Investigating the toxic effects of nyaope on the brain, liver, and kidney of Sprague-Dawley rats

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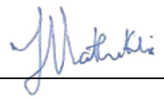
DECLARATION

I Khethani Thendo Mathikhi declare that this dissertation is my own, unaided work, which is being submitted for the degree of Master of Science in Medicine (Physiology) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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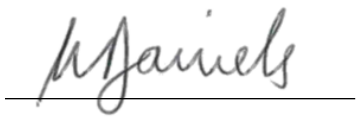

Prof. WMU Daniels

Dr. A Manilall

DEDICATION

I dedicate this thesis to the omnipotent, omniscient and omnipresent almighty God, who has afforded me the opportunity to learn, grow and live out my curiosity and talents. May this thesis glorify Him not me.

I also dedicate it to my grandparents Margret Minyuku, Elias Minyuku, Maria Thambani, Small Thambani and my late beloved great-grandmothers Vho-Muthumuni Raselabe and Vho-Masakona Mathikhi. When I think of you, Thomas Sankara's words come to mind "We must dare to invent the future" and "*Maṅa a mutukuna asi vhumatshelo hawe* – The cracked feet of a boy are not his future".

PRESENTATIONS ARISING FROM THIS STUDY

PowerPoint presentation WITS Postgraduate Symposium, September 2023, 2nd Place.

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Investigating the acute toxic effects of nyaope on the liver, kidney and brain of Sprague-Dawley rats

ABSTRACT

Background. Worldwide drug use is estimated to be 345 million, encompassing both synthetic and plant-based medications, as well as both legal and illicit opioids. The use of these drugs is on the rise and is becoming a growing concern for public health. Moreover, the emergence of heroin dominant street drugs like nyaope have gained popularity, especially in low- and middle-income countries such as South Africa. The use of nyaope exposes individuals to the risk of substance use disorders (SUDs) and the array of mental health issues, as evidenced by social and clinical studies. Nyaope, a relatively inexpensive illicit drug, is commonly found in townships and outskirts of inner cities which are predominantly saturated with African/Black ethnic communities. Its composition varies depending on the geographical area but primarily consists of ingredients like heroin, opioid derivatives, rat poison, antiretroviral drugs (ARVs), and other substances. Similar to other opioids, heroin exerts its effects on the central nervous system (CNS) by acting on opioid receptors in various brain regions, including the prefrontal cortex (PFC), nucleus accumbens, and amygdala. These regions play a critical role in regulating mood and cognitive functions. Additionally, opioids undergo processing in the liver before being excreted by the kidneys. Our work sought to evaluate the effects of acute toxicity from the unique street drug cocktail known as nyaope on the behaviour and molecular markers of the PFC, liver, and kidney in Sprague-Dawley rats, despite the abundance of material already available on opioid usage.

Methods. Twenty-five Sprague-Dawley rats were sourced from the Wits Research Animal Facility following ethical clearance and habituated for a duration of ten weeks. A pilot study involving three of these rats was conducted to determine the appropriate exposure dose of nyaope. Following the pilot study, the remaining twenty-two rats were divided into two groups; nyaope-treated (n=11) and saline-treated (n=11). Nyaope-treated rats received a single dose of nyaope at 0.4 mg/kg/bw and were subsequently exposed to the Open Field Test (OFT), which assesses various behavioural indices, including locomotor activity, mood, and exploratory behaviour, using the AnyMaze video tracking system. The saline-treated rats received a single dose of physiological saline at 0.4 mg/kg/bw and underwent the same 30-minute exposure to the OFT. After this exposure, the animals were placed in their respective home cages and qualitatively observed for an additional 30 minutes. Following this observation period, the rats were anaesthetized with isoflurane and euthanized exactly one hour after exposure to nyaope or saline. Tissues from the brain, liver, and kidney were collected, and RT-PCR was conducted to assess toxicity markers, including genes that code for proteins involved in the processes of apoptosis (*BAX* and *Bcl-2*), autophagy (*SQSTM1*/*p62*), microglial repair (*ANXA3*), and

inflammation (*IL-6*). In addition, plasma samples were collected and analysed using IDEXX catalyst technology to examine the plasma presence of liver toxicity markers; aspartate transferase and alanine transferase, along with the kidney toxicity marker; creatinine.

Results. The qualitative findings indicated that rats treated with nyaope exhibited reduced grooming behaviour. Additionally, the nyaope-treated rats experienced a phase of heightened activity followed by extreme lethargy. In contrast, the saline-treated rats displayed consistent mobility and curious behaviour. Compared to the saline-treated rats, the nyaope-treated rats exhibited clinical signs such as tremors, a rigid tail, hypoxia, and increased diuretic behaviour. When observing the track plots of the nyaope-treated rats, they tended to favour the outer zone in a thigmotaxis pattern, with few bouts into the centre, while the saline-treated rats showed more uniform movement within the OFT apparatus.

Quantitative behavioural data using the AnyMaze tracking system revealed that nyaope-treated rats had decreased locomotor activity. They covered less total distance during the test and travelled shorter distances within the centre zone compared to saline-treated rats. Nyaope-treated rats also had fewer mobility episodes and moved at slower speeds on average than the saline-treated rats. In terms of mood assessment, the nyaope-treated rats spent less time mobile overall, both in the outer and centre zones and engaged in significantly fewer grooming bouts compared to the saline-treated rats. In the assessment of exploratory behaviour, it was noted that nyaope-treated rats exhibited fewer instances of rearing, line crossing, and head entries into the centre than the saline-treated rats.

Regarding the molecular assessment of the brain and kidney, there were no significant differences in the expression of molecular markers between the two groups, except for a decreased expression of *Bcl-2* ($p < 0.001$) in the kidneys of nyaope-treated rats compared with the saline-treated rats. Additionally, plasma expression levels of AST, ALT, and creatinine were similar between the two groups.

Conclusion. These findings indicate that exposure to 0.4 mg/kg/bw of nyaope for one hour does result in behavioural changes, even though it does not immediately lead to acute molecular toxicity in the brain, liver and kidney. Conversely, nyaope exposure causes a reduction in mRNA expression of *Bcl-2*, suggesting that the drug induces cell and tissue damage in the kidney through apoptosis.

STATEMENT OF CONTRIBITON

Professor WMU Daniels played a vital role in shaping the study's concept and overseeing its execution, which is a component of a larger research project within the group. The provision of research materials was a collaborative effort involving Professor WMU, Dr A Manilall, and Dr M. Gomes. I assumed responsibility for the animal handling and the collection of behavioural data. Additionally, with the assistance of Dr A Manilall, I conducted and gathered molecular data. I conducted data analysis with guidance from Dr A Manilall and Prof WMU Daniels. The thesis was compiled with the guidance and review of Prof WMU Daniels and Dr A Manilall.

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To my fellow colleagues, comrades at SASCO and postgraduates who rode the rollercoaster of being a master's student with me, I thank you. And to "amajita"; Tshatsinde "TT" Muluvhahothe, Mpumelelo Ntuli, SRC President Kamogelo Mabe, Paballo "PB" Mothapo and Thembinkosi "Lorch" Twala, David Maatsie and Samukelisiwe Msimango, you've been a shining light and breath of fresh air especially when going got tough, I truly appreciate you guys. I further extend my thanks to the Chairman of Hurricane FC, Coach "Gunman", Coach MK, Coach Nkosi, and the players at both Hurricanes FC and Wits Junction FC for the opportunity and positive impact you've had on my Wits experience, and I carry the lessons learned from the football field with me to the world.

It is with great gratitude that I express my thanks to my family with special thanks to the Khotsimunene's (Clement Mathikhi and Jonathan Minyuku), Joyce Sadiki, Bridget Tambani and Malume Mushaathoni along with their families and the Ndidzulafhi family for the support afforded when I need a place to lay my head and receive inspiration to keep going. I reserve my heartfelt gratitude to my parents, Stanley Mathikhi-Mudimeli and Dikeledi Mathikhi, for embarking me on this journey, as the Japanese proverb says, "*Kawaii ko niwa tabi o saseyo*— Send the beloved child on a journey." My profound thanks also extend to my incredible three siblings for their enduring love, kindness, and patience, especially during the late-night calls of encouragement. Your unwavering support is a testament to a timeless familial bond.

To my beloved partner, Kopano, motho waka, I owe immeasurable thanks for your grace, commitment, and unwavering support throughout this endeavor. You have been my steadfast pillar, embodying our shared motto, "*Vhutshiloni ri tshilela u takadzana*—in life, we live to bring joy to each other." Thank you, *Makgomo wena* Sunflower *yaka*, for being precisely what I needed at every moment.

Collectively, I am reminded that it truly takes a village to raise a child, echoing the Latin phrase, "Nil satis nisi optimum—Nothing but the best is good enough.

I would like to thank the National Research Foundation, the Wits Hardship Fund, the Health and Welfare Sector Education and Training Authority, the Faculty of Health Science FRC grant and the Wits SRC100 Fund for the financial assistance received throughout this degree.

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LIST OF ABBREVIATIONS AND SYMBOLS

6-Monoacetylmorphine	6-MAM
Alanine Transferase	ALT
Animal Research Ethics Certificate	AREC
Annexin A3	*ANXA3*
Apoptosis-Inducing Factor	AIF
Aspartate Transaminase	AST
Association of Southeast Asian Nations -Narcotics Cooperation Centre	ASEAN-Narco
Autophagy Related 3/4/7 Enzyme	Atg3/Atg4/Atg7
BH3 Interacting-domain Death agonist	BID
B-cell lymphoma 2 Family protein/gene	*BAX*/BAK
B-cell lymphoma 2 Family protein/gene	*Bcl-2*
Birthweight	Bw
Blood Brain Barrier	BBB
Carboxylesterases	CEs
Central Nervous System	CNS
Complementary Deoxyribonucleic Acid	cDNA
Cyclic Adenosine Monophosphate	cAMP
Cycle Threshold	CT
c-Jun N-Terminal Kinases	JNK/c-Jun
Coronavirus Disease 2019	COVID-19
Degrees Celsius	°C
Deoxyribonucleic Acid	DNA
Delta-opioid Receptor	DOR
Delta-9-tetrahydrocannabinol	THC
Diagnostic and Statistical Manual of Mental disorders	DSM-5
Disability-adjusted Life Years	DALYs
Department of Justice and Constitutional Development	DoJ & cd
Electrocardiogram	ECG
Elevated Plus Maze	EPM
European Monitoring Centre for Drugs and Drug Addiction	EMCDDA
Gas Chromatography Mass Spectrometry	GC-MS

Glyceraldehyde 3-phosphate Dehydrogenase	GAPDH
Glucuronosyltransferase 2B7	UGTB27
Glutathione	GSH
Global Harm Reduction International	GSHR
G-protein-coupled receptor	GPCR
Human Carboxylesterase 1	hCE1
Human Carboxylesterase 2	hCE2
Human Immunodeficiency Virus	HIV
Hydroxymethylbilane Synthase	HMBS
Interferon	IFN
Interleukin	IL
International Narcotics Control Board	INCB
Intraperitoneal injection	IP
Interquartile Range	IQR
Kappa-opioid Receptor	KOR
Kilogram/s	KG
Light Chain 3	*LC-3*
Locus Coeruleus	LC
Meters	m
Meters/second	m/s
Messenger Ribonucleic Acid	mRNA
Methylenedioxy-methamphetamine	MDMA
Microgram/litre	µg/L
Microlitre	µl
Microtubule-Associated Protein	1A/1B
Milligram/decilitre	mg/dl
Milligrams	mg
Mitogen-Activated Protein Kinase	MAP-K
Morphine-3-glucuronide	M3G
Morphine-6-glucuronide	M6G
Mu-opioid Receptor	MOR
Number	n
National Department of Health	NDoH
National Institute on Drug Abuse	NIDA

Neighbour of BRCA1 Gene-1	NBR1
Not detected	n/d
Nucleus Accumbens	NAcc
Open Field Test	OFT
Organisation for Economic Co-operation and Development	OCED
Organisation of American States- Inter-American Drug Abuse Control Commission	OAS-CICAD
Prefrontal Cortex	PFC
Reactive Oxygen Species	ROS
Real Time-Polymerase Chain Reaction	RT-PCR
Ribonucleic Acid	RNA
Seconds	(s)
Standard Error of the Mean	SEM
Sequestosome 1	*p62*/*SQSTM1*
Sigma-opioid Receptor	SOR
South African Community Epidemiology Network on Drug	SACENDU
South African Medical Research Council	SAMRC
South African Rand	ZAR
Sprague-Dawley rats	SD
Substance Use Disorder	SUD
TATA-Binding Protein	*TBP*
Toll-like Receptors	TLR
Transforming Growth Factors	TGF
Tumour Necrosis Factor-Alpha	TNF-α
Tumour Necrosis Factors	TNF
Tumour Protein p53	p53
Two Delta Delta Cycle Threshold	$2^{-\Delta\Delta CT}$
United Nations Office on Drugs and Crime	UNODC
United State of America	USA
Ventral Tegmental Area	VTA
Wits Research Animal Facility	WRAF

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1. CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Impulsive behaviours are usually the cause of drug initiation for most first-time users. This can mature into compulsive drug-seeking behaviour, which can lead into poor health outcomes. Increases in frequency and quantity of drug consumption usually leads to substance abuse, which pathophysiologically facilitates progression to substance use disorder (SUD) (Kieffer & Evans, 2002). Evidence shows that alcohol and tobacco are common gateway drugs that precede the intake of more potent harmful substances (Carpenter *et al*., 2017; Kandel *et al*., 1992; Kirby & Barry, 2012; Moss *et al*., 2014). However, occasional, and single use also remain a growing challenge.

The use of drugs on a global scale has reached pandemic proportions, much like COVID-19. It is now a major public health concern that affects people from all cultures, ages, genders, and socio-economic backgrounds. According to the United Nations Office on Drugs and Crime (UNODC) in 2022, the estimated number of drug users worldwide has exceeded 345 million, representing a 34.5% increase since 2010 (ONUDC, 2021; UNODC, 2020b, 2022b; World Drug Report, 2021; World Drug report, 2021). This includes the use of synthetic amphetamines, methylenedioxy-methamphetamine (MDMA), stimulants, and plant- based drugs such as cocaine, cannabis, and opioids.

While tobacco and alcohol are more widely used (1 in 7 daily, and 1 in 5 heavy episodes, respectively), prescription and recreational or illicit drugs are also contributing to this epidemic (Peacock *et al*., 2018). Studies also show that 1 in 20 adults globally use *Cannabis* (206 million adults) making it the most popular illicit drug, followed by opioids (61 million) with the highest levels recorded in high-income countries (Degenhardt *et al*., 2008; Degenhardt & Hall, 2012). Additionally, opioid use has led to an estimated 12 million disability-adjusted life years (DALYs) lost (Harker *et al*., 2020).

However, unlike in the 1990s, drug use is skewed towards the use of illicit drugs with the introduction of cheaper and more accessible drugs. Major increases in plant-based drugs and shipping routes expanding to low- and middle-income countries have been touted for the steady rise in drug use globally (UNODC, 2022b). Furthermore, UNODC reports of 2022 also predict that population growth in these countries will be associated with high risks of drug use and SUDs, especially in Africa.

According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), SUD is classified into substance dependence and substance abuse and these

conditions may vary in clinical presentation, psychological problems and social consequences (American Psychiatric Association, 2013; Armstrong & Jane Costello, 2002; Jones *et al*., 2012). Factors such as urbanisation, increased income, and a large youthful population, are some of the driving forces steering to potentially more prevalent drug intake (INCB, 2020; Peltzer & Ramlagan, 2010; UNODC, 2020a, 2021, 2022a). Furthermore, social, peer affiliations and individual and family risk factors seem to predict early drug use trends in developing countries (W. Hall & Degenhardt, 2007).

The expansion of the distribution networks of illicit drugs in low- to middle-income countries presents a huge public health concern that is particularly worrisome in the light of a health sector that is already struggling with communicable and non-communicable diseases (Degenhardt *et al*., 2013, 2017; Degenhardt & Hall, 2012; Peacock *et al*., 2018; UNODC, 2021; World Drug report, 2021). Not only does the illicit drug industry strain the healthcare system, but it also causes a massive economic burden to affected communities. For instance, the economic liability brought on by opioid use disorder in the United States of America (USA) was estimated to be $72 billion cumulatively over a 15-year period (1998-2013) (Leslie *et al*., 2019). In 2015 heroin use alone was valued to cost the USA $51.2 billion in healthcare costs, while in Taiwan this value was estimated to be $11 791 per person per year (Jiang *et al*., 2017; S. H. Lin *et al*., 2013).

Illicit drug consumption is often associated with low income, incarceration and unemployment (Carpenter *et al*., 2017; S. H. Lin *et al*., 2013), which adds unnecessary detriment to societies. Other severe adverse events that are often associated with drug use include unintentional accidents, drunk driving, blood-borne infections and aggressive behaviour ((Degenhardt & Hall, 2012; Mack *et al*., 2017). For example, there is a positive correlation between illicit drugs and injury, with emergency rooms saturated with victims or perpetrators of violence caused by the drug user (Leslie *et al*., 2019; Lewer *et al*., 2020; Vitale & Mheen, 2006). Similarly, the escalation in unsafe injectable drug use renders users susceptible to viral infections, raising concerns regarding hepatitis B (Degenhardt *et al*., 2008, 2017) and HIV infections (Dore *et al*., 2002). Lastly, harmful drug use often co-presents with other mental disorders. A population study indicated the presence of comorbid substance use disorder with psychopathological conditions such as bipolar mood disorder, schizophrenia, personality disorder, and neurotic disorder (Degenhardt *et al*., 2013; Plana-Ripoll *et al*., 2020).

The significance of monitoring, effective policy development, and harm reduction interventions cannot be overstated due to the widespread use of drugs worldwide and its resulting consequences. While numerous studies and global surveys exist reporting on matters pertaining

to drug use and its associated detrimental consequences, it should be noted that these reports focus mainly on the impact of purified chemicals e.g., heroin, cocaine and amphetamine (ASEAN-Narco, 2021; EMCDDA, 2022; Leslie *et al*., 2019; OAS-CICAD, 2019; OCED, 2020). The growth in the consumption of cocktail, hybrid or street drugs, particularly in low- and middle-income countries, now requires additional investigations into the effects of adulterated drugs. The present study we aim to build upon our previous research on nyaope cocktail drug use to enhance the current understanding of the effects of consuming cocktail drugs in South Africa.

1.2. Overview of the African drug challenge

Africa has the fastest-growing population in the world, with more than 41% of its population being 15 years or younger. It is plausible that drug markets may be shifting to target the young and vulnerable into early initiation. The World Drug Report 2020 anticipates that health systems and law enforcement in Africa will be overburdened with an additional 14 million substance users (UNODC, 2021).

In North Africa and the Middle Eastern region, heroin and cannabis are the primary substances of abuse (Degenhardt *et al*., 2017; Global State of Harm Reduction, 2016). Remarkably, polydrug use is believed to be prevalent in Morocco and Egypt as in Western countries (Bobashev *et al*., 2018; Himmich & Madani, 2016), while the consumption of heroin and cannabis mixtures have been reported for Tunisia and Morocco (Global State of Harm Reduction, 2018). More than half a million users preferred injectable heroin use, which was associated with a significant proportion of hepatitis C infections and sexually transmitted diseases in the region (Ameri, 1999; Neale *et al*., 2008; Toufiq *et al*., 2014). These findings mirror occurrences in East African countries such as Kenya and Tanzania (Balaji *et al*., 2017; Beckerleg, 2009; Beckerleg *et al*., 2005, 2009; S. A. McCurdy *et al*., 2005; S. McCurdy & Kaduri, 2016; Mital *et al*., 2016; Tiberio *et al*., 2018).

East Africa is a major route for heroin transport and trade links associated with major drug raids in recent times (UNODC, 2020a). In the harbour city of Mombasa, Kenya, a study revealed that a distribution network had been developed to manoeuvre a low-grade heroin cocktail mixture named “white crest” that is usually consumed with tobacco or cannabis by sniffing or inhalation (Beckerleg *et al*., 2009). This study provided concrete evidence to support the notion of adulterated heroin and polydrug use being aggressively introduced into the sub-Saharan parts of the continent. Unsurprisingly, alarming rates of heroin use have also been reported in Rwanda (Riedel *et al*., 2021; Rwema *et al*., 2021), where a cross-sectional study in the capital

of Kigali showed that partner initiation, especially among women, is quite common along with communicable infections and drug-related overdose are highly prevalent. Comparable observations were recorded in Ethiopia and in Central African countries, such as Uganda (Baluku *et al*., 2019; Demissie *et al*., 2018; Dickson-Gomez *et al*., 2020). In Ghana (Kumasi), injectable heroin is the most commonly used substance of abuse, with users averaging ten injections per day (Messersmith *et al*., 2015). Heroin enters Ghana through the East African trafficking route, and both heroin and cannabis are prevalent drugs of abuse in this country (Ofori-Atta & Ohene, 2015). Social factors are significant contributors to drug use in West Africa, and drug use trends and outcomes are like those observed in East and Central Africa. For instance, there is a notable presence of tuberculosis in West Africa, suggesting a preference for smoking drugs in addition to injectable intake (Bouscaillou *et al*., 2016; Salifou *et al*., 2022).

1.3. The drug challenge in South Africa

South Africa's susceptibility to illegal narcotics has been exacerbated by its vulnerable economic and social systems, ineffective justice system, and well-established connections to European and Asian drug markets. The country's porous borders, poor policies and high unemployment rate are some of the risk factors creating a conducive environment for increasing drug use (Mokwena, 2019; Peltzer & Phaswana-Mafuya, 2018; Ramlagan *et al*., 2010). Secondary school learners at rural schools' report ease of access to drugs although prevalence in those areas was relatively low in comparison to township schools (Khoza & Shilubane, 2021; Makondo *et al*., 2017; Mohashoa & Mokoena, 2017; Moodley *et al*., 2012; Onya & Flisher, 2008; Tshitangano & Tosin, 2016). In these areas, early drug initiation is very common with the minimum age found in rehabilitation centres to be as young as nine years old (Ramlagan *et al*., 2010). The South African Community Epidemiology Network on Drug Use research unit, supported by the South African Medical Research Council, provides a comprehensive insight into the trends in drug use within the country, drawing on information obtained from treatment centres (Hornsby *et al*., 2023). The 2021 report highlights that family referrals were predominant, and the majority of users were black across all provinces except the Western Cape, which had a high number of male users of mixed ancestry (Hornsby *et al*., 2023). In line with other epidemiological studies, the report showed that a considerable proportion of drug users were between the ages of 15 and 34, except in the Western Cape, where more older drug users were identified (Hornsby *et al*., 2023; Nyabadza & Coetzee, 2017; Peltzer *et al*., 2010; Peltzer & Ramlagan, 2010; Ramlagan *et al*., 2021).

In the economically wealthy provinces with higher average income such as the Western Cape, Gauteng, and KwaZulu-Natal, there was a higher proportion of employed drug users, while

other provinces reported more secondary school attendees and unemployed individuals with drug use problems. Cannabis, methamphetamine (commonly known as "tik"), alcohol, and heroin (including nyaope or whoonga) were the most frequently used drugs across all provinces (Peltzer & Phaswana-Mafuya, 2018). The subsequent decriminalisation of cannabis has led to an increase in users resulting in more than 50% of users in all provinces declaring multiple drug consumption (Mokwena, 2019). Infection and transmission of disease were high due to unsafe drug injection practices and risky sexual behaviours, these findings are like those observed elsewhere on the continent (Scheibe *et al*., 2019). A study by Parry *et al*. (2007) revealed notable changes in the racial demographics of drug users, specifically with reference to crack cocaine and polydrug use involving cocaine, alcohol, and cannabis (Parry *et al*., 2007).

1.4. Nyaope use in South Africa

Nyaope is an adulterated heroin mixture, which is very potent and prevalent in the northern provinces of South Africa, especially in socio-economically vulnerable communities (Fernandes & Mokwena, 2021; Harker *et al*., 2020; Nyanda, 2019). Its popularity is likely due to its affordability and ease of access, with one joint costing about ZAR 20-30 (Ghosh, 2013). The constituents of nyaope vary with geographical areas. However, the common denominator is the prevailing presence of heroin (Conway-Smith, 2013; Khine *et al*., 2016; Khine & Mokwena, 2017; Mokwena, 2016). Studies have found significant amounts of morphine, morphine derivatives, codeine, caffeine, efavirenz and methyl-amphetamine (Fernandes & Mokwena, 2021; Grelotti *et al*., 2014; Khine & Mokwena, 2017; Khine A & Mokwena KE, 2018, 2019) in nyaope samples. A gas chromatography-mass spectrometry (GC-MS) quantification reported the presence of trace amounts of rat poison and cannabis active components (Khine *et al*., 2016; Mthembi *et al*., 2018, 2019). The constituents that appear in lesser quantities are used as bulking agents. However, there is a concern as the cumulative toxic effects of these compounds remain unknown. Although nyaope is commonly smoked, it is stated that intravenous intake, including blood transfusion, is also prevalent (Baloyi, 2018; Sparks, 2018). Consequently, the latter methods increase the risk of blood-borne diseases, endocarditis, and autoimmune diseases. Additionally, the use of nyaope is linked to unsafe injection methods, which in turn are associated with HIV infections. These infections can lead to immunosuppression in users, exacerbating poor health outcomes and placing a burden on public health systems (Meel & Essop, 2018).

Our laboratory recently undertook a cross-sectional study where more than 300 nyaope consumers were recruited within the Johannesburg region (Morgan *et al*., 2019). This study characterised the participants as young (15-35 years), unemployed, and homeless individuals

with poor family and societal support. The drug users were also associated with poor hygiene, fatigue and low self-esteem. Furthermore, a follow-up study (Morgan *et al*., 2019) showed that nyaope users were unable to receive adequate therapy due to the high expenses and scarcity of specialised services, a view shared by other researchers (Mokwena & Morojele, 2014). This is particularly problematic as consumers of the nyaope commonly present with other psychiatric comorbidities including major depressive disorder, generalised anxiety disorder, posttraumatic stress disorder and antisocial personality disorder (Conway-Smith, 2013; Mashaba, 2020; Morgan *et al*., 2019, 2022; Mueser *et al*., 2012). A magnetic resonance imaging study by Ndlovu *et al*. (2021) reported nyaope-induced atrophy in several brain structures including the prefrontal cortex (Ndlovu *et al*., 2021). These observations clearly point to the significant toxic effects nyaope may have on brain structure and function. However, studies focusing on nyaope are limited, and additional investigations are required to unequivocally demonstrate the deleterious impact of nyaope on the central nervous system, as well as other physiological systems in the body.

1.5. The effects of heroin on the central nervous system (CNS)

Since heroin is the major constituent of nyaope, the following section focuses on the effects of this substance on the brain. Heroin is an opioid that produces euphoria and impulsive behaviour. At higher doses, the drug also affects autonomic functions such as sleep, heart rate, and breathing rate (Gutowicz *et al*., 2011; NIDA, 2021). In clinical settings, opioids such as morphine, which is derived from heroin, are prescribed for analgesic, cough suppression and diarrhoea treatment purposes (Foley, 1993; Mercadante, 2015). *In vivo* heroin undergoes rapid metabolism, leading to the formation of 6-monoacetylmorphine and morphine through deacetylation, a process catalysed by pseudocholinesterase or carboxylesterases (Boerner *et al*., 1975; Zheng *et al*., 2013). Morphine is highly lipophilic and exhibits pharmacological effects that can significantly impact the central nervous system with increased potency (Boix *et al*., 2013). Both morphine and 6-monoacetylmorphine possess high permeability across the blood-brain barrier (BBB), enabling effective delivery to the central nervous system compared with heroin (Gottås *et al*., 2013).

Heroin and its metabolites bind to opioid receptors to elicit their effects within the body. Opioid receptors are members of the G-protein-coupled receptor (GPCR) family, mediating neurotransmitter and hormone physiology upon activation (Snyder & Pasternak, 2003; Waldhoer *et al*., 2004). Activation of the endogenous opioid system is achieved mainly via four opioid receptors, namely, the mu (MOR), kappa (KOR), delta (DOR), and nociception (NOR) receptors (McDonald & Lambert, 2005, 2013). They are predominantly located in the central

nervous system and partially in peripheral systems, such as the immune system and sensory nerves (Stein *et al*., 2009). Receptors are structurally 60% identical to each other but the most structurally diverse regions are in the extracellular N-terminal domain and intracellular C-terminal tail loops. Kappa receptors are available at high density in the brain and similar to MOR-receptors, they mediate analgesic, constipation, euphoria, nausea and vomiting effects at certain doses (Pasternak, 1993). Heroin and morphine bind with high affinity to the μ-opioid receptor and to a lesser extent to KOR- opioid receptors, thereby inducing analgesic and rewarding effects in the mesocorticolimbic system (Waldhoer *et al*., 2004; Walker & Sterious, 2005; Zheng *et al*., 2013).

