ANTIVENIN ACTIVITY OF HERBS COMMONLY USED IN KENYA AGAINST *Dendroaspis polylepis* (BLACK MAMBA) SNAKE BITE

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DECLARATION

This thesis is my original work and has not been presented for a degree at any other University.

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This work is dedicated to my daughters Tania Marie and Talei Maxine for not giving up on their mother and to my dear mum Lilies Wanjiru Mwangi, for being my beacon and strength.

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LIST OF ABBREVIATIONS

1DTK	Dendrotoxin from *Dendroaspis polylepis* venom
1FSC	Fasciculin from *Dendroaspis polylepis* venom
1NTX	Neurotoxin from *Dendroaspis polylepis* venom
1TFS	Calciseptine from *Dendroaspis polylepis* venom
5AFM	Nicotinic acetylcholine receptor
5GJV	L-Type calcium channel
5HQ3	Human acetylcholinesterase enzyme
CID	Compound identification number from PubChem
PDB	Protein Data Bank
SDF	Structure Data File
WHO	World Health Organization

ABSTRACT

The World Health Organization estimates that annually, 5.4 million snake bites occur globally resulting in up to 140,000 deaths. Most victims do not have access to health facilities and are known to resort to herbal treatments after a snake bite. Few studies have been done in Kenya to confirm activity of plant extracts in snake bite. This study was analytical experimental in design and sought to determine the median lethal doses and antivenin activity of aqueous extracts of *Zanthoxylum chalybeum*, *Solanum incanum*, *Tagetes minuta*, *Tithonia diversifolia* and *Sansevieria suffruticosa* in *Dendroaspis polylepis* (Black mamba) snake bite. Selected pharmacological activities, phytochemical analysis using X-ray fluorescence (XRF), LC-MS/MS, and GC-MS and molecular docking between the phytocompounds and proteins involved in envenomation were done of the plant extract with significant antivenin activity. The median lethal doses of the extracts were calculated using a formula, chi-square followed by Scheffé's post hoc test for the antivenin activities and ANOVA to compare differences between groups in the pharmacological study at 95% and 99% ($P<0.05$ and <0.01) confidence level respectively. Phytochemical analysis data was presented in tables and spectra, while that from molecular docking was done using AutoDock Vina®. The LD_{50} of *S. incanum* was 316mg/Kg, while that of the other four plants was 2085mg/Kg. None of the five plant extracts showed significant *in vitro* effect against the venom. However, 1500 mg/Kg of the aqueous extract of *T. diversifolia* showed significant ($P<0.05$) *in vivo* effect against *D. polylepis* venom. The *T. diversifolia* extract also showed significant anti-nociceptive, anti-pyretic and anti-inflammatory effect at a dose of 300mg/Kg in mice following both intraperitoneal and oral administration. XRF analysis of the dry powder showed high net counts of potassium, iron and calcium using while GC-MS of the crude extract detected fourteen compounds of which camphene and menthol were most abundant. Six of the compounds; menthol, d-menthol, spathulenol, methyl palmitate, citronellyl propionate, and citronellyl butyrate presented best docking results at nicotinic acetylcholine receptor, acetylcholinesterase enzyme and L-type calcium channel. Results from this study authenticate antivenin and other pharmacological claims of *T. diversifolia* in *D. polylepis* snake bite.

CHAPTER ONE
INTRODUCTION

1.1. Background of the study

Snake bite is recognized as a Category A neglected tropical disease, a potentially lifethreatening disease whose management remains confounding owing to the many species of venomous snakes and the high specificity of their veno (J. P. Chippaux *et al*., 2019). It is associated with mortality and permanent disability that occur due to the unavailability and unaffordability of safe, efficacious anti-snake venom. Neglected tropical diseases are therefore a public health concern particularly in Africa due to limited resources availed to public health budgets to cater for victims.

The World Health Organization global statistics indicate that more than five million snake bites occur annually with more than a third of these resulting in envenomation and consequently over a hundred thousand deaths. The remaining cases do not amount to envenomation despite the bites, a phenomenon known as 'dry bites.' Dry bites are thought to be one of the reasons why snake bite victims who seek medical attention in hospitals are fewer than the incidences of snake bite (Duc & DeAcetis, 2011). The other reason is thought to be the long distances that victims must cover to get to the nearest health care facilities and the associated cost of hospital care. There is also a high likelihood that the anti-venom may not even be available at the health facility as exemplified in a report in Uganda that indicates that only 4% of health facilities stocked anti-snake venom leading to most victims opting not to visit hospitals (Omara *et al.,* 2020).

Fortunately, most patients are aware and have confidence in traditional healers who live in their communities and are easier, faster and cheaper to access, noting that the most afflicted

communities are remotely located with few or no health facilities within reach. The traditional healers rely on a variety of healing aids to treat snake bite, herbal preparations being integral to the treatment (Chuat *et al.,* 2021; Pullani & Prabha, 2020; Schioldann *et al.,* 2018). Although scientific work on these herbs has not been done extensively, there are reports of their activity against snake venom.

The use of plant remedies to manage snake bite is a departure from conventional treatment which relies heavily on the administration of anti-snake venom (ASV), a specific component immunotherapy. Immunotherapy consists of injection of intravenous monovalent or polyvalent purified immunoglobulin fragments against the venom and is so far the most reliable and only scientifically proven therapy following bites by venomous snakes. Immunoglobulin is unfortunately not cheap owing to the lengthy manufacturing process that relies on harvesting of antisera (anti-snake venom, ASV) from horses inoculated with low doses of venoms leading to an immune reaction where antibodies against the venom are produced (Sajon *et al.,* 2017). The ASV is usually associated with adverse reactions due to admixture with animal proteins.

To save lives and in the context of unavailable and/or unaffordable anti-snake venom, communities throughout the world have identified local (traditional) approaches to management of snake bite. Examples of these approaches include making cuts at the site of the bite followed by sucking venom out and applying medicaments to the wound. Most of these approaches are not recommended in the conventional management of snake bite and are in fact thought to cause more harm than good (Subedi *et al.,* 2018). Traditional approaches have however not been studied enough to confirm or dispute the

recommendations against their use. Additionally, it is believed that traditional treatments cause delays in accessing conventional treatment which inadvertently worsens snake bite cases.

To date, the strongest indication that traditional approaches may have a role in management of snake bite lies in records indicating that many plant products have been and still are in use for the management of snake bite worldwide. In Myanmar for example where snake bite is prevalent, traditional healers are often consulted by victims even in communities with access to health facilities (Schioldann *et al.,* 2018). This is mirrored by the World Health Organization's (WHO) community engagement as part of the strategy to control and improve snake bite outcomes in Eswatini, Swaziland which is home to venomous snakes such as the puff adder (*Bitis arietans*), Mozambique spitting cobra (*Naja mossambica*) and the much-feared black mamba (*Dendroaspis polylepis*) living close to a rural population resulting in serious envenomation cases (Nann, 2021).

Despite such records, use of medicinal plants in management of snake bite is still unsubstantiated thus little confidence if any in their use as alternative and/or complementary therapies to anti-snake venom. The search for alternative therapies for snake bite remains an urgent matter and this gives impetus to thorough investigations into safety and efficacy of medicinal plants documented for use in snake bite.

Both traditional and conventional practitioners concur that snake bite is a medical emergency due to fast progression to life threatening manifestations such as hypovolemia, consumptive coagulopathy, tissue necrosis, cardiomyopathy, renal failure and respiratory failure (Curry, 2006; *et al.,;* Tintinalli E J Stapczynski J S, 2016). Thus, the management

is best suited in a setting where patients' airway, breathing and circulation can be stabilized and supportive management for adverse treatment outcomes can be instituted. This observation motivates efforts to integrate scientifically proven traditional therapies into conventional medicine practice, as provided for in Kenya in the Health Act, 2017 (Kenya Gazette Supplement, 2017). Thus, this study sought to determine effects of five selected herbal extracts on *D. polylepis* envenomation and other snake bite manifestations.

1.2. Statement of the problem

Snake bite is a global public health problem with an estimated 5.4 million bites occuring annually resulting in about 150,000 deaths and 400,000 permanent disabilities. Whereas complete eradication of snake bite would be an ideal solution to the snake bite problem, it is not achievable despite prevention strategies. It would therefore be expected that afflicted communities will continue to suffer losses from maiming or death, which also afflicts domestic animals, denying the same communities a source of livelihood. Due to challenges in accessing health facilities and their inadequate capacity to treat snake bite coupled with the unavailability and unaffordability of safe, efficacious anti-snake venom, there is need for alternative management approaches for snake bite that should be safe, efficacious, affordable and sustainable. For years, snake bites have been managed at village level by traditional healers who rely on herbs to heal victims, thus scientific authentication of these treatments would provide cheaper, more available, safer and sustainable treatment alternatives for snake bite.

1.3. Research Questions

This study sought to answer the following questions;

1. What is the median lethal dose of aqueous herbal extracts of *Sansevieria suffruticosa* leaves, *Solanum incanum* fruits, *Tithonia diversifolia* leaves, *Tagetes Minuta* leaves and *Zanthoxylum chalybeum* stem bark?
2. What is the *in vitro* and *in vivo* activity of aqueous herbal extracts of *S. suffruticosa* leaves, *S. incanum* fruits, *T. diversifolia* leaves, *T. minuta* leaves and *Z. chalybeum* stem bark on crude venom from *D. polylepis?*
3. What are the pharmacological activities of the most active aqueous extract(s) in 2 above on clinical wound associated with *D. polylepis* snake bite?
4. Which phytoconstituents are present in the most active herbal extract(s) in 2 above?
5. Which phytocompounds identified in 4 above are involved in molecular interactions with *D. polylepis* venom proteins and its mammalian targets?

1.4. General objective

To establish the anti-venin and pharmacological activity of herbal extracts from *S. suffruticosa* leaves, *S. incanum* fruits, *T. diversifolia* leaves, *T. minuta* leaves and *Z. chalybeum* stem bark in *D. polylepis* snake bite.

The null hypothesis (H_0) for the study was;

H_0: Herbal extracts from *S. suffruticosa* leaves, *S. incanum* fruits, *T. diversifolia* leaves, *T. minuta* leaves and *Z. chalybeum* stem bark do not have activity against *D. polylepis* venom and snake bite effects in mice.

1.5. Specific objectives

1. To determine the median lethal dose of aqueous herbal extracts of *S. suffruticosa* leaves, *S. incanum* fruits, *T. diversifolia* leaves, *T. minuta* leaves and *Z. chalybeum* stem bark in mice.
2. To establish the *in vitro* and *in vivo* antivenin activity of aqueous herbal extracts of *S. suffruticosa* leaves, *S. incanum* fruits, *T. diversifolia* leaves, *T. minuta* leaves and *Z. chalybeum* stem bark on snake venom from *D. polylepis* in mice.
3. To investigate the anti-nociceptive, anti-pyretic, anti-inflammatory and anti-microbial activities of the most active extract(s) in 2 above in laboratory animal models.
4. To determine the phytoconstituents present in the active extract(s) in 2 above using X-ray fluorescence (XRF), Liquid Chromatography-Tandem Mass Spectrometry (LCMS/MS) and Gas Chromatography Mass Spectrometry (GC-MS) techniques.
5. To identify the phytocompounds in 4 above that are involved in molecular interactions with *D. polylepis* venom proteins and their mammalian targets using molecular docking techniques.

1.6. Justification

Global annual snake bite incidences approach three million with a mortality of 5% and permanent disability in 15% of victims, a majority of whom live in impoverished communities with farming as their main economic activity. *D. polylepis* is a snake species found in sub Saharan Africa and is known to cause 100% fatality in the absence of immunotherapy. Its venom spreads rapidily through tissue due to proteins of low molecular weight which have been well characterized and known to cause neurotoxicity and

myotoxicity. Venoms from other snake species, particularly cytotoxic venoms, are more complex in composition and activity compared to that of *D. polylepis* and hence its purposeful selection for this study.

Although immunotherapy (ASV) remains the mainstay of therapy in envenomation following snake bite, it is largely unavailable and unaffordable. This is attributed partly to lack of economic incentive to manufacturers occasioned by removal of snake bite as a neglected tropical disease by WHO in 2013 (J. P. Chippaux, 2017). It was however reinstated by WHO as a neglected tropical disease in June 2017 reinforcing its importance in public health and the need for concerted efforts in the search for safe, efficacious, available and sustainable treatments. These efforts are currently underway with global anti-snake bite initiatives that recognize the urgent need to develop alternative approaches in the management of snake bite with a target of halving the deaths and disability by 2030 (J. P. Chippaux *et al.,* 2019).

Therefore, with the knowledge that the use of herbal medicine has been practiced for years worldwide and that these herbs have not been studied extensively to determine their safety and efficacy, an opportunity presented itself to subject these herbs to scientific assessments aimed at substantiating their role as safe and efficacious antivenin and pharmacological agents of benefit in snake bite management.

