to humans since the mid-19th century, where humans began to extract morphine from opium. Then at the beginning of 1900, heroin was produced and this was followed by cocaine. Development in the pharmaceutical field led to heightened and intensified production of more potent and intoxicating drugs. These new designer drugs have substantial psychological properties which cause significant abuse potential. With growing addiction problem, countries have developed severe legislation that limits the abuse of drugs (Chavan and Roy, 2015).

Conversely, the emergence of new legislation motivates clandestine laboratories to synthesize new and novel kinds of drug analogues called designer drugs. The industry of designer drugs often develops in countries that contain manpower with various skills which range from experienced chemists to cheap labor, and this yields to low overall production cost. For example, simple online search on designer drugs leads to the learning method of synthesis and use (Madras, 2012; Musselman and Hampton, 2014). Most of the current designer drugs products are synthesized in

China, Mexico, and south-east Asian countries. The main source of designer drug business is the internet, followed by nightclubs and head shops which act as potential distributors. Distributors of these designer drugs intentionally add signs showing invalid for human consumption or fraud expression on packages that deliver to users Phrases like legal high, or legal drugs used to deceive consumers consequently making series complications among societies (Corazza et al., 2007). The affordable price of the designer drugs ranges between 6 to 12 pounds for each pack and each collection has 1 to 6 tablets. Estimated profits are extremely lucrative, as one kilogram of the material cost thousands of dollars as profit returns to distribute up to $20 million (Huestis and Tyndale, 2017; Sellers, 2017). In 2010 a study done by (Davies *et al.*, 2010), illustrated the ease and simplicity of purchasing 26 brands of synthetic drugs from the popular website in the UK. Thus, the Internet makes the abuse for these designer drugs to become readily accessible to the public. There are insufficient databases or scientific literature on the pharmacology and toxicity profile of designer drugs. Additionally, healthcare professionals face huge difficulties to distinguish between many kinds of designer drugs. Most of these compounds cannot be readily detected by immunoassays, urine screens, but are detected by gas chromatography and mass spectrometry (Zamengo *et al.*, 2011; Weaver, Hopper and Gunderson, 2015; Assi *et al.*, 2017) Hence, in this chapter, we have elucidated the pharmacological effects, toxicity profile and appropriate therapy for various designer drugs.

1.2. Designer Drugs

Novel psychoactive substances are classified into two categories; based on the mental impact (stimulants, or hallucinogens) and based on their chemical structures (Liechti, 2015). The most common chemical structure for designer drugs are Phenethylamines, Piperazines, Tryptamines,

Synthetic cannabinoids, Synthetic cathinones and Synthetic opioids (Figure1.1). Statistics indicate

that numbers of new psychoactive substances in continuous raise since 2009. Percentage of newly

discovered substances between 2009-2012 are as follow 23% for Phenethylamines and Synthetic

cannabinoids, 18% Synthetic cathinones, 10% Tryptamines, and 5% piperazines (World Health

Organization, 2012). This study also concluded that the most founded substance belongs to

piperazines and cathinone compounds.
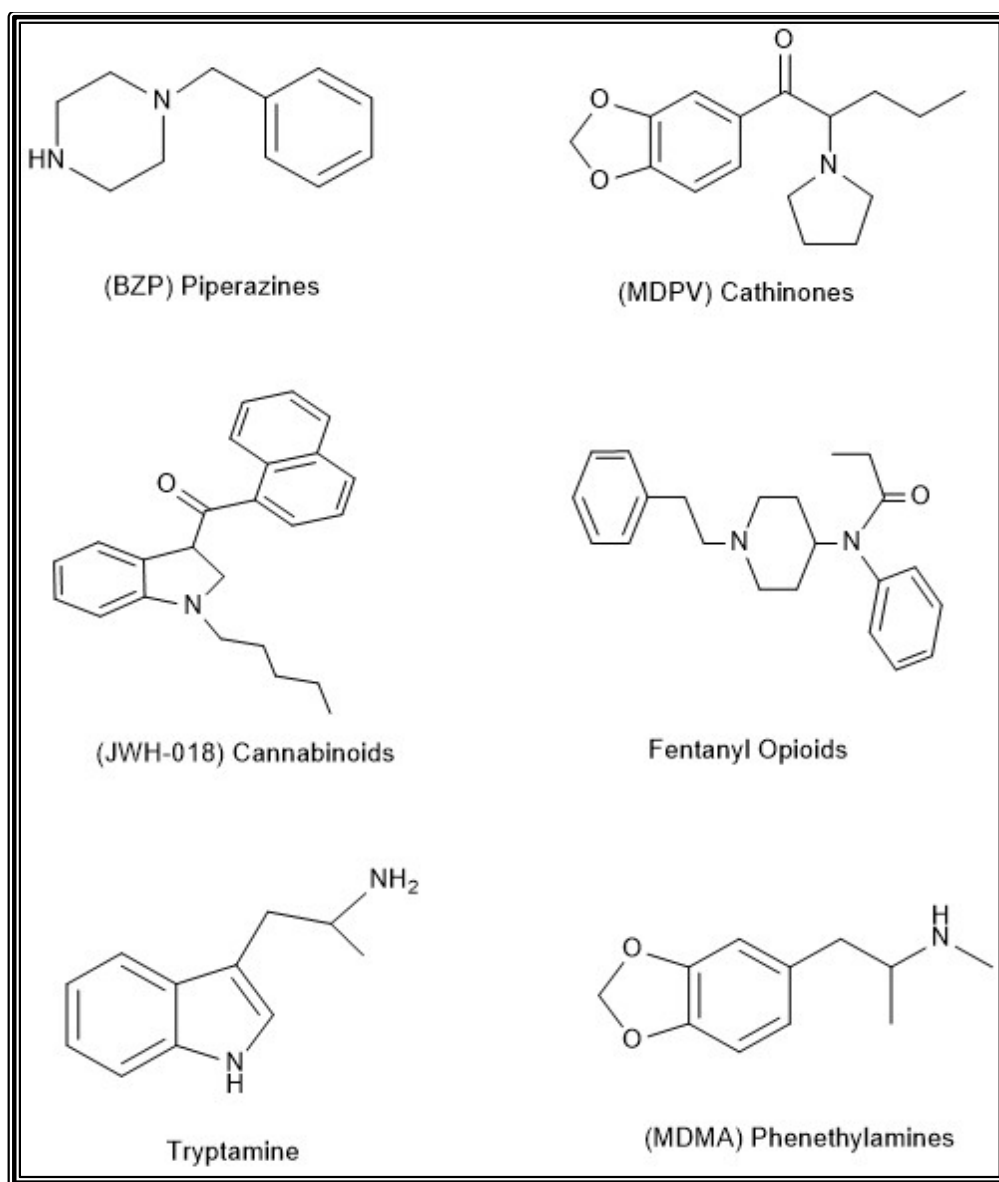
Figure 1.1 Various Designer Drugs

1.3. Piperazines Designer Drugs

At the beginning of the Millennium, Piperazines derivatives were known as a new drug of abuse since 3,4-methylenedioxymethamphetamine (MDMA) was banned by the authorities. Piperazines compounds do not exist naturally, but it is completely synthesizing in the chemical laboratories. Many industrial processes involve piperazines compounds such as insecticides, in hardener of epoxy resins, accelerators for rubber. In the medical field, piperazines were used as raw material to synthesize fluoroquinolone drugs (Dessouky and Ismaiel, 1974; Nikolova and Danchev, 2008). Piperazines have similar stimulant effects comparable to amphetamine with additional euphoric effect. Consequently, this gained the widespread popularity of piperazines around the world (Arbo, Bastos and Carmo, 2012; Rosenbaum, Carreiro and Babu, 2012). Internet is the main source of distribution for piperazines compounds under different names like; "party pills" or "legal Ecstasy", "Head Rush", "XXX"," Strong as Hell"," Herbal ecstasy"," "A2", and "Legal E." (Rosenbaum, Carreiro and Babu, 2012; Musselman and Hampton, 2014). Piperazines products are considered from the top-selling psychological drugs through the internet, especially in New Zealand, Europe, and North America. As a result, there is huge profit comes as a result of that wide distribution, in New Zealand, the annual financial revenue of the BZP sale is estimated at NZ$50 million (Wilkins *et al.*, 2006).

The most well-known drugs of abuse belong to this group are benzylpiperazines BZP , 1-(3-trifluoromethylphenyl)piperazine TFMPP, 1-(3,4-methylenedioxyphenyl) piperazine (MDBP), and 1-(3-chlorophenyl) piperazine (mCPP) (Drug Enforcement Administration (DEA), Department of Justice, 2004; Yeap *et al.*, 2010). Piperazines was abused to increase alertness, reinforce mental and physical ability (Gaia Vince, no date; Austin and Monasterio, 2004; Davies *et al.*, 2010; Cohen and Butler, 2011). The common routes of administration for piperazines

derivatives are oral as tablets, capsules, also as a powder or liquid form (Gee *et al.*, 2005). Although the United States authorities placed BZP under Schedule I controlled substance in 2004, a number of seized BZP samples continued to rise (Rosenbaum, Carreiro and Babu, 2012). Most of the reserved samples contain a mix of piperazines compounds, BZP with TFMPP, or with other psychoactive like amphetamine or cocaine (Kenyon *et al.*, 2006).

Chemical structures and Pharmacology of Piperazines

Piperazine is a cyclic organic compound with two opposing nitrogen atoms within a six-membered ring. Chemical structures of piperazines are not related to other psychoactive substances (Katz *et al.*, 2016). There are two classes for piperazine derivatives; benzylpiperazines. The benzylpiperazines include N-benzylpiperazine (BZP) and 1-(3,4-methylenedioxybenzyl)-piperazine (MDBP), the methylenedioxy analogue. And phenylpiperazines such as 1-(3-chlorophenyl) piperazine (mCPP), 1-(3-trifluoromethylphenyl)piperazine (TFMPP), and 1-(4methoxyphenyl)piperazine (MeOPP) (Arbo, Bastos and Carmo, 2012; Katz *et al.*, 2016) (Figure1.2). At first, piperazines were designed to cure intestinal roundworm and tapeworm, as anthelminthic by researchers from Burroughs Wellcome & Co. Between the 1970s and 1980s, there were few drug trials to validate the antidepressant effects following the results of addiction (Bye *et al.*, 1973; Campbell *et al.*, 1973; Musselman and Hampton, 2014). Piperazines have interaction with serotonin receptors leading to psychoactive properties. It also has stimulant and hallucinogenic effects due to increasing the levels of the monoamines (dopamine and serotonin) in CNS. BZP is a sympathomimetic stimulant (amphetamine-like effect), release dopamine, serotonin, and adrenaline in CNS, and inhibit the reuptake of dopamine. It is found as dihydrochloride salt, white powder, or as a free base with pale yellow color. The dosage of abuse

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ranges from 50 to 250mg, with the onset of duration last for 6-8 hrs. BZP can cross the blood-brain barrier and the onset of action for BZP takes 2 hours to start and has the stimulatory action for 4-8 hours the influence and this result in multiple doses by users. Elimination half-life is 5.5 hrs with 30 hrs possibility to detect in plasma. The liver is considered the main site of metabolism by hydroxylation and N-dealkylation catalyzed by cytochrome P450 (Bye *et al.*, 1973; Hill and Thomas, 2011; Curley *et al.*, 2015; Katz *et al.*, 2016). Drug interaction could happen with other drugs due to inhibiting cytochrome oxidase isoenzymes (Antia, Tingle and Russell, 2009a). Trifluoromethylphenylpiperazine (TFMPP) is phenylpiperazines which act as non-selective serotine receptors agonist and also elevate release of serotonin by blocking the reuptake(Majrashi *et al.*, 2017). TFMPP has a minimal effect on dopamine and noradrenaline release (Kennett *et al.*, 1989; Zuardi, 1990). TFMPP derivative (5-100mg) is found as powder, tablet, or capsule and usually in combination with other psychostimulants (Schep *et al.*, 2011a)(Curley *et al.*, 2015). Initial metabolism of TFMPP occurs by hydroxylation with CYP2D6 and phase 2 metabolism by glucuronidation, sulfation and acetylation (Staack, 2007). As an alternative for MDMA, BZP and TFMPP combined products (2:1) are available. In these combined products, BZP promotes the stimulant influence, while the TFMPP provide the hallucination effect (Herndon, Pierson and Glennon, 1992; Curley *et al.*, 2015).