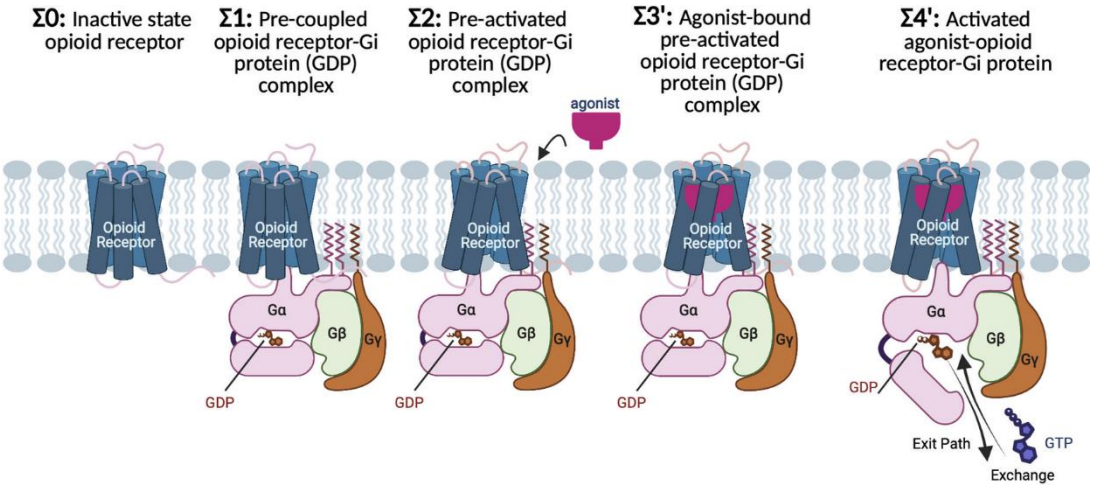

Figure 1.1: A schematic showing G protein-mediated activation of opioid receptors and their corresponding Gi protein follows a stepwise mechanism. Σ0: In the absence of ligand and Gi protein, the opioid receptors remain inactive, characterised by a strong hydrogen bond between the cytosolic ends of TM3 and TM6, which keeps the cytoplasmic region tightly sealed. Σ1: Prior to agonist binding, the inactive Gi protein, bound to GDP, interacts tightly with the inactive opioid receptor, forming a pre-coupled complex known as the opioid receptor-Gi (GDP) complex. Σ2: The interaction between the inactive opioid receptor and the inactive Gi (GDP) causes the disruption of the TM3-TM6 hydrogen bond, leading to the opening of the cytoplasmic region, enabling the accommodation of the Gi protein. This results in the emergence of the pre-activated state (Σ2), which remains in a resting state until an agonist binds to the receptor. Σ30: When an agonist binds to the pre-activated state, the Gi (GDP) protein undergoes activation. This activation is characterised by a significant opening in the cleft between the AH and Ras-like domains of Gα, creating a pathway for the release or exchange of GDP with GTP. Σ40: Upon GDP release or exchange, the agonist-opioid receptor-Gi protein complex progresses to its fully active state (Mafi *et al*., 2021).

Opioid receptors are found in many areas within the brain including those that process stressful situations such as the prefrontal cortex, amygdala, bed nucleus of stria terminalis and paraventricular nucleus (S. Lin *et al*., 2006; Y. J. Wang *et al*., 2011). Stress exposure correlates

with increases in endogenous dynorphin, which then indicates the association of opioids effects to changes in emotionality or mood (Hang *et al*., 2015; Holden *et al*., 2005; Ramos, 2008). Therefore, it seems that the effects of opioids on behaviour and emotionality may be mediated by brain circuits involving brain areas involved in stress. Furthermore, heroin use is associated with neuroadaptations, toxicity, development of tolerance and dependence (R. Brown, 2004; Cunha-Oliveira *et al*., 2007; Fu *et al*., 2008; Oliveira *et al*., 2003; Salmon *et al*., 2001; Tian *et al*., 2017).

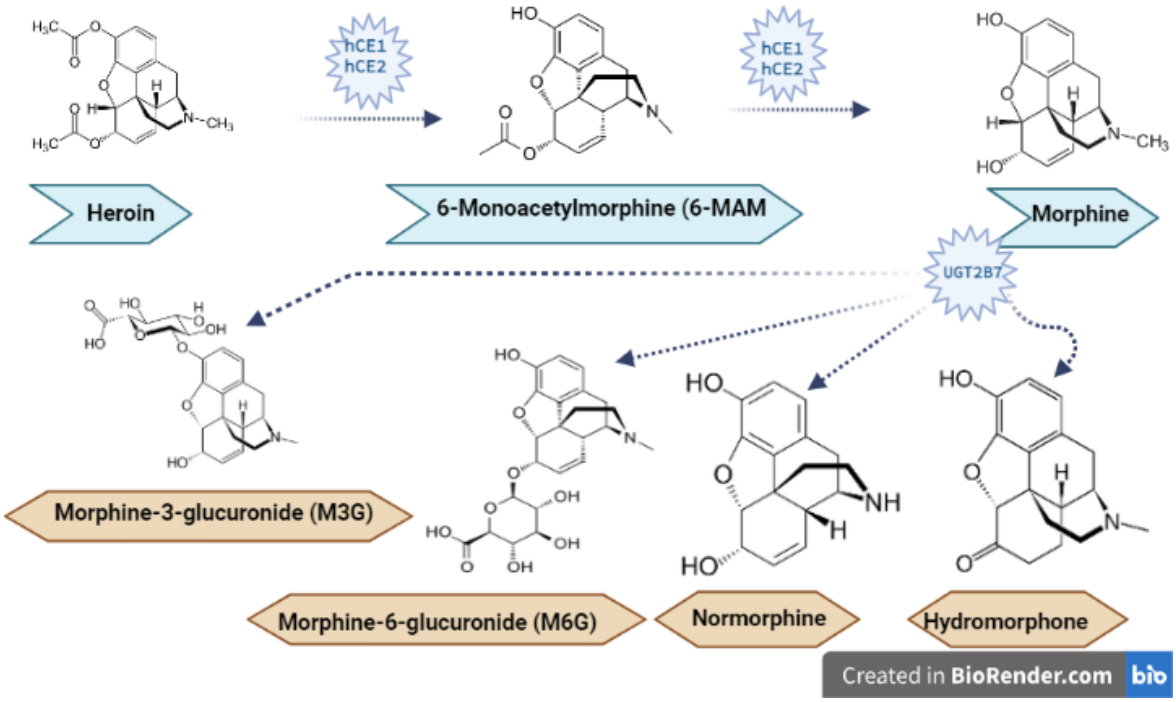

Figure 1.2: A schematic showing how lipophilic opioids compounds undergo metabolism, rendering them hydrophobic for excretion. Like other opioids, heroin is predominantly metabolised in the liver by human carboxylesterases (hCE1 & hCE2). These enzymes first perform deacetylation of heroin into 6-monoacytl-morphine, followed by another deacetylation step that forms morphine during phase 1 metabolism. In phase 2 metabolism, morphine undergoes glucuronidation with the assistance of glucuronosyltransferase 2B7 (UGTB27). This process produces metabolites such as normorphine, hydromorphone, morphine-3-glucuronide, and morphine-6-glucuronide. Among these derivatives, including 6-MAM, the latter three compounds are more active and exert opioid effects on the body's endogenous opioid system. **(Created with Biorender.com)**.

1.5.1. The Nucleus Accumbens (NAcc)

The nucleus accumbens (NAcc) is an essential brain structure in the reward system and is intimately involved in mediating the effects of psychoactive drugs (Salgado & Kaplitt, 2015). This area is known to play a critical role in circuits that modulate and regulate information processing, emotion, integration, cognition and motivated behaviours (Floresco, 2015). The

structure of the NAcc is divided into a central core which is surrounded by the ventral-lateral outer shell (Mavridis *et al*., 2011; Zahm, 1999; Zahm & Brog, 1992). These subdivisions of the NAcc differ in terms of their cellular, physiological, connection and histochemical properties (Salgado & Kaplitt, 2015). The patch organisation of the NAcc is characterised by dense μ-opioid receptors whilst the matrix organisation has weaker opioid binding sites (Hakim *et al*., 2020; Wright *et al*., 1996). These organisations receive different afferents from the cortex and midbrain. Furthermore, the core consists of low-density neurons, mostly pyramid-like, while the outer shell is mainly multipolar neurons with rich spines of secondary and tertiary cells (Russo *et al*., 2010; Sazdanovic *et al*., 2016). The two areas also vary at a molecular level with significant differences reported in the levels of neuroactive substances, receptors, dopamine and serotonin, with higher concentrations found in the shell region (Salgado & Kaplitt, 2015). Considering its similarities to other striatal tissue and intense connectivity, the NAcc is considered a transitional zone within brain circuits including connections to the extended amygdala. Evidence suggests that the NAcc receives direct and indirect input from the ventral tegmental area (VTA) to its shell, while projections from the substantia nigra innervate the core as part of the mesolimbic dopaminergic pathway (Qi *et al*., 2016; Yang *et al*., 2018). The core also receives direct glutamatergic projections from the amygdala, hippocampus, thalamus and the prefrontal cortex (PFC) (Salgado & Kaplitt, 2015).

The NAcc gives direct and indirect projections into brain circuits, which feed the autonomic and locomotor centres in the brain (Deutch *et al*., 2019). Dated studies indicate that the core connectivity is associated with premotor and supplementary motor areas of the cortex while the shell connects to the prefrontal cortical areas and subcortical motor areas (Alexander *et al*., 2002; Zahm & Brog, 1992). Furthermore, the shell sends projections to ventromedial pallidum, premotor and executive function areas in the prefrontal cortex, which send feedback to the NAcc core (Corbit *et al*., 2001). Therefore, the NAcc seems to have functional links to the limbic and motor systems driving locomotor activity, and functional and survival systems (Katsuura & Taha, 2010). Studies further indicate that the NAcc modulates goal-oriented behaviour, risk-taking or avoidance, impulsivity and sexual motivation in animals and humans (Basar *et al*., 2010; Carelli, 2002; Goto & Grace, 2005; Kuhnen & Knutson, 2005; McCullough *et al*., 1993; Wadenberg *et al*., 1990; Yun *et al*., 2004).

The NAcc is key to the natural reward system along with the emotional reinforcement of cues mediated by the limbic system and in particular the amygdala (Cardinal *et al*., 2002; Salamone *et al*., 2005). It has been shown that the prefrontal cortex regulates amygdala dopamine outputs to the NAcc, and this circuit is believed to play an important role in NAcc-mediated behaviours

(M. E. Jackson & Moghaddam, 2001). KOR- and DOR-opioid receptor signalling underpin the NAcc-induced modulation of locomotor activity as evidenced by opioid infusions that produced dose-dependent rearing behaviour in rodents (Cunningham & Kelley, 1992; Katsuura & Taha, 2010).

1.5.2. Prefrontal Cortex (PFC) and Amygdala

Cognition is broadly described as the process where information is computed and manipulated with the purpose of eliciting a particular response (Brown & Bowman, 2002; Miller & Cohen, 2001). The prefrontal cortex (PFC) has been identified as one of the pivotal brain areas involved in cognitive function. The PFC serves as a control system that coordinates functions such as task handling, information storage, working memory, attentional control, reasoning and logic, decision-making, and organised planning (Kondo *et al*., 2004; Petrides, 2000; Stuss & Knight, 2013; Tanji & Hoshi, 2001). It is further implicated in the manifestation of social, motivational, and emotional states of the individual (Dixon *et al*., 2017; Inman *et al*., 2020; Schneider *et al*., 1997).

In addition, the PFC is intensely involved in the collection and organisation of input that is further resolved to create an action or motor operation, which is required to pursue and fulfil a certain goal (Carlén, 2017; Dixon *et al*., 2017; Fuster, 2015). To perform this function, the PFC is interconnected to a set of cortical areas with overlapping connectivity patterns that include the sensory, reward and motor systems of the brain (Miller, 2007; Miller & Cohen, 2001). This infrastructure allows the PFC to influence and guide complex behaviours in a top-down bias through its convergence of input and intrinsic connections (Badre & Nee, 2018; Carlén, 2017). Therefore, the PFC performs an analysis of externally and internally collected resources that result in either the acceptance or rejection of a potential action. In this way, the PFC determines the salience of a particular desire and subsequently directs an action to obtain the original objective (Miller, 1999). Abnormalities in PFC functioning can consequently lead to the inappropriate attribution of salience resulting in an undesired outcome. For instance, giving incorrect importance to drug intake may lead to eventual substance use disorder.

The PFC is also actively involved in learning and exploratory behaviours (Daw *et al*., 2006; Mansouri *et al*., 2017). Studies have revealed that five of six premotor areas in the frontal lobe are interconnected with the dorsal PFC including the ventral premotor area. The PFC is further interconnected with the supplementary and rostral cingulate motor regions, areas that are closely linked to reward-based decision-making and learning, and the integration of emotion and cognition (Dum & Strick, 1991; Le Merre *et al*., 2018; Lu *et al*., 1994). Recently, the PFC

has also been considered as a premotor structure (Hayden & Fine, 2021). In this regard, the PFC is said to be involved in the supervision and regulation of motor function through the potentiation or depotentiation of motor cortex areas (Hayden & Fine, 2021). Hence, individuals who suffer damage to the frontal cortex tend to have intellectual, personal and social difficulties. These functions are universal as the behavioural impairment that can be observed in rats following cortical damage is like those reported in primates (G. R. Brown & Nemes, 2008; V. J. Brown & Bowman, 2002; Ragozzino *et al*., 1999).

The modulation and regulation of mood involves the activation of a range of neural circuits and different brain areas, including the amygdala and prefrontal cortex (Kenwood *et al*., 2021). Mood is described as a prolonged representation of various mental states (such as emotions, attention, and goals), sensory inputs (including our environment and interactions with others), physiological factors (including diet, exercise, and hormones), and integrated memories (Clark *et al*., 2018; Inman *et al*., 2020; Kontaris *et al*., 2020). It encompasses the conscious expression of a particular emotional state over a certain period. Therefore, mood is the culmination of influences stemming from behaviour, thoughts, learning, and other cognitive processes (Šimić *et al*., 2021). As such mood is established through the interplay of neural connections between our past experiences and the new experiences, we acquire (Sterling, 2012). For example, fear is an emotion that can be manifested through a previous experience and through associative learning it is linked to anxiety-like behaviours over time. Molecular and structural changes (synaptic plasticity) in the lateral amygdala that are associated with learning and memory, in which impaired function has been proposed as an underlying mechanism responsible for the establishment of anxiety-like behaviour (Johansen *et al*., 2011; Pittig *et al*., 2018).

Preclinical studies involving the investigations of animal responses during the open-field tests have shown a clear connection between conditioned fear stimulation and anxiety-like behaviours' (Ponder *et al*., 2007). These responses were similar in experiments where animals detected a natural danger or the presence of a predator (Roth & Katz, 1979; Tovote *et al*., 2015). Akin to these stress responses, exposure to an array of drugs also stimulated unconditioned fear and initiated anxiety-like behaviours in animal models (Prut & Belzung, 2003; White *et al*., 2016; W. Zhu *et al*., 2016).

Negative emotional states resulting from chronic opioid use have been associated with the amygdala (Koob, 2020). Hence, prefrontal cortex- and amygdala-derived pathways are deterministic of drug-induced behaviours (Morawetz *et al*., 2017). For example, the inhibition of the basolateral amygdala input to the hippocampus caused an increase in exploratory behaviour (Felix-Ortiz *et al*., 2013). A study conducted on opioid-treated rodents demonstrated

the role of kappa opioid receptors in the modulation of afferent signals from the basolateral amygdala that was associated w

ith the manifestation of anxiety-like behaviours, as was, with the reinforcement of negative experiences (Tejeda *et al*., 2015). Therefore, it would be plausible that toxic damage in these areas could have an impact on the expression of certain behaviours.

1.6. Molecular mechanisms of opioid toxicity in the brain

One of the consequences of metabolism is the generation of reactive oxygen species (ROS) or free radicals. Free radicals contain at least one or more unpaired electrons and include oxygen radicals and other non-radical oxidising agents (Bayir, 2005). In addition to cellular respiration, these partially reduced forms of oxygen are products of xenobiotics, exogenous drugs and environmental conditions. The intracellular physiological imbalance of free radicals leads to oxidative stress and consequent damage of essential biomolecules including proteins, carbohydrates, lipids and deoxyribonucleic acid (DNA) (Helmut *et al*., 2017). Exogenous drugs can elicit cell toxicity and substantially elevate free radicals with dose-dependent, time-dependent and species-specific effects resulting in compromised homeostasis, cellular malfunction and even cell death (Niles *et al*., 2008). Elevated ROS was observed to be an effector in mitochondrial-mediated metabolic dysfunction and neuronal cell death (Cunha-Oliveira *et al*., 2007; Oliveira *et al*., 2002; Tian *et al*., 2017). Furthermore, lipid and protein peroxidation has been observed in the plasma of heroin users (Pereska *et al*., 2007), while decreased levels of glutathione (GSH) were observed in the hippocampus, brain stem, white matter and cortex of heroin abusers, suggesting that these brain areas may be at risk of oxidative stress (Gutowicz *et al*., 2011). These observations indicate that heroin consumption can lead to increased ROS levels, causing oxidative stress and consequently impairing cellular function with eventual cell death.

1.6.1. Apoptosis as a mechanism of heroin toxicity

Cell death is either programmed or non-programmed. The mechanisms are based on morphological features and fragment clearance. Thus, the disruption of cell membrane integrity leads to non-programmed cell death or necrosis (Golstein & Kroemer, 2007). A local immune response and organelle swelling characterise necrosis due to the spillage of cellular contents (Festjens *et al*., 2006). Street heroin was suggested to cause necrosis-dependent neurotoxicity at concentrations above 427 μg/ml (Cunha-Oliveira *et al*., 2007).

Alternatively, programmed cell death, via the mechanisms of either apoptosis or autophagy, is a tightly regulated process. Apoptosis is associated with morphological changes, protease and

nuclease activity, mitochondrial dysfunction, and DNA fragmentation before phagocytosis (Ryter *et al*., 2006). It can be initiated through intrinsic regulation by the B-cell lymphoma 2 (*Bcl-2*) protein family, which consists of both pro- and anti-apoptotic signalling proteins (Reed, 1999). The intrinsic pathway is a mitochondria-mediated pathway that leads to the activation and translocation of *BAX*/Bak pro-apoptotic signalling via *p53* or *BH3* proteins (Edinger & Thompson, 2004; Tramullas *et al*., 2008). These proteins form part of cellular damage-mediated apoptosis through *BAX*, altering mitochondrial membrane permeability to incite the efflux of cytochrome c (Nagata, 2018). Signalling cascades activate downstream caspase-9 and initiate apoptosis-inducing factor (AIF) release (Modjtahedi *et al*., 2006). As a result, activation of executioner caspases 3, 6 & 7 facilitates proteolytic and nuclease catabolic activity to mediate apoptotic cell death (Fan *et al*., 2005; Modjtahedi *et al*., 2006; Ryter *et al*., 2006).

Alternatively, the apoptotic programme can be initiated extrinsically via ligand and extracellular membrane death factors and receptors (Meel & Essop, 2018). Intracellular formation of death-inducing domains and subsequent activation of apoptosis activator caspases can alter the Bid to *BAX* ratio, leading to mitochondrial-mediated cell death or directly activate executioner apoptotic caspases (Martin *et al*., 2012; Taylor *et al*., 2008). The extrinsic pathway, which involves the activation of caspase-8 followed by downstream caspases, can also be initiated by tumour necrosis factor-alpha (TNF-α) (Emeterio *et al*., 2006).

The altered expression levels of the apoptotic genes *Bcl-2* and *BAX* have been used as indicators of possible apoptotic activity (Nagata, 2018). Apoptosis is thought to be the main mode of cell death in response to drug-elicited forms of stress. Heroin neurotoxicity in rat cortical regions and *PC12* cells was linked to the release of cytochrome c, and *Bcl-2* was found to be downregulated following loss of mitochondrial membrane potential loss (Cunha-Oliveira *et al*., 2007, 2008, 2010; Oliveira *et al*., 2003; Tian *et al*., 2017). Chromatin condensation was observed in undifferentiated *PC12* cells following stimulation with heroin (Liu *et al*., 2007; Oliveira *et al*., 2002). Intrinsic apoptotic instigation of the programme was further supported by an upregulation in the expression of *BAX* of heroin exposed *PC12* cells (Tian *et al*., 2017).

Heroin-induced apoptosis was further associated with the activation of the JNK/c-Jun signalling cascade with downstream activation of caspase-3 (Lai *et al*., 2011; Tan *et al*., 2013). In addition, the same pathway was found to be activated in the cerebellar granule cells of Sprague-Dawley rats that were exposed to heroin. In addition, the activation of the JNK/c-Jun pathway was also associated with increased mRNA expression of apoptotic death precursors including *BAX* (Pu, Wang, Su, *et al*., 2015; Pu, Wang, Zhang, *et al*., 2015). Similarly, heroin-mediated increases in the levels of β-endorphins in cortico-limbic regions of Sprague-Dawley rats caused apoptosis

in the medial frontal cortex and NAcc of these animals (Pu, Wang, Zhang, *et al*., 2015). Finally, disturbances in the levels of endogenous opioids have been associated with patho-morphological changes in neurons, reflecting apoptotic activity (Liu *et al*., 2007).

The high incidence of HIV infection in opioid consumers is a great concern considering that the immune system of these individuals may be severely compromised. This is particularly worrisome in light of a recent rat study showing that opioids and HIV-1 envelope protein synergistically caused apoptosis through the mitogen-activated protein kinase (MAP-K) pathway (Yan *et al*., 2022). In addition, it has also been suggested that ligands to MOR-opioid receptors have the ability to promote caspase-3-activated cell death in heroin-HIV populations (Khurdayan *et al*., 2004). Therefore, it is clear that heroin may trigger neuronal death via apoptosis and this mode of cell death may explain some of its damaging effects on the brain.

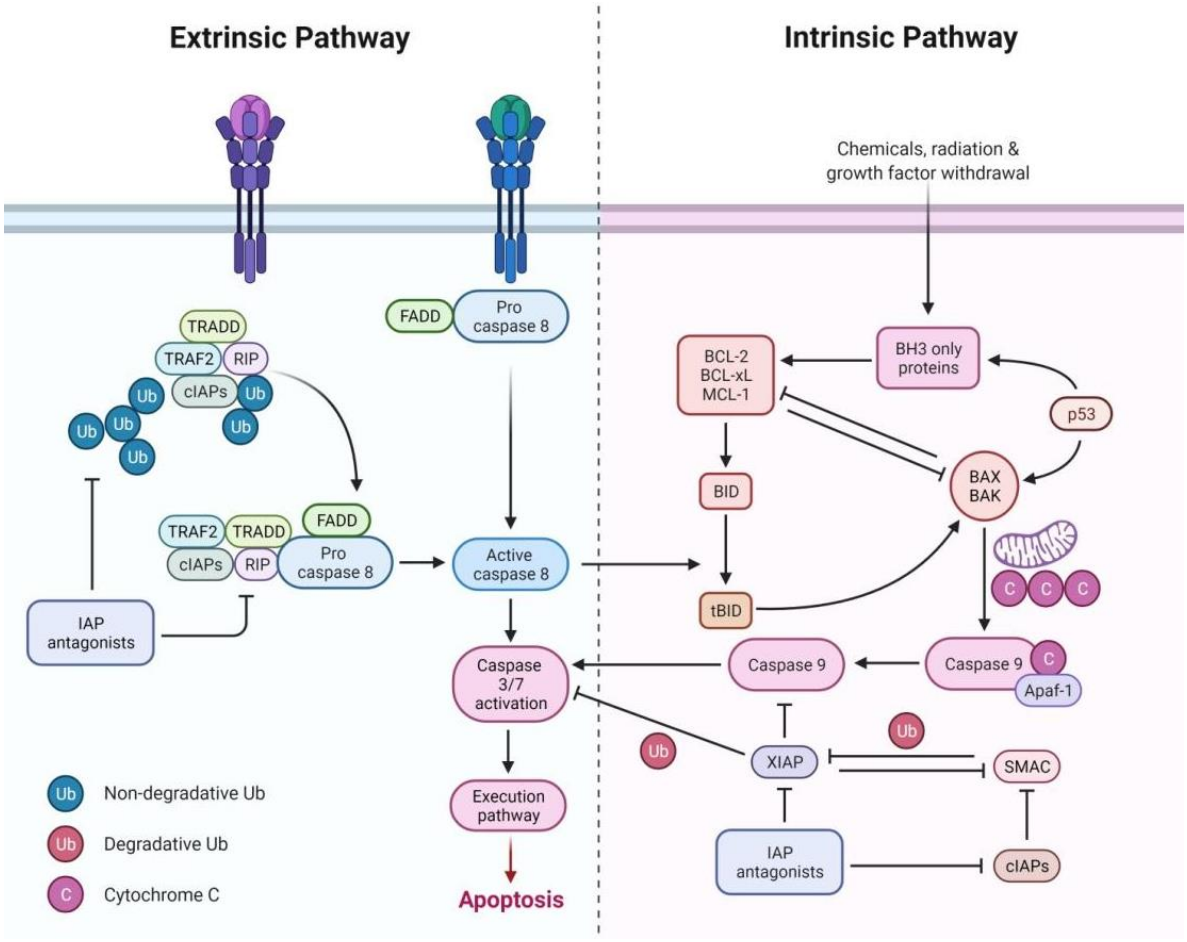

Figure 1.3: An illustration indicating the intrinsic and extrinsic apoptotic pathways (Bertheloot *et al*., 2021; Sharma *et al*., 2017).

1.6.2. Autophagy as a mechanism of heroin toxicity

Autophagy or "self-eating" is a dynamic form of programmed cell death characterised by the formation of bundled cell constituents or autophagosomes, which are delivered to lysosomes

and vacuoles (Mizushima, 2007; Mizushima *et al*., 2003; Rao, 2016). This is followed by the degradation and recycling of cellular compounds to generate nucleotides, and amino and fatty acids that may be used for the synthesis of new compounds (Galati *et al*., 2019; Glick *et al*., 2010; Kuma *et al*., 2017). Therefore, autophagy is considered a more preservative mode of cell death. The presence of autophagosomes is linked to the upregulation of microtubule-associated protein 1A/1B, light chain 3 (LC3) and *p62* (Mizushima, 2007; Mizushima *et al*., 2001; Schläfli *et al*., 2015). These proteins have a widespread presence in cells and are commonly used to monitor autophagic activity (Yoshii & Mizushima, 2017).

During the formation of autophagosomes, pro-LC3 protein is cleaved by Atg4 into LC3-l, which forms a complex with *SQSTM1*/*p62* and NBR1 on the elongating autophagosome membrane (Tanida, 2011b; Yoshii & Mizushima, 2017). LC3-l is then activated by Atg7 and Atg3 to form LC3-II complex as the autophagosome develops into a fully-fledged vesicle (Martinet *et al*., 2012). Sequestration proteins (*p62*/*NBR1*) "tag" specific ubiquitinated and non-ubiquitinated substrates for autophagic degradation (Tanida, 2011b). A phagophore initial membrane is formed around the sequestrosome, which is guided by *LC-3* (Tanida *et al*., 2008). This protein forms a complex with sequestration proteins on the membrane of the elongating autophagosome. A lysosome will then fuse with the autophagosome, a system dedicated to bulk lysosomal hydrolase degradation of substrates (Veras *et al*., 2019). Thus, *LC-3* and *p62* play key roles in the maturation of vesicles and their gene expression can be quantified for the detection of autophagic activity (Tanida *et al*., 2004), whereby an increase in expression of LC3 and a decrease in the expression of *p62* is characteristic of active autophagy (Bjørkøy *et al*., 2009; Tanida, 2011a). Interestingly, neurons exposed to opioid agonists and stress displayed neuroplastic changes that were attributed to autophagy (Karoussiotis *et al*., 2022). Furthermore, autophagy of neurons has been associated with cognitive dysfunction and loss of synaptic plasticity (X. Wang *et al*., 2020). Therefore, autophagy may play a direct role in the detrimental effects of heroin on brain function.