1.7. Significance of the study

The findings of this study provided scientific evidence on the safety, efficacy and mechanism of action of selected herbs in *D. polylepis* envenomation. The information is useful in laying a foundation for further studies towards the development of herbal

remedies as alternatives or complementary therapies to immunotherapy in the management of snake bite. The proof of efficacy for some selected pharmacological uses is an additional benefit of the herbal constituents that may be explored for similar clinical wound. Dissemination of findings of this work through publications and conference presentations would be contribution to science in the area of snake bite.

CHAPTER TWO
LITERATURE REVIEW

2.1. The burden of snake bite

The WHO defines snake bite as a potentially life-threatening disease caused by toxins in the bite of a venomous snake (WHO, 2010a). A snake bite can be life-threatening if the snake is venomous as observed in bites from snakes such as the black mamba, king cobra, banded krait, saw-scaled viper and rattlesnake.

The earliest attempt at assessing worldwide snake bite prevalence was a study published in 1954 to assess the global extent of snake bite in order to inform the World Health Organization on the need to standardize anti-venom for snakes other than those found in Europe (J.-P. Chippaux, 2008). At the time, snake bites were not recorded appropriately as medical occurrences except under the 1948 medical classification, 'E927: Stings and Bites'. It was also difficult to get information from underdeveloped countries due to under reporting. In Kenya for example, the researchers recorded 404 cases of snake bites in 1946 with one fatality. Four years later in 1950, the figure had risen to 554 with 12 fatalities. Later in 1998, (Chippaux, 1998b) in a global assessment of snake bite and mortality concluded that the largest burden was in developing countries, mainly in Asia and Sub Saharan Africa. He reported that there were 5 million bites annually in these two parts of the world that led to 2.5 million envenomations and 120,000 deaths compared to 400,000 bites, 183,000 envenomations and 5340 deaths in the rest of the world (J. P. Chippaux, 1998b). The World Health Organization (WHO) global statistics now indicate that about 5.4 million snake bites occur annually with 2.7 million of these resulting in envenomation

causing 150,000 deaths and about 400,000 permanent disabilities. Sub Saharan Africa is most severely affected with more than 20,000 deaths annually (WHO, 2018).

Envenoming resulting from snake bite is a particularly important public health problem in rural areas of tropical and subtropical countries in Africa, Asia, Oceania and Latin America. The highest burden of snake bites is in South Asia, Southeast Asia, and subSaharan Africa (Kasturiratne *et al.,* 2008) mainly affecting those involved in subsistence farming activities. Poor access to health services in these settings and, in most cases, a scarcity of anti-venom, often leads to poor outcomes and considerable morbidity and mortality. Due to these reasons, many victims fail to reach hospital in time or seek medical care after a considerable delay. It is presumed that victims first seek immediate treatment from traditional healers (Schioldann *et al.,* 2018) while some unfortunately die before reaching hospital. Hospital statistics on snake bites therefore underestimate the true burden. In addition to mortality, some snake bite victims survive with permanent physical sequelae due to local tissue necrosis and, sometimes psychological trauma. Sadly, since most victims are young, the economic impact of snake bite can be considerable(Warrell, 1995).

A community-based retrospective survey of 4712 households estimated mortality among a rural population in Kenya to be 6.7/100,000 people each year, representing 0.7% of total fatalities that year (Snow *et al.,* 1994). Although 151/100,000 people are bitten each year, only 19% of these are by potentially venomous snakes. When those who had been bitten were requested to identify the snake from photographs of a range of locally prevalent snakes, both venomous and non-venomous snakes were identified. According to this study,

68% of bite cases sought treatment from a traditional healer who invariably used local herbal preparations applied to the bite site and/or in a ring around the bitten limb. Local skin incisions were also commonly made and herbal extracts instilled.

In an assessment of the role of traditional practitioners in Africa, (Ryan *et al.,* 2011) recognized the use of traditional medicine for snake bite as a feature in most areas where venomous snakes are prevalent. Improvements in early referral and appropriate care can therefore be achieved with the integration of traditional healers into primary health care. The traditional practitioners however would need training in management of snake bites to avert complications due to some practices such as the application of tourniquets in inappropriate circumstances, incisions on skin, application of contaminants into bite sites and prompt referrals to health care facilities.

In another study, primary data were collected on the incidence, severity and species responsible for snake bites in four areas of Kenya namely western (Kakamega), the rift valley (Lake Baringo and Laikipia), coastal region (Kilifi and Malindi) and northern Kenya. The overall average frequency of snake bite was 13.8 per 100,000 populations per year (range 1.9-67.9) and the minimum rate of snake bite mortality was 0.45/100,000/year. Thirty-four of the fifty units visited reported no knowledge of death from snake bite in the preceding 5 years. Traditional treatments were common, especially the use of herbal remedies and incisions at the wound site (Coombs *et al.,* 1997).

2.2. Snake species of medical importance

According to the World Health Organization 'Guidelines for the Prevention and Clinical Management of Snake bite in Africa' (WHO, 2010a), the venomous snakes of Africa can be divided into five categories based on documented incidences of envenomation.

Category 1: Snakes that bite frequently, and are associated with serious or life- threatening envenoming. They include: saw-scaled or carpet vipers (*Genus Echis*), large african adders or vipers (*Genus Bitis*), spitting or cytotoxic cobras (*Genus Naja*), neurotoxic cobras (*Genus Naja*) and mambas (*Genus Dendroaspis*).

Category 2: Snakes that bite frequently, but rarely cause serious or life-threatening envenoming. These include African burrowing asps (*Genus Atractaspis*), African night adders (*Genus Causus*) and north african sand or desert horned vipers (*Genus Cerastes*).

Category 3: Snakes that bite rarely, but are capable of causing severe or life-threatening envenoming. These are: boomslang (*Genus Dyspholidus*), vine, bird, twig or tree snakes (*Thelotornis* spp.), other neurotoxic cobras (*Naja anchietae N. melanoleuca*), rinkhals (*Hemachatus haemachatus*), desert black snakes/cobras or Walter Innes's snakes (*Walterinnesia aegyptia*), bush or tree vipers (Genera *Atheris, Proatheris, Montatheris* and *Adenorhinos*), yellow-bellied sea snakes (*Pelamis platurus*), gaboon adders (*B. gabonica, B. rhinoceros, B. nasicornis*), small (*dwarf*) adders (*Genus Bitis*), Coral/Shield-nosed snakes (*Genus Aspidelaps*) and old-world vipers (*Macrovipera species*).

Category 4: Snakes that bite rarely, and have not caused significant envenoming. These include other minor adders (*Bitis caudalis, B. schneideri, B. worthingtoni*) and African garter snakes (*Genus Elapsoidea*).

Category 5: Other potentially venomous snakes which have not caused documented bites. These include Ethiopian mountain adder (*Bitis parviocula*), other burrowing asps (*Genus Atractaspis*), other bush vipers (G*enus Atheris*), other dwarf adders (*Genus Bitis*), other night adders (*Genus Causus*), other vine snakes (*Genus Thelotornis*), other Colubridae, other old-world vipers (*Genus Macrovipera* and *Vipera*), water cobras (Genus *Naja [Boulengerina]*), other garter snakes (*Genus Elapsoidea*), burrowing cobra (*Naja [Paranaja] multifasciata*) and tree cobras (*Genus Pseudohaje*).

It is proposed that the largest number of serious envenoming in eastern and southern Africa is due to puff adder bites. Cytotoxic spitting cobras follow suit while the mambas, though not associated with frequent bites, cause high (100%) and rapid case fatalities (Warrell & Harvey, 1995).

2.3. Dynamics of Black mamba (*D. polylepis*)

2.3.1. Characteristics of *D. polylepis*

The black mamba (*Dendroaspis polylepis*) is from the family Elapidae and is also called the common black mamba or black-mouthed mamba; *Dendroaspis* meaning ‘tree snake’ and *polylepis* meaning ‘many scaled’. It is the longest venomous snake in Africa with an average length of around 2.5 meters (8.2 feet), but there have been specimens found that have grown up to 4.3 meters (14 feet). It derives its name from the ink-black colour of the inside of its mouth and not its external colour which varies from yellowish-green to

gunmetal grey. The Kenya Reptile Atlas documents grey, almost white, olive, brown, yellow-brown and paler below as the colours of the snake as sighted in different parts of the Kenya (Spawls S. *et al.*, 2018).

The black mamba is also the fastest snake in the world with a speed of 16-20 km/h (10-12mph) and weighs up to 1.6 kg (3.5 lbs). Since its venom is made of proteins of low molecular weight, the venom and its constituents are able to spread extraordinarily fast within the bitten tissue. This makes its venom the most rapid-acting of all snake venoms, thus without effective anti-venom, the case fatality rate of its envenomation is 100% (Szalay, 2014).

Figure 2.1: Color variability of the black mamba snake as observed in different parts of Kenya

(a) Ink black lining of inner mouth and fangs of *D. polylepis* specimen from Watamu, Kilifi County; b) Metallic grey specimen from Voi, Taita-Taveta County; c) Olive specimen from Baringo County and d) Yellow brown specimen from Ngomeni, Kilifi County, Kenya *(Photos sourced from (Kenya Reptile Atlas, Website).*

2.3.2. Geographical Distribution of *Dendroaspis polylepis* in Africa

D. polylepis is habitat to Sub-Saharan and Southern Africa. There have been some sightings in Central and West Africa but these remain uncomfirmed as shown in Figure 2.2 below.

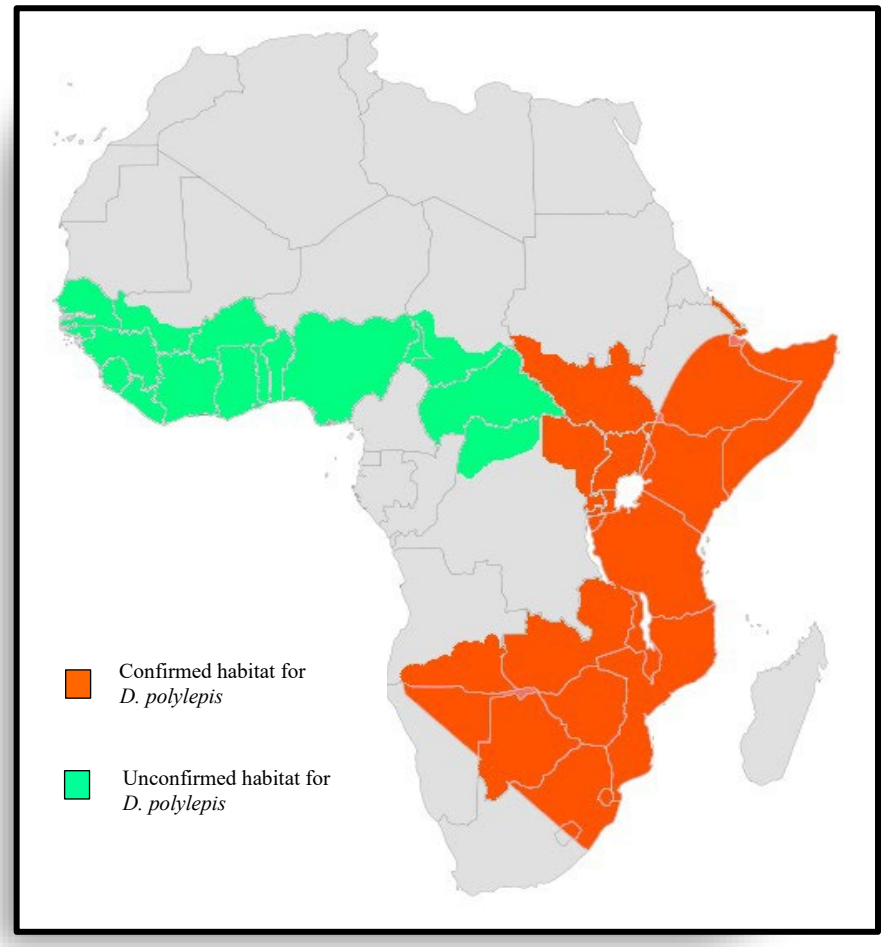


Figure 2.2: Geographical distribution of *Dendroaspis polylepis* (black mamba) in Africa (*UWL Website*, n.d.)

2.3.3. Geographical distribution of *Dendroaspis polylepis* in Kenya

According to the Kenya Reptile Atlas, *D. polylepis* is widespread in medium to low altitude savanna, woodland and coastal bush and appears absent from most of dry northern Kenya and the highlands of the southwest. The snake is mostly found in Mombasa, Watamu, Malindi, Voi, Mtito Andei, Mwingi. A few sightings have been in Kisumu.