Toxicity and treatment of Piperazines

Usually with minimal dosage piperazines lead to stimulant influence, while with high dose exhibit hallucination and sympathomimetic toxidrome (Staack and Maurer, 2005; Antia, Tingle and Russell, 2009b; Schep *et al.*, 2011b). Piperazines compounds exhibit amphetamine-like stimulant effect and with the same abuse possibility. Combination of BZP with TFMPP result in MDMA

like sympathomimetic action and this can lead to life-threatening serotonin syndrome. Due to the easy permeability through the blood-brain brier, the clinical manifestations of the CNS intoxications include anxiety, headache, paranoia, tremors, and insomnia. Piperazines synthetic drugs also affect the peripheral nervous system include vomiting, palpitations diaphoresis, sinus tachycardia, metabolic acidosis, hyperthermia, auditory and visual hallucinations, vasoconstriction, ischemia, tachycardia and arrhythmia of cardiovascular. Upon consumption of high doses of piperazine, the severe toxic effects include; multi-organ failure, seizure psychosis, renal toxicity, respiratory acidosis, hyponatremia (Gee *et al.*, 2008; Nikolova and Danchev, 2008; Gee, Jerram and Bowie, 2010). Normal detection methods such as urine immunoassay usually give a negative result. However, gas chromatography and mass spectrometry are the most useful technology to identified piperazines compounds (McNamara, 2009; Dickson *et al.*, 2010). Currently, there is no special antidote for the piperazines toxicity. Nevertheless, the current approaches are to provide supportive care and monitoring the vital sign is the first step of patient care. Benzodiazepines are the first line of therapy to treat seizure and agitation associated with piperazine toxicity. Charcoal for oral ingestion toxicity, IV fluids, and rapid cooling strategies are the other pharmacological and non-pharmacological approaches to reduce the piperazine-induced toxicity (Balmelli *et al.*, 2001; Wood *et al.*, 2007; Schep *et al.*, 2011b; Musselman and Hampton, 2014). Furthermore, as a safety precaution, patients should receive electrocardiogram test.
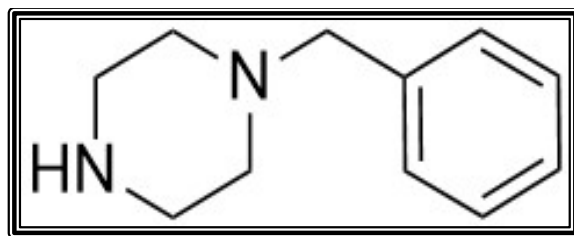
Figure 1.2 Chemical structure of BZP

1.4.Cathinones Designer Drugs

For centuries, people in Arabic peninsula and East Africa have used a green plant called as "Khat". Khat has been found in medicinal and botanical literature since the eleventh century. Remarkably, people in Yemen and Somalia are still consuming (chew the leaves) Khat for its amphetamine-like influence (Brenneisen *et al.*, 1990; Rosenbaum, Carreiro and Babu, 2012). Cathinone is the main molecule in Khat and the first synthesized compound related to cathinone was methcathinone in 1928. Synthetic cathinone (Bath Salt) and tits structurally related group of drugs gained fame in the early 1990s as drugs of abuse (German, Fleckenstein and Hanson, 2014). In 2014, around numerous patients were hospitalized in the United States related and relatively substantial number (52%) of cases were linked to cathinone (Fratantonio, 2015). The most recognized products of the Cathinone derivatives are (Figure1.3);

- 4-methyl-N-methylcathinone (mephedrone),

- 3,4-methylenedioxy-N-methylcathinone (methylone),

- 3,4-methylenedioxypyrovalerone (MDPV)

Trade names for cathinones Meow Meow, MCAT, 'Ivory Wave', 'White Lightning' and 'Vanilla Sky' (Freudenmann, Öxler and Bernschneider-Reif, 2006). The distribution process usually come in form of capsule, pills, or powder which is the most common form, with different rout of administrations (Zawilska and Wojcieszak, 2013).

Chemical structure and Pharmacology of Cathinones

Cathinone derivatives belong to phenylalkylamine and naturally appears as alkaloid beta ketoamphetamine, analogue to MDMA and methamphetamine, (Carroll *et al.*, 2012; German, Fleckenstein and Hanson, 2014). Synthetic cathenones compounds are hydrophilic due to the presence a ketone group on beta-carbon. Those chemical structures make cathinones less permeable to CNS, as result, abusers attend to raise the dose of cathinones drugs (Hill and Thomas, 2011). The potential mechanisms of action of cathinone derivatives are similar to amphetamine because of the similarity between structures. Cathinone compounds exhibit sympathomimetic action and also cause reuptake inhibition of dopamine, serotonin, and norepinephrine within the central nervous system. In addition, cathinones lead to elevated monoamines release from the presynaptic neurons (*Assessment of khat (Catha edulis Forsk)*, no date; Wood, Greene and Dargan, 2011). Orally dose of mephedrone range from 100-200mg, with the onset of effect between 30-45 minutes and extend the duration of action for 2-5hrs. MPDV has shown to exhibit more strength and extent of the abusive action.

Toxicity and treatment of Cathinones

Clinical features of bath salt toxicity are usually connected to sympathomimetic symptoms (cardiovascular and neurological). Neurological adverse effects include agitation, anorexia, insomnia, paranoia, psychosis. In addition, the patients also experience a headache, palpitation and chest pain (James *et al.*, 2011; Rosenbaum, Carreiro and Babu, 2012). The cardiovascular toxicity includes hypertension, tachycardia, hyperthermia, cardiovascular collapse and myocardial infarction (Rivera *et al.*, 2017). Cathinone compounds cannot be detected by immunoassay urine

screens but identified and detected using gas chromatography and mass spectrometry (Coppola and Mondola, 2012; Petrie *et al.*, 2013). Considering that cathinones are analog to methamphetamine, abusers after stop using cathinone compounds may have a risk of Parkinson disease as a cause of the decline in the activity of dopamine in the basial ganglia (McCann *et al.*, 1998). Management of cathinones toxicity is limited and there are limited literature currently. At this time, supportive therapy mainly provides to patients with some medications such as IV fluid, benzodiazepine (to cure hyperthermia, agitation, and seizure) to overcome problems (Prosser and Nelson, 2012; Musselman and Hampton, 2014; Rivera *et al.*, 2017).
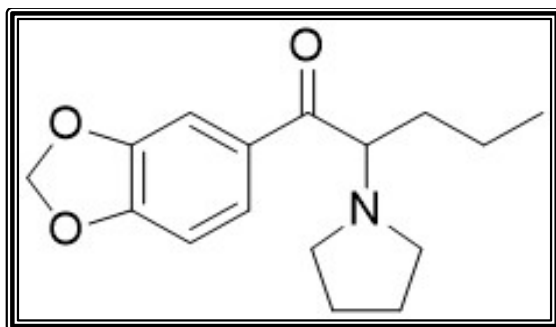
Figure 1.3 Chemical structure of MDPV

1.5. Synthetic Cannabinoids Designer Drugs

During the era of the 1960s, a group of researcher's accidentally invented the synthetic cannabinoids (Thakur, Nikas and Makriyannis, 2005a). The scientists were trying to improve the therapeutic features of the natural cannabinoids D9-tetrahydrocannabinol (D9-THC) and that yielded synthetic Cannabinoids (Musselman and Hampton, 2014). Since the early 2000s, there are hundreds of new Synthetic Cannabinoids available on the internet and like other designer

drugs; most of the synthetic cannabinoids are manufactured in China. Synthetic Drug Abuse Prevention enacted in 2012 and listed 15 of the synthetic cannabinoids as Schedule-1, and four years later the department added 47 new compounds (White, 2017). Manufacturing of synthetic cannabinoids are mostly combined with natural herbs (marijuana) to delude and the abusers smoke it (Rivera *et al.*, 2017). Synthetic cannabinoids are referred as Fake marijuana, spice, K2, (White, 2017).

Chemical structures and Pharmacology of Synthetic cannabinoids

Structurally cannabinoids are classified as seven classes; classical cannabinoids (HU-210), naphthoylindoles (JWH-018 and JWH-073), naphthylmethylindoles, napththoylpyrroles, , phenylacetylindoles (JWH-250), cyclohexylphenols (CP 47-497), and naphthylmethylindenes (Cimanga *et al.*, 2003; Thakur, Nikas and Makriyannis, 2005b; Dowling and Regan, 2011; Musselman and Hampton, 2014) (Figure1.4). The synthetic cannabinoid has a stronger effect up to 800 times by comparing to natural cannabinoids. The naturally occurring D9-THC acts as partial agonist on the Cannabinoid-1 receptor (CB1), while the synthetic cannabinoids act as a full agonist on the CB1 receptor (Seely *et al.*, 2011; White, 2017). CB1 and CB2 receptors are G protein receptors the main component of endocannabinoid system inside the brain (Baumann *et al.*, 2014). CB1 located in the central nervous system modulates GABA and glutamate neurotransmission and is responsible for the psychoactivity of cannabinoids. CB2 receptors are in the peripheral nervous system and are responsible for the immunomodulatory effect of cannabinoids (Seely *et al.*, no date; Rieder *et al.*, 2010). Since the synthetic cannabinoids are full agonist on the CB1 receptor, the onset of duration will prolong the risk of adverse effects (EVERY-PALMER, 2010; Benford and Caplan, 2011).

Toxicity and treatment of Synthetic cannabinoids

Symptoms of synthetic cannabinoids toxicity include anxiety, agitation, paranoia, delusions, aggression, paranoid thinking and anxiety. In addition, the patient usually has feelings of energy, euphoria, mild sedation, nausea, vomiting, hyperemesis, and abdominal pain (CASTELLANOS and THORNTON, 2012; Musselman and Hampton, 2014; 'The adverse health effects of synthetic cannabinoids', 2015; White, 2017).   Cannabinoids compounds cannot be detected by immunoassay in urine screens, but by using gas chromatography and mass spectrometry it can be identified (Auwärter *et al.*, 2009). Treatment for synthetic cannabinoid toxicity depends on monitoring of vital signs and providing supportive care to patients with intoxication. The drug of choice for both adverse side effects and seizures are benzodiazepines (Liechti, 2015; Mills, Yepes and Nugent, 2015).
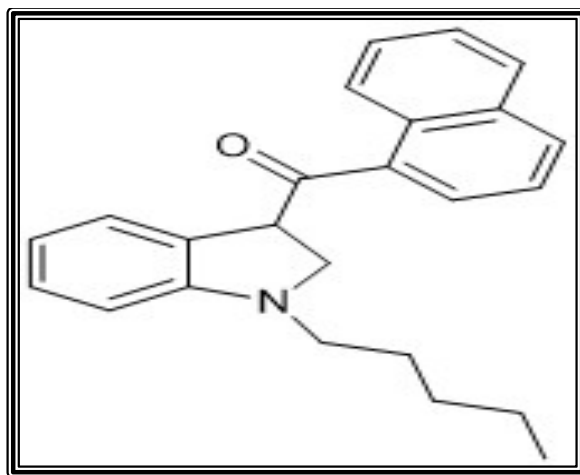
Figure 1.4 Chemical structure of JWH-018

1.6. Synthetic opioids Designer drugs

In the United States, there are approximately 12.5 million people who use pain medication incorrectly by a national survey conducted in 2015. Great demand and a huge percentage of profit, 1kg of fentanyl can make 20 $million (Armenian *et al.*, 2017). In 2014, around 29, 000 victims have been died due to this problem (Lucyk and Nelson, 2017). Sadly, opiates are responsible for 60% of overdose deaths (Prekupec, Mansky and Baumann, 2017; Rivera *et al.*, 2017). Similar to the other designer / abusive drugs, clandestine laboratories mainly in China manufacture the synthetic opioids. The most known synthetic opioids are fentanyl, fentanyl analog, and novel synthetic opioids like U-47700, which recently introduced to schedule 1 by DEA 2016 (Alzghari *et al.*, 2017). Currently, the world's focus is related to the opioid epidemic problem.