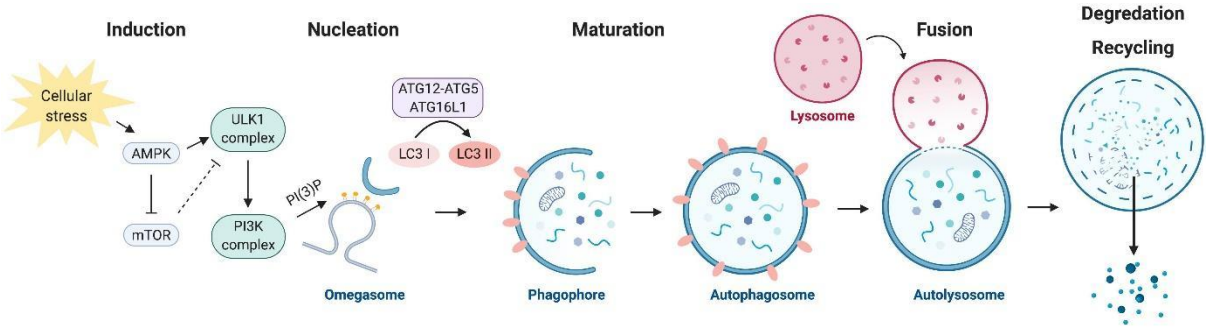

Figure 1.4: A flowchart representative of the autophagic pathway as triggered by cellular stress, which can occur as a consequence of nyaope use. When cells experience stress, numerous complexes

play a role in nucleation, including the essential ATG12-ATG15 complex in the initial step of LC3 conjugation. Additionally, ATG7 and ATG3 then mediate the second conjugation step with the ATG12-ATG15-ATG16L1 complex. This conversion of LC3-1 to LC3-II is a pivotal process, and it is part of the critical steps, along with *p62*, in the formation, elongation, and maturation of autophagosomes. Once fully developed, autophagosomes are generated, and they merge with lysosomes, commencing the phase of autophagy dedicated to degradation and recycling. Autophagy regulates and prevents the accumulation of ROS, cellular damage and mitochondrial toxicity (Chang, 2020).

1.6.3. Inflammation as a mechanism of heroin toxicity

In the maintenance of homeostasis, inflammation is extremely important in keeping us alive. Inflammation is part of our adaptive immune response to pathogens and toxic substances and aims to prevent cellular and tissue damage to maintain essential organ functionality.

Although acute inflammation is beneficial, if uncontrolled it may become chronic and deleterious to the body (Pahwa *et al*., 2018). This chemical signalling cascade stimulates the activation and recruitment of leukocyte chemotaxis from circulation to accumulate and centralise it to the area of damage or insult. The intensity of the response is dependent on the nature and location of the stimulus; however, the mechanism of action briefly entails the following: 1) cell surface receptor recognition of injury/invasion; 2) activation of inflammation pathways/endogenous signals of tissue or cell damage; 3) recruitment of immune cells; 4) inflammatory markers/cytokines are secreted; and 5) enhanced recruitment of more immune cells (Lugrin *et al*., 2014; Medzhitov, 2010). Activated intracellular signalling pathways stimulate the production of primary inflammatory mediators. These include pathogen-associated molecular patterns and an array of cytokines that trigger receptor-mediated inflammatory pathways (Chen *et al*., 2018).

Cytokines are small proteins that are primarily released by immune cells, including macrophages, lymphocytes and monocytes. There are pro- (promote) and anti- (suppress) inflammatory cytokines, which are often classified as interleukins (ILs), interferons (IFNs), tumour necrosis factors (TNFs), transforming growth factors (TGFs), chemokines and colony-stimulating factors (CSF) (Chen *et al*., 2018; Dinarello, 2000; Kaminska, 2005). Pro-inflammatory cytokines upregulate inflammation mediators and signalling molecules that increase the synthesis and activation of platelets, while chemokines facilitate the recruitment of leukocytes to inflamed areas (Abdulkhaleq *et al*., 2018). Anti-inflammatory cytokines suppress the process and intensity of the inflammation response (Dinarello, 2000), as excessive upregulation of pro-inflammatory cytokines can prolong or intensify the inflammatory response. This could lead to tissue or organ damage, which can ultimately result in death (Bindu

et al., 2020). Acute toxicity produces quick production of the acute phase protein, TNF-α, and the interleukins; *IL-6*, IL-8, IL-1β (McMaster *et al*., 2015). Cytokines are also important in the maintenance of neuroplasticity, stimulation of Toll-like Receptors and the modulation of neurogenesis (Borsini *et al*., 2015; Calabrese *et al*., 2014; Hayley, 2014; Ozato *et al*., 2002). Cytokines have been implicated in neurodegeneration through p53 dysregulation and have been identified as mediators of opioid tolerance, and dependency processes that lead to substance use disorders (Allan & Rothwell, 2001; Eidson & Murphy, 2019; Hutchinson *et al*., 2008; Turnquist *et al*., 2016). In general, neuro-inflammation, particularly *IL-6*, may cause the overproduction of ROS leading to apoptosis and/or autophagy (Sochocka *et al*., 2016).

1.7. **Toxic effects of heroin on the liver and kidney**

When heroin enters the body, it is mostly metabolised by hydrolysis into 6-monoacetylmorphine (6-MAM), 3-monoacetylmorphine and morphine. The 6-MAM and morphine are the more active metabolites with high lipid and membrane solubility facilitating easy passage through the blood-brain barrier (BBB) (Boerner *et al*., 1975; Goeldner *et al*., 2011). Hence, most opioid users experience a rapid feeling of "high" shortly after intake due to the penetrability of the BBB. The half-life of heroin is about 20 minutes depending on the tissue whilst its metabolites can remain consistently high long after administration (Cohn *et al*., 1973). It would seem that heroin does not have any direct pharmacological effects on the kidney and liver but acts as a carrier of potent morphine metabolite 6-MAM in blood and the brain (Boix *et al*., 2013). The lipid solubility further allows storage with a concomitant slow release and clearance from the body. The deacetylation of heroin happens individually in homogenate tissue (liver, brain, blood and kidney) with liver and blood exhibiting higher hydrolytic activity (Boerner *et al*., 1975; Boix *et al*., 2013). In the liver and intestine, carboxylesterases (CEs) are mainly responsible for the hydrolysis of heroin (Hatfield *et al*., 2010; Kamendulis *et al*., 1996). Hence, the high presence of these enzymes in the gut, blood and liver, especially CE-2, can account for the low presence of heroin in circulation after intravenous administration.

In general, opioids required for analgesic management do not cause liver damage. However, large doses are associated with hepatotoxicity especially for intravenously administered drug intake (Ilić *et al*., 2005, 2012). In such cases, nonspecific portal inflammation, lymph node enlargement in the porta hepatis, and subacute hepatic necrosis have been documented (Soleimanpour *et al*., 2016). The study of heroin-mediated effects on rat liver function has been limited. Cunha-Oliveira and co-workers have suggested that heroin impairs mitochondrial

energy production (Cunha-Oliveira *et al*., 2007, 2008), while Antonilli and colleagues (2012) reported that heroin reduced the glucuronidation activity of the liver (Antonilli *et al*., 2012).

Alanine transferase (ALT) and aspartate transaminase (AST) are two liver enzymes that have been validated as indicators of liver damage. ALT is found in an array of organs including the kidney and in clusters within the liver cytosol (Magnette *et al*., 2016). The elevation of ALT in blood is associated with hepatic cell injury in both viral and toxic substance damage (Gowda *et al*., 2009). AST is located in the mitochondria of hepatocytes, but it is also present in other tissues. In cases of severe tissue injury, it ultimately causes the rupture of cell membrane which leads to leakage of AST resulting in increased concentration in the circulation (Thapa & Walia, 2007). Furthermore, in humans the normal blood levels of AST range between 0-35 U/L, while for ALT it is 7-56 U/L. However, the normal values for animals may be different (Magnette *et al*., 2016).

Heroin-associated nephropathy has been identified as a complication of drug use. This has been described as even more serious when adulterated drugs are used (Cunningham *et al*., 1984). The renal complications of drug abuse are said to encompass a spectrum of glomerular, interstitial and vascular diseases (Crowe *et al*., 2000). In a recent comprehensive review, Kannan (2022) reported clinical findings related to the toxic effects of heroin on the structure of the kidney which translated to dysfunctionality (Kannan, 2022). This report expanded previous observations to include other nephrotic impairments such as renal infarction, endocarditis-associated nephritis, and rhabdomyolysis-induced acute kidney damage. Creatinine is widely used as a measure of renal function (Levey *et al*., 1988). For instance, exposure of mice to the toxin fenpropathrin, which is known to cause kidney damage, revealed increases in plasma creatinine levels that were coupled to increases in interleukin-1ß (Jaremek & Nieradko-Iwanicka, 2020). Similarly, creatinine determinations were used to investigate renal function in neonates (Filler *et al*., 2016), as well as, in monitoring the kidney function of living kidney donors (Edgren *et al*., 2010). While creatinine remains a useful proxy for kidney function, it has been described as only a crude measurement of glomerular filtration rate, which is the more preferred and accurate measurement of kidney function (Prigent, 2008).

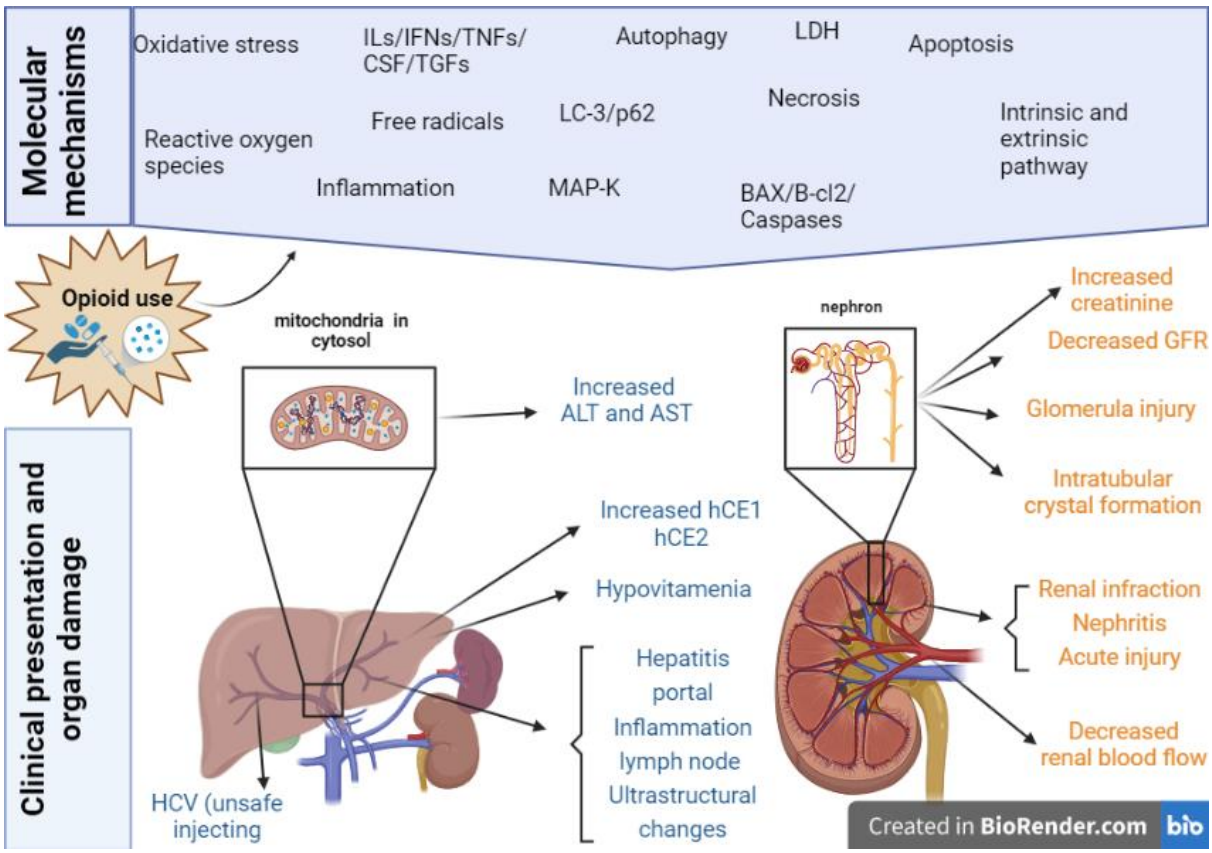

Figure 1.5: An illustration showing the various toxic effects of opioid use through the molecular mechanism of the drug's metabolised active forms. Its toxic insult begins in the liver with inflammation of the hepatic portal which contains a large concentration of hCE enzymes which mediate deacetylation of heroin to morphine. Furthermore, opioid toxicity causes increases in plasma ALT and AST levels. Heroin is also associated with kidney damage with increased creatinine being an indicator in blood for this toxic effect. Nephrotoxicity is characterised by inflammation, crystal formation and decreased glomerular filtration rate (GFR) **(Created with BioRender.com).**

1.8. Assessing the behaviour of rats

Research using rat models has provided many insights into complex disorders of the brain, and as such, they remain an important tool for investigating the intricacies of many psychopathological conditions. One of the main benefits of using rats in preclinical studies stems from the ability of the researcher to control many variables during experimentation that would otherwise present confounding factors, which make it difficult to draw firm conclusions (Gould *et al*., 2009). For example, rat studies can be well controlled for living conditions, gender, diet, weight, age, oestrous cycle, drug doses and period of medication (Kraeuter *et al*., 2019b). Furthermore, the researcher may have access to blood and brain tissue as determined by institutional ethical committees. Despite these advantages, the interpretation of data must still be carefully considered and extrapolation to the human condition remains a challenge (Lamprea *et al*., 2008).

Several rat models have been developed to study various pathologies of the central nervous system, including genetic models (Creed & Goldberg, 2018), pharmacologically manipulated models (Lai *et al*., 2011) and structurally lesioned models. Similarly, rat models for behavioural abnormalities observed in psychiatric conditions have also been validated (Ramos, 2008). For instance, the elevated plus maze (EPM) is a tool that is commonly used to evaluate anxiety-like behaviour in rodents (Kraeuter *et al*., 2019b, 2019a), while the forced swim test is used to study depressive-like behaviours (Yankelevich *et al*., 2014). The open field test (OFT) can be used to assess the locomotor activity, mood and exploratory behaviour of experimental animals and their matching controls. In performing the OFT, quantitative data such as speed, distance travelled, time spent in outer or inner zones, and episodes of grooming can be collected as measures of a rodent's mood and behaviour (Kraeuter *et al*., 2019a). For example, animals with anxiety-like behaviour in a novel first-time exposure to the OFT test are likely to avoid the exposed well-illuminated centre of the apparatus with preference to a thigmotactic behaviour in the outer walls (Holmes, 2001). In a study by Kuniishi and colleagues (2017), animals subjected to early-life adversity displayed increased high-leaning behaviour, which is when rats lean against the wall of the OFT with outstretched forepaws. This behaviour was considered a novel anxiogenic behaviour by the authors (Kuniishi *et al*., 2017). Increased defecation is a parameter associated with high emotionality and serves as another indicator of anxiety-like behaviour (C. S. Hall, 1934; Seibenhener & Wooten, 2015). Other researchers have reported general depressive behaviours with drastic decreases in locomotor activity, time spent in the centre and grooming behaviours (Haj-Mirzaian *et al*., 2019) . Dated studies propose locomotor activity to have a time-dose dependent relationship, whereby hyperactivity is observed for a certain time for a specific dose before restoration of normality or even hypoactivity is observed. As such, a dose inflection point where higher doses can result in immediate hypoactivity has been identified (Babbini & Davis, 1972; Oka & Hosoya, 1976; Schlussman *et al*., 2008).

Of relevance to the present study an initial increase in the animals' locomotor activity in the first hour following treatment with a KOR-receptor opioid agonist, indicating psychomotor responses to acute opioid dose (A. Jackson & Cooper, 1986). Acute effects of heroin administration, such as increased locomotor activity, are possibly due to the presence of 6-MAM which is swiftly metabolised and passed through the BBB to alter behaviour (Andersen *et al*., 2009). MOR-opioid receptor occupancy has been associated with locomotor hyperactivity. Interestingly, it has been shown that while naloxone, a MOR-opioid antagonist, may block anxiety-like behaviours and stress responses, it does not restore locomotor activity (Wilson & Junor, 2008).

Exploratory behaviour is an important component of the OFT, as it offers information about the apparatus and eliminates behaviours related to a sense of danger (Heinz *et al*., 2021). Rearing behaviour involves the placing of both forelimbs on the walls of the apparatus akin to leaning behaviour, when exhibiting exploratory behaviour. It is associated with stimulation of brain circuits involved in memory (Alves *et al*., 2012). Studies further showed that anxiolytic drugs may decrease the number of rearing bouts (Kuniishi *et al*., 2017; Seibenhener & Wooten, 2015), while other studies equated elevated rearing behaviour with an anxious mental state in rodents (Javani *et al*., 2022; Sturman *et al*., 2018).

1.9. Problem statement

Opioid drug use is prevalent in Western nations, and there is a notable increase in the availability of heroin and its derivatives in smaller and middle-income countries, such as South Africa. The impact of the nyaope drug, which contains heroin, on South African society has been extensively documented in clinical and social studies, even though its biochemical mode of action remains unexplained. Consequently, it is imperative to explore mechanism(s) by which nyaope affects the brain in order to explain the behavioural shifts and mental states observed in human studies. There have been reports in social studies (DOJ & CD, 2022; Mokwena, 2016; Mokwena & Makuwerere, 2021) that even a single dose of this cocktail drug can trigger cravings, initiate substance uses mechanisms, and induce neurotoxicity. Therefore, the aim of the current study was to determine if a single dose of nyaope indeed causes neurotoxic effects and to investigate its mechanism(s) of action in the brain, liver and kidney. Furthermore, it is crucial to assess the extent of toxic damage inflicted by nyaope on the liver, primarily responsible for metabolising the drug, and the kidneys, responsible for excretion. This investigation will provide insights into both the behavioural and physiological changes induced by nyaope, potentially offering valuable insights into the development of treatment and therapeutic strategies for addressing this substance use disorder.

1.10. Our hypothesis

In view of the above literature, we hypothesised that the administration of the heroin-containing nyaope to rats would affect their locomotor, mood, and exploratory behaviour; and that altered molecular mechanisms within brain areas, such as the prefrontal cortex may mediate these behavioural alterations.

We further hypothesised that the acute injection of nyaope would also have adverse effects on the liver and kidney function of treated rats.

1.11. Aim

This study aims to investigate the short-term behavioural and molecular toxic effects of nyaope in the brain, liver, and kidney of Sprague-Dawley rats

1.12. Specific objectives

The specific objectives addressed in the current study include;

1. Does the injection of a single dose of nyaope alter the locomotor activity of rats as assessed using the OFT?
2. Does the injection of a single dose of nyaope alter the mood of rats as assessed in the OFT?
3. Does the injection of a single dose of nyaope alter the exploratory behaviour of rats as assessed in the OFT?
4. Does the injection of a single dose of nyaope induce cell death in the prefrontal cortex of rats as assessed by the mRNA expression of genes involved in apoptosis (*BAX*, *Bcl-2*), autophagy (*SQSTM1*), neurotoxicity (*ANXA3*) or inflammation (*IL-6*)?
5. Does the injection of a single dose of nyaope cause acute liver (AST & ALT) and kidney (creatinine) malfunction as assessed by plasma concentration of AST, ALT and creatinine with the IDEXX Catalyst technology?

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2. CHAPTER 2: METHODOLOGY

2.1 Introduction

A previous study within our laboratory showed that nyaope, administered intraperitoneally (i.p., 1 mg/kg/bw) to 72, eight-week-old male Sprague-Dawley (SD) rats, once a day for five consecutive days, affected the animals' locomotor activity; distance travelled (*p<0.0005), mean speed (*p<0.0001) and cognitive functioning; time spent in centre (*p=0.0001) (Sekhotha, 2022). The present study subsequently investigated the acute effects of nyaope administration on the behaviour and molecular toxicity of the drug in SD rats. Nyaope substance use disorder is predominantly found in males (Morgan *et al*., 2019), hence, the decision to use males only.

Therefore, in the present study SD rats were treated with a single i.p injection of nyaope dose of 0,4 mg/kg/bw and the effects thereof on the behaviour of the animals were assessed. Tissue samples were collected and stored at -80 ºC for the subsequent determination of acute molecular toxicity caused by nyaope on the brain cognitive function, brain, liver and kidney toxicity.

2.2 Materials

Nyaope was acquired from the South African Police Forensic Services Laboratory (Pretoria, South Africa; SAPS 2019/05). Permission to possess and use nyaope for experimental purposes was obtained from the National Department of Health (NDoH), South Africa (POS 255/2022/2023; Appendix 6.3). The chemical composition of nyaope was determined by the

The chemical composition of nyaope was analysed by the South African Police Services Forensic Science Laboratory in Pretoria to determine its primary constituents. The analysis revealed that the main components were high-grade heroin and its derivatives (Appendix 6.4). Nyaope was kept in a secure cabinet in the office of the supervisor and an accurate record of its use was kept in compliance with legal requirements.

Nyaope stock was freshly prepared on each experimental day and the amount prepared depended on the weight and number of animals to be treated on the day. The nyaope powder was dissolved in physiological saline (Adcock Ingram, Johannesburg, South Africa) at a final concentration of 2 mg/ml (stock). The dose for each rat was calculated based on the weight of the animal at the time of dosing; therefore, a unique volume was extracted from the stock for each animal to yield a final dose of 0.4 mg/kg/bw. The controls were treated with a corresponding volume of physiological saline in accordance with their weight.

All other chemicals were provided by commercial suppliers in kit format as indicated in subsequent sections.

2.3 Animals

A total of twenty-five, male SD rats (~250g each), which were nurtured and bred to weight ranging from 450g to 600g, were acquired from the Wits Research Animal Facility (WRAF, University of the Witwatersrand). The animals were given 9-10 weeks to grow and mature, facilitating the transition from adolescence to young adulthood. Previous studies (McCutcheon & Marinelli, 2009; Sengupta, 2013) have shown that male rats usually reach sexual maturity between 6-8 weeks or when they reach a body weight of between 250 g and 274 g, mirroring the timing seen in the human population where nyaope use often begins in young adulthood. Following a 10-week period of growth and a pilot study (9-week-old SD), the rats were randomly assigned to either a saline-treated group or a nyaope-treated group adhering to the ethical guidelines stipulated in the Animal Research Ethics Certificate (AREC 2022/05/04/D), which was approved by the Animal Research Ethics Committee of the University of the Witwatersrand. The rats were housed in groups of three per cage with one cage having four rats in temperature-controlled (24 °C), humidity-controlled (60%) rooms with good ventilation and a 12-hour day/night cycle, where lights were turned on at 06h00. Rats were provided with free access to food and water, and cages were cleaned twice weekly in accordance with the standard operating procedure of the WRAF.

2.4 Pilot study to determine appropriate dose of nyaope

A pilot study was conducted to determine a non-lethal dose of nyaope for our experiments. Three 9-week-old SD rats were monitored for piloerection, locomotor activity, breathing rate, and muscle tone for one hour in the presence of WRAF veterinary doctors and technicians following nyaope exposure/treatment. A single rat was injected intraperitoneal with a dose of 1 mg/kg/bw of nyaope similar to the previous study. After dosing the animal, we observed extreme hypoxic perfusion in the lower limbs, ears and nose. The animal was predominantly immobile and had tremors, hence, we were unable to perform any behavioural tests. The animal's tail was very rigid 20 minutes post-treatment and tachycardia symptoms were also observed. Considering these observations, the experiment was terminated and the drug dose of 1 mg/kg/bw was considered lethal. Therefore, the initial dose was then significantly reduced to 0.4 mg/kg/bw.

Two SD rats were then administered a reduced dose of 0.4 mg/kg/bw for one hour, considering that the half-life of heroin, the main substance in nyaope, ranges from 7 to 23 minutes (Rook *et al.*, 2006).

The rats displayed hyperactivity for three to seven minutes and showed extreme hypersensitivity to sound. They briefly experienced hyperventilation and produced more urine than expected. After 10 minutes the hyperactivity subsided, and the rats became more docile but frequently pressed their noses against the cage walls. After one hour of the monitoring period the rats exhibited normal behaviour and showed signs of recovery. Hence, we concluded that 0.4 mg/kg/bw would be a suitable dose to study the acute effects of nyaope. Moreover, it was determined that the rats' behaviour would be evaluated for 30 minutes in the Open Field Test (OFT). Subsequently, they would be placed back in their home cages for another 30 minutes for qualitative monitoring of clinical signs by animal technicians. The termination of the experiment and sample collection were scheduled for 60 minutes after treatment.

2.5 Experimental design

A room separate from the housing room was set up for nyaope treatment and behavioural tests. Two groups, which consisted of 11 rats each, received i.p injections of either nyaope or physiological saline. These injections were administered by a qualified veterinary laboratory technician as per WRAF protocol. Thereafter, rats were immediately placed into the open field apparatus to assess their behaviour (Figure 2.1). This occurred both at the qualitative, as well as, at quantitative level. In particular, several parameters were recorded with the computerised AnyMaze video tracking system (Stoelting Co, Wood Dale, Illinois, USA) in order to assess the locomotor, mood, and exploratory behaviours of the rats. The AnyMaze software (Stoelting Co, Wood Dale, Illinois, USA) therefore, not only offered image track plots, but also recorded videos that allowed for subsequent analysis and description of the animal's behaviour. Before the behavioural testing, all animals had not been previously subjected to the Open Field Test or any other behavioural assessments throughout their breeding period. As a result, the animals experienced novelty-induced stress and activity in the Open Field Test, which is processed differently compared to familiar stress-inducing environments.

After the 30-minute behaviour testing protocol was completed, the animals were housed in individual home cages separate from the groups of three for 30 minutes. During this time, clinical symptoms (piloerection, locomotor activity, breathing rate, pupil dilation/constriction, urination, defection, and muscle tone) were monitored by the veterinary clinician, and steps were taken to minimize the distress associated with termination before they were moved to the

termination room. In the termination room, at 60 minutes post-treatment, rats were lightly anaesthetized using isoflurane (2.5-5%) (Bayer GmBH, Leverkusen, Germany) by inhalation exposure for 15 sec. This was accomplished by exposing the animals to cotton soaked with 2 ml of isoflurane, which was then placed inside an airtight euthanization chamber measuring 12x10x12cm. Subsequently, the animals were decapitated using a guillotine. During the termination procedure, we collected brain, liver, and kidney tissue and stored them temporarily in liquid nitrogen and RNAlater (Thermofisher, Massachusetts, USA), an RNA stabilizer that maintains the integrity of RNA in samples, for subsequent PCR analysis. Trunk blood was obtained post-decapitation using a guillotine and transferred into heparinized Vacutainer tubes(Thermofisher, Massachusetts, USA). The tubes were briefly kept on ice before being centrifuged at 8000 rpm for 10 minutes using an EBA 270 centrifuge(Hettich GmBH, Westphalia, Germany). The resulting plasma was then gathered and stored at -80 ºC for later IDEXX enzyme level assessments.

