IDENTIFICATION AND DEVELOPMENT OF URINARY PROTEIN BIOMARKER TO SCREEN FOR KRATOM USED IN REGULAR USERS

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ACN	Acetonitrile
AIDS	Acute Immune Deficiency Syndrome
APS	Ammonium Persulphate
ARDS	Acute Respiratory Distress Syndrome
BSA	Bovine Serum Albumin
C.V.	Coefficient of Variation
CBD	Catenin-Binding Domain
CDC	Centers for Disease Control and Prevention
CDH1	E-cadherin
CI	Confidence Interval
CID	Collision Induced Dissociation
DEA	Controlled Substances Act
DM	Diabetic Mellitus
DMSO	Dimethyl sulfoxide
DN	Diabetic Nephropathy
DSHEA	Dietary Supplement Health and Education Act
DTT	1,4-Dithioreitol
EDTA	Ethylenedisminetetra-acetic acid
ELISA	Enzyme-linked Immunosorbent Assay
EMT	Epithelial Mesenchymal Transition
FDA	Food and Drug Administration
FN	False Negative
FP	False Positive
GC	Chromatography

GC-MS	Gas Chromatograph Mass Spectrometry
GeLC-MS	Gel-electrophoresis-LC-MS
GPCRs	G-protein Coupled Receptors
JMD	Juxtamembrane Domain
kDa	kilo Dalton
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
m/z	Mass to charge
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MW	Molecular Weight
NIH	National Institutes of Health
NPDS	National Poison Data System
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PEA	Phenylethylamine
PTMs	Post-Translational Modifications
QC	Quality Control
RC/DC	Reducing Agent and Detergent Compatible
RDW	Red Cell Distribution
SC	Speciociliatine
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SG	Speciogynine
TBS-T	Tris-buffered saline/Tween 20

TEMED	N,N,N’,N’-tetramethyethylenediamine
THC	Tetrahydrocannabinol
TLC	Thin-Layer Chromatography
TMB	3,3’5,5’ Tetramethylbenzine
TN	True Negative
TP	True Positive
Tris	Trizma Base
TSE buffer	10 mM Tris, 1% SDS and 1 mM EDTA at pH 8.8
UGT	UDP-Glucuronosyl Transferase
WGA	Wheat Germ Agglutinin

PENGENALPASTIAN DAN PEMBANGUNAN PENANDA-BIO PROTEIN DALAM URIN UNTUK KAEDAH PENYARINGAN DALAM KALANGAN PENGGUNA KRATOM TETAP

ABSTRAK

Kratom (Mitragyna speciosa korth), telah digunakan secara tradisional di Asia Tenggara untuk sifat terapeutiknya. Alkaloid utama kratom, mitragynine mengikat kepada reseptor opioid untuk memberikan kesan seperti opioid yang menyebabkan ketagihan. Kajian kami bertujuan untuk mencirikan profil protein air kencing pengguna kratom biasa untuk menentukan kesan kratom terhadap penggunaan kratom secara tetap. Tambahan pula, untuk mengenal pasti biomarker air kencing berguna yang boleh digunakan untuk menunjukkan pergantungan dan ketagihan kratom melalui kaedah saringan menggunakan ELISA. Pendekatan proteomik digunakan untuk mengekstrak, memisahkan dan memetakan profil protein air kencing pengguna kratom dan subjek yang sihat. Apabila dibandingkan dengan jalur protein yang diekskresikan secara berbeza atau unik antara pengguna kratom dan subjek yang sihat, jalur protein sasaran dikeluarkan daripada gel dan dianalisis menggunakan LC / MS / MS untuk pengenalan protein. Keputusan kami menunjukkan pengguna Kratom mempunyai kepekatan protein air kencing yang tinggi, nisbah P/C, dan keamatan albumin serum air kencing berbanding kawalan yang sihat, walaupun kepekatan kreatinin air kencing pengguna kratom didapati berada dalam julat normal sebagai kumpulan kawalan yang sihat. Tambahan pula, jalur protein pada 80 kDa MW didapati unik pada semua pengguna kratom tetapi tiada dalam semua air kencing kawalan yang sihat. Kami membuat kesimpulan bahawa pengguna kratom biasa menunjukkan tanda-tanda kecederaan buah pinggang awal, dan CDH1 ditunjukkan dapat membezakan dengan tepat antara pengguna kratom biasa dan kawalan yang sihat.

IDENTIFICATION AND DEVELOPMENT OF URINARY PROTEIN BIOMARKER TO SCREEN FOR KRATOM USED IN REGULAR USERS

ABSTRACT

Kratom (Mitragyna speciosa korth), has been used traditionally in Southeast Asia for its therapeutic properties. The major alkaloid of kratom, mitragynine binds to opioid receptors to give opioid-like effects that cause addiction. Our study aimed to characterize the urinary protein profile of regular kratom users to determine the impact of kratom on regular use of kratom. Furthermore, to identify a useful urinary biomarker that can be used to indicate kratom dependence and addiction by means of a screening method using ELISA. A proteomic approach was used to extract, separate, and map the urinary protein profiles of kratom users and healthy subjects. Upon comparison of the protein bands that were differentially or uniquely excreted between the kratom users and healthy subjects, the target protein bands were excised from the gel and analyzed using LC/MS/MS for protein identification. Our results showed Kratom users had elevated urinary protein concentrations, P/C ratio, and urinary serum albumin intensity relative to healthy controls, although the kratom user's urinary creatinine concentration was found to be in the normal range as the healthy control group. Furthermore, a protein band at 80 kDa MW was found unique in all kratom users but absent in all healthy control urine. We concluded that habitual kratom users showed signs of early kidney injury, and CDH1 is shown to be able to differentiate accurately between habitual kratom users and healthy controls.