Chemical structures and Pharmacology of Synthetic Opioids

Paul Janssen discovered fentanyl compound in 1960, primarily to treat patients with pain. "China white" or synthetic heroin alpha-methylfentanyl (AMF) was the first analogue that was synthesized in California 1979. Fentanyl and its analogs have a different chemical structure as compared to opiates, but exhibit similar pharmacological action as of opiates. After FDA approval in 1972, Fentanyl was used in the United States as anesthetics. The potency of fentanyl is around 100 times more than morphine and it has 40 minutes duration of action (Henderson, 1998; Bremer *et al.*, 2016). Fentanyl can couple with G-protein receptors and act as a full agonist of μ-opioid receptors. It inhibits ascending pathway of pain and raise the pain threshold. (Figure1.5)

Toxicity and treatment of Synthetic opioids

Synthetic opioids can be administered by various routes, inhalation, the powder, oral, nasal insufflation, rectal, and IV injection (Helander, Bäckberg and Beck, 2014; Papsun *et al.*, 2016). The most serious toxic effects of fentanyl are respiratory depression as other opiates. In addition, fentanyl abuse leads to opioid toxidrome-bradycardia, loss of consciousness, cyanosis, and miosis (Holstege and Borek, 2012). Additional clinical features include hypotension, pulmonary edema, ileus, nausea, pruritus, cough suppression, orthostatic hypotension, urinary urgency or retention, and chest wall rigidity, particularly with IV usage (Prekupec, Mansky and Baumann, 2017). Opioids cannot be detected by immunoassay in urine screens, but by using gas chromatography and mass spectrometry can be identified (Tenore, 2010). With regard to opioid abuse, the patients are monitored for breathing (maintain proper airway). The airway maintenance is considered as the first step in providing care to patients. After the proper maintenance of airway, naloxone (opioids antagonist) is administered to reverse the opioid-induced toxicity (Armenian *et al.*, 2017; Baumann and Pasternak, 2018).
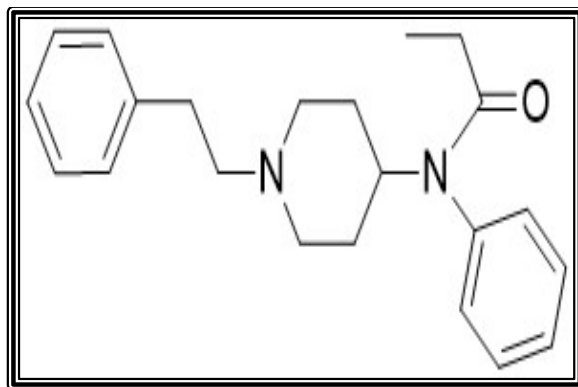

Figure 1.5 Chemical structure of Fentanyl

1.7. Tryptamines designer drugs

Serotonin is one of the most important transmitters involved in controlling many significant processes like sleep, memory, behavior, and temperature regulation. In fact, serotonin is a tryptamine derivative which is found inside the human brain with a limited amount and is significantly higher in the periphery. In 1958, scientists discovered natural sources of tryptamines compounds and this was found in fungi (*Psilocybe cubensis*) and botanicals (Araújo *et al.*, 2015). The psychoactive influence of tryptamines has been known since the ancient times through the magic mushrooms (Hill and Thomas, 2011). The synthetic tryptamines have been traced to the 1960s (alfa-Methyltryptamine-AMT) where it was used as an antidepressant by Soviets (Boland *et al.*, 2005). In recent times, in the United Kingdom, numerous cases of hallucination have been associated with the abuse of tryptamines. The most known drugs of abuse belonging to tryptamines are the alpha-methyltryptamine (AMT), 4-hydroxy-N-Methyl-N-ethyltryptamine (4-HO-MET) and Dimethyl-tryptamine (DMT). Tryptamines products are usually distributed in the form of powder or tablets. There are many different ways of tryptamines consumption like smoking, insufflation, IV or IM injection (Hill and Thomas, 2011; Corkery *et al.*, 2012). Accessible methods of synthesis from internet make tryptamines a very popular designer drug, especially among young adults (Brandt *et al.*, 2004; Schmidt *et al.*, 2011; Araújo *et al.*, 2015).

Chemical structures and Pharmacology of Tryptamines

Tryptamines are monoamine alkaloids derived from the amino acid tryptophan (Brandt *et al.*, 2004; Tittarelli *et al.*, 2015). Indole structure is the backbone of tryptamines compounds and this is the site at which the synthetic modifications occur for the scheming various designer drugs (Fantegrossi, Murnane and Reissig, 2008; Tittarelli *et al.*, 2015). Hallucination is considered as

the key effect of tryptamines abuse comparatively to stimulant actions. Tryptamines are serotonin 2A receptor agonist. Often the onset of duration for tryptamines is low, that forces the abuser to elevate the dose resulting in adverse effects (Nagai, Nonaka and Satoh Hisashi Kamimura, 2007; Fantegrossi, Murnane and Reissig, 2008; Ray, 2010) (Figure1.6).

Toxicity and treatment of Tryptamines

Clinical features of tryptamines misuse/ overdose include tachycardia, tachypnea, hypertension, trismus (lock jaw), anxiety, euphoria, sweating, diarrhea, nausea, vomiting, abdominal pain, sialorrhea, diaphoresis, palpitations, drowsiness, dysphoria, serotonin syndrome and hyperthermia (Brush, Bird and Boyer, 2004; Muller, 2004; Boland *et al.*, 2005; Alatrash, Majhail and Pile, 2006; Jovel, Felthous and Bhattacharyya, 2014). Similar to the designer drugs, there is no precise antidote to cure tryptamines intoxication. Supportive care and vital signs monitoring are the first line therapy provide to patients(ITOKAWA *et al.*, 2007; Araújo *et al.*, 2015).
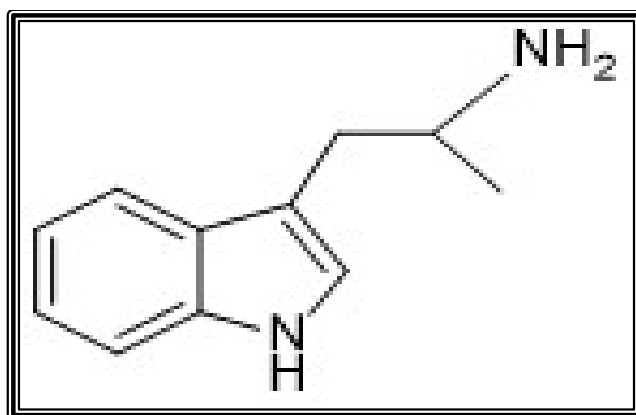
Figure 1.6 Chemical structure of Tryptamine

1.8. Phenethylamines (2Cs) designer drugs

Phenethylamines are a large group of drugs that contain different kinds of synthetic compounds. Designer drugs that belong to this group structurally have two carbon atoms located between the benzene ring and an amino group. In the late 1850s, old synthetic phenethylamines were synthesized and this included Amphetamine (α-methylphenethylamine; α-methylbenzeneethanamine), methamphetamine (α, N-dimethylphenethylamine). Many years later (in 1912), 3,4-methylenedioxymethamphetamine (MDMA) was synthesized to help people to decrease their appetite (Shulgin, 1986). These drugs are considered as old fashion phenethylamines. The new synthetic 2Cs was introduced after Alexander Shulgin released a book which started a sparkling phenomenon in designer drugs world. PIHKAL is an acronym representing "Phenethylamines I Have Known And Loved". This book explained in detail the synthesis of over 200 phenethylamine compounds. Drugs like 2,5-dimethoxy-4-ethylphenethylamine (2C-E, Europa), 2,5-dimethoxy-4-ethylthiophenethylamine (2C-T-2), 2,5-dimethoxy-4-(n)-propylthiophenethylamine , Blue Mystic, T7, Beautiful, Tripstay, Tweety-Bird Mescaline), 4-iodo-2,5-dimethoxyphenethylamine (2C-I, i), and 4-iodo-2,5dimethoxy- N-(2-methoxybenzyl) phenethylamine (25I-NBOMe) were mentioned in this book. Since 2011, 2C-I-NBOMe had spread around the world and has different streets name like Smiles, N-Bomb, Pandora and Dime. This designer drug can be found online, head shops and even gas stations (Dean *et al.*, 2013; Musselman and Hampton, 2014).

Chemical structures and Pharmacology Phenethylamines (2Cs)

The 2Cs designer drugs show high affinity to serotonin, alpha-adrenergic and dopamine receptors with different agonist and antagonist activities. MDMA is acting by elevating the release of monoamines (serotonin, adrenaline, and dopamine) from their terminal synapse, while appose their reuptake (Iravani *et al.*, 2000; Simmler *et al.*, 2014). Phenethylamines come as a powder, capsules, tablets, or in liquid form. Routes of administration are oral, inhalation, nasal insufflation, or intravenous injection. The oral route of phenethylamines is considered slower in effect than the insufflation route. The onset of action of oral phenethylamines ranges from 1 to 2.5 hours and duration of action 5-7 hours, while the insufflation takes 10-15 minutes and has a duration of action 2-4 hours (Office of Diversion Control, 2013). Phenethylamines produce stimulant effects at low doses lead to raise the activity and elevate the alertness of various (increased arousal and alertness). However, the undesirable effect of hallucination and sympathomimetic related adverse effects become after a high dose of 2Cs (Vilke *et al.*, 2012; Dean *et al.*, 2013). (Figure1.7)

Toxicity and treatment of Phenethylamines

Symptoms of phenethylamines toxicity include tachycardia, hyperthermia, hypertension, euphoria, empathy, nausea, vomiting, agitation, delirium, respiratory depression, mydriasis, paranoia, dysphoria, severe confusion, and seizures (Meyer and Maurer, 2010). Other adverse effects of phenethylamines include jaw clenching, muscular tension, tooth grinding and constant restless movement of the legs and increased muscle activity (Vollenweider *et al.*, 1998; Colado *et al.*, 1999; Sherlock *et al.*, 1999; Boot, McGregor and Hall, 2000). Phenethylamines long-term side effects also include memory deterioration, impaired mental skills, frequent paranoia and severe

depression (Parrott and Lasky, 1998; Schifano, 2000; Kalant, 2001). Treatment depends on monitoring vital signs; provide supportive care to a patient with intoxication. Drug of choice for both adverse side effects and seizures are benzodiazepines also can be used to treat agitation, hypertension, tachycardia, and hyperthermia (Taylor, Maurer and Tinklenberg, 1970; Spain *et al.*, 2008).
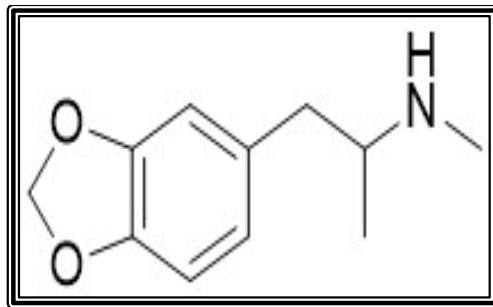

Figure 1.7 Chemical structure of MDMA

Designer Drugs	Mechanism of Actions	Adverse Drug Reactions	Therapeutic Interventions
Piperazines	BZP is a sympathomimetic stimulant (amphetamine-like effect), release dopamine, serotonin, and adrenaline in CNS, and inhibit the reuptake of dopamine TFMPP is phenylpiperazines which act as non-selective serotine receptors agonist and also elevate release of serotonin by blocking the reuptake.	Hallucination, stimulation, CNS intoxications include anxiety, headache, paranoia, tremors, and insomnia. Piperazines synthetic drugs also affect the peripheral nervous system include vomiting, palpitations diaphoresis, sinus tachycardia, metabolic acidosis, hyperthermia.	Provide supportive care and monitoring the vital sign is the first step of patient care. Benzodiazepines are the first line of therapy to treat seizure and agitation
Cathinones	Sympathomimetic action and also cause reuptake inhibition of dopamine, serotonin, and norepinephrine within the central nervous system. In addition, cathinones lead to elevated monoamines release from the presynaptic neurons	Agitation, anorexia, insomnia, paranoia, psychosis. In addition, the patients also experience a headache, palpitation and chest pain. The cardiovascular toxicity includes hypertension, tachycardia, hyperthermia, cardiovascular collapse and myocardial infarction	Supportive therapy mainly provides to patients with some medications such as IV fluid, benzodiazepine (to cure hyperthermia, agitation, and seizure) to overcome problems
Cannabinoids	Synthetic cannabinoids act as a full agonist on the CB1 receptor.	Anxiety, agitation, paranoia, delusions, aggression, paranoid thinking and anxiety. In addition, the patient usually have feelings of energy, euphoria, mild sedation, nausea, vomiting, hyperemesis, and abdominal pain	Monitoring of vital signs and providing supportive care to patients with intoxication. The drug of choice for both adverse side effects and seizures are benzodiazepines
Synthetic Opioids	Fentanyl can couple with G-protein receptors and act as a full agonist of μ-opioid receptors. It inhibits ascending pathway of	Toxidrome-bradycardia, loss of consciousness, cyanosis, and miosis Additional clinical features include hypotension, pulmonary edema, ileus,	Monitor the patient breathing is the first step in providing care to patients. After the proper maintenance of airway, naloxone (opioids

	pain and raise the pain threshold	nausea, pruritus, cough suppression, orthostatic hypotension, urinary urgency or retention, and chest wall rigidity, particularly with IV usage	antagonist) is administered to reverse the opioid-induced toxicity
Tryptamines	Serotonin 2A receptor agonist	Hallucination (Main), Less Stimulant action, tachycardia, tachypnea and hypertension, trismus, anxiety, euphoria, sweating, diarrhea, nausea, vomiting, abdominal pain, sialorrhea, diaphoresis, palpitations, drowsiness, dysphoria. In addition to serotonin syndrome and hyperthermia	No precise antidote Supportive care Vital signs monitoring
Phenethylamines	Agonist and antagonist activities at serotonin, alpha-adrenergic and dopamine receptors Elevating the release of monoamines	Hallucination, sympathomimetic related adverse effects (tachycardia, hyperthermia, hypertension, euphoria, empathy, agitation, mydriasis, paranoia, dysphoria, severe confusion, and seizures), nausea, vomiting, respiratory depression, jaw clenching, muscular tension, tooth grinding, restless leg syndrome, memory deterioration, impaired mental skills	Monitor vital signs Provide supportive care Benzodiazepines used to treat, seizures agitation, hypertension, tachycardia, and hyperthermia associated with Phenethylamine abuse