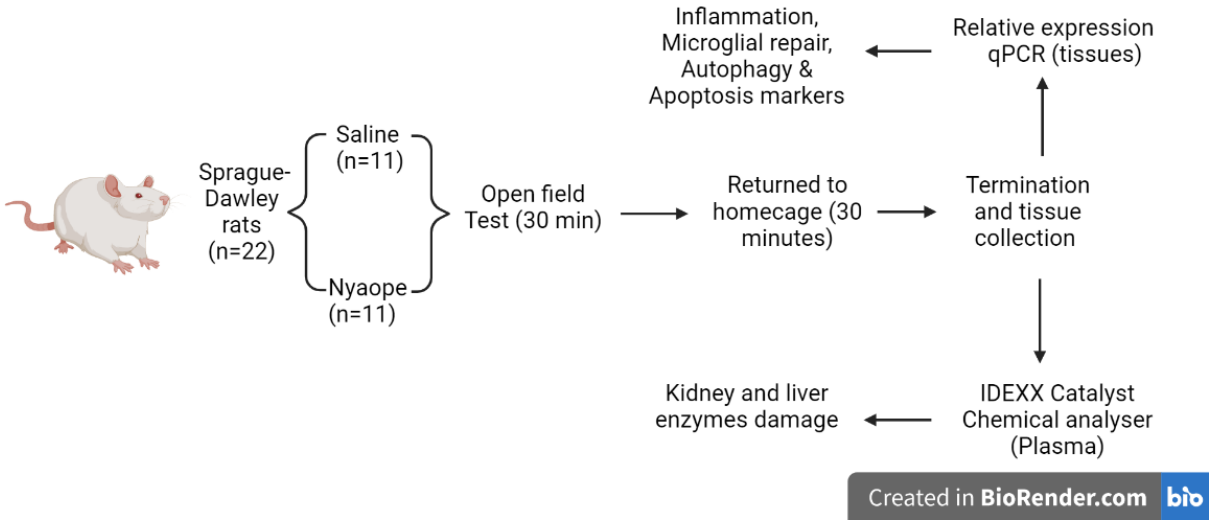

Figure 2.1: A schematic representation of the experimental design (Created with Biorender App).

2.6 Experimental procedures

2.6.1 Open Field Test (OFT)

The Open Field Test (OFT) is a widely used method for evaluating the locomotor, mood, and exploratory behaviours of rodents (Kraeuter *et al*., 2019; Sturman *et al*., 2018). When rodents are introduced to a new environment, they experience a natural balance between their fear of open spaces and their curiosity to explore. The OFT is based on the behavioural principle that anxious animals tend to avoid open spaces, while less anxious ones are more inclined to explore them. Moreover, the primary objective of the Open Field Test is to observe how animals move and behave when exposed to a new environment, which can influence various parameters such as exploratory tendencies, emotional states, grooming habits, and fear-related behaviours like

anxiety (Gould *et al*., 2009; Kraeuter *et al*., 2019). Hence, the OFT allows the researcher to investigate the level of anxiety and exploratory behaviour of rats (Kraeuter *et al*., 2019).

The open field is a 1m × 1m black Plexiglass floor area with walled sides 20 cm high. The floor is delineated into an outer zone (along the wall) and an inner zone (at the centre). In the current study, the assessment of behaviour involved placing the animal in the centre of the open field apparatus for an initial 30-second period to setup the AnyMaze program, followed by 30 minutes of monitoring in a closed room with minimal sound interference To counteract the effects of scent, olfactory cues were eliminated by wiping the apparatus with a 70% ethanol solution between individual trials.

A number of parameters were analysed to obtain an impression of the effect of nyaope administration on the locomotor activity of the animals. These included measuring;

1. the distance that was travelled by the rat in the 30 minutes,
2. the number of mobility episodes of the rat in the respective zones, and
3. the speed at which the rats travelled.

In order to assess the mood status of the animals, the following parameters were recorded;

4. time spent mobile in the various zones,
5. the time spent mobile, and
6. the number of bouts grooming.

Finally, exploratory behaviour was assessed by determining;

7. the number of line crossings
8. the number of entries into the centre zone, and
9. the number of rearing bouts.

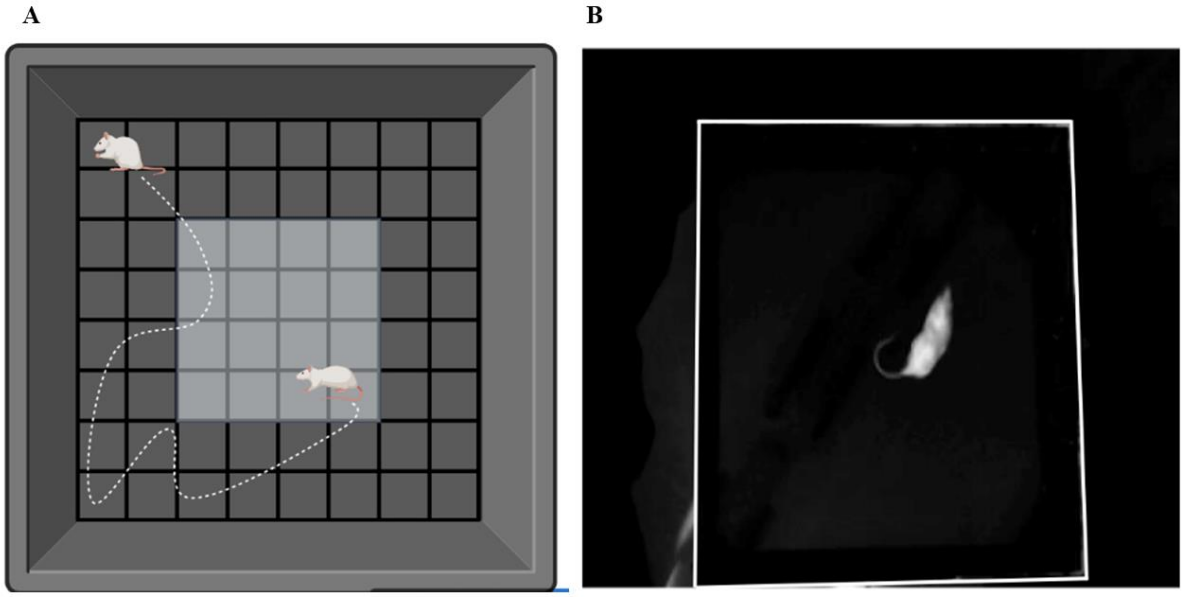

Figure 2.2: Illustration of the open field apparatus from the view of the video tracking system (Biorender App, 2023). The centre zone is represented by the lighter inner grey blocks (A), while the outer zone (peripheral area) with less light exposure is represented by the darker grey blocks. The white square in the AnyMaze video tracking system camera (Stoelting Co, Wood Dale, Illinois, USA) image (B) during testing indicates the borders, with the animal positioned in the centre at the beginning of the behavioural assessment.

2.6.2 Relative mRNA expression analysis by Real-time TaqMan® PCR

Total RNA was extracted from rat tissues (brain frontal cortex, liver, and kidney) using an Illustra™ Mini Spin RNA extraction kit (GE Healthcare, Buckinghamshire). The total RNA was then reverse-transcribed using a T100™ thermal cycler (Bio-Rad Laboratories, California, USA) and SuperScript™ IV VILO RT-cDNA Synthesis Master Mix (ThermoFisher Scientific, Life Technologies, Carlsbad, USA) kit. A NanoDrop OneC UV Spectrophotometer (ThermoFisher Scientific, Life Technologies, Carlsbad, USA) was used to determine the quality and quantity of the cDNA.

cDNA templates were used to assess gene expression of (1) a marker of inflammation; *IL-6* which codes for IL-6 protein (TaqMan® assay ID: Rn01410330_m1), (2) markers of apoptosis; *BAX* which codes for BAX protein (TaqMan® assay ID: Rn01480161_m1), *Bcl-2* which codes for Bcl-2 protein (TaqMan® assay ID: Rn99999125_m1) and (3) markers of autophagy; *ANXA3* which codes for Annexin 3 (TaqMan® assay ID: Rn00563181_m1), and *SQSTM1* which codes for p62 protein (Taqman assay ID: Rn00709977_m1) (ThermoFisher®, Life Science Technologies, Carlsbad, USA).

The assays were run in duplicate on a StepOne Plus™ thermocycler (ThermoFisher, Life Science Technologies, Carlsbad, USA) using standard conditions in 96 well plates (Manilall *et al.*, 2021, 2023). A final volume of 10µl in each well contained a reaction mix composed of TaqMan® Fast Advanced PCR MasterMix (5µl), TaqMan® target probe mixes (0.5µl), cDNA template (1µl), RNase-free water (3.25µl) and the housekeeping gene probe; *TATA box binding protein* (*TBP*) (0.25µl, TaqMan® assay ID: Rn01455646_m1) (ThermoFisher®, Life Science Technologies, Carlsbad, USA).

To determine the relative mRNA expression, the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) was used to calculate the gene expression relative to the housekeeping gene, *TBP*.

2.6.3 Plasma enzyme assays

Heparinized blood was collected from both our nyaope-treated and saline-treated rats at the time of termination. The blood samples were centrifuged (Hettich GmbH & Co, Westphalia, Germany) at 1000 rpm for 10 minutes at room temperature in order to collect plasma. IDEXX Catalyst technology (IDEXX Laboratories, Westbrook, USA) was used to determine the plasma levels of alanine transferase (ALT) and aspartate transaminase (AST) as indicators of liver damage, while creatinine concentrations were measured to estimate the level of kidney functioning. In brief, IDEXX Catalyst technology is an automated methodology whereby samples are applied to the top of a five-layered enzyme cassette. The five layers (spreading, filtering, reagent, indicator, and support layer) and optic interface pass light through the layers to detect the concentration of the enzyme/s of interest (Figure 2.3) (Laboratories IDEXX, 2019). The layers remove different contaminants and provide reagents for the enzymes to react with prior to optical detection (IDEXX Laboratories, Westbrook, USA).

During the execution of the assay, each animal's (n=22) information was entered into the IDEXX VETLAB* Station (IDEXX Laboratories, Westbrook, USA), which synchronised the data into the IDEXX Catalyst DX® chemistry analyser. Samples were gently mixed by inversion after which 200µL was pipetted into the Catalyst* sample cup and loaded into the sample drawer along with each enzyme Catalyst* cassette (AST, ALT and creatinine). No dilutions were required for the samples. Each animal sample was analysed once on the chemical analyser and the results were vetted for if they were within standard reference ranges (ALT*;* 0.1–6.0g/dL, AST*;* 0–1083 U/L, CREA; *0.1–13*.6mg/dL) as defined by the manufacturer (Laboratories IDEXX, 2019) before statistical analysis.

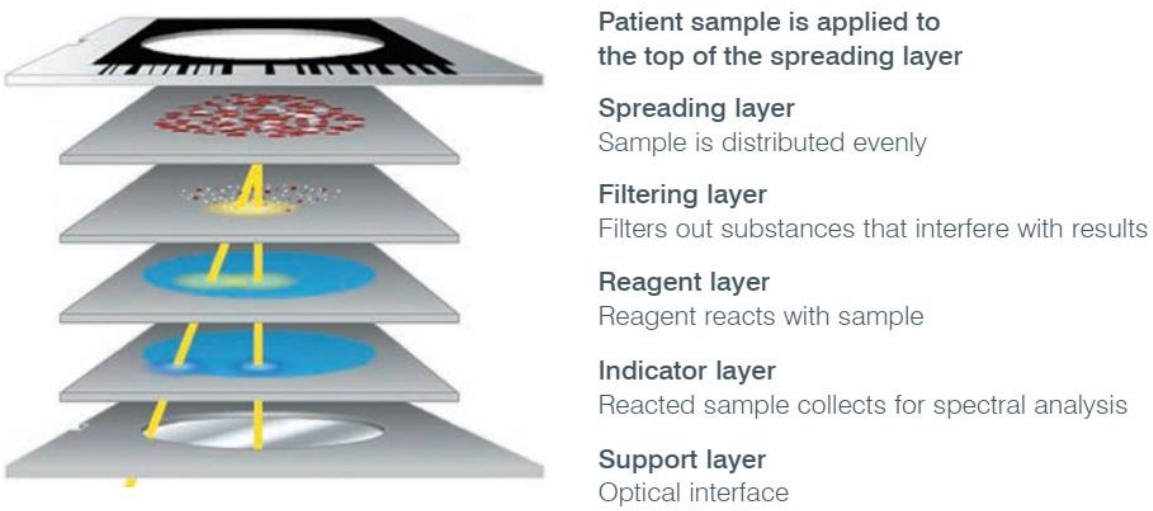

Figure 2.3: Illustration of the technical visualization process that occurs in IDEXX instruments, as a sample of plasma or whole blood being analysed. The diagram shows the five layers of an IDEXX Catalyst cassette and outlines how each layer contributes to determining enzyme levels (Source: Laboratories IDEXX, 2023).

2.7 Statistical analysis

GraphPad Prism software (version 9.5.1, La Jolla, California, USA) was used for data analysis. Individual animal behaviour was tracked using the AnyMaze system (Stoelting Co, Wood Dale, Illinois, USA), and data was collected on a single trial basis. The data extracted from the behavioural tests, PCR, and plasma assays were tested for normality using the Shapiro-Wilk test. Where data were found to be normally distributed, a student's t-test was used to analyse and make comparisons between saline-treated and nyaope-treated rats. Results are expressed as the mean ± standard error of the mean (SEM) of n samples for parametric data. In the event of non-parametric data, the Mann-Whitney U test was performed. Here the results are expressed as median with interquartile ranges (IQR) for n samples. In both cases, results were regarded as statistically significant if $p \leq 0.05$.

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3. CHAPTER 3: RESULTS

3.1 Introduction

To investigate the acute toxic effects of nyaope, rats (n=11) received a single injection of the drug (0.4 mg/kg, i.p.) and were immediately placed in an open field for behavioural monitoring for 30 minutes and then returned to their home cages for a further 30 minutes before termination of the experiment. At 60 minutes post-injection, the rats were sacrificed, and tissue and blood samples were collected for biochemical assays. Rats (n=11) that were injected with an equivalent volume of saline were handled similarly and served as controls.

3.2 Behavioural assessments

3.2.1 Qualitative description of behaviour in the open field test

Analysis of the 30-minute recordings allowed us to make the following qualitative descriptions of the behaviour of the rats in the open field. Throughout the test, saline-treated rats maintained a consistent level of movement within the apparatus, exhibiting exploratory behaviour characterised by brief (1-3 seconds) peeking out of the apparatus by placing one or both limbs on the outer wall edge. They moved easily across the centre of the apparatus, taking short breaks to groom various parts of their body including their face, limbs, tails and lower body, potentially to affirm themselves and maintain good hygiene.

Figure 3. 1: Image taken following 30 minutes of treatment and OFT experiments after the nyaope-treated rat was returned to the home cage.

Grooming behaviour increased towards the latter stages of the experiment and was more frequent when rats were in certain corners of the apparatus. In comparison to the saline-treated rats, it seemed as if nyaope-treated rats had far fewer bouts of grooming per animal with 9 out of 11 rats not attempting a single bout. Interestingly, the nyaope-treated rats showed signs of

hyperactivity in the first 5-13 minutes after being placed in the open field and primarily preferred moving around within the outer area of the open field. Nyaope-treated rats appeared hesitant to enter the centre of the apparatus, although they spent much time orienting themselves towards it. They displayed cautious behaviour and constantly scanned their surroundings.

Following the period of testing wherein the nyaope-treated rats were hyperactive, the nyaope-rats became lethargic and immobile for a period ranging from 5-18 minutes, displaying phenotypic symptoms of opioid drug use, such as tremors, hypoxia (blue limbs), and high frequency of micturition behaviour, possibly to excrete the drug or due to anxiety. The nyaope-treated rats were sensitive to ambient noise during periods of lethargy, reacting in a frightened manner followed by slow movements to another space within the outer area, before settling. Their tails were oddly rigid facing upwards, and after returning to their home cages, the rats would press their noses against the walls.

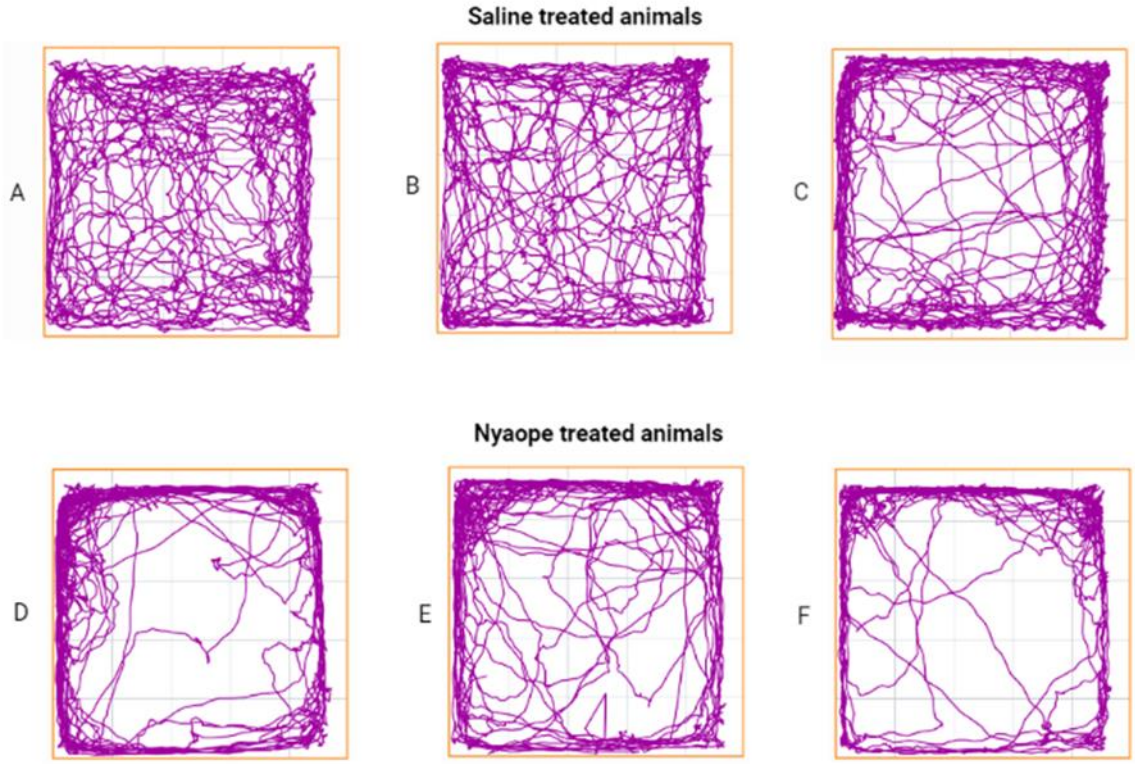


Figure 3.2: The images presented above, extracted from the AnyMaze tracking system, depict the movement track plots of rats during the open field test recorded for a duration of 30-minutes. A visual examination of the track plots for saline-treated rats (A, B, C) and nyaope-treated rats (D, E, and F).

The track plot is a scientific observational software tool used for visualising data, which was utilised to qualitatively map and assess the movement of the rats during the 30-minute OFT experiments. The randomly selected track plots provided a qualitative assessment of three saline-treated rats (figures 3.1 A, B and C) and three nyaope-treated rats (figure 3.1 D, E and

F). The saline-treated rats explored the open field apparatus freely, frequently crossing into thecentral area (figures 3.1; A, B and C). In contrast, the nyaope-treated rats exhibited a marked preference for the outer zone, as indicated by the concentrated purple colour of the tracking path in the outer area (figures 3.1; D, E and F).

3.2.2 Quantitative behaviour assessments in the open field test

The open field test was used to provide an in-depth understanding of the behavioural changes induced by nyaope with a focus on locomotor activity, mood and exploratory behaviour. The open field test was used to study how nyaope affects behaviour, particularly focusing on movement, mood, and exploration. We then measured various parameters to see if there were any differences in these behaviours between the saline-treated and nyaope-treated rats.

3.2.2.1 Assessment of locomotor behaviour

3.2.2.1.1 Distance travelled

The AnyMaze tracking system is capable of measuring the distance covered by rats during the 30-minute open field test and dividing it into different zones. The result revealed that the nyaope-treated rats covered significantly less total distance (88.30 ± 14.12 m; $p = 0.04$) than the saline-treated rats (128.20 ± 12.14 m; figure 3.3 A). Furthermore, on average the nyaope-treated rats travelled 39.33 m less than the saline-treated rats (figure 3.3 A; $p = 0.04$). The data further demonstrated differences in locomotor activity in the zones as the nyaope-treated rats (75.61 ±11.52 m; $p = 0.09$) travelled a lesser distance in the outer zone when compared to saline-treated (101.50 ± 8.94 m; figure 3.3 B) although the difference was not significant. Corresponding with the total distance travelled index, the nyaope-treated rats exhibited less interest in exploring the centre zone (12.68 ± 3.94 m; $p = 0.03$) than the saline-treated rats (26.76 ±7.76 m; figure 3.3 C) who covered almost twice the distance on average (figure 3.3 C; $p= 0.03$).

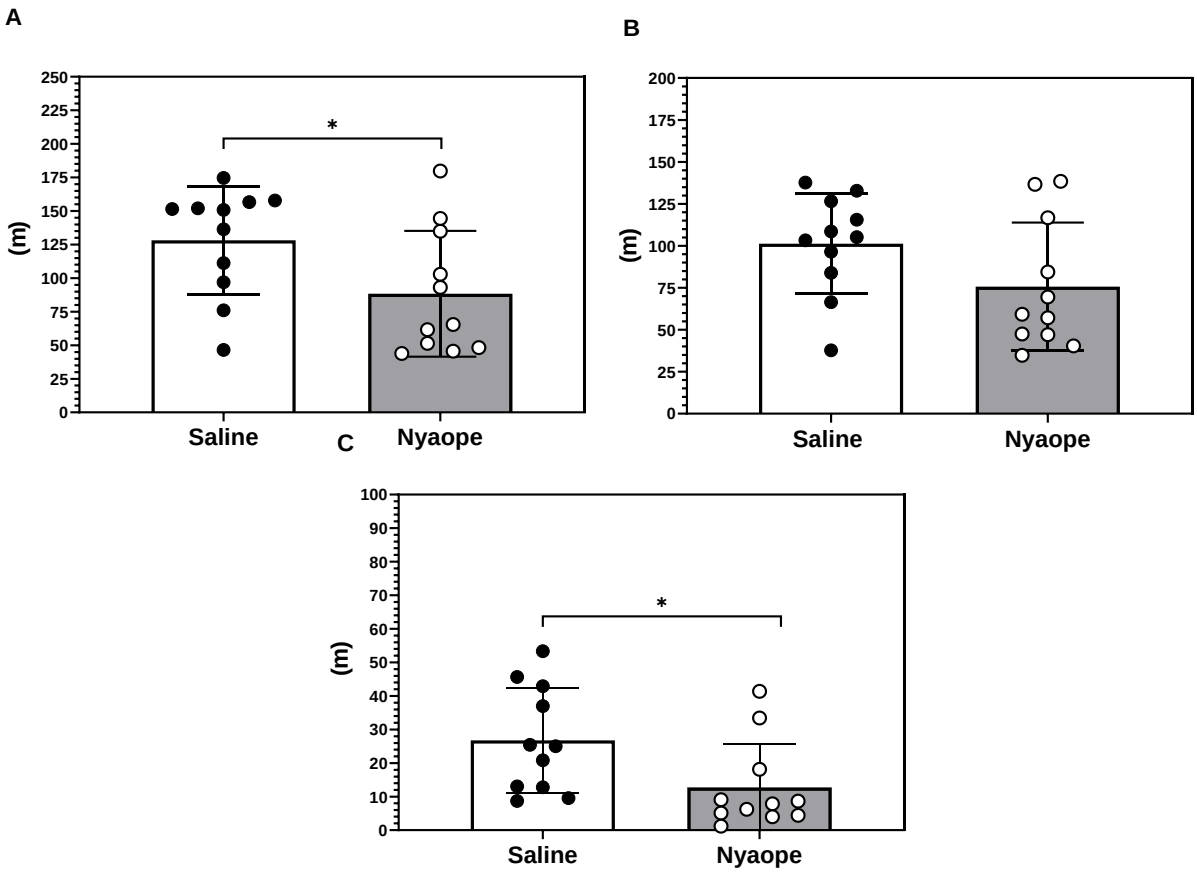


Figure 3.3: The graphs show the locomotor behaviour of the rats in the OFT apparatus as recorded for 30 minutes. Distances travelled (m) by the rats are expressed as mean ± SEM of 11 nyaope-treated (open dots) and 11 saline-treated controls (solid dots). Categorised as total distance travelled (A), Distance travelled in the outer zone (B) and distance travelled in the centre zone (C).

* $p < 0.05$: Unpaired t-test (A & C), significantly different from saline-treated rats.

3.2.2.1.2 Mobility episodes

The term mobility episode refers to an instance where the animal was completely inactive (i.e. being still and not in motion) and then transitioned into a mobile state. Therefore, mobility can be considered an inverse indicator of locomotor activity. The mobility episodes of nyaope-treated rats (85.55 ± 11.04; $p = 0.04$) were significantly lesser than the saline-treated rats (118.10 ± 8.69; figure 3.4 A) over the test duration. In addition, the data from the AnyMaze tool indicated further significant differences in the number of mobility episodes between nyaope-treated rats (83.27 ± 10.62; $p = 0.03$) in the outer zone when compared to the saline-treated rats (115 ± 114.2, figure 3.4 B). These differences also translated in the centre zone as the nyaope-treated rats [median (IQR); 1(4-0); $p = 0.02$)] had significantly fewer mobile episodes or transitions from immobile to mobile state than saline-treated rats [median (IQR); 5(23-5) (figure 3.4; C)].

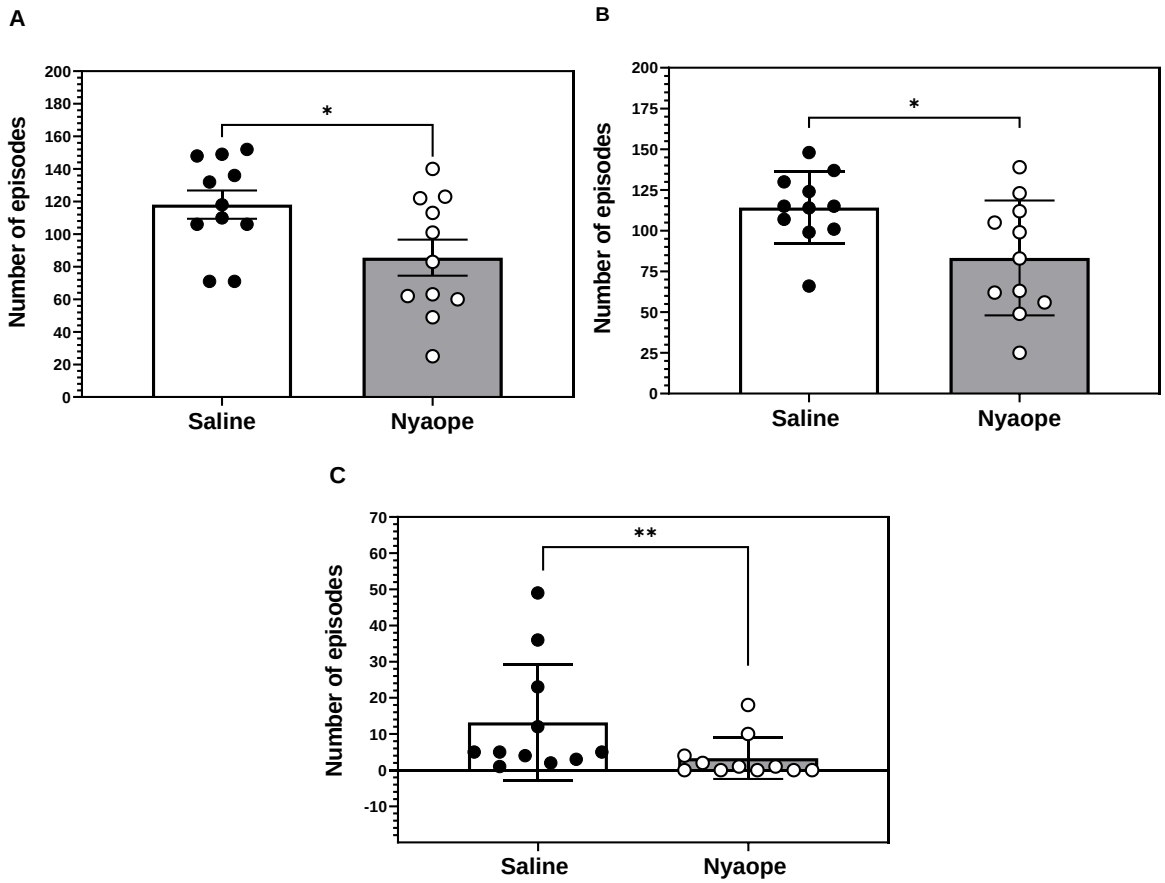


Figure 3.4: The graph above shows the graphical representation of the mobility episodes displayed by the rats during the OFT. The graphs are expressed in mean ± SEM (A & B) and median (IQR) (C) of 11 nyaope-treated (open dots) and 11 saline-treated controls (solid dots). And categorised as total number of mobile episodes (A), number of mobile episodes in the outer zone (B) and number of mobile episodes in the centre zone (C).