CHAPTER 1

INTRODUCTION

1.1 Kratom

Kratom or Mitragyna speciosa (Korth.), is a municipal Malaysian herbage (Domnic et al., 2021). Kratom is likewise known as biak-biak, ketum, or Maeng Da by local folks of various places in Asia. The word kratom refers to the tree itself and also extracts and treatments produced from the plant (Ulbricht et al., 2013). These tree leaves are known for their pharmacological effect, and this effect varies according to the white, green, or red veins in these leaves, however, these leaves may be sold in powder form in the Western countries (Brown et al., 2017). The usage of Kratom in Southeast Asia has been recorded as far back as at least 150 years and was characterized by its stimulating impact for usage throughout strenuous daywork, as well as fresh kratom leaves are chewed or fermented into a tea for its analgesic and relaxing effect (Brown et al., 2017). Folk medicine in Southeast Asia has long recognized the effectiveness of the kratom herb (Hassan et al., 2013). As an "herbal tea," Kratom is often used in the searing heat of the tropics to help workers stay alert and productive, as well as to battle weariness and wean morphine addicts off of their drug of choice (Watanabe et al., 1997). Kratom was once widely used in Malaysia and Thailand as an opium replacement and countermeasure (Veltri and Grundmann, 2019). Antispasmodic, muscle-relaxant, and antidiarrheal properties of Kratom are still in used in Southeast Asia, while its stimulant and analgesic effects are also popular home remedies (Singh et al., 2017; Suwanlert, 1975). Although the Poisons Act of 1952 makes it illegal to consume Kratom in Malaysia, the native tree and tea decoctions are abundantly available, therefore kratom is nevertheless commonly used (Singh et al.,

2016). Kratom was legalized in Thailand in 2018 for therapeutic uses after a prohibition on its usage, manufacture, and possession was overturned (Ya et al., 2019).

People in Malaysia, Thailand, and Southeast Asia have traditionally used kratom leaves to treat diarrhea, muscle discomfort, decrease blood pressure, and enhance stamina (Panjaitan and Liridah, 2021). Kratom offered in the United States as an unregulated herbal supplement. It is mostly isolated from the leaves of the Southeast Asian plant Mitragyna speciosa. For ages, manual laborers in Southeast Asia have utilized the herb for its stimulant and analgesic properties (Trakulsrichai et al., 2015). According to the United States National Poison Data System (NPDS), kratom exposures are on the rise and have previously been linked to significant opioid toxicities such as seizures, agitation, and death. Additionally, withdrawal and neonatal abstinence syndrome reports indicate that kratom, like other opioids, might cause dependency (Eggleston et al., 2019). In Thailand, the Kratom Act was passed in 1943, putting kratom under governmental supervision. This was thought to be an economic choice rather than one based on public health concerns. The opium trade was taxed at the time, and because it was expensive, individuals began to replace opium with kratom, which impacted the Thai government's earnings (Saingam et al., 2013; Singh et al., 2016). Increased sales of Kratom in Europe along with North America raised worries about substance's safety prompted some European governments to prohibit the plant and its active alkaloids (Cinosi et al., 2015). Kratom's status as a dietary supplement remains a matter of debater in the American States, the Food and Drug Administration (FDA) does not consider kratom as a recognized supplement that was available on the American States market prior to the legislation of the Dietary Supplement Health and Education Act (DSHEA) of 1994, which would have permitted such a provision (Henningfield et al., 2018). On the contrary, the FDA designated

mitragynine and 7-hydroxymitragynine as substances with analgesic effects and suggested to the US Drug Enforcement Administration (DEA) that they be placed on Schedule I of the Controlled Substances Act (Grundmann et al., 2018). Opioids were responsible for more than 42,000 deaths in 2016, according to the US Department of Health and Human Services, this is the highest figure ever recorded (Todd et al., 2020). Prescription opioids were responsible for more than 40% of these deaths. Many who were suffering from chronic pain are resorting to alternative treatments, one of which is kratom. (Todd et al., 2020). Nevertheless, despite the FDA's repeated calls to criminalize kratom under the Control Substances Act, there is no solid decisive proof that kratom usage has caused significant health implications as classical opioids (Singh et al., 2019).

Figure 1.1 Kratom plant at different stages.

1.2 Problem Statement and Scope of the Study

Despite of the fact that kratom toxicity insignificant, this plant was nonetheless misused. This eventually led to addiction. Since 2005, the rise of kratom misuse in Malaysia has generated a slew of issues for the community, including crimes, family neglect, poor work performance, and even loss of consciousness. Extensive kratom consumption results in longer sleep. Hostility, anger, tearfulness, muscular discomfort, and difficulty to work are among the withdrawal symptoms.

As a result, it is critical to not only restrict kratom usage but also to detect and monitor the users. However, using kratom alkaloid as a detection marker can only suggest that such a person has consumed kratom, since it is widely known that many people utilize kratom for medical purposes. Long-term kratom usage may devolve into outright addiction, and there has been an increase in cases of kratom addiction and toxicity, therefore there is a need to discover a useful biomarker that can be used to diagnose kratom addiction.

1.3 Research Question

Kratom, which was originally used for therapeutic purposes, is now mostly utilized as a recreational drug. Its increased use and lack of checks and balances has resulted in several major issues. Kratom is metabolized in the liver and eliminated through the kidneys. Any issue with metabolism or excretion might lead to problems. Problems develop when the liver or kidneys are ill, or when Kratom causes harm to any of these essential organs.

In view of the harm of long term kratom usage to human body, monitoring of kratom abuse is essential. Such monitoring device will make use of human bodily fluid while urine may be the best source for identification of marker or indicator to show the abuse of kratom. Does urine contain such marker or indicator?

1.4 Hypothesis and Study Objectives

Our hypothesis of the study is: long term consumption of kratom will cause physiological changes to the body and result in excretion of protein(s) in kratom addicts' urine which may not be common to those of healthy people. Therefore, it is highly possible to detect unique protein biomarker in the urine of kratom addicts that can be used to indicate kratom dependence.