Table 1 Different kinds of designer drugs and its effects

Based on the current literature, there are no studies on the effect of the new piperazines such as TFmBZPPs on the dopaminergic neurotoxicity. Therefore, in the present study, we investigated the dopaminergic c neurotoxic effects of TFmBZPPs.

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2. Materials and Methods

2.1. Chemicals and Reagents

Thiazolyl Blue Tetrazolium Bromide (MTT) reagent was purchased from Tokyo Chemical Industry America. Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin Solution and Trypsin-EDTA solution were purchased from ATCC. Phosphate buffer saline (PBS), Dimethylsulfoxide (DMSO), Nicotinamide adenine dinucleotide (NADH), 2`, 7-dichlorofluorescindiacetate (DCF-DA), Pyrogallol, Hydrogen Peroxide (H_2O_2), Phosphoric acid, o-phthalaldehyde (OPA), L-Glutathione reduced, Trichloroacetic acid, Thiobarbituric acid and Phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Aldrich (St. Louis, MO). For protein quantification, Thermo Scientific Pierce 660 nm Protein Assay reagent kit was purchased (Pierce, Rockford, IL).

2.2. Rat dopaminergic neuron cells (N27)

DMEM medium was supplemented with Fetal Bovine Serum (10%) and Penicillin-Streptomycin Solution (1%) to culture the N27 cells (Kovalevich and Langford, 2013). For MTT assay, N27 cells were cultured in 75 cm^2 flasks at 37°C and 5% CO_2. After the cells reached 80% confluency, they were detached by trypsinization and seeded in a 96 well plate at a density of 1 x 10^5 cells/well. Cells were used in 14 passages (Zheng *et al.*, 2014).

2.3.Synthesis of new piperazines derivatives

Four novel compounds of piperazine were designed and synthesized by Dr. C. Randall Clark and Dr. Jack Deruiter. The starting material phenylpiperazine acted as the nucleophile and it attacks the carbonyl to form amino carbinol. The nucleophilic addition followed by reduction to form the new piperazine derivatives.
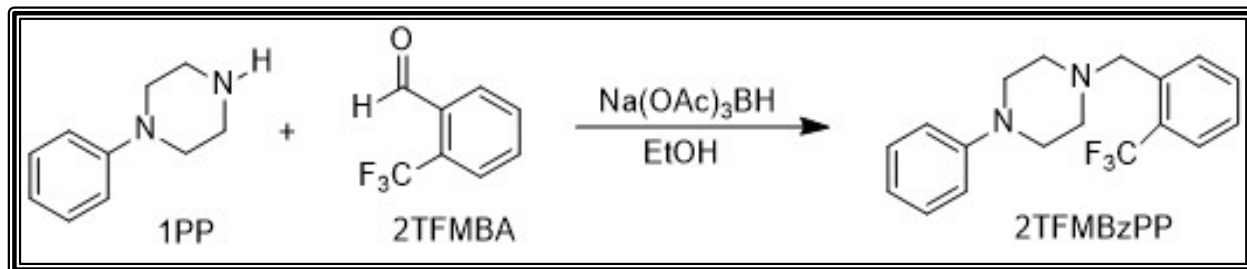

Figure 2.1 Synthesis of 2-TFMBzPP

NA(OAc)$_3$BH: selective reductive amination
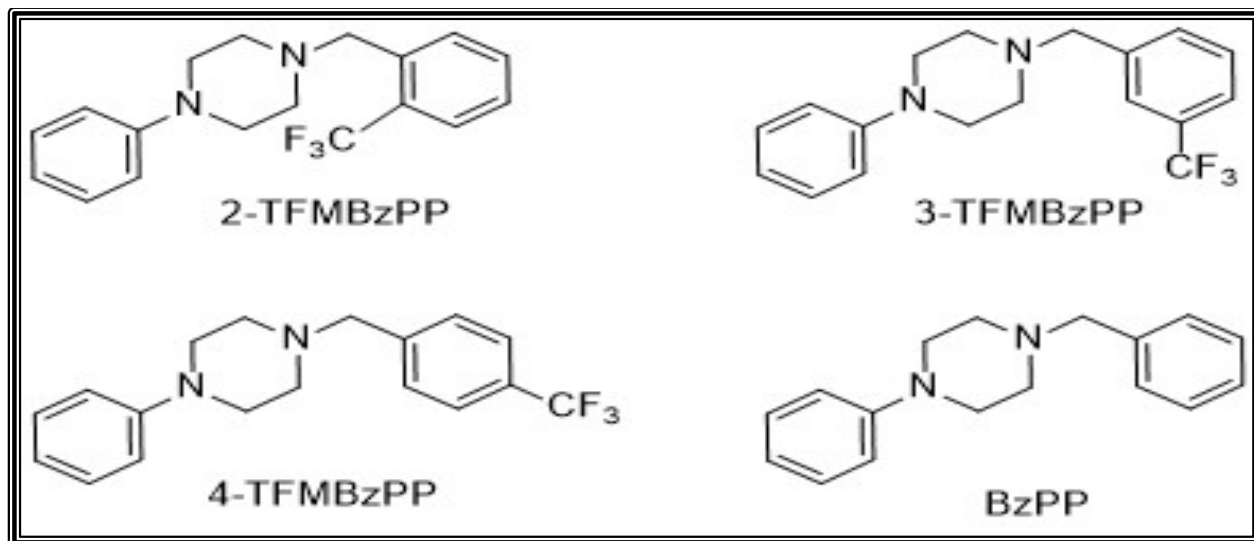2TMFBA: Trifluoro methyl benzaldehyde

Figure 2.2 Novel piperazine derivatives

2.4. Treatment design

2-TFMBzPP, 3-TFMBzPP, 4-TFMBzPP and BzPP were dissolved in DMSO to make 100mM stock solution. Later on, the stock solution was diluted to get the concentrations required for the experiments. For the evaluation of neurotoxicity, four different concentrations of 2-TFMBzPP, 3-TFMBzPP, 4-TFMBzPP and BzPP (0.1, 1, 10, 100µM) were achieved by serial dilution in serum-enriched fresh culture medium. N27 cells were exposed to different concentrations for 24, 48 and 72 hours. For the elucidation of the mechanism of neurotoxicity of the novel piperazines derivatives, the N27 cells were exposed to 100nM and 100uM for 48 and 72 hours. Each experiment was done by using freshly prepared designer drugs. Hydrogen peroxide, a well-known endogenous neurotoxin served as the positive control.

2.5. Cytotoxicity Assay

MTT cell viability assay was used for cytotoxicity assessment. In MTT assay, the mitochondria of viable cells reduce the yellow-colored water-soluble tetrazole reagent, MTT (3-(4,5-dimethylthiazol-2- 31 yl)-2,5-diphenyltetrazolium bromide) to an insoluble blue crystal formazan through succinate dehydrogenases. The resulted crystal formazan can be measured colorimetrically at 570nm (Mosmann, 1983; Berridge, Herst and Tan, 2005). After 24, 48 and 72 hours incubation with 2-TFMBzPP, 3-TFMBzPP, 4-TFMBzPP, and BzPP in serum-fed and serum-free medium, 12 mM MTT stock solutions were prepared and then added on each well along with fresh culture medium. After 2 hours of incubation at 37°C, the medium was aspirated and 200µl of DMSO was added to solubilize the formazan crystal and kept for 10 minutes. The absorbance was measured at 570 nm using a microtiter plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA). Results were expressed graphically as % cell viability.

Cells were imaged using an Axiovert 25 inverted microscope equipped with a Nikon Coolpix 4500 camera (Zhang *et al.*, 2014).

2.6. Protein quantification

Protein quantification was accomplished using Thermo Scientific Pierce 660 nm Protein Assay reagent kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard for protein measurement.

2.7. Quantifying Reactive Oxygen Species

The evaluation of reactive oxygen species in the N27 rat dopaminergic cells treated with TFMBzPP derivatives was measured by the transformation of non-fluorescent chloromethyl-DCF-DA (2′, 7- 32 dichlorofluorescindiacetate, DCF-DA) to fluorescent DCF. ROS was estimated spectrofluorometrically at excitation wavelength of 492 nm and emission wavelength of 527 nm. The control and designer drugs treated cell homogenates were incubated with 0.05% w/v solution of DCFDA in ethanol (10 μl), and phosphate buffer (150 μl) at 37°C for I hour. The fluorescent product DCF was measured using BioTek Synergy HT plate reader (BioTek, VT, USA). Results were expressed as percentage change from the control (Dhanasekaran, Tharakan and Manyam, 2008).

2.8. Nitrite assay

The final products of nitric oxide oxidation pathways are nitrite and nitrate, which used as an expression of nitric oxide production. The nitrite assay was performed with Griess reagent, NO_2 reacts with sulfanilamide under acidic condition leading to the production of diazonium ion. This

diazonium ion association with N-(1-naphthyl) ethylenediamine to form 36 chromophoric azo product which can be measured spectrophotometrically at 545 nm (Giustarini *et al.*, 2008).

2.9. Lipid Peroxide Content

Lipid peroxidation occurs due to the oxidative breakdown of lipids when ROS attack the polyunsaturated fatty acids in progression reaction process. Estimation of the lipid peroxidation content was done by quantifying malondialdehyde (MDA) content in the form of Thiobarbituric acid-reactive substances (TBARS) (Ohkawa, Ohishi, & Yagi, 1979). Control and designer drug-treated cell homogenate (100μl) were incubated with ice-cold 100μl Trichloroacetic acid (TCA, 20 % w/v), 400 μl Thiobarbituric acid (TBA, 0.5 % w/v) and 500μl deionized water. Following the incubation, the samples were kept at 80ºC in a water bath for 15 minutes. Centrifugation at 10,000 RPM was done after cooling for 5 minutes. The absorbance of the supernatant was measured at 532 by using plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA). MDA levels were calculated as TBARS reactive substances per mg protein (Dhanasekaran *et al.*, 2007).

2.10. Catalase Activity

Catalase is an antioxidant enzyme that catalyzes the breakdown of hydrogen peroxide into water and oxygen. Catalase activity was done by mixing the cell homogenate with PBS in the presence of 30mM of hydrogen peroxide. Breakdown of hydrogen peroxide was measured spectrophotometrically at 240nm for 1 minute. The decrease in absorbance was observed and the enzyme activity was calculated as hydrogen peroxide decomposition/mg protein (Muralikrishnan and Mohanakumar, 1998).