*p <0.05, Unpaired t-test (A & B), **p< 0.01, Mann-Whitney U test (C), significantly different from saline-treated rats.

3.2.2.2 Speed measures

Overall, the average speed of the nyaope-treated rats (0.05 ± 0.001 m/s; p = 0.04) was significantly lesser when compared to the saline-treated rats (0.07 ± 0.001 m/s; figure 3.5 A). The average speed in the centre zone (figure 3.5 C; p = 0.10) of nyaope-treated animals showed a decrease compared to the speed in the same zone for animals treated with saline, albeit this difference was not statistically significant. However, nyaope-treated animals achieved the maximum speed (figure 3.5 B; p = 0.52), and higher the average speed in the outer zone (figure 3.5 D; p = 0. 054), when compared with saline-treated animal but did not show any significant differences between the two treated groups.

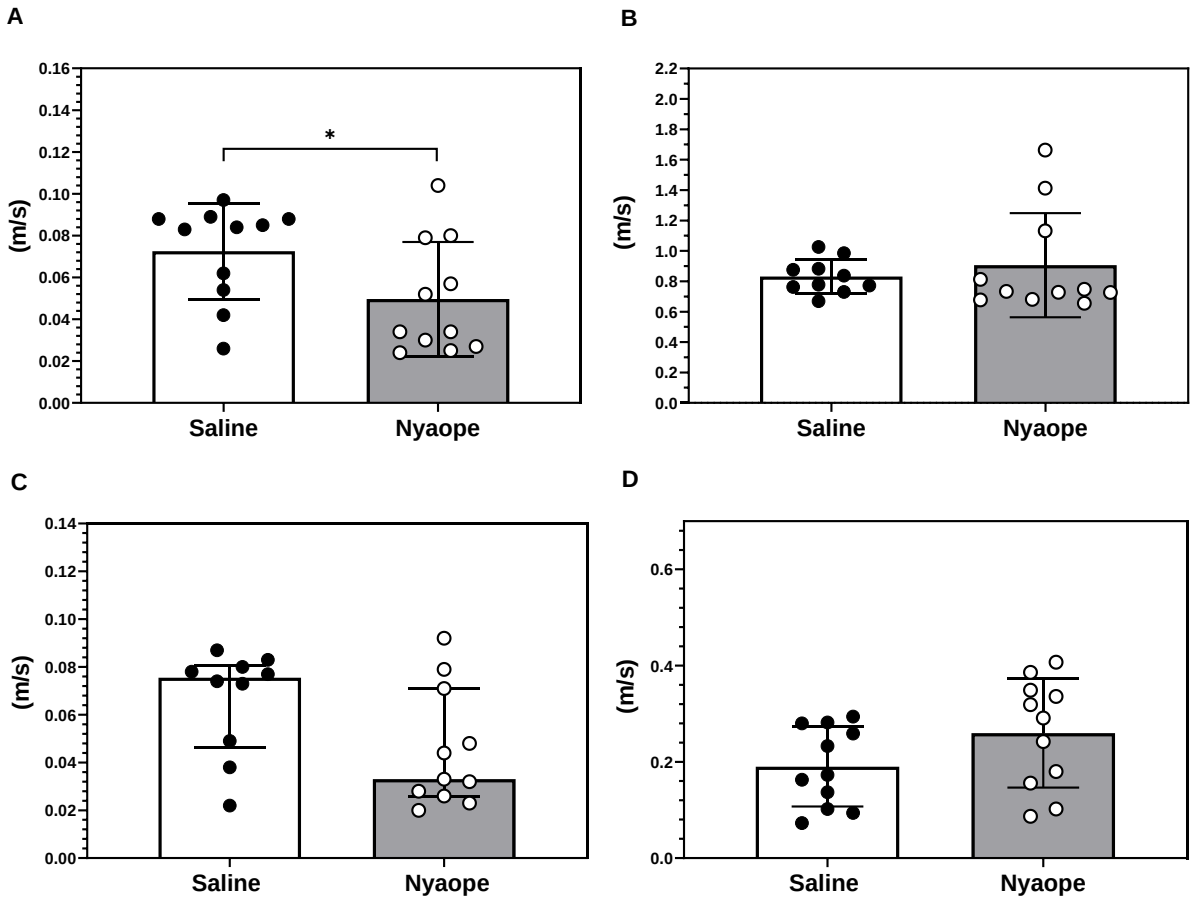


Figure 3.5: Graphs depicting the speed measures of rats during the OFT. The graphs are expressed in mean ± SEM (A, B & D) and median (IQR) (C) of 11 nyaope-treated (open dots) and 11 saline-treated controls (solid dots). The graphs are represented as overall average speed (A), maximum speed (B), average speed in the outer zone (D) and average speed in the centre zone (C) achieved in the duration of the OFT.

*p <0.05, Unpaired t-test (A), significantly different from saline-treated rats.

3.2.3 Assessment of Mood Status

3.2.3.1 Time measures in respective zones to assess the level of anxious-like behaviour.

In total, the nyaope-treated rats (444.70 ± 62.70 seconds (s); p=0.0077) spent significantly less time being mobile compared to the saline-treated rats (702.71 ± 60.42 s, figure 3.6 A). Similarly, the nyaope-treated rats (395.40 ± 52.34 s, p=0.0097) seconds showed reduced locomotor activity spending less time mobile in the outer zone than saline-treated (587.50 ± 42.12, figure 3.6 B) seconds and centre zone (figure 3.6 C) when compared to the saline-treated rats. Additionally, the nyaope-treated [median (IQR); 26.00(69.60-13.20); p = 0.20] spent 67.58 seconds in the centre zone which was nearly 3-fold less than the saline-treated rats [median (IQR); 66.60(188.10-38.00); figure 3.6 C)]. Likewise, nyaope-treated rats spent 192.17 seconds

less time mobile in the protected outer zone when compared with saline-treated rats (figure 3.5 6). Noticeably, both treated rat groups showed more time spent mobile in the outer zone than the centre zone (figure 3.6 B & C).

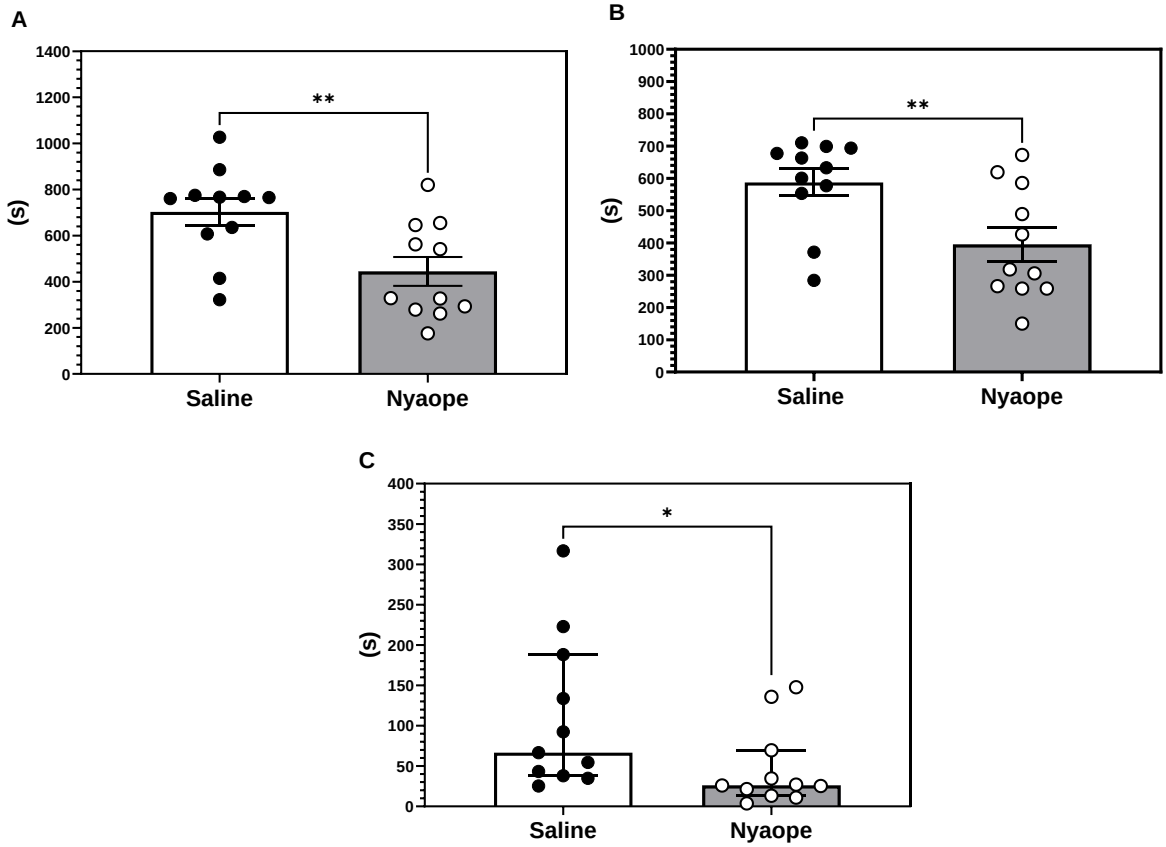


Figure 3.6: The graphs depict the time spent mobile by the rats in different zones of the open field apparatus. The graphs are expressed in mean ± SEM (A, B & D) and median (IQR) (C) of 11 nyaope-treated (open dots) and 11 saline-treated controls (solid dots). The data is represented as total time spent mobile (A), total time spent mobile in the outer zone (B) and total time spent mobile in the centre zone (C).

**p<0.01, Unpaired t-test (A & B), *p < 0.05, Mann-Whitney U test (C), significantly different from saline-treated rats.

3.2.3.2 Grooming

Grooming behaviour is a parameter of emotionality that can be used to assess the overall mood status of the animals. In this study, the AnyMaze tool provided data which indicated that nyaope-treated rats displayed significantly fewer bouts of grooming (13.09±1.18, p<0.0001) when compared with the saline-treated rats (1.20 ± 0.55, figure 3.7). Furthermore, nine of the eleven nyaope-treated rats did not attempt a single grooming bout in the 30-minute duration of the test.

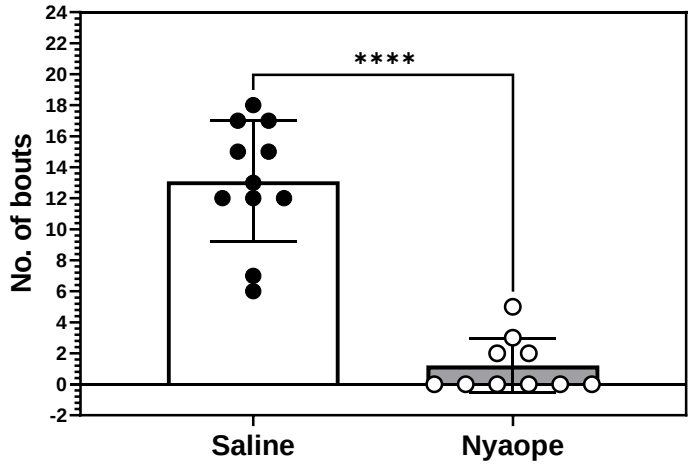


Figure 3.7: A graph showing the number of grooming bouts of the rats during the OFT 30-minute test as recorded by the AnyMaze tool. The values above are expressed in mean ± SEM of 11 nyaope-treated (open dots) and 11 saline-treated controls (solid dots).

****$p < 0.001$, unpaired t-test, significantly different from saline-treated rats.

3.2.4 Exploratory behaviour

3.2.4.1 The number of line crossings

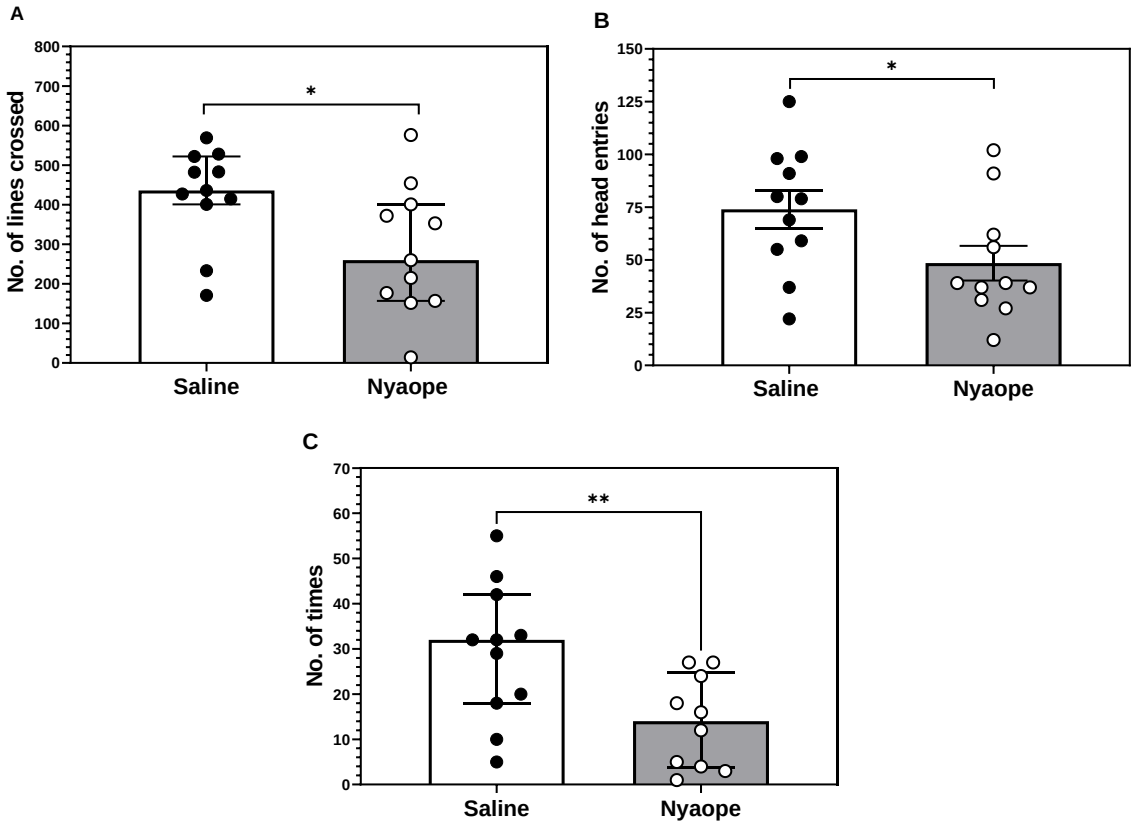
The qualitative visual inspection of the track plots in figure 3.1, showed observational differences in the rat exploration of the open field between nyaope-treated and saline-treated rats. Subsequently, in a complementary manner, the number of line crossings recorded supported this observation. Similarly, the qualitative data indicated the rats treated with nyaope (284.6 ± 48.95; p= 0.038) had a significantly few number of line crossings in the apparatus than the saline (424.3 ± 36.87; figure 3.8 A).

3.2.4.2 The number of head entries into the centre zone

The nyaope-treated rats (48.45 ± 8.23, p=0.048) had significantly fewer number of head entries into the centre zone compared to saline-treated rats (74.00 ± 8.96; figure 3.8 B).

3.2.3.3 Rearing behaviour

Spontaneous rearing behaviour is considered when the rat explores the environment by placing both forelimbs on the edge of the walls of the apparatus. The results showed that on average nyaope-treated rats [median (IQR); 26.00 (54.40); p=0.0094] had significantly lower bouts of rearing than the saline-treated [median (IQR); 66.61(150.12), figure 3.8 C] rats which had an average of 29.3 rearing bouts.



168

169 **Figure 3.8: Graphs showing the exploratory behaviours of saline and nyaope-treated rats in the**
170 **open field.** The graphs represent the number of line crossings (A), the number of head entries into the
171 centre zone (B) and the number of bouts of rearing (C) recorded. The values are expressed as mean
172 ±SEM (A & B), or median and IQR (C) of 11 nyaope-treated (open dots) and 11 saline-treated controls
173 (solid dots).

174 *$p < 0.05$, Unpaired t-test (A and B), significantly different from saline-treated rats. ** $p < 0.01$, Mann-
175 Whitney U test, significantly different from saline-treated rats.

176 ## 3.3 Relative mRNA expression analysis by Real-time TaqMan® PCR

177 The mRNA expression of genes that are typically expressed during acute toxicity was measured
178 in the frontal cortex of the brain, liver, and kidney. For relative gene expression analysis, a
179 housekeeping gene is used, which is expected to have consistent expression regardless of tissue
180 health (Rapacz, 2013). This is employed to compare and calculate whether the expression of
181 the gene of interest has shown an increase, decrease, or remains unchanged relative to the
182 expression of the housekeeping gene. To yield meaningful results, a housekeeping gene should
183 ideally exhibit a moderate level of expression, indicated by a cycle threshold (Ct) value that
184 should not change irrespective of experimental conditions. The Ct is defined as the number of
185 cycles required for fluorescence to reach a specific threshold level of detection and is inversely
186 related to the quantity of gene transcript present in the sample being investigated (Wisc, 2023).

3.3.1 Expression levels of the housekeeping gene, *TBP*, in brain, liver and kidney tissue

The values in Table 3.1 are a summary of the Ct value from the duplicate samples of the 11 saline-treated and 11 nyaope-treated rats' brain PFC, liver and kidney tissue. This data can be used to describe the extent of the variability in the Ct value of our housekeeping gene, *TBP*. The Ct values obtained from brain tissue showed that the expression levels of *TBP* were similar for both the nyaope-treated rats (20.68 ± 0.18; $p = 0.12$) and the saline-treated rats (20.15 ± 0.10; Table 3.1). These values were also within the acceptable range of Ct values for reference genes, i.e., 14 ± 30 (Caraguel *et al*., 2011; Wisc, 2023). In contrast, the Ct values obtained from liver tissue showed a marked difference between *TBP* expression levels of the saline-treated rats (33.21 ± 0.49, $p \leq 0.0001$) and nyaope-treated rats (22.34 ± 0.27, table 3.1). The Ct value of 33.21 was outside the acceptable range for reference genes (Caraguel *et al*., 2011; Wisc, 2023). Furthermore, the liver had the most failed readings which the instrument reported as not detected (n/d) collectively in both groups. Analogous to brain PFC data, the Ct values obtained from kidney tissue reflected comparable expression levels of *TBP* for the saline-treated (30.09 ± 0.51, $p = 0.46$) and the nyaope-treated rats (30.62 ± 0.50, table 3.1).

Table 3. 1: Ct value summary for reference gene, *TBP* in the brain, liver and kidney tissues following RT-PCR.

	Brain tissue samples n=11 per plate		Liver tissue samples n=11 per plate		Kidney tissue samples n=11 per plate	
	Average Ct saline	Average Ct Nyaope	Average Ct saline	Average Ct Nyaope	Average Ct saline	Average Ct Nyaope
Mean ± SD	20.15 ± 0.77	20.68 ± 1.24	33.21 ±3.13	22.34 ± 1.43	30.09 ± 3.80	30.62 ± 3.59
Median (IQR)	20.11 (20.63-19.76)	20.36 (21.27-19.71)	34.36 (35.20-32.10	21.98 (23.55-21.12)	30.05 (37.03-27.07)	30.50 (36.30-28.10)
Ct not detected	0	5	3	10	0	3

Data are expressed as mean ± SEM for five independent PCR experiments, performed in duplicate for each sample. **N/d = not detected**

3.3.2 Expression levels of target genes in brain and kidney tissue

Due to the *TBP*. Ct data in liver tissue having been outside the acceptable range for reference/housekeeping genes, the gene expression data for liver tissue was excluded from further analysis, which was restricted to brain and kidney tissues only.

The results obtained from the brain frontal cortex samples indicated that rats similar expression levels were observed for *Bcl-2* (figure 3.9 A, p = 0.64), *BAX* (figure 3.9 B, p = 0.11), *P62* (figure 3.9 C, p = 0.94), *ANXA3* (figure 3.9 D, p = 0.11), and *IL-6* (figure 3.9 E, p = 0.16) between nyaope-treated and saline-treated rats.

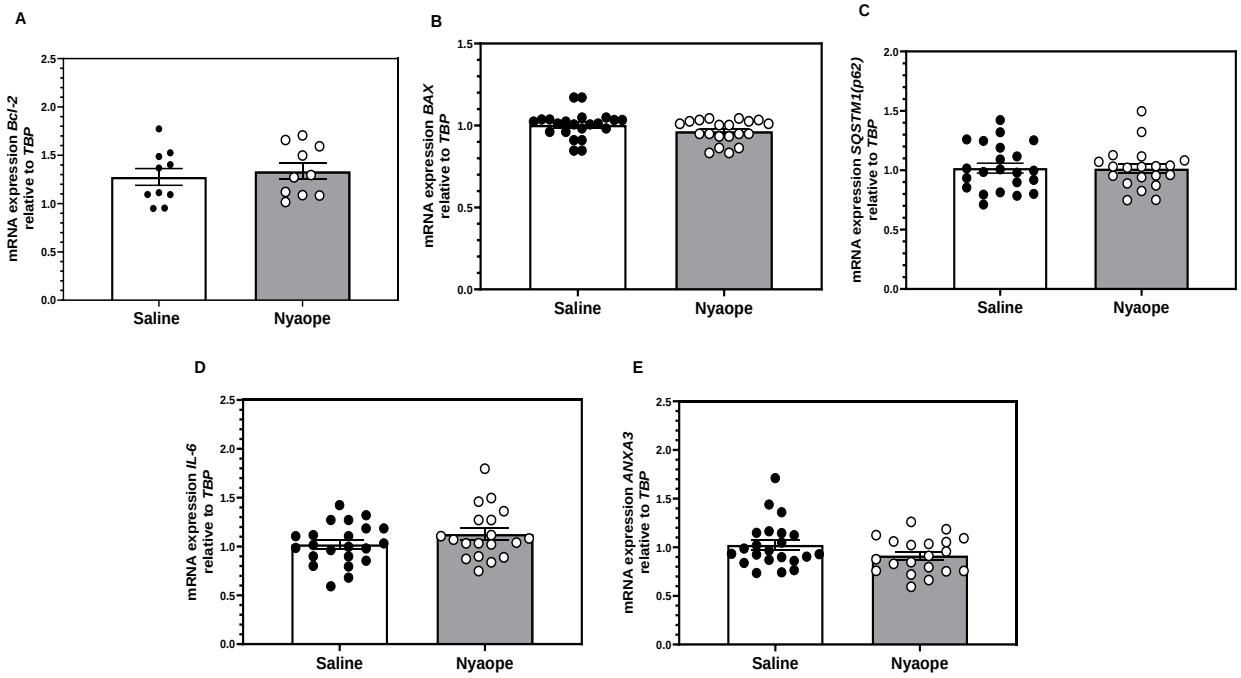


Figure 3.9: Graphs showing mRNA expression of molecular markers in brain frontal cortex tissue. Sprague-Dawley rats were treated with saline (n = 11, solid circles) or nyaope (0.4 mg/kg, i.p.; n = 11, open circles). Total RNA was isolated from brain frontal cortex tissue to determine the relative mRNA expression of genes involved in apoptosis: *Bcl-2* (A) and *BAX* (B), genes involved in autophagy: *p62* (C,) and microglial repair: *ANXA3* (D), and a gene that codes for the pro-inflammatory cytokine: *IL-6* (E). Results are presented as arbitrary units normalized for expression of the endogenous reference gene, *TBP*, using the delta-delta CT method. Data is expressed as mean ± SEM (A, B, C, D), or median and IQR (E). All experiments were performed in duplicate.

Similarly, the kidney samples showed no differences in expression levels of *BAX* (figure 3.10 B, p = 0.37), *p62* (figure 3.10 C, p = 0.44), *ANXA3* (figure 3.9D, p = 0.38), and *IL-6* (figure 3.10 E, p = 0.40) between nyaope-treated and saline-treated rats. In contrast, the expression level of *Bcl-2* was significantly decreased in nyaope-treated rats (0.40±0.05, p<0.0001) compared to saline-treated rats (1.00 ± 0.06, figure 3.10 A). Nyaope-treated animals exhibited a higher BAX/Bcl-2 ratio of 12.02 compared to the mRNA ratio of 2.57 that was reported for saline-treated rats, which suggests that nyaope exposure induces pro-apoptotic activity. The expression of the pro-apoptotic gene BAX was similar between the two groups, while the expression of the anti-apoptotic gene Bcl-2 was significantly different (Figure 3.10 A). Consequently, the reduction in Bcl-2 expression in the mitochondria resulted to a higher BAX/Bcl-2 ratio (nyaope-treated rats), which an indicator of potential pro-apoptotic activity,

as the hepatic cells' resistance to apoptosis has been altered. Therefore, the nyaope-treated rats *BAX/Bcl-2* ratio was 4-fold higher than the saline-treated *BAX/Bcl-2* ratio ($p<0.0001$).

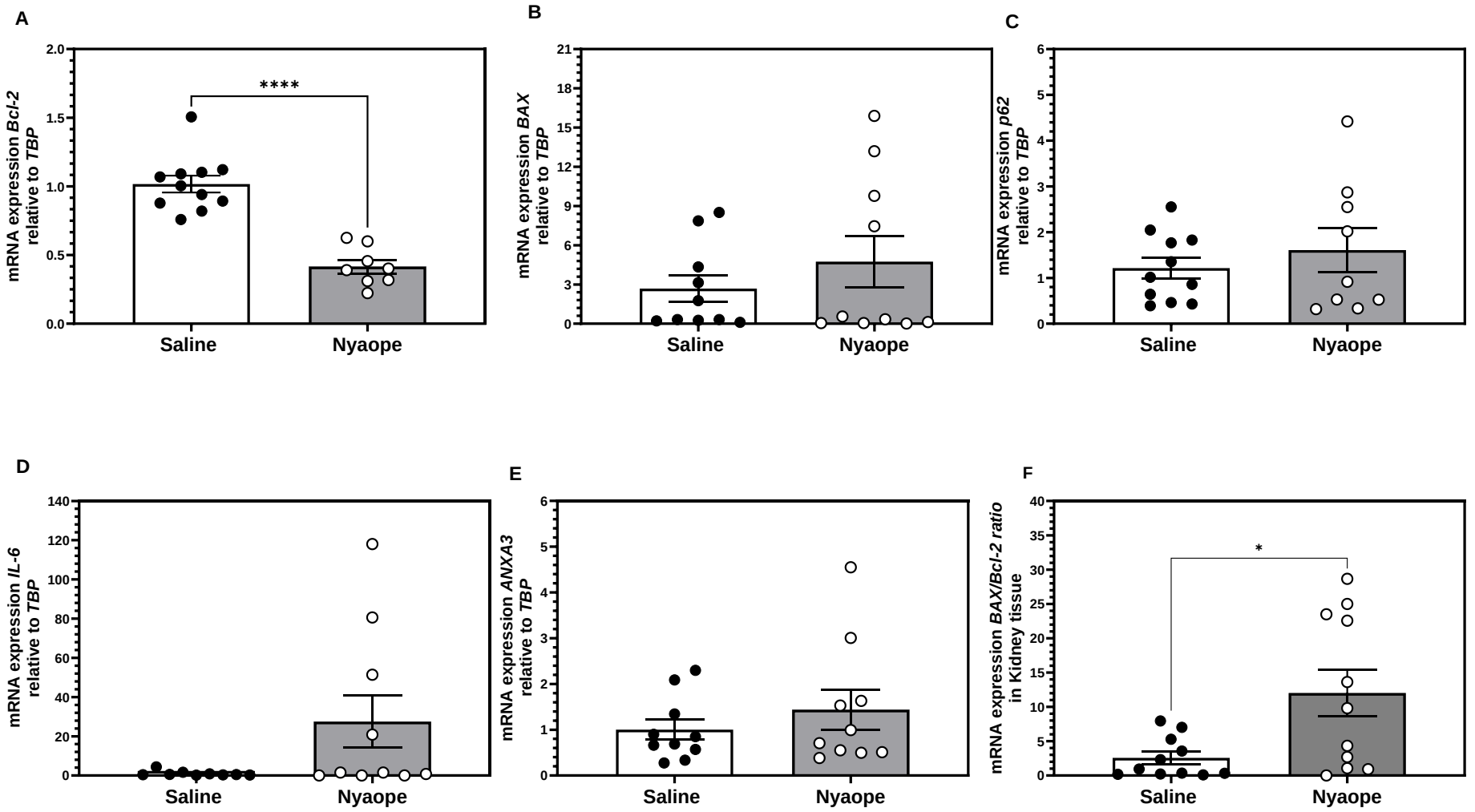


Figure 3.10: Graphs showing mRNA expression of molecular markers in kidney tissue. Sprague Dawley rats were treated with saline (n = 11, solid dots) or nyaope (0.4 mg/kg, i.p.; n = 11, open dots). Total RNA was isolated from kidney tissue to determine the relative mRNA expression of genes involved in apoptosis:

Bcl-2 (A) and *BAX* (B), genes involved in autophagy: *p62* (C) and microglial repair: *ANXA3* (D), and a gene of a pro-inflammatory cytokine: *IL-6* (E). Results are presented as arbitrary units normalised for expression of the endogenous reference gene, *TBP*, using the delta-delta CT method. Data is given as mean ± SEM (A, B, C, D), or median and IQR (E) of 11 nyaope-treated (open dots) and 11 saline-treated controls (solid dots). All experiments were performed in duplicate.