Currently, there is no reliable biomarker that can be used to indicate kratom dependency or addiction, in view of the rise of kratom addiction cases, there is an urgent need for such a biomarker. The general objective of this study is to apply the proteomics approach by means of gel electrophoresis and tandem mass spectrometry for the identification of urinary protein biomarker for kratom addiction. Subsequently, such biomarker will be employed as a detecting marker in the development and validation of a non-invasive screening method by means of ELISA for the detection of kratom addiction.

The specific objectives of the project are:

1. To analyze the urinary protein profiles of 88 healthy controls and 88 regular kratom users.

2. To compare the urinary protein proteomes of healthy control and Kratom users.

3. To identify a useful urinary biomarker that can be used to indicate kratom dependency.

4. To develop and validate a non-invasive screening method for kratom addiction.

1.5 Framework of the Study

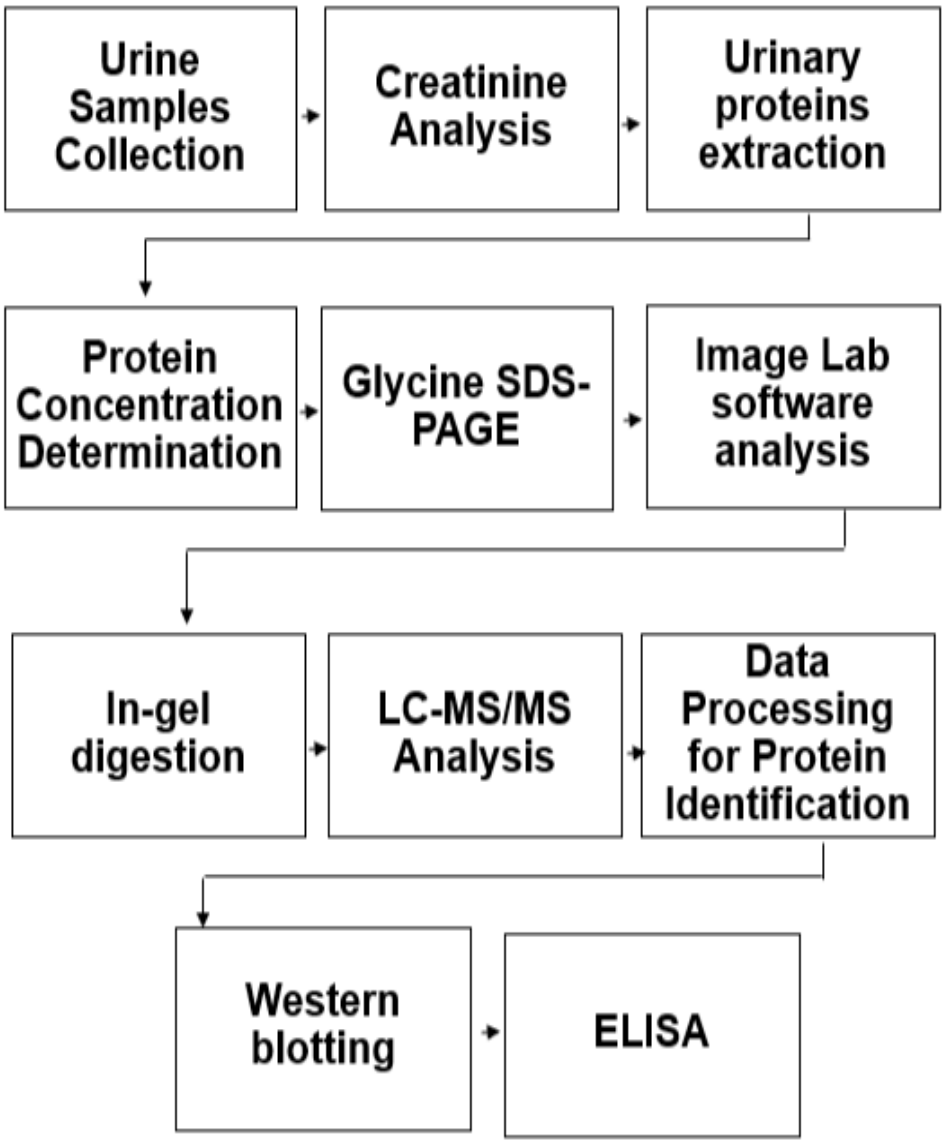

Figure 1.2 Flowchart of research protocol.

CHAPTER 2

LITERATURE REVIEW

2.1 Social Functioning in Kratom Users

Rural Malay communities in northern Malaysia frequently employed kratom for recovery from diabetes mellitus and hyperpiesia (Hassan et al., 2013). Kratom abuse is a global issue impacted socioeconomically of rural Malay customs (Singh et al., 2014). Kratom was said to have opium- and cocaine-like effects and was utilized to alleviate opiate withdrawal symptoms in the Malaysian community amid an opium scarcity (Jansen and Prast, 1988). In Malaysia, manual laborers commonly employed kratom for its stimulant properties, which aid in increasing physical endurance during strenuous work. When consumed for an extended length of time, Kratom is said to "quiet the mind" (Suwanlert, 1975). Despite the addictive characteristic of kratom, a survey reported that most of the respondents claimed that it did not pose the same social and health hazards as narcotic medications or cannabis. Although majority of respondents reported being dependent on kratom, they were able to regulate their usage when they took it as a substitute for other substances or to alleviate opioid withdrawal symptoms (Vicknasingam et al., 2010). Kratom formulations are used recreationally in the United States and Europe, for example, to self-manage alcoholism and opioid withdrawal symptoms (Boyer et al. 2007; 2008). Most people who have used kratom for an extended period reported being unable to discontinue use it due to because of the withdrawal symptoms which are unpleasant and may interfere with everyday functioning (Suwanlert 1975; Vicknasingam et al. 2010; Singh et al. 2014). Regular kratom users are more likely to gradually increase their dose. Likewise, they are certain to notice a drop in energy because of extended kratom use. To replenish energy exhaustion, People who use kratom often increase their intake of the herb to maintain

normal function (Suwanlert 1975).