2.11. Glutathione Content

Fluorescence that produced as a result of the reaction between GSH and 34 o-phthalaldehyde (OPT) can be evaluated spectrofluorometrically (Cohn and Lyle, 1966). The assay samples contain cell homogenate, 0.1 M phosphoric acid, and 0.01 M phosphate buffer. First, mixing the cell homogenate with 0.1 M phosphoric acid to induce protein precipitate, then the samples were centrifuged at 12000 RPM for 10 minutes. Later, the supernatant was incubated with 0.1% OPT (dissolved in methanol) for 20 minutes at room temperature. Fluorimetric readings were taken at an excitation wavelength of 340 nm and an emission wavelength of 420 nm. A GSH standard curve was prepared from commercially acquired GSH. The GSH content was calculated as mmol of GSH/μg protein (Muralikrishnan and Mohanakumar, 1998).

2.12. Glutathione peroxidase (GPx)

Catalyzes the reduction of hydrogen peroxides and functions to protect the cell from oxidative damage. Oxidized glutathione (GSSG) is produced upon reduction of an organic hydrogen peroxide by GPx. GPx is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340nm. The rate of decrease in absorbance is directly proportional to GPx activity in the cell homogenate (Muralikrishnan and Mohanakumar, 1998; Hui *et al.*, 2002).

2.13. Mitochondrial Complex-I Activity

Complex-I (NADH dehydrogenase) catalyzes the oxidation of NADH to NAD^+. The determination of NADH dehydrogenase activity was done spectrophotometrically at 340 nm, by mixing the cell homogenate with phosphate buffered saline and NADH. NADH oxidation was measured by the

decrease in absorbance at 340 nm for 3 minutes. A standard curve was composed of commercially obtained NADH (Ramsay *et al.*, 1986).

2.14. Mitochondrial complex IV activity

Complex IV assay was performed by mixing the cell homogenate, phosphate buffered saline and Cytochrome C. Activity of the Cytochrome C oxidase was measured spectrophotometrically at 550 nm. Change in absorbance for 3 minutes was used to determine the cytochrome C activity. A standard curve was created from commercially obtained Cytochrome C (Wharton and Tzagoloff, 1967; Ramsay *et al.*, 1986).

2.15. Monoamine oxidase (MAO) activity

The activity of the total monoamine oxidase was measured fluorometrically by measuring the amount of 4-hydroxyquinoline formed as a result of kynuramine oxidation (Morinan and Garratt, 1985). MAO activity was calculated as 4-hydroxyquinoline formed/hour/mg protein (Muralikrishnan and Mohanakumar, 1998; Albano, Muralikrishnan and Ebadi, 2002).

2.16. Statistical Analysis

Data were reported as mean ± SEM. Statistical analysis was accomplished using one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparisons test ($p < 0.05$ was considered to be statistically significant). Statistical analysis was performed using Prism-V software (La Jolla, CA, USA).

3. Results

3.1. TFMBzPP derivatives induced Dose-Dependent and Time-Dependent reduction of dopaminergic (N27) Cell viability

N27 dopaminergic cells were treated with different doses (0.1 µM, 1µM, 10µM, 100µM) of TFMBzPP derivatives (2-TFMBzPP, 3-TFMBzPP, 4-TFMBzPP, and BzPP) for 24, 48 and 72 hours. The endogenous neurotoxin hydrogen peroxide was used as a positive control. TFMBzPP derivatives and BzPP caused significant dose-dependent and time-dependent reduction of dopaminergic neuronal viability compared to control (n=12, p<0.0001, Figure 3.1.a., Figure 3.2.a., Figure 3.3.a. and Figure 3.4.a.). Treatment of N27 cells with TFMBzPP derivatives for 24 hours did not show significant dose and time-dependent of neurotoxicity. However, the treatment of N27 for 48 hours exhibited dose-dependent decrease in dopaminergic neuronal viability (20-30% reduction). On the other hand, the 72 hours treatment of N27dopaminergic neuronal cells with TFMBzPP derivatives illustrated the significant reduction in cell viability (40-60 %). The new piperazines derivatives (TFMBzPP) caused well defined morphological changes in N27 dopaminergic neuronal cells. Dopaminergic neurons were structurally deformed, shrunken and also showed considerably less synaptic connections due to which cells became more rounded leading to neuronal death. The endotoxin hydrogen peroxide (the positive control) induced significant dose-dependent neurotoxicity (Figure 3.5).

Figure 3.1 Effect of 2-TFMBzPP on dopaminergic (N27) neuronal viability

a) Neuronal Viability:
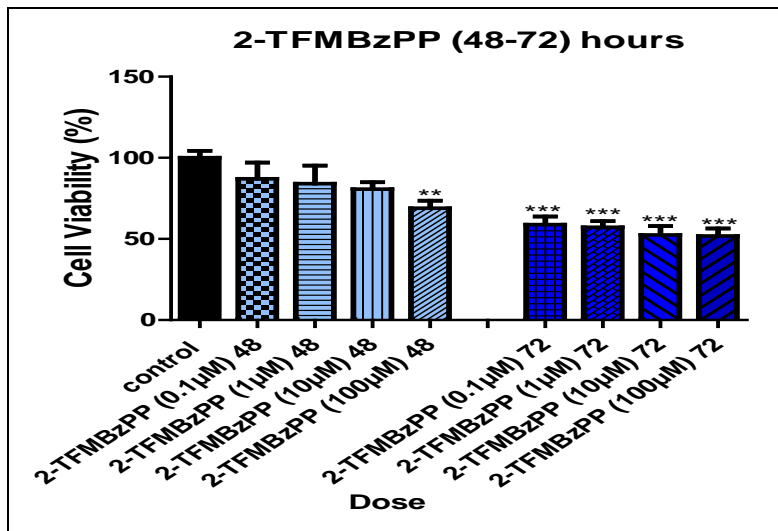
b) Morphological

Control   2-TFMBzPP (100µM)

3.1. Cells were treated with different doses of 2-TFMBzPP for 48 hours and 72 hours.

Cell viability was evaluated through the MTT reduction assay . After incubation, the cells were washed with PBS and visualized under a microscope (magnification 10x). Results are expressed as percentage control ± SEM. Statistical comparisons were made using oneway ANOVA/ Dunnet's multiple comparison tests. Note (*) indicates a statistically significant difference when compared to controls ($p < 0.0001$, n=12)

Figure 3.2 Effect of 3-TFMBzPP on dopaminergic (N27) neuronal viability

a) Neuronal Viability

b) Morphological features

Control   3-TFMBzPP (100μM)

3.2. Cells were treated with different doses of 3-TFMBzPP for 48 hours and 72 hours.

Cell viability was evaluated through the MTT reduction assay . After incubation, the cells were washed with PBS and visualized under a microscope (magnification 10x). Results are expressed as percentage control ± SEM. Statistical comparisons were made using oneway ANOVA/ Dunnet's multiple comparison tests. Note (*) indicates a statistically significant difference when compared to controls ($p < 0.0001$, n=12)

Figure 3.3 Effect of 4-TFMBzPP on dopaminergic (N27) neuronal viability

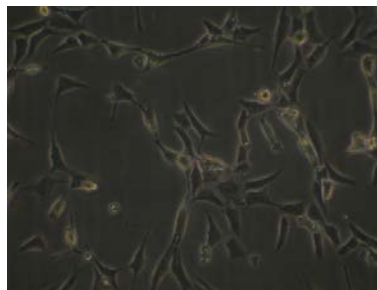a) Neuronal Viability

b) Morphological features

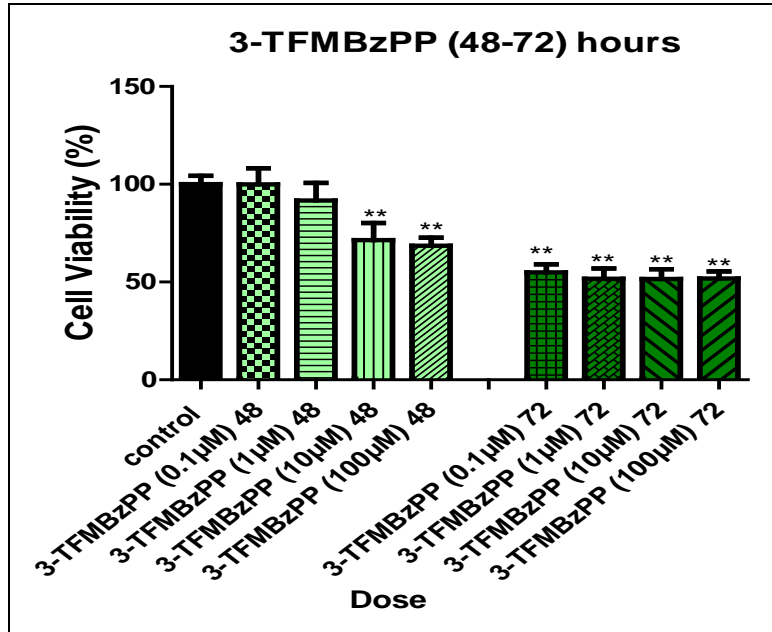Control   4-TFMBzPP (100μM)
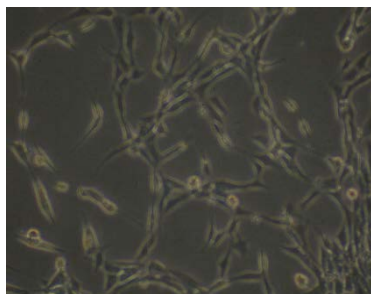
3.3. Cells were treated with different doses of 4-TFMBzPP for 48 hours and 72 hours.

Cell viability was evaluated through the MTT reduction assay. After incubation, the cells were washed with PBS and visualized under a microscope (magnification 10x). Results are expressed as percentage control ± SEM. Statistical comparisons were made using oneway ANOVA/ Dunnet's multiple comparison tests. Note (*) indicates a statistically significant difference when compared to controls ($p < 0.0001$, n=12)
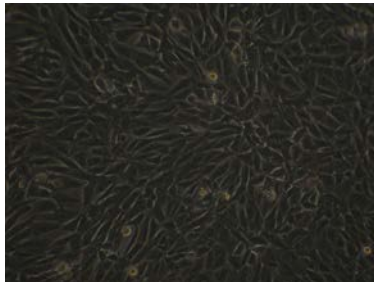
Figure 3.4 Effect of BzPP on dopaminergic (N27) neuronal viability

a) Neuronal Viability
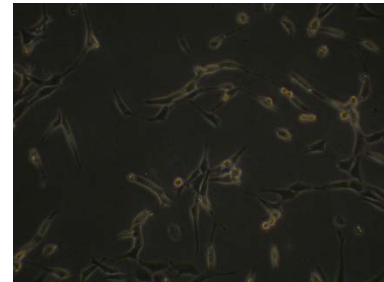

b) Morphological features

Control   BzPP (100μM)
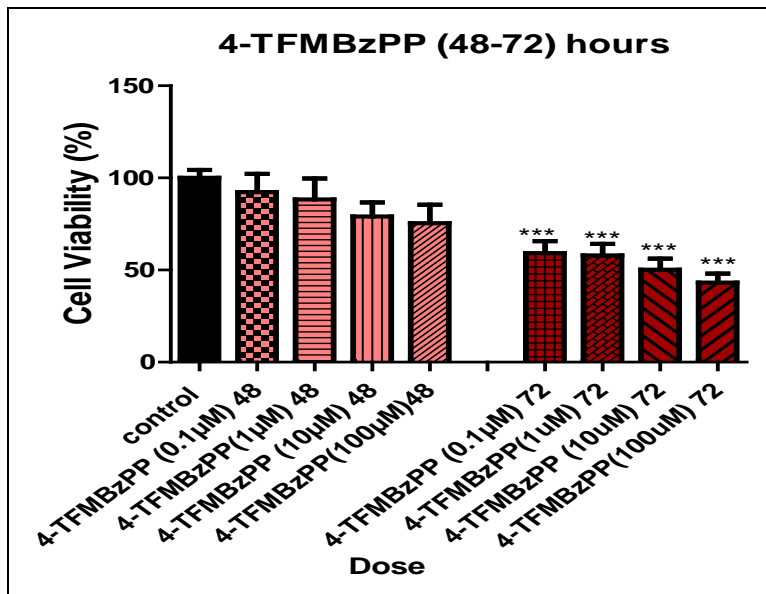

3.4. Cells were treated with different doses of BzPP for 48 hours and 72 hours.

Cell viability was evaluated through the MTT reduction assay. After incubation, the cells were washed with PBS and visualized under a microscope (magnification 10x). Results are expressed as percentage control ± SEM. Statistical comparisons were made using oneway ANOVA/ Dunnet's multiple comparison tests. Note (*) indicates a statistically significant difference when compared to control ($p < 0.0001$, n=12)