****$p \leq 0.001$ (C), unpaired t-test, significantly different from saline-treated rats.

3.4 Plasma enzyme assays

Plasma markers that circulate in the body can serve as potential indicators of end-organ damage in cases of acute dose toxicity. In our acute study, we recorded no significant differences in the plasma levels AST between nyaope-treated rats (157.30 ± 6.02 µg/L; p = 0.68) and saline-treated rats (161.50 ± 8.14 µg/L; figure 3.11 A). Likewise, there was no significant difference in the plasma levels of AST between the nyaope-treated rats (61.90±1.60 µg/L: p = 0.82) compared to the saline-treated rats (62.55 ± 2.30; figure 3.11 B). Additionally, the plasma levels of kidney toxicity marker, creatinine were also similar in both nyaope-treated rats (0.28 ± 0.04 µg/L; p = 0.59) and saline-treated rats (0.23 ± 0.03 µg/L, figure 3.11 C).

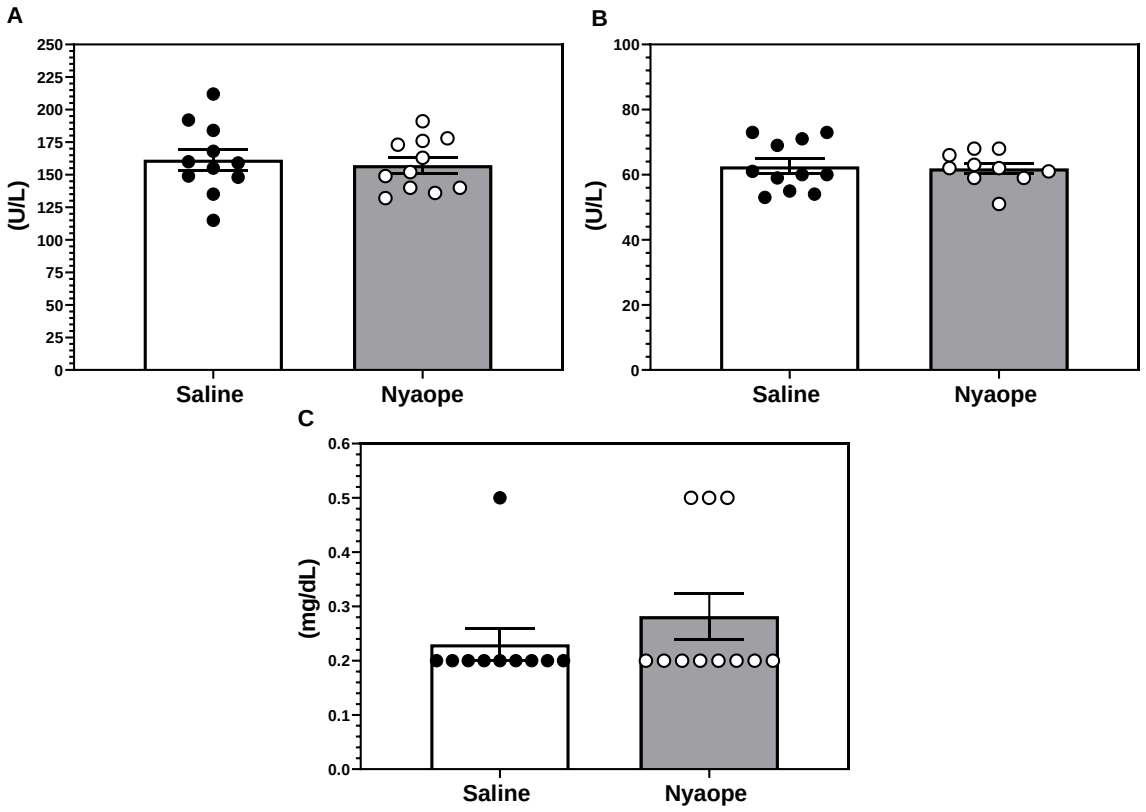


Figure 3.11: Plasma biochemical indicators were used to estimate the extent of organ damage caused by administering 0.4 mg/kg nyaope. The levels of liver enzymes aspartate aminotransferase (A), alanine aminotransferase (B), and the kidney biochemical marker creatinine (C) that were released into the blood circulation of Sprague. Data is given mean ± SEM (A, B, C). All determinations were performed in duplicate.

4. CHAPTER 4: DISCUSSION

4.1 Introduction

The progressive increase in the use of illegal substances is a worldwide challenge (UNODC, 2020b, 2022b). Nyaope is a cocktail drug that contains mainly heroin of a very high grade (Sekhotha, 2022). Consumption of nyaope has been associated with severe detrimental consequences for young adults in terms of their physical and mental health, as well as their roles in society (Fernandes & Mokwena, 2021; Varshney *et al*., 2023). Previous studies within our laboratory (Morgan *et al*., 2019, 2022), characterised a cohort of nyaope users and reported acute neurobehavioral abnormalities; such as generalised anxiety disorder, major depressive episodes and suicidality. A similar study in Johannesburg, South Africa reported neurostructural alterations that included extensive grey matter atrophy in brain areas linked to impulse control, decision-making, and cognitive functioning (Ndlovu *et al*., 2021). These findings confirm that nyaope exerts widespread damage to the central nervous system and reflect the devastating cumulative effects of nyaope use over an extended period of time. The aim of the present study was to investigate the acute effects of nyaope. Therefore, we examined the effects of a single dose of nyaope on brain, kidney and liver function, using a rat model.

In summary, rats were administered a single dose of nyaope, followed by a 30-minute period of behavioural monitoring and analysis utilising the AnyMaze video-tracking system. Both qualitative and quantitative methods were employed to assess behaviour. Qualitative descriptions aligned well with the quantitative measures across parameters such as locomotor activity, mood, and exploratory behaviour. The results demonstrated that rats treated with nyaope exhibited decreased locomotor activity, including reduced distance travelled. Additionally, the presence of heroin in nyaope impacted the mood of the animals when administered at a dosage of 0.4 mg/kg/bw and altered their exploratory behaviour in the open field test. After an additional 30 minutes, blood and tissue samples were collected from the brain frontal cortex, liver, and kidney for biochemical analyses. This allowed for an investigation into the gene expression levels of markers associated with acute toxicity, including *Bcl-2*, *BAX*, *p62*, ANXA-3, and *IL-6*. Our findings indicated no significant differences in the gene expression in kidney tissue of these marker between the saline-treated and nyaope-treated rat groups, with the exception of the downregulation of Bcl-2 gene expression of nyaope-treated rats. Furthermore, we observed no significant variations in the expression of markers associated with liver and kidney toxicity, namely ALT, AST, and creatinine, as determined using the IDEXX test.

4.2 Qualitative description of behaviour

Qualitative analysis of behaviour is not a common technique employed in neurobehavioural experiments. However, it allows the researcher to make a detailed recording of behaviours and clinical observation not usually captured by automated tracking systems. Furthermore, it provides a multi-perspective and descriptive analysis of the animal's behaviour, which complements quantitative data obtained from automated tracking systems, in this instance; the AnyMaze tool.

Changes in the behaviour of the nyaope-treated rats by qualitative OFT evaluation were immediately evident in comparison to the saline-treated rats. The track plots revealed apparent differences in the movement and preferred paths, as there was a dark purple presence in the outer zone of track plots of the nyaope-treated rats, which is a marker of higher activity in the zone. This preference for the outer area of the OFT was also observed in the saline-treated rats, however, the saline-treated rats also showed consistent exploration of the unprotected open centre of the OFT. In addition, the rats treated with nyaope had initial bursts of hyperactivity within the first 13 minutes that was different from the saline-treated rats. This behaviour was followed by extreme lethargy, tremors and hypoactivity. These observations correspond well with multiple human social studies describing nyaope to cause immediate euphoric "high" effects with heavy cravings reported even after first-time usage (Cronje, 2015; DOJ & CD, 2022). Also, nyaope users were further described as having half-dazed expressions associated with slow movement following the high (Mokwena, 2016; Mokwena & Makuwerere, 2021; Moroatshehla *et al*., 2020). In contrast, rats treated with saline displayed sustained activity over the entire 30-minute observation period, primarily dedicating their time to exploring the apparatus. This conduct aligns with the typical human behavioural tendencies of curiosity and exploratory actions which is part of the learning process (Poli *et al*., 2022; Shin & Kim, 2019). Psychologically, curiosity is characterised as a motivational state that instils a thirst for knowledge and serves as a fundamental factor in stimulating exploratory behaviour, which is observed in both humans and animals, are both involved in goal-driven behaviour (Buschman & Miller, 2014; Edelman, 1997; Haziza, 2022; Le Merre *et al*., 2018).

The nyaope-treated rats further developed hypoxic limbs, which was likely due to the respiratory depression observed in these rats. This observation is coherent with human studies that reported dose-dependent brain hypoxia that results from opioid-induced respiratory depression (Kiyatkin, 2019; Mashiloane *et al*., 2021; Oelhaf & Azadfard, 2022; Stowe *et al*., 2020; Strauss, 2022; Varshney *et al*., 2023). Furthermore, opioids have capacity reduce the responsiveness of chemoreceptors to elevated carbon dioxide and induce respiratory depression

interacting with μ-opioid receptors found in the respiratory centres within the medulla and pons (McDonald & Lambert, 2005).

Nyaope-treated rats also displayed tachycardia possibly as a compensatory mechanism to the lack of oxygen. Synthetic opioids have been shown to induce arrhythmias by prolonging the time taken for ventricular depolarisation and repolarization (Q-T interval, ECG) or time taken from ventricular systole to occur, thereby causing ventricular tachycardiac events (Behzadi *et al*., 2018; H. Smith *et al*., 2014; Szeto *et al*., 1990; Y. S. Zhu & Szeto, 1989). This phenomenon is readily apparent in human investigations of individuals who use heroin, wherein an extended Q-T interval is observed, a condition typically correlated with cardiac palpitations, feelings of light-headedness, anxiety and muscular weakness (Fanoe *et al*., 2007; Yildirim *et al*., 2021). It is also possible that the tachycardia observed in the nyaope-treated rats could be a direct effect of exposure to nyaope. Several human studies have linked opioid-induced tachycardia to withdrawal symptoms in individuals with opioid use disorder and clinical cases of overdose (Hayley, 2014; Hoffman *et al*., 2021; Hyun *et al*., 2020; Jovanovic *et al*., 2015; Torres-Lockhart *et al*., 2022). Another study proposed an alternative mechanism, suggesting that tachycardia is a result of chronically elevated cyclic adenosine monophosphate (cAMP), which leads to a heightened autonomic system activation (hyperadrenergic state) characterised by increased levels of norepinephrine and epinephrine (Chan & Lutfy, 2016; Torres-Lockhart *et al*., 2022). This response is believed to promote wakefulness and remains elevated after cessation and is also associated with symptoms such as anxiety, abdominal cramping, nausea, and vomiting in withdrawal (McDonald & Lambert, 2005, 2013; Torres-Lockhart *et al*., 2022). The primary locus coeruleus (LC) projections to the PFC, amygdala, and hippocampus interact with opioids to inhibit the cAMP pathway (Kosten & Baxter, 2019). Following opioid cessation, the LC experiences an overproduction of cAMP, resulting in elevated levels of norepinephrine, which is proposed to be the fundamental neurobiological genealogy behind opioid withdrawal symptoms. However, this explanation seems unlikely to account for the tachycardia observed in the current study owing to the acute nature of the experiment. Additionally, after the administration of nyaope and subsequent behavioural tests (Figure 3.1), the tails of the treated rats displayed stiffness, and it is worth noting that rats rely on their tails for purposes like proprioception and temperature regulation (Lehnen *et al*., 2015). Past research has established a link between opioids, temperature irregularities, and neurotoxic mechanisms in the brain (Kiyatkin, 2013). Therefore, the changes in temperature regulation and bodily equilibrium could potentially account for the observed tail rigidity in nyaope treated rats (Figure 3.1).

In the 30-minute period wherein rats were placed in the cage before termination, he nyaope-treated rats further exhibited tremors and high reactivity to sudden ambient noise, which was absent in saline-treated control rats. Furthermore, saline-treated rats consistently displayed more bouts of licking, scratching and face-washing throughout the duration of the test when compared to nyaope-treated rats. In essence, we observed certain clinical signs and also behaviour dissimilarities between the nyaope-treated rats and saline-treated rats which was possibly influenced by the acute nyaope dose.

4.3 Quantitative analysis of behaviour

In addition to the visual qualitative descriptions, the recorded videos of the rats' behaviour were also subjected to quantitative analysis using AnyMaze software. The OFT apparatus is a widely utilized tool for assessing locomotor activity, mood, and exploratory behaviour in animal models (Kraeuter *et al*., 2019b; Walsh & Cummins, 1976). The primary focus of this test revolves around monitoring the movement of animals, a parameter susceptible to influences like their exploratory drive, emotional states, exposure to novel environments, and fear-related behaviours, such as anxiety (Gould *et al*., 2009). Additionally, it serves as a popular method for evaluating the impact of drugs on the motor behaviour of animals (Gould *et al*., 2009). Furthermore, the video-tracking system creates provision to track movement parameter in defined zones (outer and centre zone) of the parameters, detecting moving sequences and immobility.

The quantitative is complementary to the quantitative observations as, the track plots showed that the nyaope-treated rats displayed a lack of interest in exploring the centre of the open field with intense thigmotaxis behaviour (staying close to walls when exploring open spaces). In contrast, saline-treated rat activity was much more evenly spread across the apparatus with multiple centre zone crossings. Therefore, the track plots presented early indications of exploratory behaviour in the saline-treated rats when compared to nyaope-treated rats. Since the nyaope-treated rats restricted most of their movement to the outer zone and walls of the open field, this behaviour was considered anxious-like and hence suggested that nyaope was able to induce a mood change when consumed. This finding was coherent with data from our clinical study whereby nyaope consumers displayed high levels of generalised anxiety disorder (Morgan *et al*., 2019). Furthermore, it is also well known that the development of negative mood states are also associated with of the deleterious consequences of heroin intake (Reid *et al*., 2018).

When assessing locomotor activity, we use the AnyMaze tool to measure the total distance covered by the rats within the whole OFT apparatus in the 30-minute test period. Additionally,

we segmented the total distance travelled into the distance travelled in the outer zone and centre zone in both rat groups. Our findings indicate a noteworthy decrease in the total distance travelled by rats exposed to nyaope during the 30-minute test. Moreover, these differences were also evident in the distances covered within the unprotected and open centre zones. However, similar distinctions were not observed when comparing the nyaope-treated rats with the saline-treated control rats in the outer zone. It is evident that the variance in the overall distance travelled between the two groups can be attributed to the curiosity of saline-treated rats to explore the centre area. The inclination towards thigmotactic behaviour, characterised by a tendency to seek refuge or remain close to vertical surfaces, as well as a preference for the outer zone, is a behaviour observed in both rats and insects. This behaviour serves as a defence mechanism against potential predators, as reported in other animal studies (Lamprea *et al*., 2008; Martinez & Morato, 2004; Salazar *et al*., 2018). While the preference for staying within the thigmotactic area is likely an initial response to the animal's danger aversion in unfamiliar environments (Padilla *et al*., 2010), it is worth noting that following familiarisation, the saline-treated rats in the current study covered more than twice the distance within the centre zone compared to the nyaope-treated rats. Furthermore, our findings show that nyaope-treated rats exhibited a diminished probability of transitioning from a state of immobility to mobility, both in the central and outer zones. This discovery enhances our understanding of why they covered a shorter distance during the test, as less likelihood to transition from immobility to mobile will lead to decreased movement and distance travelled. In terms of visual video analysis, we observed a sudden and intense bout of hyperactivity in the nyaope-treated rats within the initial 13 minutes. This observation likely accounts for the similarities in the average speed of animals in both the central and outer zones. In contrast, rats treated with saline consistently displayed a notably higher overall movement speed compared to their nyaope-treated counterparts. Nyaope mainly consists of heroin, which was found to quickly metabolise into 6-monoacetate-morphine (6-MAM), which in a similar animal study its concentration was shown to increase rapidly in brain tissue after the first hour of administration, it was further associated with the immediate stimulation of a psychomotor behavioural response (Andersen *et al*., 2009). Cortical areas such as the prefrontal cortex primarily express Sigma- and -MOR opioid receptors, which could be responsible for changes in animal behaviour following nyaope treatment. Furthermore, opioid receptors in the nucleus accumbens are suggested to contribute to heroin-induced hyperactivity (Amalric & Koob, 1985; Koob, 2020; Koob & Volkow, 2009). Therefore, the acute hyperactivity in the nyaope-treated rats may be a behavioural indication of the heroin prodrug interacting with the mesocorticolimbic pathways and related structures in the brain. The heightened capacity of heroin to convey 6-MAM, a compound with a strong affinity to μ-opioid

receptors within the brain, is linked to the induction of acute hyperactivity in rats (Eriksen *et al*., 2014, 2016; Seleman *et al*., 2014; J. J. Zhang & Kong, 2017). Additionally, the configuration of heroin's hydrogen bonds contributes significantly to a hundred-fold acceleration in its ability to swiftly breach the blood-brain barrier (Oldendorf *et al*., 1972; Pimentel *et al*., 2020). The increased lipophilicity and temporary storage further amplify its potential for toxicity and addiction (Pimentel *et al*., 2020).

Taken together, these findings show that the reduced average speed and a significantly lower frequency of transitions from immobility to mobility were associated with the decreased distance travelled by the nyaope-treated rats compared to those treated with saline. Thus, the administration of nyaope induced overall hypo-locomotor activity in the rats during the OFT. Our findings are not isolated as other heroin animal studies had similar findings concerning the effect of the drug on locomotor activity and passivity (Gutierrez *et al*., 2022). Furthermore, Gutierrez and colleagues found that the co-administration of heroin and delta-9-tetrahydrocannabinol (THC) had an additive effect with suspected physiological side effects. This is a cause of concern considering that the major route of intake of nyaope by its users is by smoking it in combination with Cannabis (Ellgren *et al*., 2006; Morgan *et al*., 2019; Mthembi *et al*., 2018, 2019; Varshney *et al*., 2023).

The open-field apparatus is designed to assess the emotionality of rodents, which is regulated by multiple areas of the brain (Kraeuter *et al*., 2019b). Several measures which include time spent in the various zones of the apparatus, urination, defecation, grooming and washing (Walsh & Cummins, 1976), are considered when the behaviour of the animal is assessed in the open field (Kumar *et al*., 2013). Our results showed that nyaope-treated rats spent less time mobile in both the outer zone and centre zone. Furthermore, the saline-treated rats spent twice as much time mobile in the open centre zone compared to the nyaope-treated rats. This evidence supports the suggestion that acute injection of heroin containing nyaope has an effect on the affinity of the animals towards the highly illuminated centre and decreases overall mobility with the exception of the hyperactive phase.

In mice, active avoidance of the centre zone of the open field has been accepted as an indication of anxiety-like behaviour even though some criticism was raised in that the apparatus lacked sensitivity to validate anxiety-like effects from a clinical perspective (Prut & Belzung, 2003; Seibenhener & Wooten, 2015). In our study, we qualitatively observed that nyaope-treated rats were more likely to defecate and urinate in the duration of experiment which suggested a heightened level of emotionality and anxiety-like behaviour (Gould *et al*., 2009; Seibenhener & Wooten, 2015; A. L. Smith *et al*., 2011; Walsh & Cummins, 1976). A dated study on rats

revealed that animals with high mobility had low defecation during tests under bright lighting, which we also observed as our apparatus contained a highly illuminated centre and the saline-treated rats had low defecation (Igarashp *et al*., 1995). Grooming behaviour in rodents is closely linked to their emotional state and mood (Archer, 1973; Bolles, 1960). This behaviour encompasses various actions, such as licking different parts of their body (licking their paws, feet, and genitals, washing their face and ears). Grooming behaviours have been associated with self-care, maintaining good hygiene and being comfortable in the apparatus (Haj-Mirzaian *et al*., 2019; Kalueff & Stewart, 2015), while bad hygiene and lack of self-care are some of the hallmarks of substance users (Masombuka & Mathibela, 2022; Mokwena, 2016; Mokwena & Makuwerere, 2021; Tetarwal *et al*., 2019). Our findings relating to grooming habits and self-care are therefore coherent with clinical (Estanislau *et al*., 2019; Kalueff *et al*., 2016; Niesink & Van Ree, 1989). There is strong evidence in the literature reporting rodent observations models of depression exhibiting reduced grooming behaviour that was associated with poor hygiene and despair (Kalueff *et al*., 2016). In our study, nyaope-treated rats had a significantly lower number of grooming bouts with nine out of eleven rats not attempting a single grooming bout. Self- grooming is modulated by the limbic system with the amygdala and hypothalamus playing primary roles in regulating this behaviour (Kalueff & Stewart, 2015). These brain structures are also involved in the control of anxiety-like behaviour, fear responses, and depression. Furthermore, the decrease in dopamine in the amygdala is associated with anxiety-like behaviour (Kalueff *et al*., 2016; Kalueff & Stewart, 2015). Therefore, it is possible that in our nyaope-treated rats, the heroin-containing drug most likely caused an initial increase in dopamine neurotransmission (Diana, 2011; George *et al*., 2021; Gottås *et al*., 2013, 2014; Koob & Volkow, 2009; Niesink & Van Ree, 1989; Volkow *et al*., 2007). However, once the drug's effects wear off, dopamine levels plunge below baseline in various brain regions that are involved in mediating depressive and anxiety-like behaviours.

In unfamiliar settings or when encountering unfamiliar objects, animals often exhibit behaviours' such as orienting themselves towards novel objects or surroundings by moving around the environment and engaging in sniffing activities (G. R. Brown & Nemes, 2008). And the open-field apparent has in rat studies shown to be a tool that can be used to assess anxiety-like behaviours (Belovicova *et al*., 2017; Heinz *et al*., 2021).

Therefore, we utilised the OFT to assess exploratory behaviour through measure such as the number of line crossings, number of animal head entries into the centre zone and rearing as indicators. These indices are complementary to the locomotor activity and mood parameters. We observed that nyaope-treated rats had a significantly lower number of line crossings overall

in the 30-minute duration of the test. The number of line crossings is an indicator of the rat's natural drive to explore the novel environment. Nyaope-treated rats also had fewer rearing episodes and fewer head entries into the centre zone compared to saline-treated rats. Rearing is a dependent variable of both locomotor and exploratory behaviour. Rearing includes two types, wherein it can be described as the rodent placing both limbs on the apparatus/vertical wall (also referred to as wall leaning), or it is when the animal briefly stands on both hind legs without support. This behaviour is subject to experimental context with studies relating low rearing to be associated with avoidance of perceived potential environmental dangers (Djiogue *et al*., 2018; Sturman *et al*., 2018).

The decrease in both the number of line crossings and rearing in the nyaope-treated rats when compared with saline-treated rats, may be indicative of possible cognitive impairment and change in mood state (Djiogue *et al*., 2018). These findings correspond with the locomotor and mood results we have observed, as changes in the mood states of the animal can mediate a low exploratory drive within nyaope-treated rats and further result in lower locomotor activity when compared with saline-treated rats. It is also worth noting that exogenous opioids act on innate analgesic receptors of endorphins, which also mediate sedation and other undesirable effects (Holden *et al*., 2005; John *et al*., 2007). In clinical settings, morphine is employed as an anti-nociceptive and co- anaesthetic agent for brief procedures, albeit with side effects like lethargy, drowsiness, and dizziness (McDonald & Lambert, 2013; H. Smith *et al*., 2014). Opioids are linked to the disturbance of the ascending reticular activating system, which oversees functions such as sleep-wake cycles, attention, and arousal, within the trinity of the thalamus, brainstem, and hypothalamus (Kosten & Baxter, 2019; H. Smith *et al*., 2014). Therefore, the decreased wakefulness and lethargy of the nyaope-treated rats are likely to have also been influenced by the sedative properties associated with opioid-containing nyaope, possibly accounting for the nyaope-treated animal's reduced exploratory drive and locomotor activity.

4.4 Identifying a molecular basis for nyaope effects

Opioids are primarily metabolised in the liver through a group of enzymes known as carboxylesterases. Additionally, there is evidence suggesting that heroin acts as a prodrug, easily crossing the blood-brain barrier, to be converted into 6-MAM. This metabolite then interacts with opioid receptors located in various regions of the brain. The overall toxicity of opioids is mainly mediated by the generation of nitric oxide, reactive oxygen species, leading to oxidative stress, inflammation, DNA damage and eventual cell death (Y. Wang *et al*., 2023; Xu *et al*., 2006; Y. Zhang, 2018). In the present study, we investigated the mRNA expression levels of markers of inflammation (*IL-6*), cell death by apoptosis (*BAX* and *Bcl-2*) or autophagy

(p62/*SQSTM1*), and the activation of microglia (*ANXA3*). We also examined the impact of nyaope on liver and kidney functioning by assessing the drug's effect on the enzyme markers aspartate aminotransferase (AST) and alanine transaminase (ALT) for the liver, and creatinine levels for the kidney.

The validity of any mRNA expression experiment depends on the reliability of the reference gene included in the study. For this reason, we evaluated the suitability of our reference gene, *TBP*, in brain, liver and kidney tissue in both rat groups. Analysing our mRNA expression data, we observed that the expression of our reference gene remained stable in both brain and kidney tissues, as indicated by the Ct values falling within the acceptable range of 18 ± 30 (Caraguel *et al*., 2011; Wisc, 2023). However, in the case of liver tissue treated with saline, the average CT value for *TBP* was above this acceptable range, rendering the data unreliable for further analysis.

A study conducted in mice by Tatsumi *et al*. (2008) is in agreement with our findings that *TBP* is not a suitable housekeeping gene for liver tissue (Tatsumi *et al*., 2008). Other studies have shown that TPB expression is considerably variable across various adult rat tissues (including the liver, adrenal glands, prostate, fat pad, testis, and ovaries), as well as in cattle liver tissues under both physiological and toxicological conditions (Lisowski *et al*., 2008; Svingen *et al*., 2015). Furthermore, in the presence of inflammatory conditions, TBP was found to be one of the least stable reference gene markers in hepatic cell lines and liver tissue samples, as the TBP data fluctuated and was therefore deemed inconclusive for gene analysis. However, GAPDH emerged as a better candidate for normalising gene expression data in liver tissues (Hashemi *et al*., 2012). We were therefore confident in our decision to exclude the liver gene expression data from the current study.

Comparing saline-treated and nyaope-treated rats, the analysis of samples from both the frontal cortex and kidney showed no significant differences in the mRNA expression levels of the majority of gene markers investigated. The expression levels of *BAX*, *SQSTM1*, *ANXA3* and IL-6 genes were similar in both groups of rats. The only significant difference was obtained in kidney tissue where the comparative mRNA expression of *Bcl-2* was decreased in nyaope-treated rats compared with saline-treated control rats.