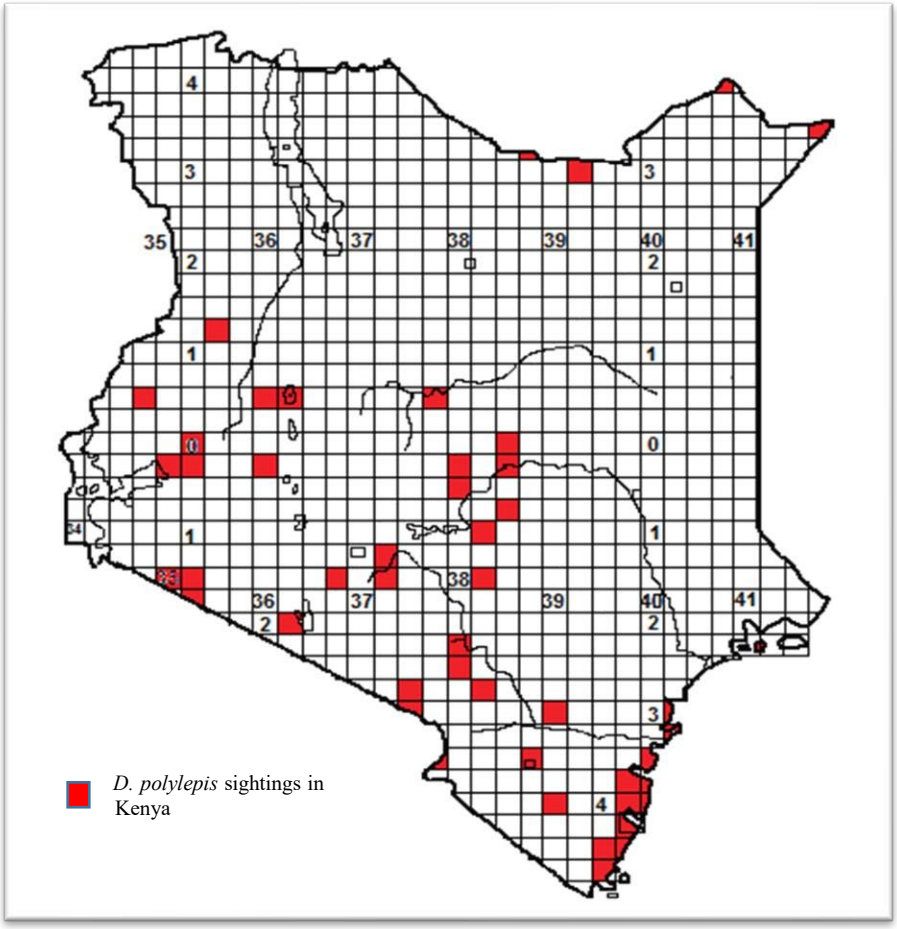


Figure 2.3: Mapping of *D. polylepis* sightings in various parts of Kenya. Sourced from the Kenya Reptile Atlas (Kenya Reptile Atlas, n.d.)

The Kenya Reptile Atlas provides local names for the black mamba as; Koboko (Swahili), Isembelei, Ikuuwa, Ivuu, Ivwau (Kikamba), Rai Ikombe (Dholuo), Kiplogoyon (Kalenjin), Matangi (Luhya), Kiria Munyore (Tharaka), Tsatsapala (Mijikenda), Cuthu (Kikuyu), Ilimanjiwu, Imilambimbi (Kitaita). It also describes the black mamba found in Kenya as a very large snake, up to 3.2 m, usually, 1.5 – 2.5 m with a long head and body often barred with dry country specimens often heavily speckled towards the tail.

2.3.4. Composition of *D. polylepis* venom

The venom of the black mamba is mainly neurotoxic and about 100 to 120 mg of venom on average is delivered per bite, however up to 400 mg can be delivered and a human being can die from a bite delivering 10 to 15 mg. The venom can potentially kill a human within 20 minutes depending on the health, size, age, and psychological state of the person; but death usually occurs after 30 to 60 minutes, sometimes taking up to three hours (Laustsen *et al.*, 2015).

In general, elapid toxins act at the neuromuscular junction and are classified according to their pharmacological activity as pre-synaptic and postsynaptic toxins (Mirajkar *et al.,* 2006). Post-synaptically active toxins bind to the acetylcholine receptors at the postsynaptic membrane thus preventing the binding of acetylcholine. The effect is a nondepolarizing type of neuromuscular blockade. The classical example is α-bungarotoxin from the venom of *Bungarus multicinctus*. Pre- synaptically active toxins act on motor end terminals to either facilitate or block neurotransmitter release, e.g., β-bungarotoxin, also from the venom of *Bungarus multicinctus* (Gawade, 2008).

In 2015, Laustsen and others evaluated the black mamba venom and reported that it was a complex venom constituted predominantly of proteins belonging to the Kunitz-type proteinase inhibitor family, which comprises the dendrotoxins and α-neurotoxins of the three-finger toxin family (Laustsen *et al.,* 2015). This was corroborated by (Závada *et al.,* 2011), who reported that the venom contains three principal substances; dendrotoxins, calciseptine and mambalgines. Generally, there are approximately 20 known enzymes found in snake venom. Venom from any venomous snake usually comprises of 6 to 12 of these enzymes in their venom; fasciculins, proteases, hyaluronidases, phosphatases, phospholipases and peptides. The abundance of some of these venom components over others is observed within snake families and clinical presentations in what is referred to as syndromic wound of snake bite. Hemolytic, hemorrhagic and coagulation components for example are almost completely absent from mamba venom (Warrell, 1995). *D. polylepis* venom proteins showing highest acute toxicity according to Laustsen were α-neurotoxins, which induce post-synaptic blockade of the neuromuscular junctions, followed by dendrotoxins, which inhibit the voltage-dependent potassium channels presynaptically. The combination of the two types of toxins in the venom underscores the presence of a dual strategy that results in a highly effective mechanism for prey subduction. Additionally, fasciculins which are acetylcholinesterase inhibitors prevent the breakdown of acetylcholine at the synapse while calciseptine, an L-type calcium channel blocker prevents the excitation contraction coupling of skeletal, smooth and cardiac muscle and secretion of aldosterone in endocrine cells of the adrenal cortex.

2.3.5. Mechanism of action of *D. polylepis* venom components

a) Dendrotoxins

Dendrotoxins act on the pre-synaptic membrane of nicotinic acetylcholine receptor by preventing the release of potassium ions through blockade of ion channels. This in turn stimulates the increased release of acetylcholine from the pre-synaptic membrane causing an initial stimulation, however the pre-synaptic neuron is then unable to repolarise and hence signals are blocked. This blockade leads to uncontrolled neurotransmitter release, seizure activity and eventually paralysis (Kularatne & Senanayake, 2014b).

Dendrotoxins are ~7kDa proteins consisting of a single peptide chain of approximately 5760 aminoacids. Several homologues of dendrotoxin have been isolated, all possessing a slightly different sequence. δ-Dendrotoxin is a member of the dendrotoxin family of neurotoxins isolated from neurotoxic snake venom of black mamba (Harvey & Robertson, 2004). These toxins contain three disulphide bridges that give it its structural stability and contribute to its conformation.

The association between a single delta dendrotoxin molecule with a potassium channel is reversible and is thought to result from the electrostatic interactions between the positively charged amino acid residues in the cationic domain of dendrotoxin and the negatively charged residues in the ion channel pore. Potassium channels, similar to other cationselective channels, are believed to have a cloud of negative charges that precede the opening to the channel pore that help conduct potassium ions through the permeation pathway. It is proposed that dendrotoxin molecules bind to anionic sites near the

extracellular surface of the channel and physically occlude the pore, thereby preventing ion conductance. Some studies suggest that dendrotoxins inhibit potassium channels by altering their structure (Imredy & MacKinnon, 2000).

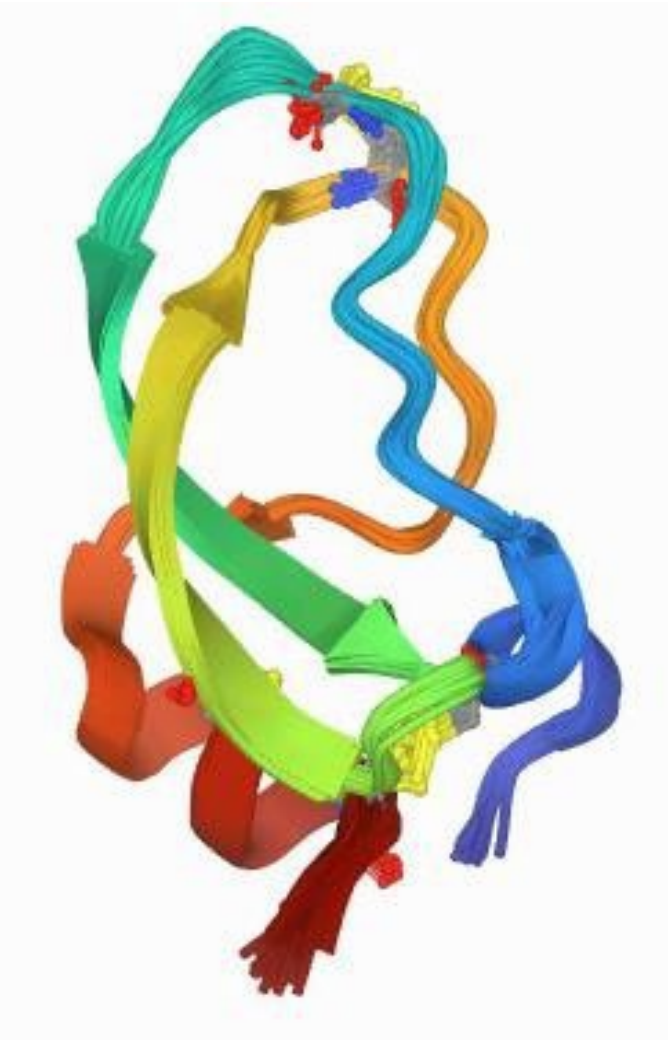


Figure 2.4a: NMR solution structure of dendrotoxin k (1DTK) from the venom of *Dendroaspis polylepis polylepis* (Pettersen *et al*., 2004).

b) Neurotoxic peptides

These are α-neurotoxins that competitively bind and block acetylcholine from stimulating nicotinic acetylcholine receptors at the postsynaptic membrane of the neuromuscular junction thus causing paralysis (Barber *et al.,* 2013).

Figure 2.4b: NMR solution structure of α-neurotoxin (1NTX) from *Dendroaspis polylepis polylepis* based on sequence specific proton nuclear magnetic resonance assignments (Pettersen *et al*., 2004)

c) Fasciculins

Fasciculins (Fas-1, Fas-2, Fas-3) represent a type of acetylcholinesterase inhibitors that provoke muscle fasciculation through a powerful inhibition of enzyme activity at the neuromuscular end-plate, interfering with the normal degradative activity of the acetylcholine molecule into choline and acetic acid. Initially, skeletal muscle fasciculation is observed, followed by flaccid paralysis. Fasciculins are also powerful inhibitors of brain acetylcholinesterases and of the three sub-types, Fas-2 is the most abundant in *D. polylepis* venom (Riva, 2013).

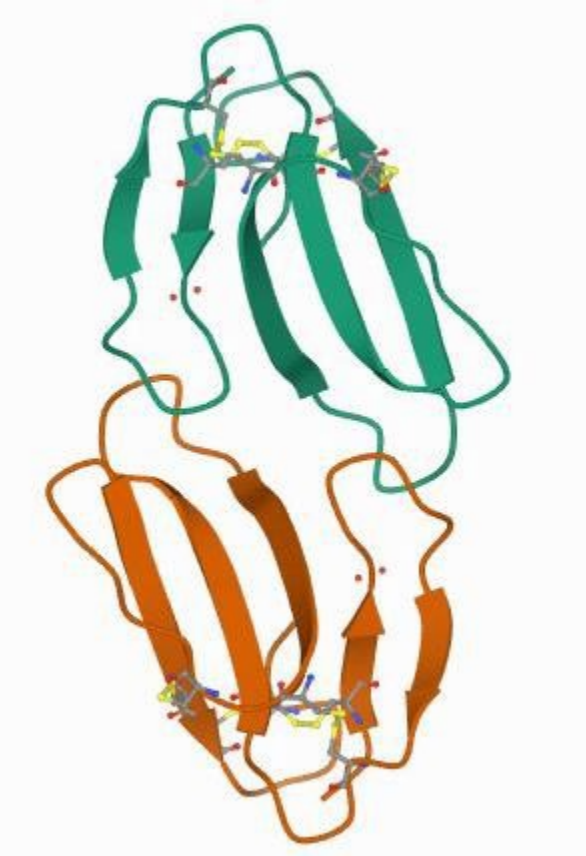


Figure 2.4c: Crystal Structure of Fasciculin 2 (1FSC) (Pettersen *et al*., 2004)

d) Calciseptine

This toxin consists of 60 amino acids with four disulfide bonds which blocks L-type calcium channels, but not other voltage-dependent calcium channels such as N-type and Ttype. L-type calcium channels are responsible for the excitation contraction coupling of vascular, skeletal, smooth and cardiac muscle and for aldosterone secretion in endocrine cells of the adrenal cortex (Hofmann *et al*., 1994). Its action resembles that of 1,4-dihydropyridines which are important in the treatment of cardiovascular diseases (Riva, 2013).