Kratom is less costly than stimulants like amphetamines and heroin. Users of Kratom do not have to resort to illegal activity to maintain their addiction. In the community, illicit kratom traffickers and growers sell their wares. Non-destructive and socially acceptable, kratom usage permits kratom users to mingle and speak with their coworkers. The majority of kratom users are socially and familiarly well-adjusted. However, the majority of kratom users' families have an ambivalent attitude, believing that kratom usage would insidiously develop into severe addiction issues (Singh et al., 2015).

2.2 Kratom Pharmacology

Mitragyna speciosa components have been isolated and chemically characterized since the 1960s (Suhaimi et al., 2016). Thus far, more than 40 compounds have been discovered, but only four are renowned to have pharmacological activity, namely mitragynine, 7-hydroxymitragynine, speciociliatine, and corynantheidine (Feng et al., 2017; Takayama, 2004). Mitragynine is the most common alkaloid in kratom plants, (Eastlack et al., 2020) and it can be easily oxidized (Eastlack et al., 2020), it consists of 66% of the alkaloid content of kratom, and actually this depends on the country of origin of kratom. On the other hand, 7-Hydroxymitragynine was identified as a minor ingredient of kratom leaves extracts (Hassan et al., 2013) which is made of 0.04% of the alkaloid (Kikura-Hanajiri et al., 2009). Speciogynine, paynantheine, and mitraphylline are also indole alkaloids in Kratom (Chittrakarn et al., 2012). These compounds are not known to have pharmacological activity, but they contribute synergistically to kratom's overall effect. Given the wide variety of alkaloids found in kratom after extraction and their individual pharmacodynamic properties, the substance's net physiological effect is

made up of combining effects of stimulant and opioid-like that occur in a dosage-dependently manner. Mitragynine may inhibit the activity of cytochrome P450 enzymes, notably CYP2D6. As a result, combining mitragynine with herbal or contemporary medications that use the same metabolic route may lead to herb-drug interactions (Hanapi et al., 2013).

Mitragynine and 7-hydroxymitragynine have the ability to target opioid receptors, yet their binding affinity to opioid receptors is significantly different (Prozialeck et al., 2012). Mitragynine has a lesser binding affinity to opioid receptors than morphine, but 7-hydroxymitragynine is substantially more strong than either, which is around forty-six times the strength of mitragynine and thirteen times the potency of morphine (Matsumoto et al., 2004; Yamamoto et al., 1999). Both mitragynine and 7-hydroxymitragynine have been demonstrated to work as agonists, with mitragynine activating primarily μ- and δ-receptors and 7-hydroxymitragynine activating primarily μ- and κ-receptors (Matsumoto et al., 2004, 2006; Matsumoto et al., 2005). Contrary to popular belief, however, data reveals that mitragynine and 7-hydroxymitragynine work differently on various receptors than simple agonists (Eastlack et al., 2020). Both mitragynine and 7-hydroxymitragynine are mixed opioid receptor agonists/antagonists, acting as fractional agonists at μ-receptors and competitive antagonists at δ-receptors, with relatively minor effects on κ-receptors (Kruegel et al., 2016).

Kratom contains indole alkaloids, these indole alkaloids are structurally and pharmacodynamically unlike its opioid rival, therefore there were identified as atypical opioids in order to differentiate them from morphine, semisynthetic opiates, and endogenous ligands (Raffa et al., 2018). Upon binding to opioid receptors the indole alkaloids activate G-protein-coupled receptors (GPCRs), however, unlike

conventional opioids, indole alkaloids do not start off the β-arrestin pathway when they activate GPCRs (Suwanlert, 1975). This process referred to as biased agonism or ligand-directed signaling, permits a single receptor to exert numerous distinct intracellular effects by selectively disabling the receptor's various signaling cascades (Wisler et al., 2014). Interestingly, symptoms of opioid use like respiratory depression, sleepiness, and constipation are due to β-arrestin recruitment (Raehal and Bohn, 2011). The ability to inhibit β-arrestins selectively is a desired quality in an opioid, and mitragynine may serve as a valuable paradigm for the creation of new opioids with more bearable side effects. (Eastlack et al., 2020).

Apart from its opioid-like analgesic actions, mitragynine may be involved to inhibit pain signals via other pathways, implying a multimodal involvement in pain perceptiveness regulation. For example, mitragynine bears a high degree of structural similarity to yohimbine, another indole alkaloid with well-documented adrenergic effects (Prozialeck et al., 2012). One of Mitragynine analgesic properties appears to act as yohimbine, through activating the α-2 adrenergic postsynaptic receptors (Matsumoto et al., 1996). α-2 receptors are found in pain modulatory "descending" pathways, these pathways constitute a significant improvement in complicated neurobiological knowledge of pain (Giovannitti et al., 2015; Ismail et al., 2017). Mitragynine inhibits neuronal pain transmission via Ca2+ channel blockage (Matsumoto et al., 2005). The indirect analgesic qualities have been ascribed to Mitragynin’s potential anti-inflammatory activities, which are thought to be mediated through the suppression of COX-2 and prostaglandin E2 mRNA expression (Mossadeq et al., 2009; Utar et al., 2011). Apart from these antinociceptive properties, mitragynine exhibits some affinity for central nervous system receptors, including the 5-HT2C and 5-HT7 serotonin receptors, the D2 dopamine receptors, and the A2A adenosine

receptors (Matsumoto et al., 2005).

Mitragynine and 7-hydroxymitragynine have a G-protein-biased signaling mode of action, which causes kratom to have the effects of a partial agonist in terms of its respiratory depressive properties (Henningfield et al., 2019; Kruegel et al., 2016; Kruegel and Grundmann, 2018). The net physiological impact of kratom is complicated, including stimulant and opioid-like properties in a dose-dependent way due to the assortment of alkaloids shown in kratom extricates and the one of a kind potential pharmacodynamic properties of each (essentially stimulant-like at a low dosage, with opiate impacts prevailing at greater doses) (Babu et al., 2008; Singh et al., 2016). At larger doses, Kratom possesses unique narcotic qualities that blend psychostimulant and opiate-like effects (Harun et al., 2015).