Figure 3.5 Effect of established dopaminergic neurotoxin hydrogen peroxide on dopaminergic
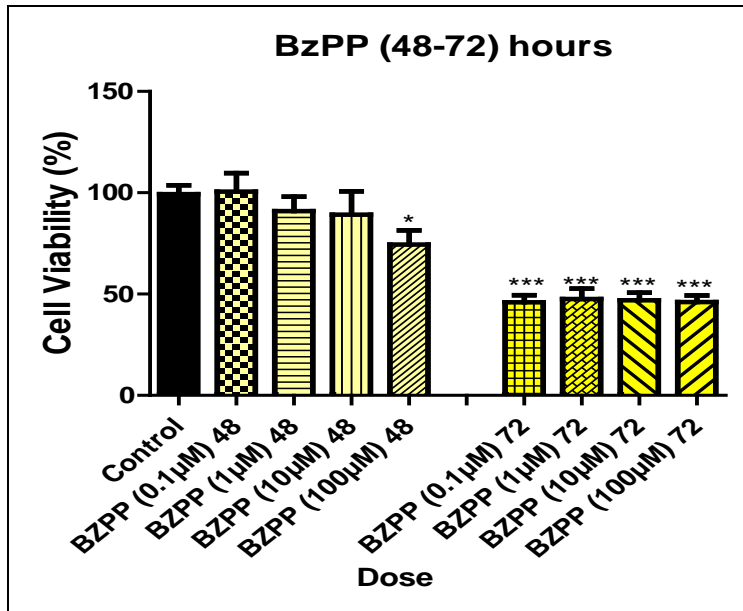
 (N27) neuronal viability
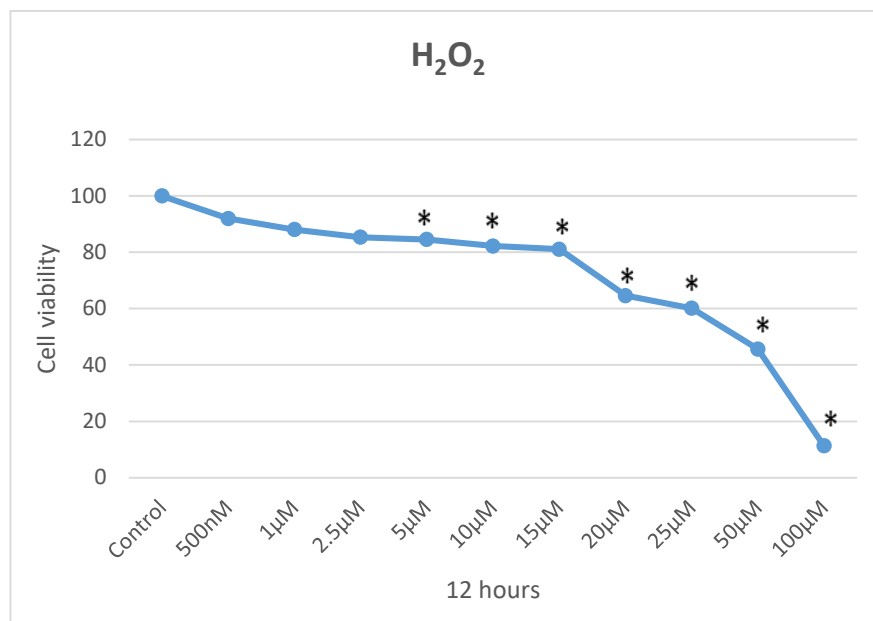
3.5. Dopaminergic neuronal cells were treated with different doses of hydrogen peroxide for 12 hours.

 Cell viability was evaluated through the MTT reduction assay. After incubation, the cells were washed with PBS and visualized under a microscope (magnification 10x). Results are expressed as percentage control ± SEM. Statistical comparisons were made using oneway ANOVA/ Dunnet's multiple comparison tests. Note (*) indicates a statistically significant difference when compared to controls ($p < 0.0001$, n=12)

With regard to the mechanism of neurotoxicity of piperazine derivatives, we evaluated the neurotoxic mechanisms of 2-TFMBzPP and BzPP. We evaluated the role of oxidative stress and mitochondrial dysfunction associated with the dopaminergic neurotoxicity induced by piperazine derivatives (2-TFMBzPP and BzPP) after 24 hours of exposure.

3.2. TFMBzPP derivatives generate ROS

Generally, human illnesses are related to the generation of ROS. Aging and diseases like atherosclerosis, cancer and neurodegenerative disorders are associated with ROS generation. The destruction of biological molecules such as DNA, protein and lipids are produced by oxidative stress that resulted from the generation of ROS. Antioxidants such as catalase, superoxide dismutase and glutathione neutralize the harmful effects of ROS (Freeman and Crapo, 1982; Halliwell, Gutteridge and Cross, 1992). TFMBzPP (100μM) significantly induced dose-dependent increase in ROS generation in N27 dopaminergic neuronal cells as compared to the control (n = 5, p < 0.0005; Figure 3.6).
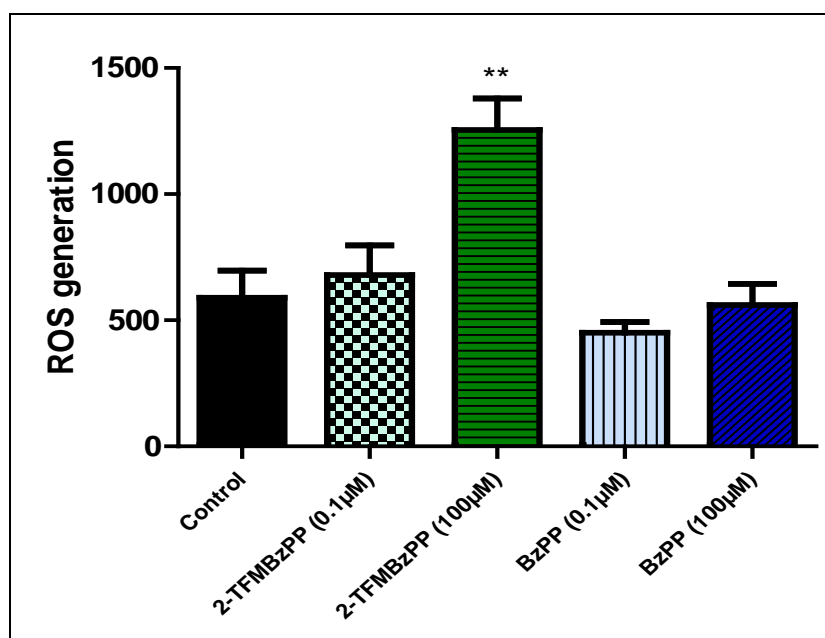
Figure 3.6 Effect of TFMBzPP derivatives on ROS generation in N27 dopaminergic neuronal cells

2-TFMBzPP and BzPP generate oxidative stress by generating reactive oxygen species in N27 dopaminergic neuronal cells after 24 hours. The fluorescent product DCF was measured spectrofluorometrically. 2-TFMBzPP (100μM) showed a significant increase in ROS generation (p < 0.0005, n=5). Results are expressed as control ± SEM. Statistical comparisons were made using oneway ANOVA/Dunnet's multiple comparison tests

3.3. TFMBzPP derivatives increase nitrite production

Many studies revealed that patient with Parkinson's disease has a high percent of nitric oxide production. This nitric oxide may lead to oxidative stress eventually causing dopaminergic neuronal damage (Qureshi *et al.*, 1995). 2-TFMBzPP (100µM) caused a significant increase in nitrite formation (n=5, p<0.05; Figure 3.7).
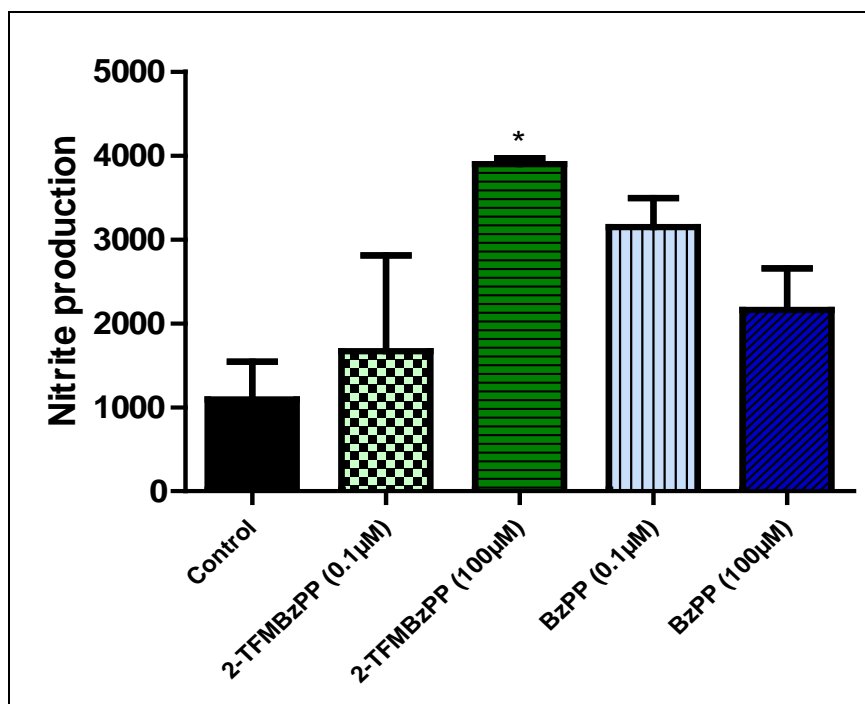
Figure 3.7 Effect of TFMBzPP derivatives on Nitrite production in N27 cells

TFMBzPP (100µM) caused an increase in nitrite production (n=5, p<0.05). However, the increase in nitrite production was not statistically significant at the lower dose (0.1µM). Nitrite production was determined spectrophotometrically at 540 nm. Results are expressed as control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison tests

3.4. TFMBzPP derivatives induce lipid peroxidation

Lipid peroxidation occurs due to degradation of lipids increased by the effect of free radicals (ROS). When compared to the control, 2-TFMBzPP and BzPP at a dose of 100μM significantly increased lipid peroxidation (n=5, p< 0.0005; Figure 3.8).
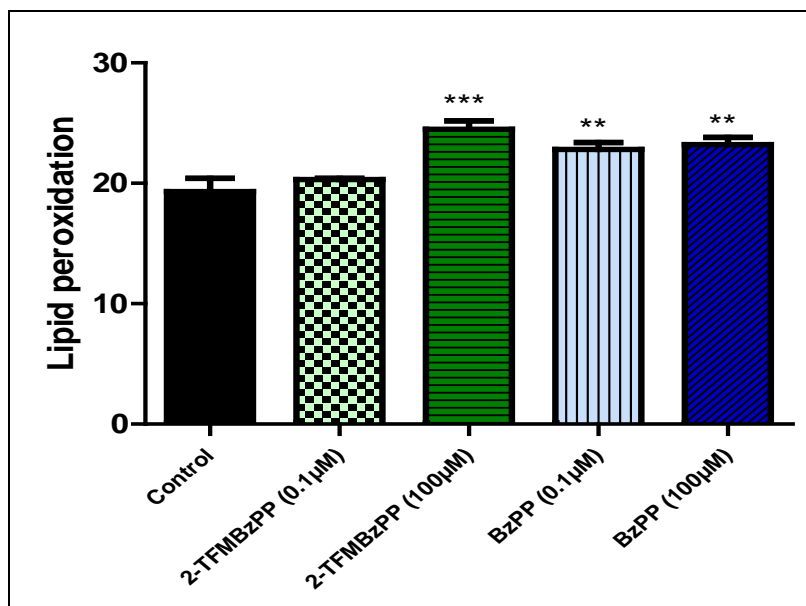
Figure 3.8 Effect of TFMBzPP derivatives on lipid peroxidation in N27 dopaminergic cells

2-TFMPP (100μM) and BzPP (both doses) significantly increased lipid peroxidation in dose-dependent manner (n=5, p < 0.0005). Lipid peroxidation was measured colorimetrically as TBARS, a marker of cellular membrane damage. Results are expressed as control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison tests.

3.5. Effect of TFMBzPP derivatives on GSH content and GSH-Px activity

TFMBzPP derivatives dose-dependently increased GSH in N27 dopaminergic neuronal cells as compared to the control (n=5, p< 0.0001; Figure 3.9). Regarding the Glutathione peroxidase activity, TFMBzPP derivatives did not have a significant effect on Glutathione peroxidase activity.