Gene expression markers in frontal cortical tissue showed that the processes of apoptosis, autophagy, inflammation, and microglial activation, may not yet have been initiated. This could be due to the dosage of 0.4 mg/kg/bw of heroin-containing nyaope administered having been too low to induce the production of reactive oxygen species that would impact signalling

pathways responsible for altering the gene expression. Alternatively, it may be possible that the time period post-treatment (60 minutes) was not enough to induce gene expression changes at the molecular level. Other studies conducted using a heroin-induced murine model demonstrated an increase in pro-apoptotic pathway proteins in various brain regions, including the cortex and hippocampus (Emeterio *et al*., 2006; Li *et al*., 2017; Tramullas *et al*., 2008). Furthermore, chronic prenatal exposure to heroin caused impaired learning and memory in offspring of the treated group and increased *BAX* and caspase-3 protein expression (Pu, Wang, Su, *et al*., 2015), while the *ANXA3* gene exhibited a significant upregulation 12 hours after the final morphine injection, following a four-day incremental treatment (10-40 mg/kg) of Sprague-Dawley rats (Desjardins *et al*., 2008). Additionally, Sil and colleagues (2018) have demonstrated that regions such as the PFC, basal ganglia, and cerebellum may potentially experience ER-stress-associated autophagy (Sil *et al*., 2018). This was indicated by the upregulation of LC3 and *p62* gene expression following human brain A-172 cell line exposure to morphine, which in turn led to neuroinflammation and heightened astrocyte activation (Sil *et al*., 2018). This suggests that heroin indeed exerts an impact on the brain and can lead to toxicity by triggering apoptotic and autophagic pathways. It's important to note, however, that the referenced studies differ in their experimental conditions from our model. These studies involved prolonged treatment durations and notably higher doses of heroin when compared to the model used in the current study.

It is crucial to note that there was a significant decrease in the expression of the *Bcl-2* gene within the kidneys of rats exposed to nyaope in the present study. This shift in gene expression had a substantial impact on the *BAX*/*Bcl-2* ratio, favouring a fourfold increase in the nyaope-treated group when compared to saline-treated rats. This elevated ratio points to an increased likelihood of mitochondria-mediated apoptosis in the kidneys of the nyaope-treated rats (Teijido & Dejean, 2010). The decline in *Bcl-2* gene expression and the resulting adjustment in the *BAX* to *Bcl-2* ratio is known cause to modifications in the mitochondrial membrane potential (Edlich, 2018; Havasi & Borkan, 2011). Consequently, this cascade triggers the release of cytochrome-c, which subsequently initiates the activation of effector caspases (Edlich, 2018). Although there were no significant differences in the expression of *BAX* itself between the two groups, the alteration in the ratio due to opioid intake has been previously associated with apoptotic cell death in liver and kidney tissue in chronic dosage studies (Awadalla & Salah-Eldin, 2015; Nasiraei-Moghadam *et al*., 2010). The ratio of BAX to Bcl-2 expression acts as a cell death switch, determining whether cells live or die in response to apoptotic stimuli (Gao & Wang, 2009). An increased BAX/Bcl-2 ratio lowers cellular resistance to apoptotic stimuli,

resulting in higher cell death and a reduced incidence of cell toxicity. These findings are not isolated, as street heroin cell treatment has been shown to lead to mitochondrial dysfunction and consequently apoptosis in neural and human embryonic kidney cell lines (Cunha-Oliveira *et al*., 2007; Domingues *et al*., 2006; Oliveira *et al*., 2002, 2003).

Examination of the expression of *ANXA3* and *IL-6* as potential indicators of microglial activation and pro-inflammation respectively, tissue as well as in the kidney, showed no significant differences between the two groups of rats. The data therefore suggested that inflammatory pathways have not yet been activated. This was an unexpected result, as *IL-6* is known to cause the upregulation of μ-opioid receptor transcription which mediates multiple physiological functions that include respiration, mood, memory, temperature and motivation (Kraus, 2009). The incongruence between the sets of results could again be ascribed to differences in methodology. In the study of Houghtling and colleagues (2002) rodents were treated with 10 mg/kg of morphine, which is 25-fold higher than the dose that was used in the current study (Houghtling & Bayer, 2002). Furthermore, Kraus and colleagues (2004) also found elevated gene expression of *IL-6* after opioid treatment in an *SH-SY5Y* neuroblastoma cell-line *in vitro* study (Börner *et al*., 2004; Kraus, 2009).

Since the liver is responsible for drug metabolism and the kidney for its excretion, we also investigated the effects of acute nyaope administration on the functioning of these two organs. As such, we determined the levels of specific enzymes *ALT* and *AST* and metabolic end-products (creatinine) that are considered biomarkers of organ damage, liver and kidney, respectively. In the current study, *ALT* and *AST* activity in the nyaope-treated rats was similar to the control saline-treated rats. Increased plasma concentration and activity of ALT have been observed in rodents following heroin treatment, an observation that was correlated with increased oxidative stress and decreased anti-oxidative markers (Junhua *et al*., 2008; Y. T. Zhang *et al*., 2004). In these experiments, the rodents were subjected to doses exceeding 10 mg/kg of morphine or heroin. Furthermore, Zhang and colleagues (2004) administered 15 doses to their rats over a ten-day period. Similarly, other studies that showcased heightened levels of *ALT, AST*, and creatinine differed in terms of their methodological and experimental conditions compared to our study (Y. T. Zhang *et al*., 2004). These differences included the administration of higher doses of heroin or morphine and extended durations of drug treatment (Anbar *et al*., 2018; Atici *et al*., 2005; Samarghandian *et al*., 2014; Yue *et al*., 2023).

Clinical studies have supported the notion of opioid-induced alterations in liver function. Elevated *ALT* and *AST* plasma levels were reported for opioid users when compared to a control group (Alshamsi & Ahmed, 2021; Łangowska-Grodzka *et al*., 2016; Pawan *et al*., 2011;

Quraishi *et al*., 2021; Sanli *et al*., 2015). The participants of these clinical studies were described as chronic users with several confounding factors present including hepatitis infections and poly-drug use. Due to the complexity of human studies, comparison between clinical and preclinical findings must be carefully considered.

Creatinine is a kidney marker for renal glomerular dysfunction and toxicity. Our results showed that there was no significant difference in plasma creatinine levels between the nyaope-treated and saline-treated rats. The literature on opioid use and kidney function as reflected by creatinine levels, remains unclear. One clinical study found creatinine levels to be elevated in opioid-dependent individuals (Afarinesh *et al*., 2014; Sanli *et al*., 2015), while Quraishi and colleagues (2021) reported opioid users to have creatinine levels like controls (Quraishi *et al*., 2021). A review article investigating the potential link between opioid usage for pain management in cancer patients and the development of renal failure underscores the significant discrepancies found within the current literature (King *et al*., 2011; Sande *et al*., 2017). Moreover, these inconsistencies were documented in reviews of clinical studies (King *et al*., 2011; Sande *et al*., 2017), involving a range of opioids including morphine, oxycodone, hydromorphone, and others. Some of these studies reported elevated kidney toxicity markers such as creatinine levels and instances of renal failure in certain patients. In contrast, in other similar studies, there were no discernible differences between the opioid-treated group and the control group (King *et al*., 2011; Sande *et al*., 2017). These disparities may be explained by whether opioid use leads to an increase or decrease in creatinine levels and the subsequent development of renal impairment. Consequently, the current literature describing the association between opioids and creatinine levels remains inconclusive, and hence the relationship between opioid utilisation and renal impairment continues to be uncertain. Nevertheless, it has been reported that chronic use of opioids may potentially contribute to kidney damage, depending on the dosage and duration of treatment (Awadalla & Salah-Eldin, 2015; King *et al*., 2011; Sande *et al*., 2017). The results of the current study show that acute nyaope treatment may have no significant impact on kidney function, despite the increased mRNA expression of *Bcl-2*, suggesting early signs of apoptotic activity.

4.5 Conclusions

The study null hypothesis (H0) suggests that in our sample data, the acute administration of 0,4 mg/kg/bw of nyaope would not cause differences in the behaviour of the nyaope-treated rats and saline-treated rats. In our model, we investigated if H0 is true using qualitative observation and quantitative parameters of the open field test which were segmented into 3 broad categories; (1) locomotor activity, (2) mood, and (3) exploratory behaviour. Of note, in the assessment of

behaviour, all animals had never been exposed to the OFT apparatus to encourage novelty-evoked activity, which is processed differently in the brain compared to familiar stimuli in response to stress (Padilla *et al*., 2010).

The introduction of a single dose of nyaope significantly alters the locomotor activity of rats compared to those receiving saline treatment. Additionally, given that nyaope contains heroin, an opioid known for its sedative properties, this factor may have contributed to the observed decrease in locomotor activity. Our study also observed changes in the limbs, breathing patterns, and eye dilation in the nyaope-treated rats following the testing procedure. Additionally, we observed tail rigidity, which could be associated with nyaope's rapid conversion of heroin to 6-MAM, suggesting acute effects of the drug on the brain's mood-regulating centres involved in stimulating psychomotor activities. The nyaope-treated rats displayed reduced mobility within the thigmotaxic area, a behaviour associated with anxiety-like tendencies in rodents and insects. Moreover, the heightened emotional response of the nyaope-treated rats may have influenced their exploratory behaviour, which is typically stimulated by a novel environment, as seen in our OFT apparatus. In contrast, the saline-treated rats were more stimulated by the novelty of the environment and explored the OFT apparatus more extensively, resulting in higher locomotor activity. Since, heroin affects various brain regions and dopamine release, it influences memory, mood, sleep, learning, concentration, movement, and other bodily functions. Therefore, our study's findings on nyaope use align with the behavioural patterns and effects of heroin use on the dopamine-reward system, including changes in mood states.

Our study aimed to establish a connection between behavioural data, such as cognition and mood, and their correlation with molecular changes caused by acute nyaope doses. Therefore, we examined gene expression related to apoptosis, autophagy, microglial activation, and pro-inflammatory markers in the brain and kidney. Notably, we excluded the liver from our assessment due to unsuitable Ct values for the *TBP* housekeeping gene (Table 3.1 and 6.1) and the most failed readings (not detected) amongst all tissues.

The results obtained from RT-PCR showed that the acute administration of nyaope to rats did not induce molecular changes associated with toxicity within the one-hour experiment duration in the prefrontal cortex. Similarly, in the kidney, we observed similar gene marker expression between the nyaope-treated and saline-treated rats, with the exception of the downregulation of the anti-apoptotic marker *Bcl-2* in the nyaope-treated rats. This change in mRNA gene expression level of *Bcl-2* in nyaope-treated rats resulted in a 4-fold increase in *BAX*/*Bcl-2* ratio of nyaope-treated animal when compared to saline-treated rats ($p<0.0001$). An increase in this

ratio therefore suggests activation of pro-apoptotic mechanisms. When assessing plasma concentrations of liver enzymes *AST* and *ALT* for signs of acute toxicity, we found no significant differences between the rat groups. Creatinine, a kidney marker, also did not show substantial variations, although the *BAX/Bcl-2* ratio was altered in nyaope-treated rats.

In conclusion, our experiment revealed that an acute dose of nyaope induced impairments in cognition, locomotor activity, mood, and exploratory behaviour of Sprague-Dawley rat, resulting in anxiety-like behaviours. However, this acute effect did not translate into significant molecular changes in the brain, liver, and kidney, except for the observed alteration in the *Bcl-2* marker.

4.6 Limitations and recommendations

In the present study, we designed our research as an acute investigation, aiming to assess the immediate impacts of nyaope on both behaviour and molecular toxicity. It is important to note that much of the existing research on drug use, including heroin, *in vivo* experiments often involve repeated dosing of rodents. Moreover, these studies frequently employ significantly higher dosages compared to the levels used in our study. This divergence in dosing regimens creates a challenge in making direct, acute-to-acute comparisons.

The limitations within the existing scientific literature extend to the study of nyaope, especially within the context of South Africa and other street-heroin cocktails. Available research primarily consists of social studies, clinical observations, quantitative assessments of nyaope content, and questionnaire-based reports. Given that nyaope is a complex mixture of substances, the body of heroin-related research provides the most relevant comparative basis for our work. Nevertheless, such comparisons may not be entirely precise, and the scarcity of published literature specifically focusing on *in vivo* or *in vitro* studies related to nyaope, irrespective of geographical origin, further complicates this endeavour.

While noticeable behavioural changes occurred within the initial hour after nyaope treatment, it is possible that the experimental one-hour procedure may not be sufficient to produce molecular changes that correspond to the observed behavioural effects following nyaope treatment. Another notable limitation of our study is the inconsistency observed in the expression of the *TBP* housekeeping gene within the liver tissue, which hindered our ability to assess toxicity accurately. We recommend the utilisation of alternative housekeeping genes, such as *GAPDH*, for future studies, particularly as the liver stands as one of the most vulnerable organs to opioid toxicity. Another methodology improvement would be the measuring of protein expression in addition to gene expression. Additionally, we suggest that future

735 investigations consider employing a dosing regimen more representative of repeated injection
736 studies to better mirror the real-life experiences of individuals grappling with nyaope substance
737 use disorder over the course of years, which often leads to the development of mental disorders,
738 including heightened anxiety, and depression. Therefore, exploring other behavioural tools may
739 also be advantageous.

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6. APPENDICES

6.1 RT-PCR cycle threshold raw data

Table 6. 1: Ct values for the reference gene (*TBP)* raw RT-PCR data in brain, liver and kidney tissues.

Plate no	Brain tissue samples n=11 per plate		Liver tissue samples n=11 per plate		Kidney tissue samples n=11 per plate	
	Average Ct saline	Average Ct Nyaope	Average Ct saline	Average Ct Nyaope	Average Ct saline	Average Ct Nyaope
1	20.1	21.3	n/d	25.0	33.2	35.2
	20.3	22.7	34.9	22.0	32.6	28.1
	21.5	21.0	31.8	24.1	33.0	31.6
	20.0	20.1	26.5	21.8	32.0	31.5
	20.2	21.1	36.3	22.7	29.2	30.1
	19.7	20.2	35.7	22.7	27.2	34.7
	21.4	21.3	34.8	23.7	27.8	32.1
	16.5	19.3	37.0	24.2	26.3	30.8
	20.6	22.4	32.1	23.4	26.8	30.5
	19.6	24.6	35.2	n/d	30.0	29.4
	21.6	n/d	n/d	n/d	26.2	28.5
2	19.71	21.20	34.42	26.74	33.33	35.26
	20.13	22.55	36.97	21.98	33.33	26.30
	19.45	20.73	n/d	23.75	33.78	34.38
	19.80	19.71	26.22	21.65	35.82	26.21
	20.02	19.71	34.63	22.27	30.96	29.80
	19.91	19.97	34.45	22.27	27.56	34.92
	19.90	19.38	34.61	24.02	27.61	32.39
	19.93	19.03	36.99	22.47	27.24	33.17
	19.91	19.04	31.81	23.80	26.73	24.81
	19.60	21.21	35.07	n/d	33.55	28.89

	20.27	n/d	34.98	n/d	22.41	27.94
3	19.92	21.11	34.52	27.00	32.92	n/d
	20.29	22.48	33.43	20.71	33.24	27.08
	20.40	20.85	n/d	22.64	34.08	35.12
	19.76	20.02	24.98	20.67	35.58	n/d
	20.12	19.71	32.63	21.23	31.45	29.99
	20.11	20.10	33.40	20.62	27.12	33.90
	20.19	19.84	31.58	21.74	27.80	31.40
	20.16	19.63	35.40	21.12	26.91	33.06
	19.88	19.40	29.05	21.18	27.23	23.88
	19.12	21.66	32.80	n/d	33.02	29.22
	20.69	n/d	34.53	n/d	22.66	28.06
4	20.66	21.26	33.83	27.30	34.42	35.60
	21.14	23.39	35.67	21.25	32.80	26.23
	20.33	21.04	35.43	22.71	34.79	34.40
	20.63	20.52	25.45	22.13	37.03	n/d
	21.12	21.13	35.05	21.11	32.41	30.43
	20.78	20.84	34.51	20.83	26.85	36.13
	20.96	20.81	35.38	22.11	28.50	32.79
	20.77	20.12	36.27	21.56	27.07	34.11
	20.80	20.13	30.99	21.87	27.34	24.53
	20.32	21.64	34.36	n/d	34.46	29.59
	21.57	n/d	36.40	n/d	23.16	28.91
5	19.60	19.65	32.30	25.21	33.00	36.30
	19.70	22.05	33.60	20.15	33.75	25.15
	19.05	19.80	36.70	21.50	32.75	32.85
	19.75	19.65	24.65	19.50	35.55	n/d
	20.00	19.95	33.20	20.10	30.05	30.15

	20.20	19.75	32.30	20.00	26.25	35.55
	20.00	19.45	31.00	21.30	27.55	31.75
	19.80	19.25	32.80	20.35	26.90	32.10
	19.75	19.35	29.55	20.70	26.60	24.00
	19.60	22.95	33.90	n/d	32.40	29.15
	20.70	n/d	33.70	n/d	22.64	23.55
Mean ± SD	**20.15 ± 0.77**	**20.68 ± 1.24**	**33.21 ±3.13**	**22.34 ± 1.43**	**30.09 ± 3.80**	**30.62 ± 3.59**
Ct range	**16.50-21.60**	**19.03-24.60**	**24.65-35.70**	**19.50-27.30**	**22.41-37.03**	**23.55-36.30**
Ct not detected	**0**	**5**	**3**	**10**	**0**	**3**

6.2 Animal Ethics Certificate

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2022/05/04/D

APPLICANT: Mr K Mathikhi
School: School of Physiology; Department: N/A; Location: WRAF

PROJECT TITLE: Investigating the toxic effects of nyaope on the liver and brain of Wistar rats
Category: D; Species and Numbers involved: *30X, 250g, Male Wistar Rats*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 31 May 2022. This approval remains valid until 10 Jun 2024 and is conditional to the following (if blank there are no special conditions):

Condition 1	Condition 2	Condition 3	Condition 4
Pilot study	Necessary authorisation prior commencement of the study		

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____ _____ Date: _11/07/2022_____

(Chairperson of the AREC)

I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

Signed: ____ _____ Date: __11 July 2022_____

(Registered Veterinarian)

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Wits Research Animal Facility (WRAF): Dr Kim Jardine

6.3 South African Police's Forensic Science Laboratory Nyaope analysis

G.P.-S. 002-0222

SAP 21

SUID-AFRIKAANSE POLISIEDIENS

SOUTH AFRICAN POLICE SERVICE

Private Bag X620

Verwysing Reference	Lab No 610716/22
Navrae Enquiries	Lt Col P.M. MTHEMBI
Telefoon Telephone	(012) 845 5924
Faksnommer Fax number	(012) 845-5903

THE HEAD
FORENSIC SCIENCE LABORATORY
PRIVATE BAG X620
PRETORIA
0001

2022-12-08

Office of the Unit Commander
South African Police Services
NAT HEAD OFFICE CHEMISTRY

FORENSIC INVESTIGATION REGARDING:

COMPONENT / INSTANCE : NAT HEAD OFFICE CHEMISTRY
REFERENCE NUMBER : OTHER NUMBER 1/11/2022
CRIME / SUBJECT : DEPENDENCE PRODUCING SUBSTANCES

1. Attached a statement according to Section 212 of the Criminal Procedure Act, 1977(Act 51 of 1977) regarding the above mentioned case.

2. The attached SAP 83(A) must be completed when the case is finalised and returned to this office. **Under no circumstances should the SAP 83(A) be returned to this office without mentioning whether the exhibit material can be destroyed.**

3. The exhibit material will be under this office's safe-keeping until notification in accordance with paragraph 2.

4. If exhibit material is needed for court purposes, a written request, signed by the commanding officer, should be forwarded to this office.

5. Arrangements must be made with this office, at least 14 days before the exhibit material is required, so as to prevent any delay.

6. Mention above mentioned Lab Number and entry in any further correspondence / enquiries. (ex. summons)

.....: LIEUTENANT COLONEL
F/HEAD: FORENSIC SCIENCE LABORATORY
(P.M. MTHEMBI)

LAB NO. 610716/22

NAT HEAD OFFICE CHEMISTRY OTHER NUMBER 01/11/2022

AFFIDAVIT IN TERMS OF SECTION 212 OF THE CRIMINAL PROCEDURE ACT, 1977 (ACT 51 OF 1977) :

PABALALA MESHACK MTHEMBI declares under oath, in accordance with Section 212 subsections (4)(a) and (8)(a) of the Criminal Procedure Act, 1977 (Act 51of 1977), as follows:

1.

I, number **0542763-1,** am a **Lieutenant Colonel** in the South African Police Service, attached to the Chemistry Unit of the Forensic Science Laboratory, 218 Visagie Street, Pretoria with telephone 012 845 5924. I am a **Chief Forensic Analyst**, and I am in the service of the State.

2.

I have been attached to the Chemistry Section since 2002 as a Forensic Analyst. I am in possession of a B.Sc. degree with majors in Chemistry and Mathematics, and a B.Sc. Honours degree in Chemistry both from the University of South Africa. I am also in possession of a M.Sc. degree in chemistry from the University of Pretoria and a PhD degree in Forensic Science from the University of the Free State. I have attended internal and external training including identification of drug and medicine, GCMS and FTIR. I have 31 years' experience in chemical analysis of which 18 years are at the Forensic Science Laboratory. I am currently competent in forensic drug analysis.

3.

During the execution of my official duties, on 2022-12-05, I received one sealed evidence bag with unique number PA6004336240, from the Drug Analysis Subsection Administration. The seal was intact. The bag was opened by me. The evidence bag was marked inter alia **"NAT HQ: FSL ENQ 1/11/2022**" and contained one glass bottle. The glass bottle was marked inter alia **"27/9/22"** and contained an amount of powder.

4.

I was requested to examine the exhibit material in order to determine whether it contains any substances as listed in the Schedules of the Medicines and Related Substances Control Act, 1965 (Act 101 of 1965) and/or the Drugs and Drug Trafficking Act, 1992 (Act 140 of 1992).

5.

I, during the execution of my official duties, examined the exhibit material(s) with processes requiring skill in Chemistry. The following technique was used Coupled Gas Chromatography Mass Spectrometry (GC-MS). GC-MS is an internationally accepted analytical comparative technique where compounds in the gas phase are separated and a characteristic pattern (mass spectrum) of the separate compounds is obtained. The identity of the compounds was thus established by comparing their separation characteristics (retention time) and characteristic patterns (mass spectra) with that of known reference material collected on the same instrument under the same conditions.

The system suitability of instrumentation used during the analyses of the above mentioned exhibit material was verified and deemed suitable for use.

The instrumentation used during mass determinations of the above mentioned exhibit material was verified and deemed suitable for use.

6.

I examined the exhibit material by using the above mentioned technique and by comparison with reference material determined that it contains diacetylmorphine (heroin). Diacetylmorphine (heroin) is listed Part III of Schedule 2 of the Drugs and Drug Trafficking Act, 1992 (Act 140 of 1992). The mass of the exhibit material was 0.15 g.

7.

During the execution of my official duties, I kept the exhibit material exclusively under my safekeeping for purposes of examination thereof, by placing it behind lock and key from 2022-12-05 to 2022-12-07. After examination the exhibit material was sealed in an evidence bag, with the unique number PA6004336292, and archived.

8.

I know and understand the contents of this declaration.
I have no objection to taking the prescribed oath.
I consider the prescribed oath to be binding on my conscience.

..... **LIEUTENANT COLONEL**
PABALALA MESHACK MTHEMBI

I certify that the deponent has acknowledged that **he** knows and understands the contents of this declaration which was sworn to me and the deponent's signature was placed thereon in my presence at **PRETORIA** on 2022-12-11.

7213171-3
TERECIA TLHOLOGELO LEBYANE
COMMISSIONER OF OATH
Forensic Science Laboratory
218 Visagie Street
PRETORIA 0001

SOUTH AFRICAN POLICE SERVICE

SUID-AFRIKAANSE POLISIEDIENS
FORENSIESE WETENSKAP LABORATORIUM
PRIVAATSAK / PRIVATE BAG X620
11 DEC 2022
FORENSIC SCIENCE LABORATORY
PRETORIA 0001
SOUTH AFRICAN POLICE SERVICE

LAB 610716-22

1. **Background**

   A powder sample from the University of the Witwatersrand, suspected to contain heroin was submitted to the South African Police's Forensic Science Laboratory (SAPS-FSL) for analysis. It was requested to determine if the sample indeed contains diamorphine (heroin) and any other adulterants.

2. **Chemicals**

   Dichloromethane (for HPLC, ≥99.8%) and tetracosane (99%) were purchased from Sigma-Aldrich. Certified reference diamorphine (both 1 mg/mL) standard was purchased from Ceriliant-Sigma Aldrich. The standard was prepared as a cocktail mixture of methaqualone, cocaine, tetrahydrocannabinol and diamorphine standards (MCTH). All chemicals and solvents used without further purification.

3. **Preparation of internal standards and samples**

   The internal standard solution, tetracosane (C_{24}), was prepared at a final concentration of 0.02 mg/mL in dichloromethane. The internal standard solution was used to prepare samples, before analysis using gas chromatography-mass spectrometry (GC-MS). For GC-MS sample preparation, 10mg sample was dissolved in 50ml dichloromethane sonicated for 15 minute and filtered. 2.0 mL of solution was transferred into a GC-MS vial and submitted for analysis without further dilution. The samples were analysed qualitatively in triplicate.

4. **Instrumentation**

   GC-MS analysis was carried out using an Agilent Technologies system (Chemetrix, RSA) consisting of a gas chromatograph (GC), Agilent 7890A, and mass selective (MS) detector (Agilent 5975 CVL MSD) with an auto sampler 7683 B series (1 µL injection). Chromatographic separation was performed on a computer controlled auto sampler used with a fused-silica capillary column HP-5MS (30 m x 0.25 mm film thickness 0.25 µm; J&W Scientific, Folsom, CA, USA). Splitless injection was used at 280 ^{0}C. The GC oven temperature consisted of an initial temperature of 100 ^{0}C for 0.4 min, raised to 290^{0}C at rate of 60^{0}C/min, maintained at this temperature for 2.4 min, raised to reach 320^{0}C at 60^{0}C/min and finally maintained at this 320 ^{0}C for 1.03 min. The total run time was 7.35 minutes. High-purity helium (99.9995%) was used as the carrier gas, at flow rate of 1 mL/min. The MS parameters used was performed as follows: the interface temperature (280 °C), the inlet temperature (250 °C), the ion-source temperature (230 ^{0}C), electron ionization (EI) at 70 eV and the mass spectrometer (quadrupole) used in scan mode. The spectra were recorded in the scan range of mass particles (m/z) from 35 to 550 amu, at a scan time of 1 scan/sec (scan rate).

5. **Results**

Table: **Retention times, peak areas and relative response ratio of detected compounds**

Retention time	Peak area	Relative response ratio	Compound ID
4.030	1.03E+08	1	Tetracosane
4.476	3390655	0.032863	Acetylcodeine
4.592	7920293	0.076906	6-monoacetylmorphine
4.773	1.01E+08	0.979948	Diamorphine sample

The sample was determined to contain diamorphine, acetylcodeine and 6-monoactylmorphine. Acetylcodeine is a by-product in the clandestine synthesis of diamorphine while 6-monoactylmorphine is both a by-product in the clandestine synthesis of diamorphine as well as a diamorphine degradation product. No adulterants were detected in the sample provided. Table 1 shows the retention times of the internal standard, acetylcodeine, 6-monoacetlmorphine and diamorphine as well as their respective average peak areas and relative response ratio(calculated by dividing average peak area of sample by average peak are of the internal standard) . Diamorphine was identified on the basis of its retention time and mass spectral data using certified reference material (CRM). The total ion chromatograph of the CRM and diamorphine sample is shown in Figures 1 and 2 respectively. The acceptance criteria using the SAPS-FSL's standard operating procedure is a retention time which does not differ by not more than 0.06 minutes and a comparable mass spectra. Acetylcodeine and 6-monoacetylmorphine were identified by comparing the experimental mass spectral data with the designer drug mass spectral library, 2014 (DD2014). The total ion chromatograph of the acetylcodeine, 6-monoactylmorphine and their respective mass spectral data are shown in Figures 5 to 8 respectively.