Kratom metabolism is primarily hepatic, and it can influence the metabolism and efficacy of another medicines by inducing drug-metabolizing enzymes including CYP450s and UDP-glucuronosyl transferase (UGT) (Meireles et al., 2019). The effects of Mitragyna speciosa on human recombinant CYP450 enzyme activity have been studied in various research (Kong et al., 2011). Herb-drug interactions were observed when mitragynine is used with herbaceous or up-to-date medications that share the same metabolic path (Hanapi et al., 2013). Mitragynine has been claimed to have a halflife of as little as three hours, however, s ome research suggests it may be much longer (Trakulsrichai et al., 2015; Manda et al., 2014). Significant advancement in kratom pharmacology conception revealed that mitragynine is transformed in vivo via hepatic metabolism into 7-hydroxymitragynine (Kamble et al., 2019; Kruegel et al., 2019; Yusof et al., 2019). This leads to the hypothesis that the physiologically active mitragynine component is the 7-hydroxymitragynine, which is responsible for the majority (if not all) of the effects

normally attributed to the precursor mitragynine. There are findings established that mitragynine is activated by CYP34A-mediated dehydrogenation, a mechanism akin to how opiates such as codeine are activated via CYP2D6-mediated dehydrogenation, in spite of the fact that 7-hydroxymitragynine is found in kratom extracts at minimal levels, the authors concluded that any consumed 7-hydroxymitragynine is insignificant in comparison to the endogenous synthesis of 7-hydroxymitragynine from mitragynine (Kamble et al., 2019; Kruegel et al., 2019; Singh, Narayanan, et al., 2020).

Kratom has sparked a slew of preliminary studies, including those looking at the potential for dependency and addiction caused by mitragynine and its close relatives. For example, prolonged mitragynine consumption in mice and rats has shown addiction potential and cognitive impairment (Hassan et al., 2019; Hemby et al., 2019; Yusoff et al., 2016). 7-hydroxymitragynine has been determined to be the most important factor in the development of addiction and toxicity, with mitragynine posing just a small danger (Hemby et al., 2019; Sabetghadam et al., 2013). Furthermore, chronic usage has been linked to dependency (Ismail et al., 2017).

2.3 Risks of Kratom Abuse and Toxicology

kratom dependence and addiction are legitimate concerns in mankind (Matsumoto et al., 2005; Singh et al., 2014; Yusoff et al., 2016). However, for many frequent users, the primary objective is merely to avoid weariness and boost energy. In such instances, frequent usage may not be defined as dependency or addiction, but rather as a desire to increase productivity (Singh et al., 2019). This is consistent with "drug instrumentation" hypotheses, according to which a substance is used for a specific plan and aim (Hassan et al., 2013; Müller and Schumann, 2011). Long-term usage of kratom may lead to adaptation, which can lead to outright addiction under certain conditions (Singh et al., 2014). Additionally, it has been suggested that a

considerable percentage of kratom usage happens as a substitution for more hazardous drugs particularly opioids in individuals who already have a history of substance misuse, in which case kratom use is considered harm reduction rather than drug abuse (Hassan et al., 2013; Swogger and Walsh, 2018). As an unregulated supplement, kratom presents several extra risks to patients, many of which stem from its abuse potential. There is nothing that can be done to assure the veridicality, pureness, grade, and safety of commercially accessible kratom formulations in the absence of governmental control (Hanna, 2003). As a result, it is impossible to determine exactly what is contained in commercially obtainable kratom formulations, and the quantity of mitragynine can vary significantly (Kikura-Hanajiri et al., 2009). There have been reports that kratom products can be enhanced in potency by intentionally raising the quantity of 7-hydroxymitragynine (Lydecker et al., 2016). Additionally, many cases of purposeful adulteration of kratom have been observed, including the insertion of synthetic drugs such as phenylethylamine (PEA) or O-desmethyltramadol, both of which led to the death of affected patients (Arndt et al., 2011; Nacca et al., 2020). Additional dangers include purposeful or accidental product contamination; for instance, laboratory and epidemiological evidence in 2018 specified kratom to be the cause of salmonella infestation (CDC, 2018; Dixon et al., 2019). Besides that, there have been instances of kratom products being sold that were later shown to have dangerous heavy metal impurities (Kuehn, 2019).

2.4 The Adverse Effects of Kratom Addiction to Human Health

Among the symptoms reported to the National Poison Data System, agitation was the most prevalent symptom of kratom consumption (18.6%), followed by tachycardia (16.9%), sleepiness (13.6%), and disorientation (8.1%) (Eggleston et al., 2019). Seizures occurred in 6.1 percent of patients, hallucinations in 4.8 percent, and

coma in 2.3 percent. Toxicity was dose-dependent, especially when kratom powder dosages surpassed 8 g. (Eggleston et al., 2019). Kratom is typically utilized for its stimulating properties at low doses (Raffa et al., 2013). Opioid-like effects can be seen in greater doses. when abstaining from intake, negative withdrawal symptoms such as hostility, violence, muscle and bone soreness, jerky limb, anorexia, weight reduction, sleeplessness, as well as psychosis were recorded (Singh et al., 2014; Yusoff et al., 2016). Sickness, weight reduction, weariness, stasis of the lower bowel, sleeplessness, xerostomia, urinary frequency, and melasma are all possible side effects, especially for heavy users (Warner et al., 2016). The pharmacological research and epidemiology explorations of kratom in South-East Asia, showed that unlike morphine-like opiate, kratom does not cause life-threatening respiratory complication and is not linked to the personal and in-society harm that morphine-like opioids are linked to (Prozialeck et al., 2019; Singh et al., 2017; Veltri and Grundmann, 2019). It was reported that people who were reliant on kratom for extended durations (Singh et al., 2015) exhibited little signs of social dysfunction. It is now being offered as "legal highs" in the United States and Europe, despite the lack of research on the drug's addiction potential or hazardous consequences at extremely high dosages, and it is being used to treat constant pain and opiate analgesia (Boyer et al., 2008, 2007). Concern about kratom and mitragynine, its major psychoactive ingredient, is growing across the world because of its increasingly documented harmful effects on humans (Forrester, 2013; Holler et al., 2011; Kapp et al., 2011; Neerman et al., 2013; Nelsen et al., 2010; Trakulsrichai et al., 2013).