Figure 2.4d: NMR solution structure of calciseptine (1TFS), a specific blocker of the L-type calcium channel, isolated from *D. polylepis* venom (Pettersen *et al.,* 2004).

e) Mambalgins

These toxins have a strong analgesic effect in both central and peripheral nerves, reported to be as potent as morphine but better because they cause less tolerance and no respiratory distress. This is because they avoid pain using a completely different route, which is potentially capable of causing fewer side effects. Mambalgins have been found to take away pain by inhibiting acid-sensing ion channels (ASIC) in the peripheral and central nervous system. The opening of ASCI facilitates pain signals to the brain (Riva, 2013).

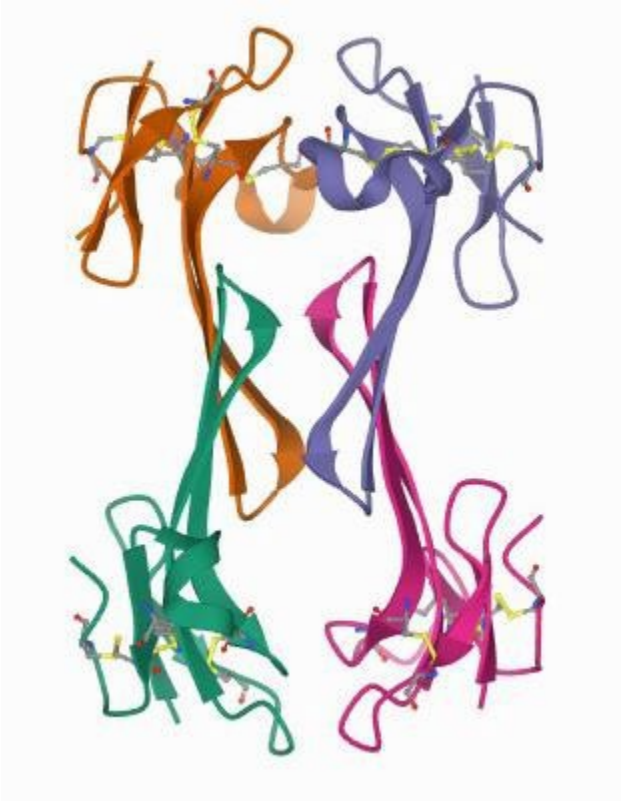


Figure 2.4e: The crystal structure of *Dendroaspis polylepis* mambalgin-1 wild-type (5DU1) in P21 space group (Mourier *et al.,* 2016)

f) Enzymes

Hyaluronidases break up extracellular components between cells facilitating rapid propagation of venom components in the organism. Proteases break up proteins destroying blood haemoglobin and important enzymes. Phosphatases fragment adenosine triphosphate (ATP), disrupting the cellular currency from working properly. Phospholipases destroy the ester bonds which are important in making the cell membrane, causing the contents of the cells to pour out (Kularatne & Senanayake, 2014a).

2.4. Clinical presentation of *D. polylepis* envenomation

The venom attacks the nervous system, causing paralysis of vital organs, most susceptible the respiratory muscles. If bitten, severe neurotoxicity often occurs with common symptoms being rapid onset of dizziness, drowsiness, coughing or difficulty in breathing, convulsions, and an irregular heartbeat. Other common effects are neuromuscular symptoms, shock, loss of consciousness, hypotension, pallor, ataxia, excessive salivation (oral secretions may become profuse and thick), limb paralysis, nausea and vomiting, fever, and very severe abdominal pain. Hemolytic, hemorrhagic and coagulation activities are almost completely absent from mamba venom (WHO, 2010a).

Amin *et al.* in a study of patients with neurotoxic envenomation at a medical college hospital in Bangladesh reported that all the patients developed ptosis, 94% had external opthalmoplegia, 77% dysphagia, 68% dysphonia and over 80% had broken neck sign caused by paralysis of neck flexor muscles (Amin *et al*., 2008).

There are also local effects at the site of the bite which include redness, swelling, bruising, bleeding, or blistering. There may be infection of the wound usually by commensals, *Staphylococcus aureus* and *Streptococcal pyogenes* among others (WHO, 2010b).

2.5. Medical management of *D. polylepis* envenomation

It is now known that symptoms of envenomation occur in a syndromic fashion depending on the type of venom involved. Patients present majorly with either neurotoxic, haemotoxic, cytotoxic and/or myotoxic symptoms (WHO, 2010a). From the foregoing and with the knowledge that victims rarely can or do identify the snake, it would be assumed that herbal preparations are administered indiscriminately, with little reference to the identity of venomous snake suspected or confirmed to have caused the bite. This means that the overall choice of herbs is standard irrespective of the type of venom, similar to administration of polyvalent anti-venom which is a cocktail manufactured to neutralize a number of snake venoms prevalent in a geographical region. It would seem however that theoretically, the herbal extracts act in a nonspecific manner, unlike the highly specific immunotherapy and would theoretically be effective against a wide variety of snake venoms. An alternative submission would be that different herbs have activity against certain venoms and none or little activity against others. These theories remain scientifically unsubstantiated.

Currently, snake bite treatment relies on anti-snake venom (ASV) serum as the only available specific approach which has been available since its development by Calmette in 1894, but its use comes with certain challenges. It is costly, mostly unavailable, often requires refrigeration and is associated with life threatening allergic reactions. As such

most rural facilities are unlikely to stock it (Cannon *et al*., 2008). Interestingly, despite ASV being the most reliable treatment for snake bite, it unfortunately has not been found to protect from snake venom induced necrosis, hemorrhage, renal failure, cardiotoxicity, pain, inflammation and infections at the site of the bite amongst other organ effects (Richard & White Julian, 2020).

Adverse reactions associated with the use of the serum appear mostly as anaphylactic reactions caused by the immunoglobulin E (Ig E) and anaphylactoid reactions mediated by the complement system. They can range from relatively less serious symptoms such as chills, nausea and fever, to more life-threatening wound such as bronchospasms and anaphylactic shock which has been observed even when the most refined antiserum has been administered. Amin *et al*., 2008 observed patients who received the serum after neurotoxic envenomation and noted that over 88% developed adverse drug reactions (ADRs) in thse form of pyrogenic reactions (80%) and anaphylaxis (65%). Efforts to develop products that have minimal adverse effects are encouraged and is achievable by improving the purification of the serum or seeking alternative therapies. The adverse effects are brought about by the animal antigens present in the serum, an unavoidable occurrence since it is derived from animal serum, mostly horses (Morais & Massaldi, 2009).

The antibodies in ASV are specific to the venom injected into the horses and are not effective for other venoms. Some are produced as monovalent for one species of venomous snake while others as polyvalent to cover several snake species.

Unfortunately, this life saving product is not always available and affordable at the point of use, usually in public health facilities serving afflicted communities. These public health facilities are not always accessible in rural communities owing to challenges with transport and long distances from these communities (Morais & Massaldi, 2009).

Neurotoxic symptoms should be managed with an intravenous initial dose of 10 ml ASV serum administered at a slow rate of 1-2 ml per minute (Williams *et al.,* 2007). Subsequent doses may be added to normal saline and administered at a strength of no more than 20ml per 500ml of normal saline and should run fairly slowly at a rate of no more than 5ml per minute for about 30 minutes. Breathing and other vital signs should be monitored continuously and mechanical ventilation instituted until the patient regains respiratory muscle tone and is able to breathe on their own. Proper wound management may necessitate use of antibiotics to prevent local infection while pain, fever and inflammation should be managed appropriately.

2.6. Strategies for overcoming challenges in the use of anti snake venom

2.6.1. Global Efforts for overcoming challenges in the use of anti snake venom

In 2013, snake bite suffered a blow when it was removed from the WHO list of neglected tropical diseases (NTDs). This meant that funding for research and production of life saving anti-snake venom significantly reduced, culminating in the cessation of the production of the lifesaving product and subsequent crisis in the Sub-Saharan region whose last batch of specific ASV, at the time FavAfrique®, was due to expire in December 2016. Opportunities opened up for other antivenom manufacturers to penetrate the Sub-Saharan market with new products whose specificity for venoms in the region had not been

established. As the situation worsened and with increased mortality and disability, scientists and conservationists lobbied for the reinstatement of snake bite as a NTD, the aim being to encourage production of ASV that was safe and efficacious. Fortunately, due to concerted efforts of the Global Snake bite Initiative (GSI), there was a breakthrough in June 2017, when the WHO reinstated snake bite as a NTD creating opportunity for funding for research and publicity matters snake bite (Lancet, 2017).

Since then, there have been global and local efforts towards providing alternative therapies while pre-qualifying anti-snake venoms already in the market on the basis of safety and efficacy. The WHO in its part has embarked on assessment of ASV currently in the markets of the affected WHO snake bite regions, Sub Saharan Africa being one of them. The results of the assessment will be critical in decision making for various governments but most importantly shall ensure that only safe and efficacious ASV is stocked and used.

2.6.2. Local solutions for overcoming challenges in the use of anti snake venom

As global efforts continue, there is need to explore homegrown solutions that would be equally safe and efficacious in the management of snake bite which would also be more affordable, available and sustainable. One of the potential solutions lies in local approaches to management of snake bite by traditional healers. A survey carried out by Mwangi *et al*., 2009 in a peripheral health facility in Kenya revealed that the number of cases of snake bite recorded in a peripheral health facility that was located in a snake bite prone area were surprisingly very few, 107 in an eight-and-a-half-year period from January 2003 to June 2009. The health care providers thought that this was due to management of snake bites within communities by herbal practitioners. Further interrogation of this claim resulted in interviews of traditional healers from Machakos, Kitui and Makueni counties which were

selected on the basis of reports of frequent snake bite cases in the chosen counties as well as their proximity to each other. The interviews revealed that indeed there were traditional healers who specialize in management of snake bite, although these were very few compared to those specializing in other ailments. These herbalists were able to list plants and parts used to prepare concoctions for managing snake bite. Most them reported positive outcomes of their patients but also reported that they are aware that complicated cases were best referred to health facilities after initial management.

2.7. Overview of medicinal plants used in management of snake bite

2.7.1. Plants with antivenin properties

In a study of African plants used in snake bite treatment, (Kaushik *et al.,* 2013) submit that chemical constituents such as alkaloids, flavonoids, phenols, glycosides and many others found in alcoholic and water extracts have potency against snake venom. They further suggest that scientific proof of efficacy would contribute to new alternative therapies. *Hibiscus aethiopicus* for example completely neutralizes haemorrhagic activity of *Echis ocellatus* venom while extracts of *Pterocarpus indicus* exhibits its anti-platelet aggregation activity via its inhibitory effect on phospholipase A (PLA2) activity. PLA2 is a key component of most snake venoms, an enzyme that catalyzes the hydrolysis of the sn-2 position of membrane glycerophospholipids to liberate arachidonic acid which is the precursor of eicasonoids including prostaglandins and leukotrienes. The same reaction produces lysophospholipids which are bioactive signaling lipids via G-protein coupled pathways. PLA2 therefore interacts with various phospholipids making it a critical component in snake venom for prey digestion but also causes myotoxic, neurotoxic,

cytotoxic, hemolytic, edematogenic, hypotensive, anticoagulant, platelet aggregation inhibition and activation, bactericidal and proinflammatory effects (Cedro *et al.,* 2018).

Gomes (2010) in a review of 'herbal antidotes' gave a long list of plants used in India, Brazil and China as anti-snake venom agents. Most of the herbs are prepared as ethanol, methanol or water extracts and are chewed or topically applied as saps to snake bite wounds. Plant species that were found to have activity against neurotoxic venom are shown in Table 2.1 below.

Table 2.1: Some plants used in India, Brazil and China with activity against neurotoxic snake venom

S. No.	***Name of plant***	***Part of plant***	***Type of extract***
1.	*Strychnos nux vomica*	Seeds	Ethanol
2.	*Withania somnifera*	Leaves	Ethanol
3.	*Andragraphis panniculata*	Stem and Leaves	Pet ether, methanol, ethyl acetate and water
4.	*Mimosa pudica*	Roots	Aqueous
5.	*Hemidesmus indica*	Roots	Methanol
6.	*Pluchea indica*	Roots	Methanol
7.	*Vitex negundo*	Roots	Methanol
8.	*Embilica officinalis*	Roots	Methanol
9.	*Mucuna pruriens*	Seeds	Distilled water

In addition, studies using natural products such as glycoproteins from *Withania somnifera,* aqueous root extract of *Mimosa pudica* and ethanolic extract of *Tamarindus indica* have been found to neutralize hyaluronidases of snake venom of *Echis carinatus, Naja naja* and *Daboia russellii.* Other plants that have been documented around the world for use in snake bite include; *Aristolochia* sp., *Casearia sylvestris*, *Curcuma* sp., *Eclipta prostrata*, *Mangifera indica*, *Mikania glomerata*, *Schumanniophyton magnificum*, *Tabernaemontana catharinensis* (Dey & De, 2012).