A. Glutathione content
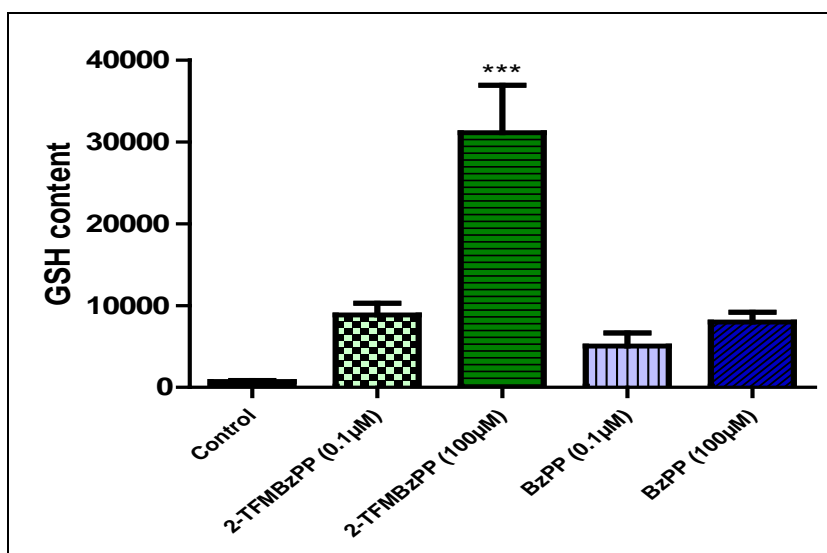
Figure 3.9 .A. Effect of TFMBzPP derivatives on GSH content in N27 cells dopaminergic neuronal cells

2-TFMBzPP (100μM) increased GSH content in N27 dopaminergic neuronal cells after 24 hours (n=5, p<0.0001). The condensation reaction between GSH and ophthalaldehyde (OPT) produce fluorescence at pH 8.0 that was measured spectrofluorometrically. Results are expressed as control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison tests.

B. Glutathione peroxidase Activity
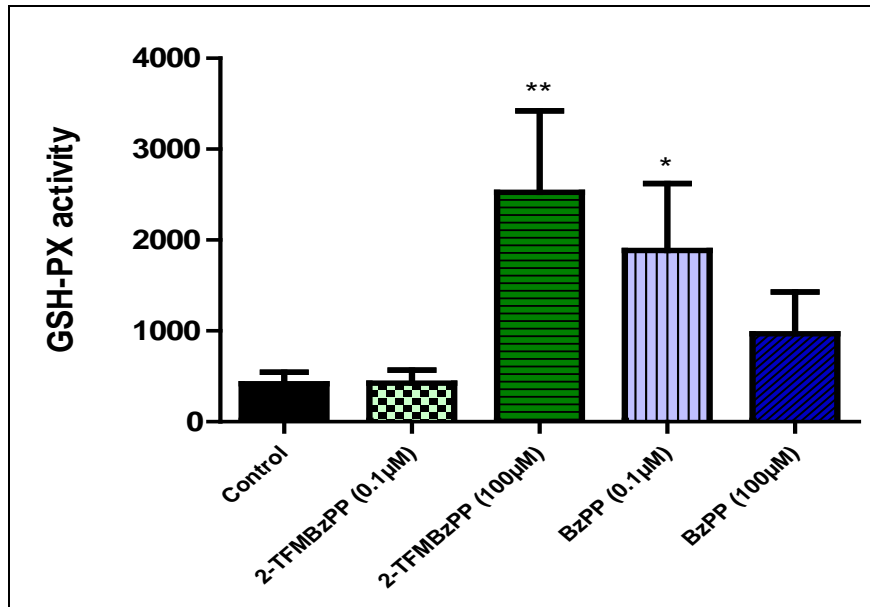
Figure 3.9.B. Effect of TFMBzPP derivatives on GSH-Px activity in N27 dopaminergic neuronal cells

2-TFMBzPP caused an increase in glutathione peroxidase activity ($n=5$, $p<0.05$). Results are expressed as control $\pm$ SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison tests.

3.6. TFMBzPP derivatives alter antioxidant enzymes activities

TFMBzPP derivatives (100µM) increased the catalase activity as compared to the control (n=5; $p < 0.05$; Figure 3.10).
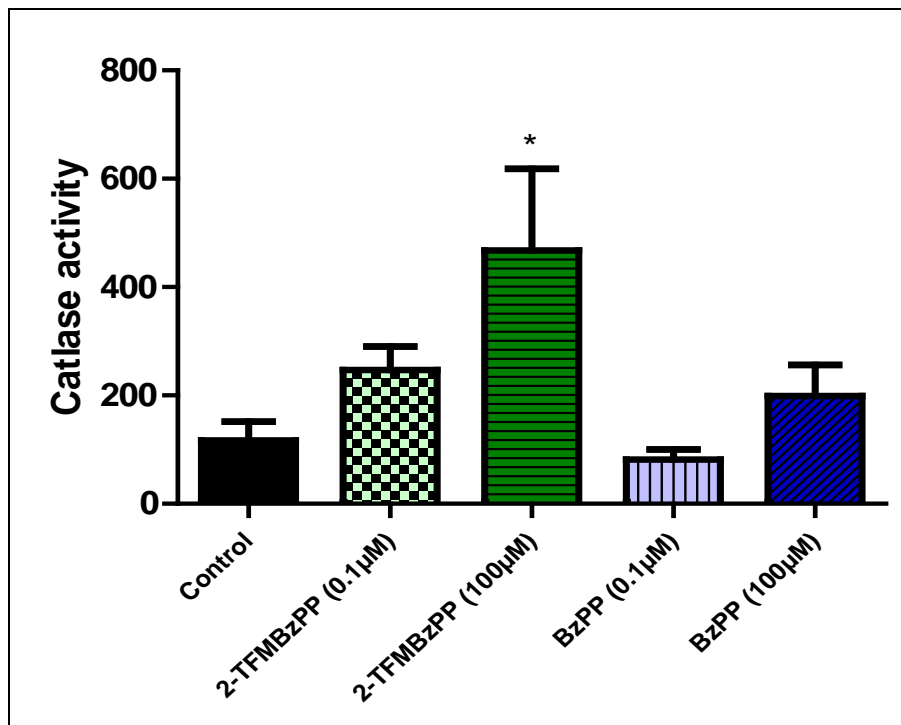
Figure 3.10. Effect of TFMBzPP derivatives on Catalase activity in N27 dopaminergic neuronal cells

2-TFMBzPP showed a significant increase in catalase activity (n=5; $p < 0.05$). Results are expressed as control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison tests.

3.7.TFMBzPP derivatives increase Monoamine oxidase activity (MAO) in N27 cells

Monoamine oxidase activity influences oxidative stress, apoptosis, glial activation and the aggregated protein clearance. Furthermore, MAO activity has been connected to neurodegenerative diseases. (Youdim and Lavie, 1994; Merad-Boudia *et al.*, 1998; Siddiqui *et al.*, 2010). 2-TFMBzPP (100µM) derivatives dose-dependently increased MAO activity in N27 dopaminergic neuronal cells as compared to the control (n=5, p< 0.0005; Figure 3.11).
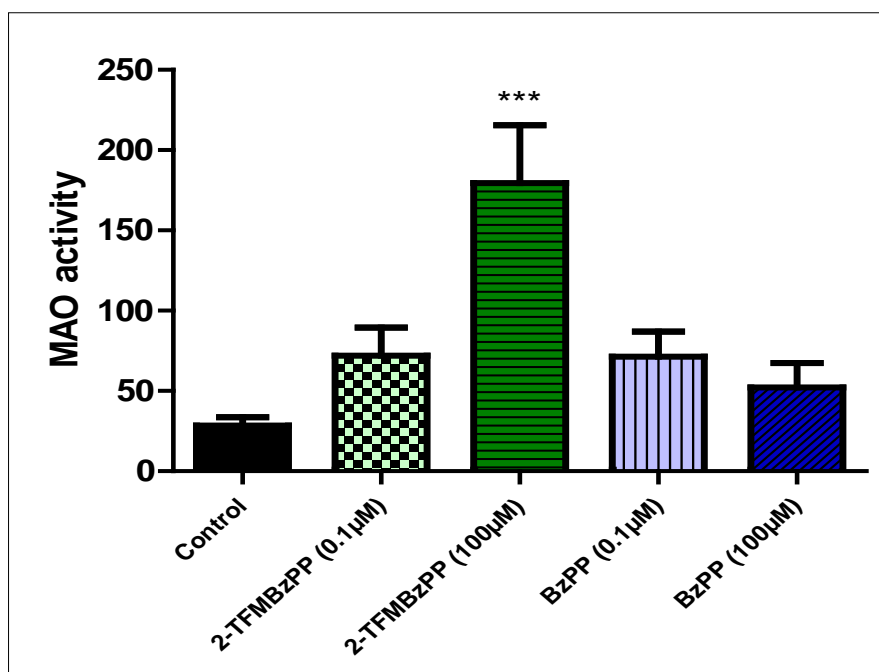
Figure 3.11 Effect of TFMBzPP derivatives on mitochondrial monoamine oxidase (MAO) activity in N27

2-TFMBzPP (high dose) caused a significant increase in MAO activity (n=5, p<0.0005). Total MAO activity was determined fluorimetrically at 315 nm excitation / 380 nm emission. Results are expressed as control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison tests

3.8.TFMBzPP derivatives do not affect Mitochondrial Complex-I activity and Complex IV activity

Mitochondrial is considered as a regulator of cell viability because respiration of mitochondrial is responsible for the production of energy (ATP). Many disorders like Parkinson's disease, Alzheimer's disease and Huntington's disease are related to deficiency of Mitochondrial Complex-I and complex-IV (Michael T Lin and Beal, 2006). TFMBzPP derivatives did not affect mitochondrial function as compared to control (n=5, p< 0.5; Figure 3.12. a, b).

3.8.a Complex I Activity
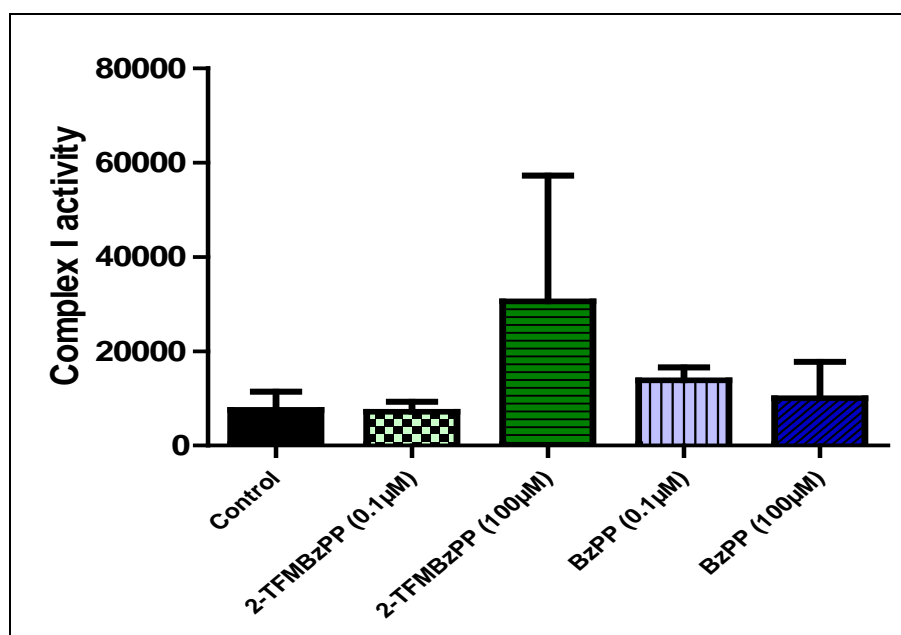
Figure 3.12. a. Effect of TFMBzPP derivatives on Mitochondrial Complex-I activity in N27 cells

TFMBzPP derivatives did not affect the Complex I activity (n=5; p< 0.5). Mitochondrial Complex-I activity was measured spectrophotometrically. Results are expressed as control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison tests.