Additionally, Kratom has been linked to organ malfunction and toxicity (Ilmie et al., 2015). Drug-drug interactions have been demonstrated in animal investigations, namely by modulation of hepatic P450 activity and drug metabolism (Kong et al., 2011; Meireles et al., 2019). Mitragynine appears to inhibit hepatic demethylases and

transferases, in addition to the glucuronidation reaction spurred by UDP-glucuronosyltransferases (UGT) like UGT2B7 and UGT1A1 (Anwar et al., 2012; Azizi et al., 2010, 2013; Lim et al., 2013). This has a major indication of the possibility of interaction among kratom and other UGT substrates, such as buprenorphine and ketamine, which are metabolized by UGT2B7 (Lim et al., 2013). These explanations have been cited as a possible clarification of the toxicity associated with co-administration of kratom and another drug, including a death associated with supratherapeutic doses of a prescription antipsychotic concomitant with kratom absorption (Hughes, 2019). The authors ascribe this result to a significant decrease in quetiapine, which is a CYP3A4 substrate, clearance because of kratom's acute inhibition of hepatic metabolism.

Case studies unfold the fact of kratom impact body organs for instances of kidney damage (Ilmie et al., 2015), cardiotoxicity, and arrhythmia (Abdullah et al., 2019; Lu et al., 2014), thyroid injury and hypothyroidism (Sheleg and Collins, 2011) lung injury/acute respiratory distress syndrome (ARDS) (Pathak et al., 2014; Jaliawala et al., 2018), neonatal abstinence syndrome, (Davidson et al., 2019; Eldridge et al., 2018; Mackay and Abrahams, 2018; Murthy and Clark, 2019; Smid et al., 2018) and hepatic injury (Dorman et al., 2015; Kapp et al., 2011; Osborne et al., 2019; Waters et al., 2018) have all been related to kratom. Damage to the liver is a very prevalent manifestation, and it frequently manifests as cholestatic hepatitis comparable to other drug-related illnesses (Antony and Lee, 2019). Several neurological problems associated with kratom impact have also been brought to light, along with severe brain damage and coma (Antony and Lee, 2019), as well as the risk of seizures in both the acute and chronic settings (Tatum et al., 2018; Burke et al., 2019).

According to a study published in 2020 by Singh et al, kratom consumption may raise hematological risk, particularly when used in significant amounts for a lengthy length of time (Singh, Narayanan, et al., 2020). The red cell distribution (RDW) score was somewhat higher than the reference value, which could indicate hematological risk or serve as a marker for inflammatory cytokines (Felker et al., 2007). By a quantitative analysis of urine, Nelsen and his colleague provided a case study of severe human toxicity following kratom use, which detailed regular kratom usage that was augmented with an additional xenobiotic, resulting in severe toxicity characterized by seizure activity (Nelsen et al., 2010). Their findings are comparable to those of Boyer et al., (2008), who found a relationship between kratom usage and seizures. Seizures and coma have also been reported as possible unintended consequences of kratom use (Swogger et al., 2015). In humans, there have been instances of kratom toxicity (Kapp et al., 2011; Prozialeck et al., 2012). Individuals who have consumed kratom for a long time or who have had an acute overdose are more likely to have seizures and addiction. Significant kratom overdose has also been connected to liver damage (Kapp et al., 2011; Prozialeck et al., 2012). Intrahepatic cholestasis, in particular, has been reported (Kapp et al., 2011). Although this has only been confirmed in animal experiments (Kapp et al., 2011), studies reveal that glutathione-S-transferase is enhanced in people who consume big quantities of kratom. The stimulant and opioid actions of kratom appear to be a primary cause of the initial unpleasant effects experienced by many consumers (Kapp et al., 2011; Nelsen et al., 2010). Individuals with long-term addiction have been found to have melasma, tremors, anorexia nervosa, and weight reduction. The usage of relatively high dosages of kratom has frequently resulted in reports of major adverse consequences (Prozialeck et al., 2012). In a study conducted by Saref et al., (2019), kratom usage was linked to a lower prevalence and intensity of

self-reported adverse effects when compared to illicit opioids among Malaysian drug users.

Kratom toxicity has resulted in death in certain severe situations. Indeed, the prevalence of kratom-related mortality increased, on the authority of the Centers for Disease Control and Prevention (CDC), which connected kratom to 152 fatalities between 2016 and 2017 (Kuehn, 2019). Notably, the presence of polysubstance usage is a considerable threat for toxicity and fatality, occurring in around 87 percent of cases (Corkery et al., 2019). This has helm to the idea that mortality purely because of kratom use is infrequent, and maybe beyond the bounds of possibility. Although this is the case, a study conducted in Colorado found that the fatality rate associated with kratom use was significant, with mitragynine toxicity accounting for four of the fifteen fatalities recorded between 1999 and 2017 (Gershman et al., 2019).

2.5 Kratom Consumption Clinical Presentation

As far as kratom's clinical manifestations are concerned, they aren't well-defined or thoroughly researched. In most cases, the symptoms are gathered from individual polls and online forums (Suwanlert, 1975). Positive and negative symptoms are two categories of clinical presentations (Swogger et al., 2015). Amongst the beneficial side effects of kratom were energized feeling, socially attached, and happiness. Kratom was said to enhance sensory sensitivity and causes no discomfort in its users (Swogger et al., 2015). On the contrary, there were a number of psychological and physical side effects that have been linked to kratom use, such as anxiety and depression (Fluyau and Revadigar, 2017), irritability and restlessness (Singh et al., 2016), and low sex desire (Saingam et al., 2013). These effects are thought to be a result of the drug's stimulant properties, which include a feeling of euphoria and a heightened sense of well-being (Suwanlert, 1975). Symptoms such as excessive sweating, drowsiness,

nausea, vomiting, rashes on the lips or throat, and sedation have been reported by some users (Singh et al., 2016). According to Saingam et al., (2013), coughing, sneezing, and shaking as if chilled to the bone were among the symptoms that were reported.