Kenyan plants documented to have anti-snake venom activity include *Allium cepa* and *Tagetes Minuta* (used by the Kamba) while the Luo in Nyanza have *Senna siamea* and *Tithonia diversifolia* (Owuor & Kisangau, 2006) among many others. The herbs are administered as infusions; decoctions or macerations. In some treatments, the snake teeth are mixed with the herbs as is the case with *Opilia amentecea*, a woody vine.

2.7.2. Overview of plants under study

Sansevieria suffruticosa, Solanum incanum, Tithonia diversifolia, Tagetes Minuta and *Zanthoxylum chalybeumn* which were selected for this study are known and utilized in folklore in various ways including as medicinal plants.

a) Sansevieria suffruticosa

This sisal like short stemmed plant is from the family *Asparagaceae*. It is semi-erect having branches with leaves of up to 60 cm long that grow in a rosette or sometimes in two spiraling rows (spiral distichous). The spine-tipped leaves are 12-16mm thick, elliptical in cross-section and pale to dark green depending on sun exposure. It is one of the species of *Sansevieria* native to Africa. The first description of the species was made in 1915 by N. E. Brown, hence its name, *Sansevieria suffruticosa* N.E.Br. It is also considered to be endemic to Kenya. (Maundu *et al.,* 2001).

Figure 2.5: *Sansevieria suffruticosa* in its natural habitat

a) Semi erect stems and spine-tipped leaves; b) In its natural habitat.
Pictures by Gladys Mwangi.

Uses of *Sansevieria suffruticosa*

In the highlands of Kenya, the plant is used to produce fiber and medicinal uses where its leaves are valued for treatment of ear-ache and open fresh wounds, while the rhizomes are used to treat snake bites and stomach ulcers. The Loita Maasai use the leaf sap to treat gonorrhea while the fibers can be used for weaving (Beentje *et al*., 2003).

b) *Solanum incanum*

Solanum incanum L. from the family Solanaceae is commonly known in Kenya as sodom apple, bitter apple, poison apple, snake apple, thorn apple, mutongu (kikuyu), mtunguja mwitu (swahili), ochok (luo) among others. It is thought to be naturalized to East Africa, Australia, Mauritius, Madagascar and is also found in the Middle East and India having originated from Africa.

The plant is a soft wooded shrub that grows to a height of 1.8m in height with spines on the stem, stalks and calyces and with velvet hairs on the leaves. The leaves are alternate, egg shaped in outline with broad ends and wavy margins. The upper surface is grey-green and the lower green-white. Its fruits are globular and yellow when ripe. It is normally a weed of disturbed and overgrazed areas and road sides, in woodlands, evergreen and riverine forests (Fukuhara & Kubo, 1991).

Figure 2.6: *Solanum incanum* at different stages
a) Young shrub just before flowering; b) Flowering plant
Pictures by Gladys Mwangi

Uses of *Solanum incanum*

Although the plant is toxic to livestock, it has not been listed as noxious in any east African country. It is used for sore throat, stomach-ache, head-ache, painful menstruation, liver pain, malaria, hypertension, stomach problem, asthma, diabetes, common cold and pain caused by onchocerciasis, pneumonia and rheumatism and its aerial parts are used in compost (Dakone & Alemu, 2016). Omara (2020) listed the plant as one among those frequently used for treatment of snake bite in rural Kenya.

c) *Tagetes Minuta*

T. minuta L. (1753) belongs to the Asteraceae family and was originally from South and North America. It is commonly known as African marigold, khaki weed, stinking roger, khaki bush. It is an erect, woody, annual, herbaceous weed that grows to about 2 meters tall. There are no branches on its stem but will have repeated branching in its upper parts. The stem changes colour from green to brown or yellow after flowering.

Figure 2.7: *T. Minuta* growing in its natural habitat on the roadside at Gilgil Toll Station *Picture by Gladys Mwangi.*

T. minuta is an invasive weed that grows in areas where human activity has previously been such as old farm lands, abandoned gardens, roadsides and waste places. It prefers dry or moist soils. In some parts of the world like South Africa, France and North America, it is cultivated commercially for its essential oil. In Kenya, *T. minuta* was first recorded as an alien weed during the 1920s. It was originally restricted to the higher altitudes, but has since spread to lower altitudes as a result of increasing agricultural activities (Stadler *et al*., 1998).

Uses of *Tagetes Minuta*

The plant is best known for its oil called *tagetes* oil which is citrus-smelling and amber to red in colour, obtained through steam distillation of the leaves, stalks and flowers. Although the oil has an unpleasant smell after production, the aroma improves to a fruity mango smell over a 1–5-year period during which period the colour darkens and it thickens

after which it deteriorates. The oil may be used in perfumery industry and blended with massage oil or used as a bath oil for relief of wounds, cuts, chest infections, coughs and fungal and parasitic infestations. In a 5% dilution, Tagetes oil has been used to kill maggots in open wounds.

The boiled leaves of the *Tagetes Minuta* are used to treat infections in the respiratory system or stomach problems, by drinking the boiled mixture. When used in a cream or lotion, it can have a beneficial effect on fungal and microbial infections and especially helpful when treating weeping wounds or athletes' foot. In burners, vaporizers or steamers, the vapour from the leaves can treat headaches, coughs, bronchitis and chest infections or can be used as an insect repellent for a wide variety of insects. The vapour also acts as an antiseptic in a room. The leaves and flowers are a good insect repellent and are often seen hanging in dwellings to deter swarms of flies and mosquitoes. The oil is also used as a repellent to the bowfly and is useful as a bowfly dressing. The plant is listed as one among those considered to have antivenom properties against snake venom (Omara *et al.,* 2020).

d) Tithonia diversifolia

Tithonia diversifolia (Hemsl.) A. Gray from the family Asteraceae is native to Mexico and, is commonly known as 'mexican flower' and also grows in parts of Africa, Australia, Asia, and other countries of North America. It is a herbaceous flowering plant is used for ornamental purposes. It is invasive in disturbed sites, along roadsides and areas near cultivation. It invades new habitats through its tolerance to heat and drought, its rapid growth rates and its large production of lightweight seeds which are easily dispersed by wind, water and animals.

Figure 2.8: *T. diversifolia* growing in natural habitat
a) Non-flowering and b) Flowering *T. diversifolia* growing in natural habitat.
Pictures by Gladys Mwangi.

Uses of *T. diversifolia*

T. diversifolia extracts are traditionally used for the treatment of diabetes, diarrhea, menstrual pain, malaria, hematomas, hepatitis, hepatomas, and wound healing. These medicinal properties are thought to be the effects of terpenoids and flavonoids contained in the aerial parts of the plant. Several studies investigated anti-inflammatory, analgesic, antimalarial, antimicrobial and antidiabetic activities and although these investigations revealed the potential of this plant and its constituents for different therapeutic activities, studies are needed in order to understand the molecular modes of action of its extracts (Giacomo *et al.,* 2015).

e) Zanthoxylum chalybeum

Zanthoxylum chalybeum is a tree or shrub from the family Rutaceae. It is deciduous and can grow to about 10 meters tall, has a round crown and large woody spikes about 2cm

long. Its habitats dry bushland and wooded grassland and can also be found in drained slopes, riverine forest and bushland at approximately 1600 meters above sea level.

Figure 2.9: *Z. chalybeum* growing in natural habitat along Njoro River, Nakuru County, Kenya. *Pictures by Gladys Mwangi.*

Figure 2.10: Harvested stem bark of *Z. chalybeum*
Picture by Gladys Mwangi

The plant is native to East tropical Africa from Ethiopia and Somalia and stretching south to Zambia, Zimbabwe and Mozambique. In East Africa, it is traditionally harvested in the wild for use as medicine, toothbrush, tea and timber.

Uses of *Z. chalybeum*

Z. chalybeum leaves are cooked and used as a vegetable or dried, pounded and sieved to make a thick vegetable paste which is mixed with groundnut paste and eaten with ugali, a popular mize staple food. Aromatic tea can also be made from a brew of dried leaves. It is a well-known source of traditional medicine in Africa; n Somalia the leaves have been reported to be used against stomach pain and urinary retention; decoctions of the bark and roots are used to cure malaria. The wood is very hard, heavy, elastic and highly durable. It

has been used for carving, turnery, building poles, bedsteads, spoons, stools, drums, walking and other wood work items. The stem and bark powder of the plant is mixed with cold water and drank three times daily for at least three days and applied topically (Okot *et al.*, 2020).

2.8. Phytochemical analysis of herbal extracts

Various analytical techniques are available for detection and quantification of constituents in herbal preparations. Some of the techniques such as X-ray fluorescence detect the presence of mineral content in plant powders while GC-MS and LC-MS techniques are applicable to mixtures of extracts in which detection and semi quantification of phytocompounds is desired.

2.8.1. X-ray fluorescence (XRF)

XRF (X-ray fluorescence) is a non-destructive analytical technique used to determine the elemental composition and quantities of materials. XRF analyzers determine the chemistry of a sample by measuring the fluorescent (or secondary) X-ray emitted from a sample when it is excited by a primary X-ray source. Each of the elements present in a sample produces a set of characteristic fluorescent X-rays ("a fingerprint") that is unique for that specific element, which is why XRF spectroscopy is an excellent technology for qualitative and quantitative analysis of material composition (Nas *et al.,* 1993).

The identification of mineral components in plant powders is important for quality control purposes in the recommendation of such products for human consumption. Plant powders with heavy metals present may not be recommended for human and animal consumption (Ayaz *et al.,* 2014; de Souza *et al.,* 2019). The mineral content can also guide on

interactions of herbal products and their biological targets. A study on the phytochemical and nutraceutical potentials of beach bean, *Canavalia rosea* in Kogi State Nigeria revealed that the percentage of ash content, and indicator of mineral component, was higher in the leaves than in the seeds and recommended the use of various parts of the plant for nutraceutical purposes in the treatment of various diseases (Tijani *et al.,* 2020). The mineral composition on *Ajuga iva* subsp. Pseudoiva (dc.) bric revealed the most abundant minerals were iron (Fe), potassium (K) and sodium (Na) further suggesting that these play an inhibitory role in three enzymes; α-amylase, α-glucosidase and β-galactosidase thus contributing to its hypoglycemic effects (Senhaji *et al.,* 2022).

2.8.2. Gas chromatography mass spectrometry technique (GC-MS)

GC-MS is a combination of two analytical techniques, GC which can separate volatile and semi volatile compounds with great resolution but cannot identify them and MS which can give detailed structural information of compounds and hence their identity (Chauhan, 2014; Hussain & Maqbool, 2014) such as reported by Meshack O *et al.,* (2021) who used the technique in phytochemical analysis of the ethyl acetate extract of the leaves and stem bark of *Warbugia ugadensis* in which various compounds were identified as being consistent and form part of the monograph for assessing quality, purity and authenticity of *W. ugadensis* products. Other studies done using GC-MS include the establishment of male attractants for fruit flies in the leaf extract of *Schinus mole* (N. Gikonyo & Lux, 2006), determination of variation in volatiles from fruits of mango and marula attractive to fruit fly, *Ceratitis cosyra* (N. Gikonyo *et al.,* 2005) and the evaluation of the odor composition

of preferred (buffalo and ox) and non preferred (waterbuck) hosts of some savanna tsetse flies (N. K. Gikonyo *et al.,* 2002).

Phytochemical analysis of herbal extracts using GC-MS is an important approach that is used to detect the presence of phytoconstituents that are known to be responsible for biological activities that bring about healing such as constituents of the essential oil of *Suregada zanzibariensis* leaves that are repellent to the mosquito, *Anopheles gambiae* (Innocent *et al.,* 2010) . Identification of the phytoconstituents is critical in the pursuit of knowledge of molecular interactions that are involved in the biological activities. Such bioactive constituents have been detected in *Caulerpa peltata* (green algae) using GC-MS analysis which showed 28 compounds among which dibutylphthalate, n-hexadecanoic acid, and 1,2-Benzene dicarboxylic acid was found in high percentage (Nayaka *et al.,* 2020). Some of these compounds have anti-cancer, antioxidant, anti-inflammatory and anti-diabetic activities. Similar reports of bioactive compounds that have been detected using GC-MS include those detected in *Taraxaccum officinale* (L) (Sasikala *et al.,* 2019), *Asparagus racemosus* (Janani & Singaravadivel, 2014a), *Ximenia Americana* (Shettar *et al*., 2017) and *Thaumatococcus daniellii* (Iwueke *et al.*, 2020).

2.8.3. Liquid chromatography Tandem Mass Spectrometry (LC-MS/MS)

Liquid Chromatography with tandem mass spectrometry (LC-MS-MS) is a powerful analytical technique that combines the separating power of liquid chromatography with the highly sensitive and selective mass analysis capability of triple quadrupole mass spectrometry (LC-MS-MS | Liquid Chromatography | EAG Laboratories). Despite the highly specific and sensitive quantification by GC-MS of thermal stable volatile molecules of molecular weight of about 500, many molecules that do not conform to these

specifications cannot be analyzed. These include proteins, carbohydrates, DNA, drugs and metabolites, thus necessitating alternative analytical techniques.