3.9.b. Complex IV Activity
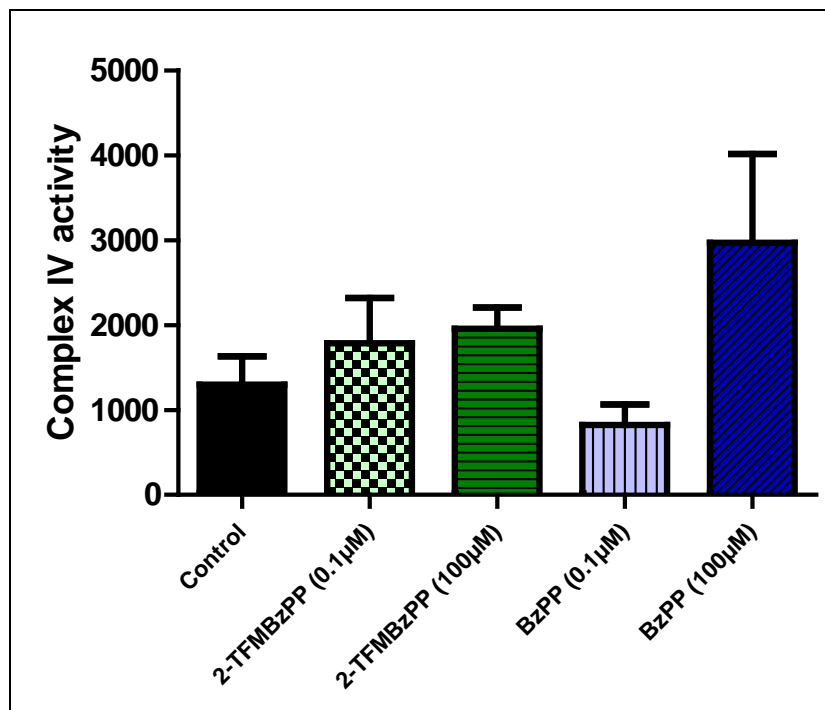
Figure 3.12. b. Effect of TFMBzPP derivatives on Mitochondrial Complex-IV activity in N27 cells

TFMBzPP derivatives did not affect the Complex IV activity (n=5; p< 0.5). Mitochondrial Complex-IV activity was measured spectrophotometrically. Results are expressed as control ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison tests.

4. Discussion

People used different kinds of designer drugs such as Phenethylamines, Piperazines, Tryptamines, Synthetic cannabinoids, Synthetic cathinones and Synthetic opioids to obtain an escalation in physical and mental activity, and ecstasy. The chronic use of the above drugs can increase the risk for neurodegeneration. Neurodegenerative diseases detrimentally affect the central nervous system and are characterized by significant loss of neuron and axons. Lately, these phenomena gained huge consideration due to the morbidity, disability features and incurring a magnanimous cost which has a big impact on the current and future health care. Parkinson's disease (PD), Alzheimer's disease, neurotropic viral infections, Huntington's disease (HD), stroke, paraneoplastic disorders, traumatic brain injury and multiple sclerosis are the most known diseases that related to neuronal death. Usually, elderly people are the most vulnerable to these kinds of diseases. The exact principle that leads to neurodegenerative diseases is still under investigating. However, there are some factors that found connected to neuronal cell death (Wyss-coray and Mucke, 2002; Cappellano *et al.*, 2013). The factors associated with neurodegeneration are oxidative stress, mitochondrial dysfunction, inflammation, apoptosis, excitotoxicity, and exposure to endogenous and exogenous neurotoxins (environmental, exposure to pesticide/herbicide & metals, head injury, cigarette smoking and rural living) (Brown, Lockwood and Sonawane, 2005). Inflammation is a complex reaction that protects the body from endogenous and exogenous neurotoxins and aids to eliminate and inhibits toxic material and overcome the negative health impact. In neurodegenerative diseases, the abnormalities in protein conformations and signals from harmful neurons are generated by inflammation. This inflammation can be caused by various

factors such virus, bacteria, chemicals (endogenous and exogenous). These factors can trigger chronic immune activation of microglia in the central nervous system. The irregularity of inflammatory control mechanism usually causes accumulation of abnormal proteins in the CNS. Neuroglia is the primary homoeostatic and defense elements of the central nervous system (CNS) (Amor *et al.*, 2010; Stephenson *et al.*, 2018). A combined dysregulation of microglial activation and pro-inflammatory cytokine production may be an important driver in the pathogenesis of neurodegenerative diseases.

In Parkinson's disease, the substantial nigra produces less dopamine which leads to decrease the movement disorder. The patient with PD usually has chronic neuroinflammation which characterized by the presence of glial cell activation and astrocytes. Increase in activation of microglia and astrocyte result in the increased expression of pro-inflammatory mediators (TNF-α, IL-1β, IL-6, and interferon-γ). A lot of studies connect the production of these pro-inflammatory mediators to the degeneration of dopamine neurons in the brain(Cappellano *et al.*, 2013; Amor and Woodroofe, 2014). The role of apoptosis in neurodegenerative diseases has gained wide attention in recent years. The apoptotic mechanism (programmed cell death) is characterized by cell shrinkage, chromatin condensation, apoptotic bodies and DNA fragmentation. Cysteine aspartyl-specific proteases (caspases) have a significant role in control the apoptosis process inside cells. The caspases family has a huge role in neuronal death in various neurodegenerative disorders. There are 14 different caspases which grouped to the upstream initiator and downstream effector. Apoptosis in neurodegenerative disorders is mediated by activating the caspases that lead to neuronal cell death. Protease enzyme is associated with the formation of β-amyloid which is linked to Alzheimer disease. These β-amyloid causes neurotoxicity by producing intracellular oxidative

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stress and increase the concentration of Ca^{2+}. Interestingly, in Parkinson's disease, some studies report the presence of DNA fragmentation and apoptotic cells (Kermer *et al.*, 2004). Glutamate is the principal excitatory neurotransmitter in the central nervous system. Glutamate is usually released from the presynaptic neurons to activate the postsynaptic glutamate receptors. There are low amounts of glutamate found in synaptic cleft in the normal physiological condition. However, in case of the excessive amount of glutamate in the synapse, will lead to increase in glutamate receptors stimulation and produce excitotoxicity. To avoid the extra stimulation of post synoptic glutamate receptors, excitatory amino acids transporters (EAATs) mediate the uptake of excessive glutamate in the synaptic cleft. Many studies illustrated that toxic effect of glutamate excitatory linked to dopaminergic neuronal death in substantial nigra in Parkinson's disease (Zhang *et al.*, 2016).

Oxidative stress is defined as an imbalance between the excessive generation of reactive oxygen species (ROS) and antioxidant elements. The oxidative stress is considered one of an early sign of pathogenesis of neurodegenerative diseases that affect brain due to the high demand for oxygen in brain tissue. The involvement of oxidative stress in neurodegenerative diseases represent in causing intracellular damage, altering the DNA mutation and leading to mitochondrial function disturbances. Furthermore, ROS cause impairment in lipid production and amino acid modification for protein synthesis inside cells. The mitochondrion is organelle located in the cytoplasm responsible for energy production in most eukaryotic cells. Dysfunction of mitochondria plays a crucial role in the aging process that leads to neuronal death through the alertness DNA mutation and production of reactive oxygen species (ROS) (Kim *et al.*, 2015). DNA of mitochondria has specific genome code to produce unique protein inside the

mitochondria. Disturbance in mitochondria DNA mutation is connected to many disease pathogeneses especially that influence brain.   Based on the current literature, our TFMBzPP derivatives also exerted its neurotoxicity in dopaminergic cells by inducing oxidative stress as seen by increased ROS and nitrite production leading to lipid peroxidation. Interestingly, TFMBzPP derivatives also affected the antioxidant molecule (glutathione) and antioxidant enzyme activities (catalase and glutathione peroxidase)(Michael T. Lin and Beal, 2006).

The central nervous system (CNS) has a specific immune system that prevents or limits the pathogenesis from entering the CNS. This system called immune privilege which relays on the blood-brain barrier (BBB). The (BBB) main function is to prevent foreign and harmful substances to penetrate the CNS. However, these operations impeded also antibodies and immune cells to enter and make the CNS unable to produce an adaptive immune reaction (Amor *et al.*, 2010).

Stimulants are psychomotor substances that increase the activity of the central nervous system by enhancing the monoaminergic neurotransmission.   Pharmacological actions of stimulants include increase alertness, enhanced memory, euphoria, excitation and decreased appetite. In addition, stimulants are used to treat many diseases like fatigue, chronic fatigue syndrome, Attention-deficit/hyperactivity disorder ADD / ADHD and eating disorders (Holman, 1994). In general, there are two kinds of stimulants; natural stimulants (caffeine and khat) and synthetic stimulants (amphetamine, methyl amphetamine and Methylphenidate). Due to the high demands of these kinds of drugs especially among school and college students, there is a concern around the world regarding the adverse effects of stimulants. The largest and most important risks of using this type of medication are addiction. The influence of stimulants drugs can be divided into two categories; behavioral and physical(Craig *et al.*, 2015). The behavior characteristics of long-term use include;

paranoia, hallucinations, violent behavior, cravings for the drug, compulsive drug-seeking behavior, convulsions and obsessive behavior. The physical side effects include cardiovascular complications (tachycardia), increased blood pressure (hypertension), headache, hyperthermia, euphoria, empathy, nausea, vomiting, agitation, delirium, respiratory depression, mydriasis, paranoia, dysphoria, severe confusion, and seizures (Vollenweider *et al.*, 1998; Sherlock *et al.*, 1999; Mato *et al.*, 2011).

Designer drugs are abused in order to experience psychostimulant effects similar the legally banned substances of abuse (heroin or cocaine). Numerous people around the world are currently abusing the designer drugs. Substantial health impairments have been observed globally due to the abuse of designer drugs. Hence, if this epidemic is not controlled appropriately, it can cause a huge economic impact and decline in the health of the current and future generation. These products are presently produced by clandestine labs in the U.S and other countries around the world. Designer drugs are currently sold by independent dealers in different formulations (powdered form, in single-component tablets, capsules, or in combination combined with MDMA or other illicit controlled substances through the internet and retail stores. The most common route of administration by the abusers are an oral route (ingest), inhale, inject, smoke, or snort. Due to the structural and chemical characteristic features (lipophilicity), these designer drugs readily cross the blood-brain barrier and also be readily distributed throughout the body. Hence, it exerts an effect throughout the body on different organ systems. The biogenic monoaminergic neuronal tract and peripheral sympathetic nervous system are extensively affected by the designer drugs abuse which can lead to behavioral changes (memory deficit, mental disorders and movement impairment) and further increase the risk for neurological disorders such as Parkinson's disease

(movement disorder), dementia (memory disorder) and various other mental disorders (psychosis, ADD and depression). Stimulants used for the treatment of various different peripheral and CNS disorders have shown to exhibit several adverse drug reactions. Thus, a drug with stimulatory effect and minimal adverse drug reaction will be extremely beneficial for patients with fatigue, chronic fatigue syndrome, Attention-deficit/hyperactivity disorder ADD / ADHD and eating disorders. Thus, the designer drugs with minimal adverse effects will be a new therapeutic avenue to venture in the future.

5. Conclusion

Designer drugs are abused in order to experience psychostimulant effects similar the legally banned substances of abuse. Numerous people around the world are currently abusing the designer drugs. Substantial health impairments have been observed globally due to the abuse of designer drugs. These products are presently produced by clandestine labs in the U.S and other countries around the world. Designer drugs are currently sold by independent dealers in different formulations (powdered form, in single-component tablets, capsules, or in combination combined with MDMA or other illicit controlled substances through the internet and retail stores. Due to the structural and chemical characteristic features (lipophilicity), these designer drugs readily cross the blood-brain barrier and also be readily distributed throughout the body. Hence, it exerts an effect throughout the body on different organ systems. The biogenic monoaminergic neuronal tract and peripheral sympathetic nervous system are extensively affected by the designer drugs abuse which can lead to behavioral changes (memory deficit, mental disorders and movement impairment) and further increase the risk for neurological disorders such as Parkinson's disease. Hence, if this epidemic is not controlled appropriately, it can cause a huge economic impact and decline in the health of the current and future generation. TFMBzPP derivatives dose-dependently induced dopaminergic neurotoxicity as seen by the morphological characterization and the cell viability. Our results were further validated with the use of positive control, hydrogen peroxide. With regard to the neurotoxic mechanisms of action of TFMBzPP derivatives, they induced oxidative stress but had no effect on the mitochondrial functions.

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