2.6 Kratom Treatment and Management

Reversal medications are considered the standard of treatment in instances of opioid overdose, thus far, proper clinical study is yet to be conducted on management of kratom overdose (Diep et al., 2018; Overbeek et al., 2019). Recommending treatment is depending on the organ system involved (Rech et al., 2015; Rosenbaum et al., 2012). For example N-acetylcysteine was be used to treat acute hepatitis in a way similar to that used to treat drug-induced hepatitis (Mousa et al., 2018). Antiepileptic medication can be used in attendance of seizures or neurological signs (Nelsen et al., 2010). Kidney damage, cardiovascular issues, and other emergency presentations were managed accordingly to the affected organs.

Ingestion of more than 15 g of kratom may cause kratom overdose with the symptomology mirror to the opioid toxidrome (Mousa et al., 2018). Patients experiencing kratom withdrawal symptoms frequently have a clinical profile similar to that of opioid withdrawal (Stanciu et al., 2019). These kratom withdrawal symptoms during abstinence includes sickness/vomiting, chills, looseness of the bowels (diarrhea), rhinorrheanoun, body pains, anxiety, and irritability (Singh et al., 2014). Mydriasis, hypothermia, tremors, and diaphoresis are all physical exam results, besides a sizable proportion of patients state having mental symptoms, the most prevalent of which are uneasiness, worry, and sadness (Khazaeli et al., 2019; Kruegel and Grundmann, 2018). Acute withdrawal patients are handled conservatively, a combination of buprenorphine and naloxone may help ease both physical and psychological symptoms related with kratom withdrawal (Khazaeli et al., 2019).

Additionally, favorable results have been reported when higher dosage of clonidine or other α-2 agonists combined with hydroxyzine are used (Suwanlert, 1975). A long-dated pharmaceutical replacement remedy may be necessary for people with persistent kratom addiction. Kratom-dependent opiate may be resulted from dependency, the reason is that Kratom is a less expensive and natural alternative to buprenorphine or methadone for people seeking to discontinue narcotic consumption. However, as noted, no solid clinical proof that kratom is a potent substitute for this purpose (Boyer et al., 2008, 2007). Consequently, such individuals face the risk of acquiring a kratom addiction while their underlying chronic addiction goes untreated. Given the scarcity of empirical remediation recommendations for kratom dependence, therapy regimens for stimulated kratom-dependent persons assiduously pursuing continuing management of drug cravings in a medical setting are the same as those utilized for opioid consumption. Management of kratom dependence include buprenorphine-naloxone (Subuxone) and clonidine regimens are employed (Agapoff and Kilaru, 2019; Khazaeli et al., 2018).

2.7 Qualification and Quantitation Analysis Methods for Kratom

There are presently no widely accepted analytical covering methodologies for kratom and its metabolites following administration, restricting detection to more advanced manners such as liquid chromatography-mass spectrometry and, more recently, IMS and Liquid chromatography/mass spectrometry was applied (Warner et al., 2016). A unique approach for screening along with detection of mitragynine and 7-hydroxymitragynine in urine specimens collected from humans using high-performance liquid chromatography-tandem mass spectrometry has been documented. This approach was swifter and more selective, and it may be used in ordinary clinical examinations as well as forensic investigations. The technique has been claimed to be

more efficient and selective than others (Fluyau and Revadigar, 2017). Prior to this method, numerous methods for determining kratom have been investigated, including capillary electrophoresis, gas chromatography, mass spectrometry coupling, and other approaches. The authors claimed that past techniques were not employed optimally to designate kratom as the single constituent in the specimen provided, making the tandem mass spectrometry more selective (Fluyau and Revadigar, 2017). Fuenffinger et al. (2017) recently proposed IMS as a novel approach for detecting Mitragynine in Kratom products (Fuenffinger et al., 2017). The researchers stated that 13 of 15 samples of the sample set of mitragynine with concentrations more than the IMS detection limit, resulted in a 100% positive success rate for Mitragyna identification and no false positives. The group asserted that IMS is a good approach for rapidly screening kratom items containing mitragynine (Fuenffinger et al., 2017).

2.8 Proteomic Analysis

Proteins are essential components in cellular function. Expression, localization, and activity of protein vary depending on cell type and function. Therefore, studying protein in different cell types or conditions is critical for identifying and understanding cellular biological information (Amiri-Dashatan et al., 2018). In 1995, the term "proteomics" was coined (Wilkins et al., 1995). Proteomics is a highly complex and rapidly enhanced science for apprehensive the expression and function of proteins (Hedl et al., 2019). Proteomics aims to acquire a more comprehensive and integrated biological picture of a cell, by analyzing overall cell's proteins rather than individual proteins (Graves and Haystead, 2002). Proteomics provides information on early illness diagnosis, prognosis, and disease monitoring. Furthermore, proteins play an important role in drug research as target molecules. In general, proteomics is the concept of the proteome, which encompasses all aspects of protein production,

architecture, function, interaction, and change through time. (Aslam et al., 2017). Proteomics methods include gel-based electrophoresis (Van Den Bergh and Arckens, 2005) and liquid chromatography tandem mass spectrometry (LC/MS/MS). Electrophoresis on a gel support is typically utilized to separate a mixture of proteins while mass spectrometry analysis is used to identify and identify proteins. Proteomics approach makes it a viable biomarker finding tool for a variety of disorders (Kim et al., 2021). This approach has been used to identify biomarkers in several disorders, including cancer (Peyvandi et al., 2018; Zamanian-Azodi et al., 2015), cardiovascular disease, acute immune deficiency syndrome (AIDS) and renal illness, diabetes (Safari-Alighiarloo et al., 2017). Proteomics is also a valuable tool in drug development, which is a huge phenomenon that includes genomics, proteomics, metabolomics, bioinformatics, and system biology. Proteomics investigations are also helpful in studying the action, toxicity, resistance, and effectiveness of drugs (Amiri-Dashatan et al., 2018). In general, proteomic techniques Figure (2.1) have been used to (a) profile the proteome, (b) compare the expression of two or more proteins, colocalize and identify post-translational modifications, additionally (d) analyze protein-protein interactions (Chandramouli and Qian, 2009).