LC-MS has undergone technological improvements with the introduction of atmospheric pressure ionization (API) techniques and powerful ion analyses technologies; tandem MS, time of flight MS and Ion trap MS; which have increased the capacity of MS analyzers (J.-S. Kang, 2012). This technique is best exemplified in its role in Traditional Chinese Medicine which uses complex mixtures of plant products to treat diseases, and has remained undisputed for thousands of years. It is however difficult to scientifically explain the mechanisms of action of these herbal components without first identifying them and processing the identified components through various analytical techniques for determining molecular interactions or through bio assays. LC-MS/MS has emerged as a popular technique that has high capability of separating complex mixtures and strong qualitative ability of MS (Pang *et al.,* 2016). Similar approaches that have been used include quantification of kratom alkaloids in leaf extracts of *Mitragyna speciose* (Sharma *et al.,* 2019), authentication of grape-based and cranberry-based natural and pharmaceutical products (Puigventós *et al.,* 2015)) and analysis of fermented extracts of *Viscum album L*. which are used in treatment of cancer (Peñaloza *et al.,* 2020).

2.9. Molecular docking techniques for prediction of molecular interactions

Molecular docking, also referred to simply as ‘docking’, is a bioinformatics technique used to predict how a protein interacts with a ligand such as a drug. It is a computational tool used in drug discovery originally developed for prediction of interactions between small and large molecules and is now used for various purposes including prediction of adverse

effects of drugs, polypharmacology, drug repurposing, target fishing and drug profiling (Pinzi & Rastelli, 2019). Virtual screening of compounds using large library databases such as PubChem and protein data bank (PDB), which provide information such as names, molecular formulae, structures and other details such as physical chemical properties, biological activities, safety and toxicity of compounds enables virtual approaches for discrimination of binding from non-binding entities which in effect reduces the number of physical experiments required to determine potential interactions in the process leading to drug discovery. Visual examination of predicted binding geometries (docking poses) further enhances the search of lead compounds by predictions that may enhance binding affinities of target molecules, reduce side effects or avoid mutation interactions. One such visualization tool used widely in docking techniques is PyMOL, a molecular graphics system that is a powerful molecular visualization tool with exceptional 3dimensional capabilities. A number of molecular docking software utilize PyMOL for this purpose; Autodock Vina for example uses rectangular boxes to define binding sites which are defined using coordinates or using a PyMOL selection such as a reference ligand.

The input data requires that compounds are converted to formats acceptable by the software such as those from PDB for proteins which also gives atom types, charges and definitions; and for ligands, topological information giving rotational bonds such as that generated in spatial data format (SDF). For each binding site, the ligand receptor interaction energy is calculated and collated into a grid map that is readable by PyMOL which gives electron density maps with iso surfaces and iso meshes. Each docking pose is accompanied by a score that is ranked and thus provides a priority list in terms of reliability of interactions. (Seeliger & Groot, 2010; Sethi *et al.,* 2020).

This approach has been used for the aqueous root extract of *Cynodon dactylon* which has been found to have significant antihemolytic activity against venom from the Indian cobra *Naja naja* venom based on in vitro data. The extract was subjected to mass spectrometry and the forty-six compounds identified were subjected to molecular docking against cardiotoxin 1, the venom protein of interest, for determination of lead compounds with anti-hemolytic activity. Ten of these compounds showed potential based on interaction scores out of which compound 1 and 2 had reliable binding affinities that could be investigated further as lead compounds for drugs that have hemolytic activity against cardiotoxin 1 and hence of potential use in *Naja naja* envenomation (Gnanaselvan & Sivaraman, 2020). Similar approaches have led to the identification of antivenin components from plant extracts such as anti-myotoxic activity of gallic acid from *Anacardium humile* and its mechanism of action against phospholipase A2 found in snake venom (Costa *et al.,* 2021).

CHAPTER THREE
MATERIALS AND METHODS

3.1. Study Design

The study design was analytic experimental and drew data from a combination of fieldwork, laboratory and bioinformatics sources as illustrated below.

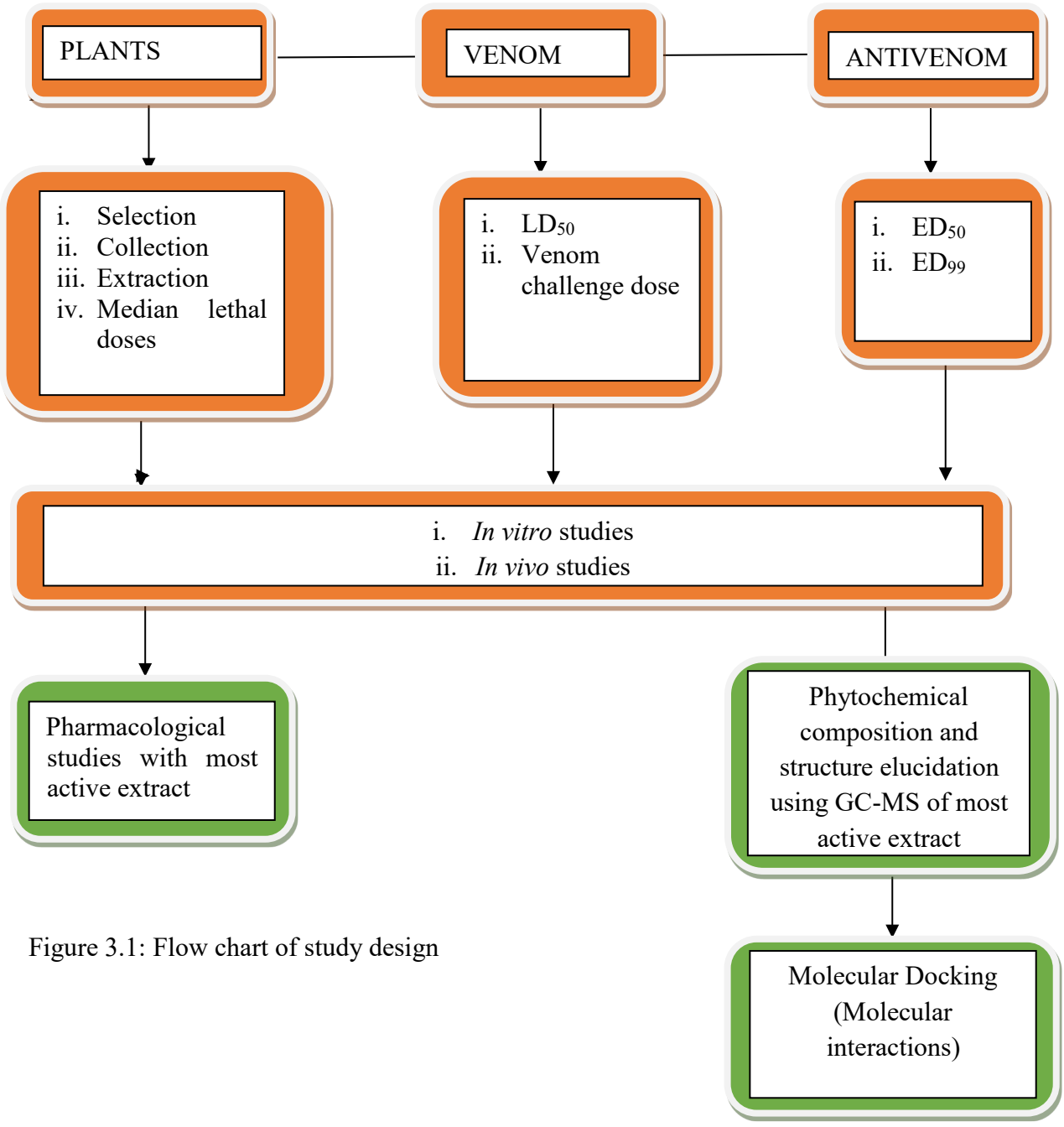


Figure 3.1: Flow chart of study design

3.2. Selection, collection and preparation of herbal extracts

3.2.1. Selection of plants for the study

The plants included in this study were selected from a list derived from interviewing herbalists from selected counties in Eastern Kenya namely Machakos, Makueni and Kitui, which are known for snake bite incidences and are not very far from the study site. Four herbalists who specialize in snake bite treatment were identified by the Chairman of Traditional Healers Group based in Machakos County. Two meetings were arranged with the herbalists, the first one sought to establish the suitability for recruitment of the herbalists as sources of information for the study and in the second meeting, interview sessions were conducted aimed at collecting specific information about the treatments including names of plants, preparation, administration and success rate of the treatments.

The plants mentioned were listed and their botanical names searched from internet sources (Table 3.1). A familiarity index (Chinsembu *et al.,* 2015), was used to rank plants that had the highest mentions from the herbalists (Table 3.2). From this list, plants that scored at least fifty percent (50%) were considered where convenient sampling was done to select those that were readily available in areas of close proximity to the study site and whose harvesting would not compromise the biodiversity of the species.

3.2.2. Collection and preparation of herbal materials

The plant materials were collected in the period between September and November of 2015 during morning hours. The herbal materials were placed in cellulose paper carrier bags immediately after collection and transported to the Pharmacognosy laboratory at Kenyatta University for sorting, cleaning under running tap water and drying under shade. The dry plant parts were then ground into powders using a disc mill and stored in air tight containers at room temperature.

Table 3.1: Collection sites, GPS co-ordinates and voucher specimen number of medicinal plants

S. No	***Botanical name and Common name of plant***	***Family name***	***Collection site and GPS co-ordinates***	***Voucher specimen number***
1.	*Solanum incanum* Sodom apple, Thorn apple, Bitter apple, Bitter Tomato, Mutongu	Solanaceae	Kenyatta University Medicinal Garden. -1.183337, 36.924553	GM/001/2015
2.	*Tagetes Minuta* Wild marigold, Stinking roger, Muster John Henry	Asteraceae	Gilgil Toll station -0.522250, 36.328754	GM/002/2015
3.	*Tithonia diversifolia* Daisy family, Mexican sunflower	Asteraceae	Kenyatta University stream -1.186927, 36.930651	GM/003/2015
4.	*Zanthoxylum chalybeum* Knob wood, Yellow wood	Rutaceae	Egerton, Along Njoro River -0.369069, 35.941857	GM/004/2015
5.	*Sanseviera suffruticosa* Snake Plant, Mother-In-Law's Tongue, Viper's bowstring hemp	Asparagaceae	Gilgil Toll station -0.522250, 36.328754	GM/005/2015

50

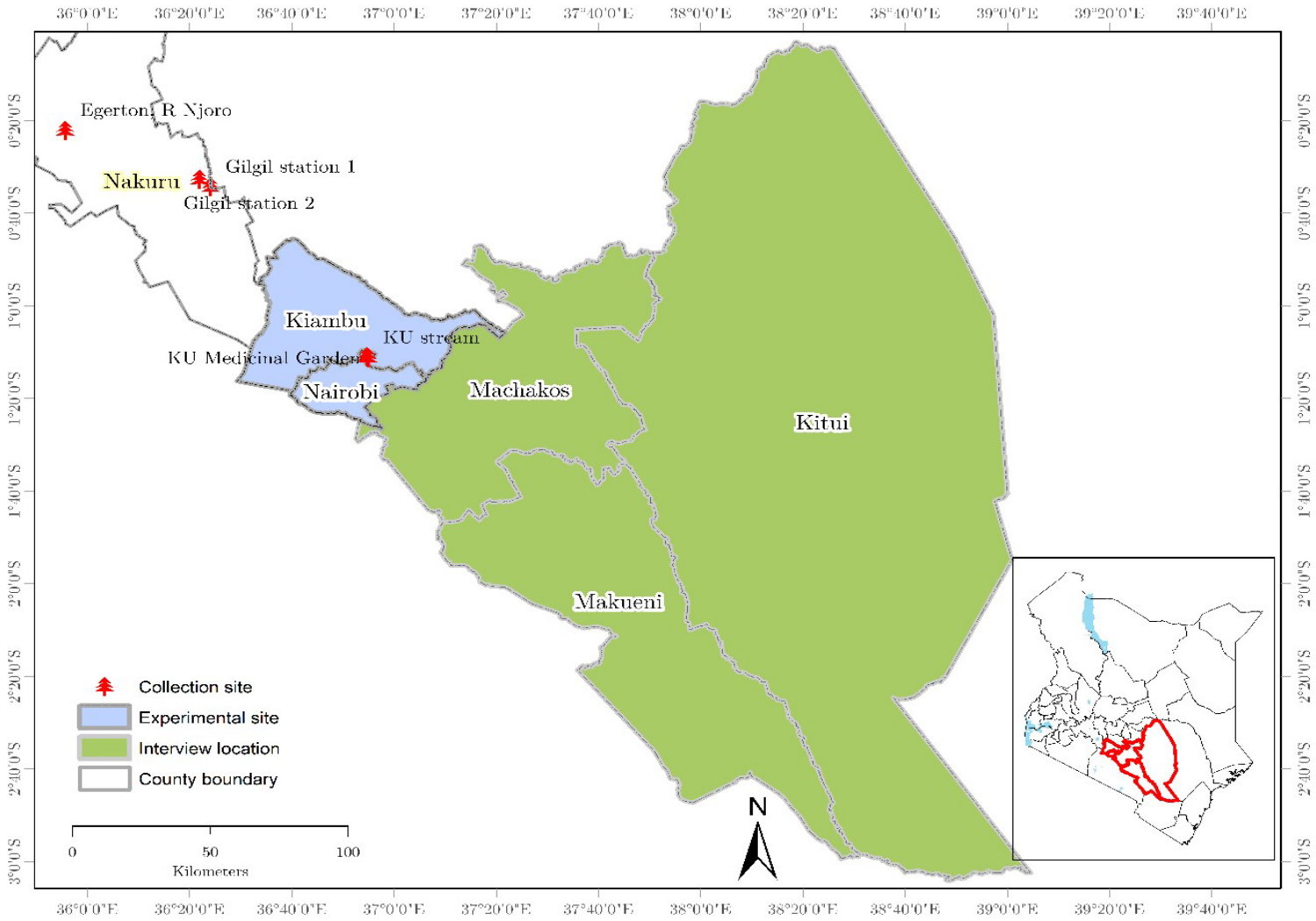


Figure 3.2: Map showing collection sites of five plants, experimental sites and location of interviews with herbalists.
Map by James Muthoka and Gladys Mwangi

51

a

b

c

d

Figure 3.3: Collection and preparation of herbal materials
a) Collection b) Packing c) Drying after sorting and cleaning d) Grinding, weighing and packing
Pictures by Gladys Mwangi

3.2.3. Extraction and preparation of test solutions

Extraction was done using methanol HPLC grade (Sigma Aldrich®) after which hexane, analytical grade (Sigma Aldrich®) and dichloromethane HPLC grade 99.8 (Sigma Aldrich®) were used to partition the crude extract into mid and non-polar fractions.