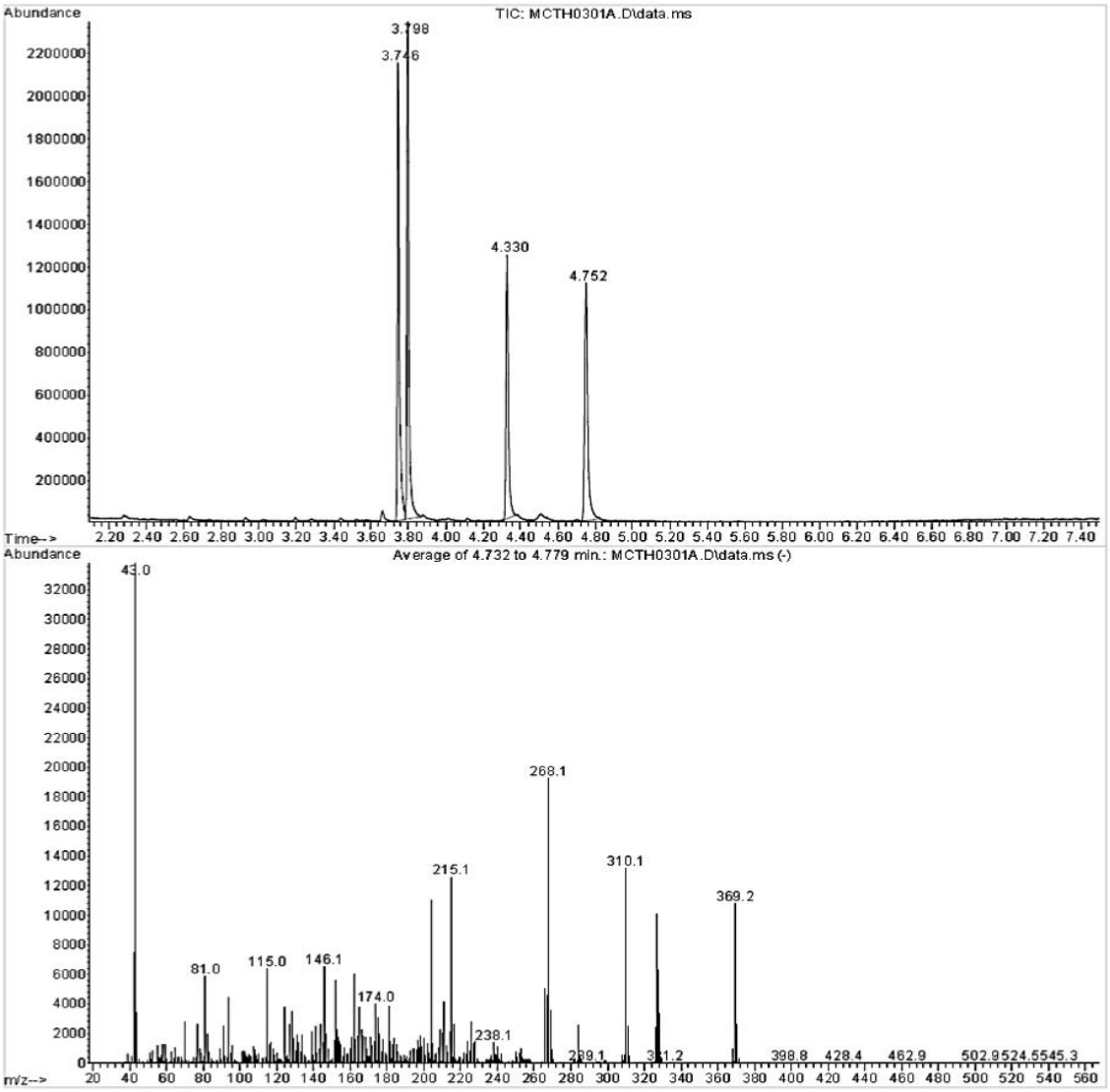

Figure 1: A total ion chromatograph and mass spectrum of diamorphine CRM, mthaqualone retention time, 3.746 min.; cocaine retention time, 3.798 min.; THC retention time, 4.330 min. and diamorphine retention time, 4.752.

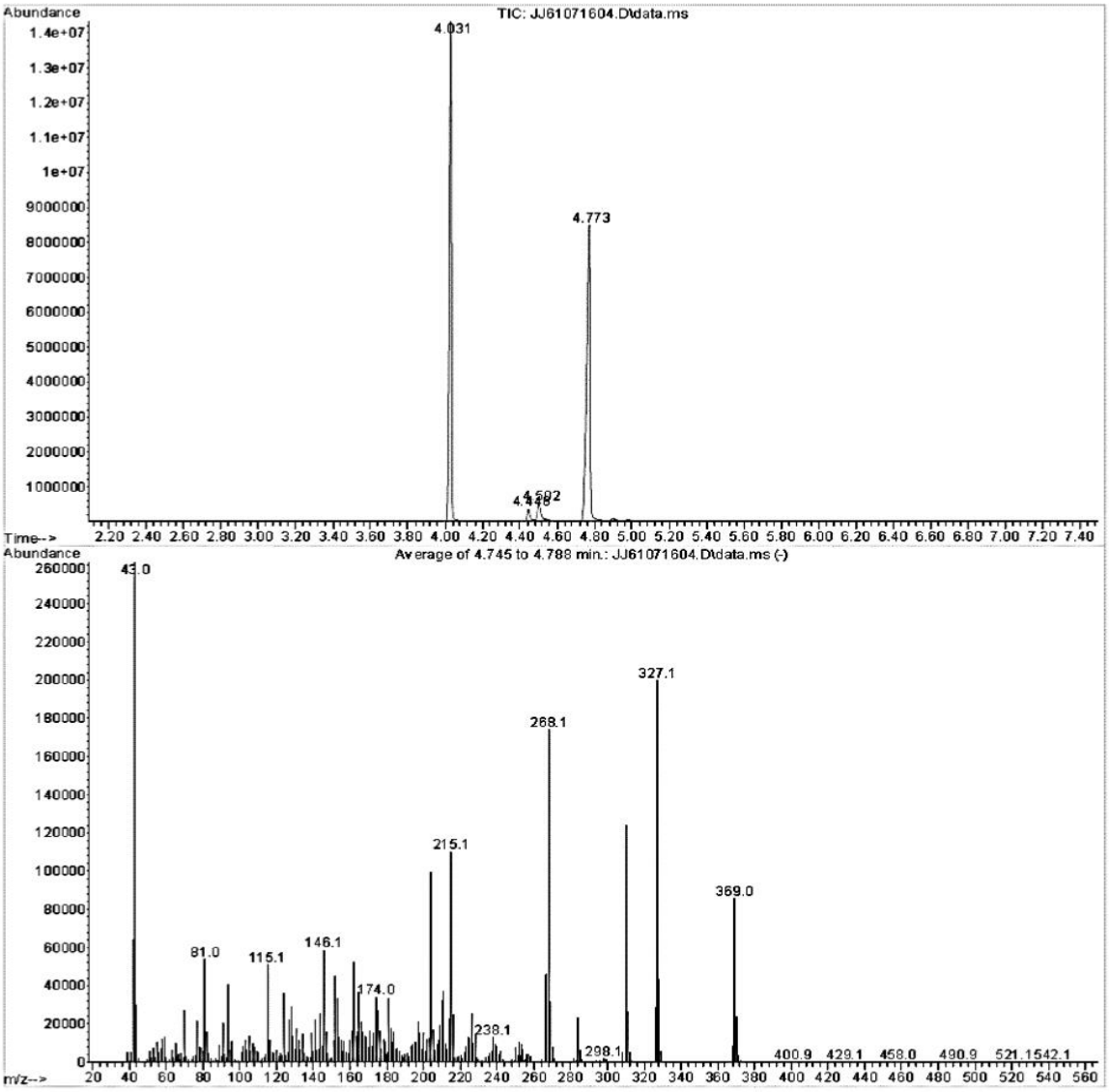

Figure 2: A total ion chromatograph and mass spectrum of diamorphine from the samples

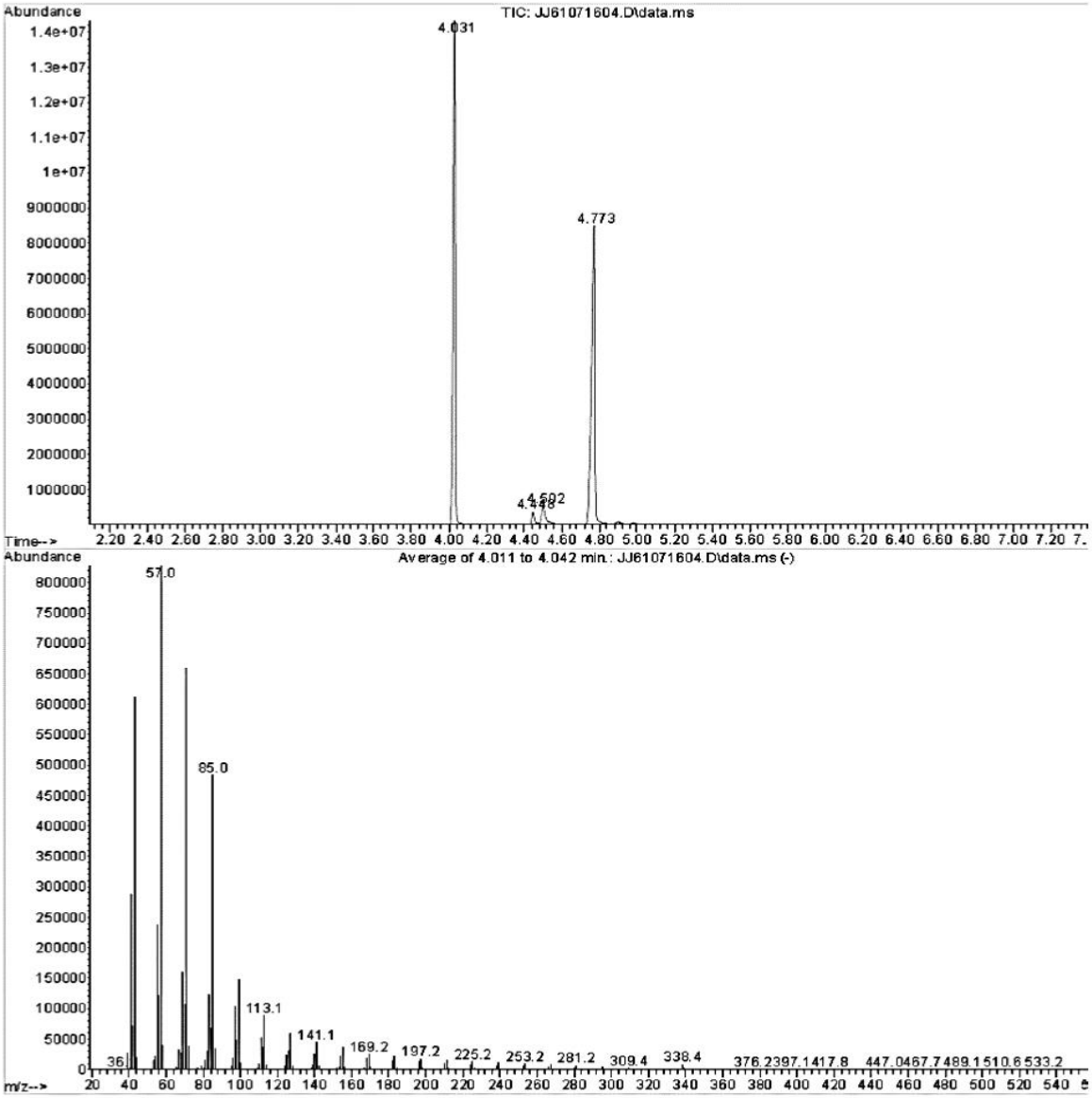

Figure 3: A total ion chromatograph and mass spectrum of the internal standard.

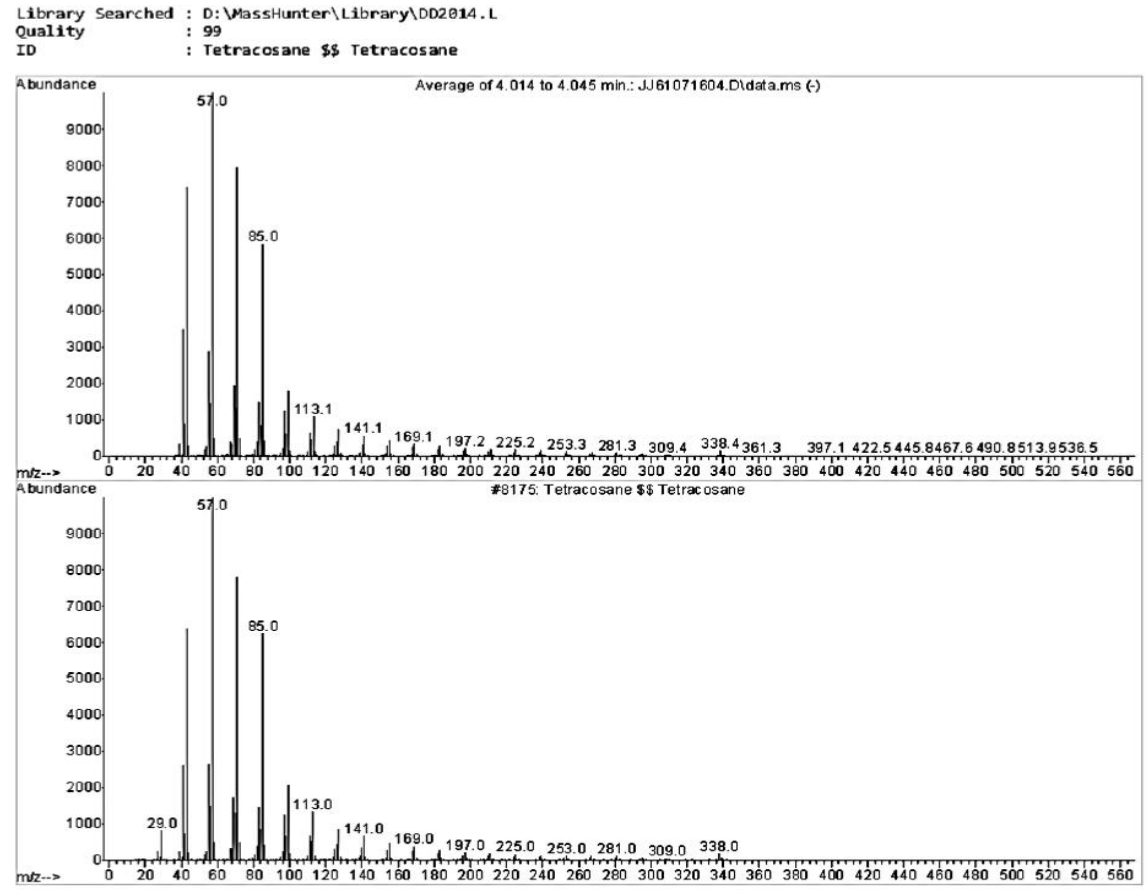

Figure 4: Mass spectral library data of tetracosane.

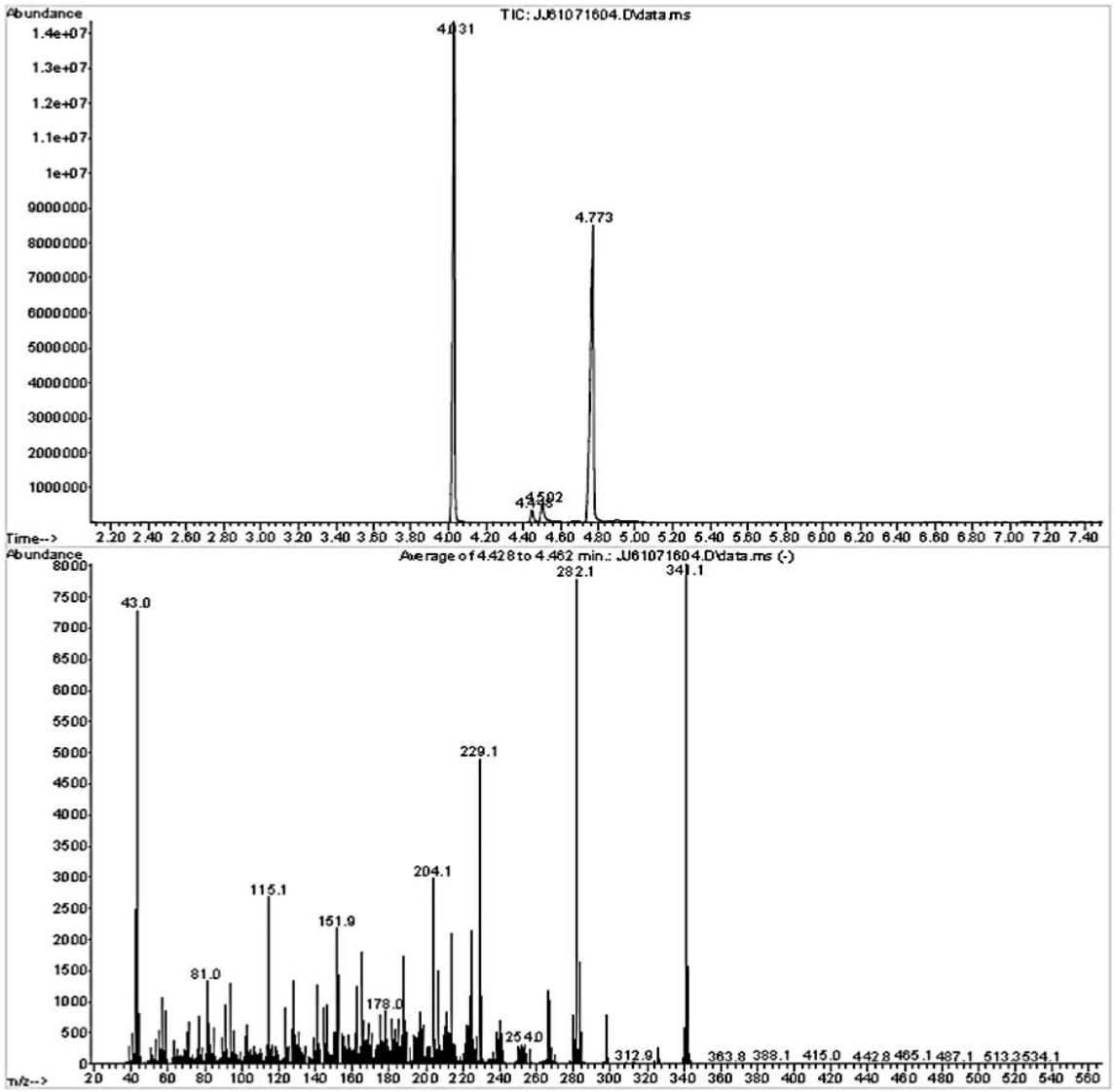

Figure 5: A total ion chromatograph and mass spectrum of acetylcodeine from the samples

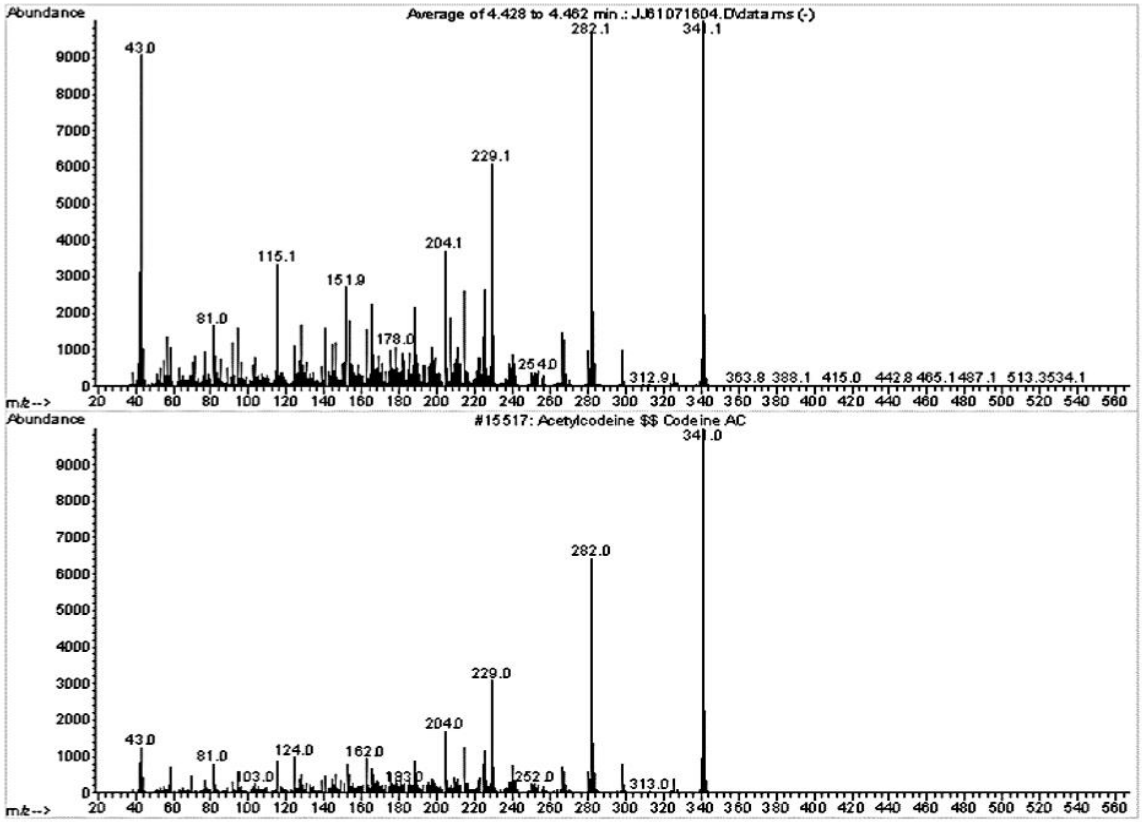

Figure 6: Mass spectral library data of acetylcodeine.

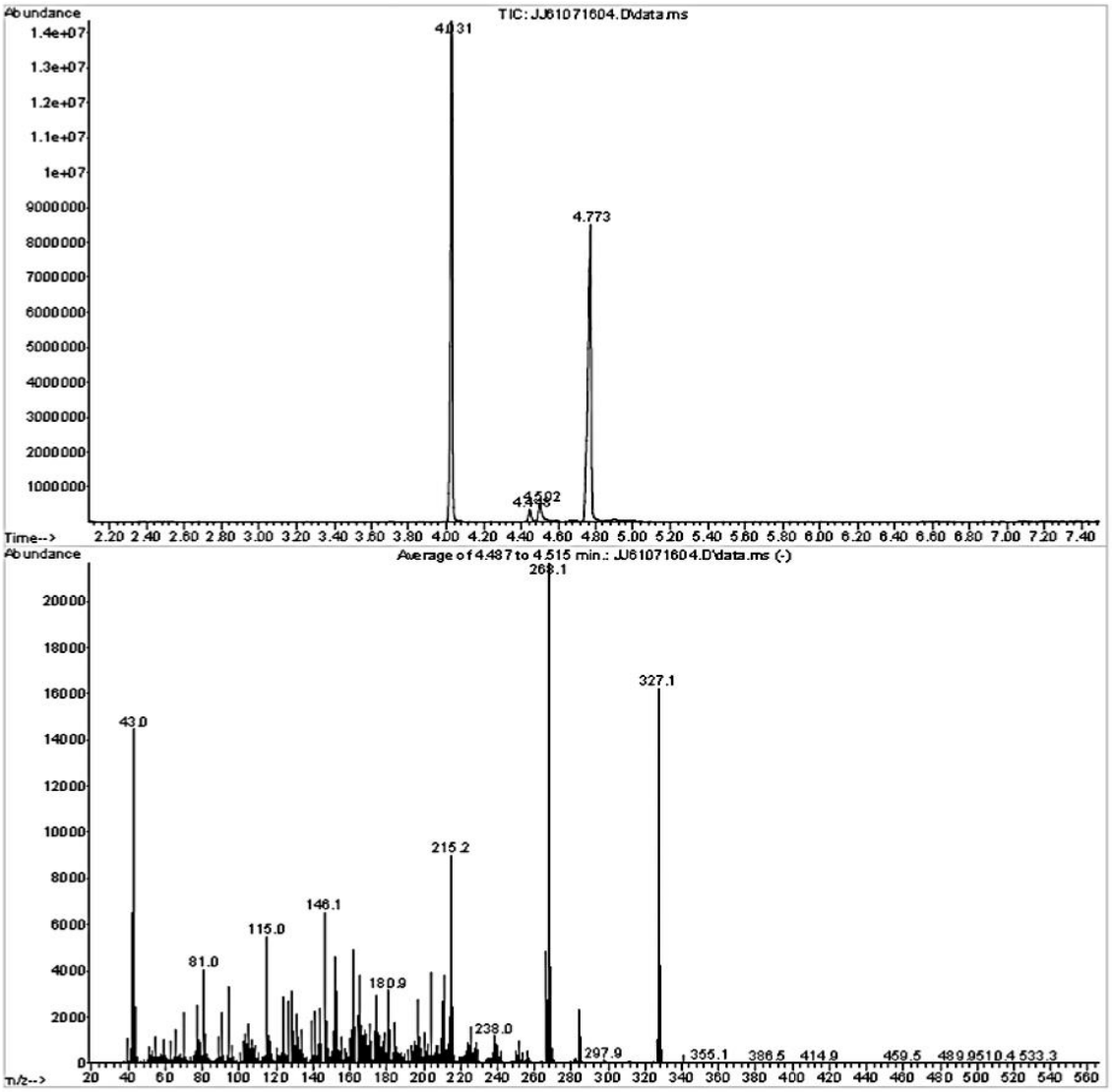

Figure 7: A total ion chromatograph and mass spectrum of 6-monoacetylmorphine from the samples

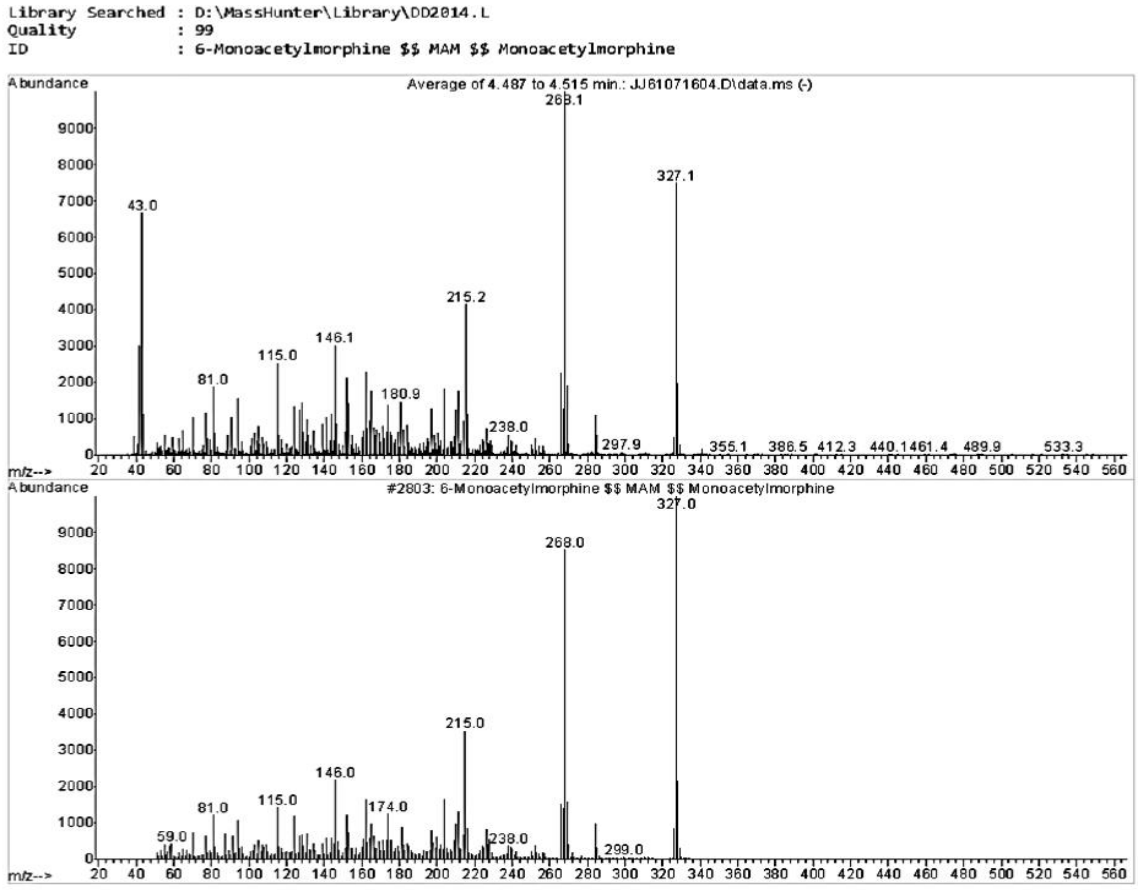

Figure 8: Mass spectral library data of 6-monoacetylmorphine.