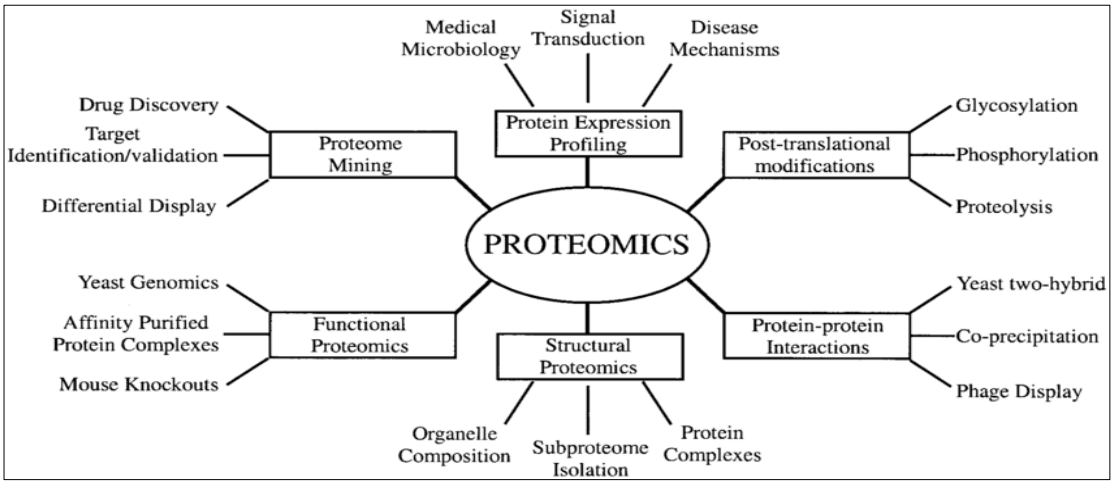

Figure 2.1 Applications and types of proteomics techniques (Graves and Haystead, 2002).

Electrophoresis is based on the principle of the migration of a charged particle, including big molecules such as proteins in a liquid phase under an electric field. Bigger proteins move faster while proteins with a lower net charge move slower. Furthermore, when a protein is in its natural (compact) form, it migrates faster than when it is denatured and stretched, where it encounters higher frictional resistance with the surrounding medium (Gallagher, 2012). Gel electrophoresis is a well-established method for separating macromolecules such as DNA, RNA, or proteins depending on bulk, shape, or isoelectric point (Cai, 2020). When an electric field is applied to a gel matrix, electrophoresis may be used to separate molecules into their individual components, charged molecules flow across the gel matrix under the externally applied field of force electric. This method is now widely used in biological chemistry, molecular biology, analytical chemistry, and proteomics concept. Gel electrophoresis is often used for analysis, but it may also be employed as a preparatory manner to partly refine molecules previous to use other methods, primarily mass spectrometry, to undertake proteome analysis (Wasinger et al., 1995). Protein gel electrophoresis by using polyacrylamide matrix, often known as polyacrylamide gel electrophoresis (PAGE), is the most frequently used procedure for characterizing complicated protein combinations. It is an easy, quick, and low-cost procedure (Garca-Descalzo et al., 2012). In two-dimensional gel electrophoresis, in the beginning, proteins are separated based on their respective isoelectric points, If the proteins are in a medium with a pH dissimilar to their isoelectric point, they have a net electrical charge and can move when exposed to the field of force. The migratory velocity is related to the charge-to-mass ratio of the protein. The faster the movement, the bigger the charge per unit of mass. They will stop moving when they are at their isoelectric point even if electromotive force is applied. Subsequently, the proteins will be denatured by the

addition of a detergent such as sodium dodecyl sulfate (SDS), and then they will be separated according to their molecular weight in the second dimension of separation. Shapiro et al. was the first to use this approach (1967). SDS is a reducing agent that shatters disulfide bonds, splitting the protein molecule into its subunits, and leaving it with an overall negative charge, allowing it to move freely within the gel in proportion to its size. Furthermore, The tertiary structure is also lost during this denaturation (Shapiro et al.,1967).

2.9 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is an essential technique for protein separation and molecular weight determination. Laemmli described the most widely used SDS-PAGE method in 1970 (Laemmli, 1970). Protocols for SDS electrophoresis of proteins differ in buffer formulation and gel permeability based on the size of the proteins to be separated. The capacity of SDS to combine proteins underpins all versions of the SDS-PAGE method. SDS connects with non-polar portions of the protein molecule via hydrophobic interactions due to the existence of a twelve-carbon tail, whilst the polar head of the SDS molecule provides the SDS-protein complexes a net negative charge (Pavlova et al., 2018). Gels of SDS-PAGE are made up of long polymers arranged in a cross-linked mesh. The pores are the spaces between the polymers. Higher polymer concentrations result in narrower average pore diameters. Polyacrylamide gels are created by covalently connecting acrylamide monomers with bis-acrylamide using a free radical such as persulfate (SO_4). The acrylamide polymers are cross-linked, resulting in 'pores' of a certain size. The average pore size is determined by the total acrylamide concentration and the ratio of bis-acrylamide to acrylamide. Polyacrylamide gels are made by polymerizing acrylamide with the cross-linking agents N, N'-methylene-bisacrylamide. The ammonium persulfate (APS) catalyst, and N, N, N', N' -