The method described by Parekh (2006) was adapted where two hundred grams of each plant powder was extracted three times with methanol. The first extract was obtained by filtration after 48 hours of soaking in 500ml after which the residue was re-soaked in 300ml of fresh methanol for a further 24 hours to obtain the second extract and the procedure

repeated for a further 24 hours to obtain a third extract. The three portions were combined and the methanol removed using a rotary evaporator to obtain a gummy product. The obtained product was then subjected to sequential partitioning using hexane, dichloromethane and finally distilled water. The non-polar fractions were labeled and stored for future studies while the aqueous fractions were lyophilized, the percentage dry weight calculated and stored in airtight containers at -20^{0}C. Preparations for injection were made by dissolving 0.2g of aqueous extract in 20ml of distilled water to make a 1% w/v solution.

3.3. Preparation of test solutions

After extraction and lyophilization, the percentage dry weight of extracts was calculated before storage at 2-8^{0}C pending further studies. The plant with the highest dry weight was *S. incanum* followed by *T. minuta* while *T. diversifolia* gave the least percentage dry weight of the five plants.

3.4. Study animals

Thirty albino mice (*Mus musculus*), 15-30g body weight, 6-10 weeks old which were obtained from Kabete Veterinary Laboratory animal house, Upper Kabete (Nairobi) were used. They were transferred to the Small Animal House at the College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology (JKUAT). The mice were kept in a 12/12-hour normal photoperiod and fed on standard laboratory chow and water *ad libitum* for one week.

Wistar rats (*Rattus norvegicus*) were obtained from the Department of Biochemistry and Biotechnology animal house at Kenyatta University and were housed under standard

laboratory conditions and fed with standard laboratory chow and water *ad libitum* for one week in a 12/12-hour normal photoperiod.

3.5. Median lethal dose of aqueous herbal extracts in laboratory mice

This study adopted an up down approach and was conducted as described by (Ezeonu & Ejikeme, 2016; Santhi & Sengottuvel, 2016) using sixty mice. The study was conducted in two phases; the first phase required nine mice divided into three groups of three mice each. Each group of mice received a different dose intraperitoneally (10, 100 and 1000 mg/kg) of reconstituted plant extract (Appendix IV, Table IVa, b, c, d and e).

The mice were then placed under observation for 24 hours and monitored for mortality. The second phase involved the use higher concentrations of extracts where three mice were used for each plant. The mice received a dose of intra-peritoneal injection of reconstituted plant extract (1600, 2900 and 5000 mg/kg) and observed for 24 hours with mortality as the end point. The medium lethal dose (LD_{50}) of the extracts was calculated using the formula:

$LD_{50} = \surd\ (D_0 \times D_{100})$

D_0 = Highest dose that gave no mortality

D_{100} = Lowest dose that produced 100% mortality

For this study, the highest dose with 0% mortality for each extract was taken as the working dose of that extract that would maximize the effect of the extract.

3.6. *In vitro* and *in vivo* effect of aqueous herbal extracts against *D. polylepis* venom

3.6.1. Medium lethal dose (LD_{50}) dose of crude venom

The median lethal dose (LD_{50}) is defined as the least amount of venom injected intra-peritoneal to animals resulting in 50% and 99% death of the animals within 24h respectively. The LD_{50} was determined as described by Parveen (2017) which is a modification of the method recommended by the WHO (2010) in assessment of lethal venoms.

Freshly milked crude *D. polylepis* venom (3.8mg) was donated for research by Bio-Ken Snake Farm, Watamu, Kilifi County, Kenya. It was immediately refrigerated at 2-8^{o}C and transported to JKUAT SAFARI laboratory in a cooler box before being stored in a refregirator at 2-8^{o}C. A stock solution was prepared by dissolving 3.8mg of the venom in 5ml of water for injection (0.76mg/mL). 1 mL of the stock solution was then diluted to 100 mL with water for injection resulting in a 1% solution (0.0076 mg/mL/7.6ug/mL) of crude venom. The potency of the venom was determined before each experiment and remained the same throughout the study.

Six groups of five mice each were injected intraperitoneally with venom at increasing doses (Appendix V, Table Va) (1.25, 2.5, 5, 7.5, 10 and 20mL/Kg) corresponding to (0.0095, 0.019, 0.038, 0.057, 0.076 and 0.152 mg/Kg). A 24-hour observation period was allowed with the end point taken as Alive (A) or Dead (D). Probit analysis of the survival rate within groups was done with LD_{50} taken at probit 5.

3.6.2. Venom challenge dose

The method by Gutiérrez (2017) was used in which crude venom was administered intraperitoneally to groups of mice at multiples of the established LD_{50} (0.024, 0.048, 0.072, 0.096, 0.120, 0.144mg/Kg) (Appendix V, Table Vb) until 100% mortality was recorded within 24 hours, as expected in *D. polylepis* envenomation if no anti-snake venom is administered (Szalay, 2014).

3.6.3. Effective dose (ED_{99}) of anti-snake venom (ASV)

A duly registered polyvalent anti-snake venom (Manufacturer: Veteria Labs; Mexico City; Batch Number:91T06001; Expiry Date June 2021; was purchased from Laborex Kenya Limited in Nairobi, Kenya. The label claim indicated that each 10 ml of the lyophilized, equine (Fab'_2) contained not more than 1000mg of total proteins that neutralize at least 500 LD_{50} of the venom of *Echis ocellatus*, *Bitis arietans*, *Naja nigricollis* and *Dendroaspis polylepis*.

The method described in WHO (2010) was used where mice were put into five groups of five mice each and injected with the venom challenge dose immediately after injection of anti-snake venom at increasing doses (10, 20, 30, 40 and 50 mL/Kg; 1000, 2000, 3000, 4000 and 5000mg/Kg) (Appendix V, Table Vc) for each subsequent group. The survival rate after 48hours was recorded and the lowest dose of ASV (30ml/Kg; 3000mg/Kg) that resulted in 100% survival was taken as the working dose.

3.6.4. *In vitro* activity of aqueous herbal extracts against *D. polylepis* venom

The method was adapted from the one described by Alam & Gomes (2003) where mice were divided into six groups of six mice each. The venom challenge dose (0.0240mg/Kg)

for each mouse was mixed with the working dose of each plant extract; *S. incanum* (100mg/Kg), *S. suffruticosa, T. minuta, T. diversifolia and Z. chalybeum* (1500mg/Kg); and the mixture incubated for 1 hour at 37°C. The incubated mixture was injected through the intraperitoneal route into the mice which were observed for 24 hours with death as the end point. The negative control group received an incubated mixture of venom challenge dose (0.0240mg/Kg) and water for injection. The positive control group received an incubated mixture of venom challenge dose (0.0240mg/Kg) and ED_{99}; 30ml/Kg of anti-snake venom (3000mg/Kg) (Appendix V, Table Vd).

3.6.5. *In vivo* activity of aqueous herbal extracts against *D. polylepis* snake venom

In vivo activity of selected plant extracts was determined using the method adapted from the one described by Alam & Gomes (2003) in which mice were divided into seven groups of six mice each. Mice in the treatment groups received intraperitoneal injections of working dose of the plant extracts; *S. incanum* (100mg/Kg), *S. suffruticosa, T. minuta, T. diversifolia and Z. chalybeum* (1500mg/Kg); immediately after injection of venom challenge dose (0.0240mg/Kg). The negative control group received water for injection immediately after injection of venom challenge dose and the positive control group received ED_{99} (3000mg/Kg) of ASV immediately after injection of venom challenge dose (Appendix VI, Table Ve). The survival rate for all the groups was recorded after 24 hours.

3.7. Pharmacological effects of aqueous leaf extract of *T. diversifolia* on *D. polylepis* snake bite wound

Out of the five plant extracts, that from *T. diversifolia* leaves showed a significant *in vivo* effect against *D. polylepis* snake venom and was further investigated for antinociceptive,

anti-pyretic, anti-inflammatory and anti-microbial effects considered beneficial in snake bite.

3.7.1. Anti-nociceptive effect of aqueous leaf extract of *T. diversifolia*

The hot plate method described by Fan *et al.,* (2014) was used in which twenty-five mice were used. A 12h fast was instituted before the mice were weighed and randomly assigned to five groups of five animals each. The five groups were treated orally as follows; the negative control group received normal saline (10 mL/kg), the positive control group were given 50mg/Kg diclofenac sodium and those in the remaining three groups were administered plant extract at 100, 200 and 300mg/kg (Appendix VI, Table VIa and VIb).

The mice were then placed on a hot plate maintained at 55°C and the time taken for them to react to the thermal pain by licking their paws or jumping was recorded with the maximum possible analgesia (MPA) taken to be at 45 seconds of exposure. The experiment was replicated using the intraperitoneal route of administration.

3.7.2. Anti-pyretic effect of aqueous leaf extract of *T. diversifolia*

Anti-pyretic activity was assessed using the method described by Charles Akapa (2014) in which wistar rats were deprived of feeds for 12 hours but were allowed adequate water *ad libitum* before anal basal temperature for each was recorded using a digital thermometer inserted 3-4 cm into the rectum. Pyrexia was then induced in each rat by injecting 20ml/Kg of turpentine subcutaneously near the groin. The rats were then kept at room temperature for one hour after which confirmation of pyrexia was done and only rats that had an increase of at least 0.6 ^{0}C from basal reading were included in the study. The rats were grouped into five groups of six rats each. Rats in the negative control group were given

20mL/Kg of distilled water orally, rats in the three test groups were treated orally with the plant extract at 100, 200 and 300mg/Kg and those in the positive control group were treated orally with 100 mg/Kg of paracetamol (Appendix VI, Table VIc and VId). Rectal temperatures were recorded at 1, 2 and 3 hours after the respective treatments. The procedure was repeated using the intraperitoneal route.

3.7.3. Anti-inflammatory effect aqueous leaf extract of *T. diversifolia*

Anti-inflammatory effect was determined as described by (Galehdar *et al*., 2018) where the right hind basal paw circumference of each mouse was measured using a digital caliper followed by sub plantar injection of 0.1 mL of λ-carrageenan (1% in NaCl 0.9%) into the same paw. The mice were then placed randomly into five groups of six mice each. The groups were subjected to a gavage with the following solutions; Group I got sterile saline solution (0.9% NaCl) at 10 mL/kg; Group II, III and IV got oral plant extract at 100, 200 and 300 mg/kg respectively and mice in Group V received diclofenac sodium at 50 mg/kg (Appendix VI, Table VIe and VIf). The paw circumference was then measured at 1.5, 3 and 6 hours after λ-carrageenan injection. Increase in the linear circumference of the right hind paw was taken as an indicator of paw edema. The procedure was repeated using the intraperitoneal route.

3.7.4. Antimicrobial effects of aqueous leaf extract of *T. diversifolia* against selected bacteria and fungi

The method as described (Saranya *et al.,* 2019) was adapted for determination of viability and sensitivity of standard organisms to herbal extract.

3.7.4.1. Viability of standard organisms

Standard organisms; Staphylococcus aureus (ATTC 25923), Streptococcus agalactiae (ATTC 13813), *Streptococcus pyogenes* (ATTC 19615), *Pseudomonas aeruginosa* (ATTC 27853), *Escherichia coli* (ATTC 25922) and *Candida albicans* (ATTC 14053); were obtained from the Medical Laboratory Sciences repository at Kenyatta University. The viability of the standard organisms was done by inoculating them in freshly prepared and appropriate nutrient broths followed by incubation for 24 hours at 37^0C. Examination of turbidity on nutrient broth to confirm growth was done after the incubation period. The nutrient broths that showed turbidity were then sub cultured in nutrient agar plates for *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* and Sabouraud dextrose agar plates for *Candida albicans* and incubated at 37ºc for 24 hours. Confirmation of growth of the pure standard cultures was done by observation after the incubation period following which the pure broths were standardized by adjusting their turbidity to that of McFarland's standard turbidity solution equivalent to 1.5 x 10^8 CFU/ml.

3.7.4.2. Sensitivity of extract to standard organisms

The pour plate method was used in which serial dilutions of the herbal extract (25, 50, 100, 200, 400, 800 and 1000 mg/ml) were prepared using distilled water. The growth media was prepared by reconstituting 38 grams of Mueller Hinton powder in 1-liter distilled water, sterilizing it by autoclaving at 121ºc for 15 minutes and cooling it to 40ºc in water bath. Transfer of 100ul of bacteria standard organisms was done into sterile petri dishes which had been marked into four quadrants using a permanent marker. Approximately 20 mls of the Mueller Hinton broth was then carefully poured into each petri dish making sure that

the mouth of the conical flask was sterile. The contents of the petri dishes were then mixed gently to achieve uniform distribution of the standard organisms into the media after which the plate was allowed to stand for 15 minutes to achieve solidification of the contents.

A gel puncture was then used to punch 6mm diameter wells into each quadrant that were then filled with 50ul of plant extract dilutions as indicated above. The positive control used was an appropriate antimicrobial and antifungal agent for each bacterium and fungus under study respectively. The negative control, Dimethylsulphoxide (DMSO), was the solvent used to reconstitute the herbal extracts (Appendix VI, Table VIg). Each of the experiments was done in triplicate and the contents incubated with the plate upright at 37^{o}c for 24 hours at the end of which the plates were checked for growth and susceptibility to the plant extracts and control drugs. Zones of inhibition indicated that the organisms were susceptible to the extract and/or the control agents. The measurements were done using a zone reader and the results presented as tables of zones of inhibition.

3.7.4.3. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The MIC and MBC was determined for the organisms that showed susceptibility to the extract. A conical flask was used to weigh 13g nutrient broth powder in which 50g of glucose and 10g of phenol red were added. These were dissolved in 1 litre of distilled water and heated gently on a hot plate to mix followed by sterilization by autoclaving at 121^{o}c for 15 minutes and then cooled to 40^{o}c in water bath. The turbidity of the standard organisms was adjusted to that of McFarland's standard turbidity solution equivalent to 1.5x 10^{8} CFU/ml.

Ninety-six well microtiter plates were labeled appropriately in duplicate and 50ul of nutrient broth/glucose/phenol red suspension pipetted into the first well for each concentration of extract that exhibited the least zone of inhibition. Serial dilutions were then done up to well No. 11 with well No. 12 reserved for the negative control (50 uL of DMSO) and was replicated for the positive controls as well. This was followed by addition of 50uL of standard organisms into all the wells and the contents gently mixed and incubated in the plate at 37^{o}c for 24 hours. The results were determined by observing colour change in the wells where yellow colour indicated growth (G) and red colour indicated no growth (NG) (Appendix VI, Table VIh).

The MIC and MBC was further confirmed by sub-culturing on Mueller Hinton the contents of the last well with growth i.e. the least dilution that allowed growth and the two preceding wells with no growth. The sub cultures were incubated at 37^{o}c for 24 hours in an incubator. The MIC was taken as the least concentration that had zones of inhibition while the MBC was taken as the least concentration that had no zones of inhibition. The results were presented in tables of zones of inhibition. All the used and waste materials from the study were sterilized before safe disposal.

3.8. Phytochemical analysis of herbal materials

3.8.1. Qualitative phytochemical analysis of plants under study

The crude methanol extracts of the five plants were subjected to qualitative phytochemical screening for alkaloids, flavonoids, glycosides, saponins, tannins and terpenoids using standard qualitative procedures as described by Ezeonu & Ejikeme (2016).

Test for Alkaloids

Iodide (1.2g) was dissolved in 2.0g of sulphuric acid and the solution was diluted to 100mL with distilled water. Two mL of the extract was acidified by adding 1.5% v/v HCl and a few drops of Wagner's reagent was added. Formation of a yellow or brown precipitate confirmed the presence of alkaloids.

Test for Flavonoids

To 1mL of the extract, a few drops of dilute sodium hydroxide were added. An intense yellow color was observed, which became colorless on the addition of few drops of dilute HCl acid, which indicated the presence of flavonoids. The presence of flavonoids was also confirmed by adding a few drops of 10% ferric chloride solution to 1mL extract. A green or blue color indicated presence of phenolic nucleus.

Test for Glycosides

0.5 mg of extract was dissolved in 1 ml of water and then aqueous NaOH solution was added. Formation of a yellow color indicated presence of glycosides.

Test for Saponins

In a test tube containing about 5mL of extract, a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 minutes. A honey comb like froth formation confirmed presence of saponins.

Test for Tannins

A few drops of 1% lead acetate and 5 mL of the extract were mixed. A yellow precipitate indicated presence of tannins.

Test for Terpenoids

Five mL of extract was mixed with 2mL of chloroform and 3mL of concentrated sulphuric acid. Formation of a reddish-brown coloration at the interface indicated presence of terpenoids.

3.8.2. Mineral composition using x-ray fluorescence (XRF) of *T. diversifolia* leaf powder

T. diversifolia leaf samples were finely ground and placed in polypropylene sample cups using 4µm prolene at the base. No vacuum conditions were used. Samples were analyzed under ambient conditions using an X-ray fluorescence instrument; Bruker SI Titan 600 which uses spectrometric scan mode for data acquisition. The XRF generator Voltage was 50 kV and the generator current 35 µA. The acquisition time was 40 seconds.

3.8.3. Liquid Chromatography Tandem Mass Spectroscopy (LCMS/MS) of aqueous fraction of *T. diversifolia* leaf extract

The aqueous fraction of *T. diversifolia* leaf extract was obtained through sequential fractionation of the crude methanol extract using hexane, dichloromethane, methanol and water. The water (aqueous) fraction was diluted in the mobile phase solvents; 50 % acetonitrile (0.1 % Formic acid) followed by filtration through 0.22 µm polytetrafluoroethylene (PTFE) syringe filter into a 2ml glass auto-sampler vial for LCMS/MS analysis. Qualitative phytochemical analysis was done using multiple reaction monitoring (MRM) in which the Shimadzu method package for primary metabolites was used to screen the sample for primary metabolites, in both positive and negative electrospray ionization (ESI) modes using the following parameters of the LC MS/MS Shimadzu Scientific Instruments;

LC parameters

The parameters for the liquid chromatography were a Mobile phase A: Deionized water (0.1 % Formic acid; Mobile phase B: Acetonitrile (0.1 % Formic acid); Flow rate: 0.25 ml/minute and Flow mode: Gradient.

Time	% B
2	0
5	25
11	35
15	95
20	95
20.1	0
25	0

The column was Kinetex C-18, 2.6 µm, 150 mm by 3 mm and the column oven temperature was 40 ^{0}C.

MS parameters

The mass spectroscopy parameters were; Nebulizing gas flow of 2 L/min; Drying gas flow of 15 L/min; Desolvation line temperature of 250 ^{0} C and heat block temperature of 400^{0}C.

3.8.4. Gas chromatography-Mass spectroscopy (GC-MS) analysis of crude methanol extracts of *T. diversifolia* leaves

The crude plant extract of methanol was prepared by re-extraction using Dichloromethane (DCM) and ethyl acetate in the ratio of 1:1. In each case one gram of the plant extract was re-extracted in 50ml of DCM and ethyl acetate. After re-extraction, the sample was cleaned by soaking it in activated charcoal. The soaked plant sample extract was filtered to obtain a clear colourless sample which was loaded to the GC-MS. Combined gas chromatography and mass spectrometry analysis for the extracted plant samples was carried out by a use of GC-MS Shimadzu Scientific Instruments Agilent Equipment 7890A installed with a

software by Mass Hunter to facilitate identification of the volatile compounds from the plant sample extract. The equipment comprised an inert capillary tube with a diameter of 0.32mm with a 0.2 µm film-stationary phase; Helium flowing at 1.0 mL/min was used as a carrier gas. The injector was operated at 250ºC and the oven temperature was increased from 50 - 300ºC gradually for 10 minutes.

The identity of phytocompounds in the plant extracts were generally proposed in relation to their fragmentation behavior and in reference to the spectra published in the National Institute of Standards and Technology (NIST) library - MS database. To identify the phytocompounds of the spectra, a match of above 80% with the library - MS database was required. The phytocompounds' name, molecular formula and molecular mass were determined. The peak area normalization percentage was used to determine the relative concentration of the phytocompounds (Appendix VII, (c)).

3.9. Molecular interactions of phytocompounds from methanol extract of *T. diversifolia* with venom proteins from *D. polylepis* and its mammalian protein targets

The structure data file (sdf) format of the fourteen compounds identified using GC-MS were retrieved from PubChem database (Kim *et al*., 2016). They were used for molecular docking simulations to understand the protein-ligand interacting behaviors. The twodimensional structures of these chemical compounds were also drawn using ChemDraw Ultra 10.0 (Mills, 2006) while the target protein 3-dimensional structures were downloaded from Protein Data Bank (PDB).

Molecular docking analysis was done using the software AutoDock Vina® where the target protein structures as downloaded from PDB were searched for appropriate active binding

sites and a grid box designed with specific parameters for docking against each of the identified phytocompounds using Autodock Vina Tool (Trott & Schroer, 2010). The ten best poses for each protein-phytocompound complex were retrieved and the complex that scored highest by virtue of least energy (kcal/mol) required for bonding was used for the molecular interaction analysis to better understand the structural features of proteins involved in the interaction with selected chemical compound. Pymol tool was used to convert the out file into a top scored complex file (Seeliger & Groot, 2010). This resulted in fourteen complexes for each venom toxin and human target protein.

Interaction Analysis involved subjecting the docked complexes to LigPlot+ tool for the interaction analysis of protein-ligand complex (Laskowski & Swindells, 2011). This generated the two-dimensional plot of each of the docked complexes showing the molecular interactions of protein residues with the selected ligand compound. The network of interactions presented the hydrophobic bonding with red color spikes and hydrogen bonding in green color along with the bond distance within 4 angstroms (Å).

3.10. Ethical considerations

Throughout the study, the three Rs in animal research; Replacement, Reduction and Refinement were followed to ensure humane research in animals. Ethical approval for use of animal subjects (Appendix I) was issued by the Faculty Biosafety, Animal use and Biosafety Committee, Faculty of Veterinary Medicine, University of Nairobi while the research permit (Appendix II) was granted by National Council for Science, Technology and Innovation (NACOSTI/P/17/32416/16118).

3.11. Limitations of the study

The study was prolonged by the unavailability, high cost and tight regulatory control of aqueous venoms, hence the eventual use of crude venom which had the challenge of standardization prior to commencement of the experiments. Duly registered anti-snake venom was also unavailable at the incept of this study since ASV production for the WHO snake bite region had ceased leading to delay as new ones had to be registered for sale and use in Kenya. The route of administration chosen for the study was intraperitoneal, to allow for comparison with the ASV which was the positive control agent for this study; this is however not the conventionally used route by traditional healers, who mainly administer the herbs orally and/or topically.

3.12. Data management and analysis

Selection of plants that were commonly used by the herbalists in management of snake bite was done using a familiarity index (F_i) which was calculated using the formula; $F_i = N_a/N_b$ x 100 (where N_a was the number of herbalists who mentioned a plant as being used for snake bite and N_b was the total number of herbalists interviewed). To determine the median lethal dose levels of plant extracts, a formula was used on survival data sets that were recorded in tables. Probit analysis was used for establishing the median lethal dose (LD_{50}) while the bottom-up approach was used to establish the venom challenge dose and effective dose (ED_{99}) of antivenom. Data for the *in vitro* and *in vivo* studies was recorded in tables and using SPSS version 28.0.1 was presented as mean ± standard deviation. Chi square test was then used to compare results of each group of mice with the negative control group. For the pharmacological studies, one-way analysis of variance (ANOVA) was used to compare data across treatment groups followed by Scheffe post hoc test for significance

testing where P-values less than 0.05 ($P<0.05$) and 0.01 ($P<0.01$) were considered significant at 95 and 99 percent confidence intervals respectively. Data from phytochemical analysis obtained from XRF was processed in the Bruker Artax® software where the K-α lines of the selected essential elements were used to process the mineral counts for the plant powder while quantitative and qualitative data obtained from LCMS/MS and GC-MC analysis was presented in the form of spectra and tables. Bioinformatics using AutoDock Vina® software was used to eliminate non-viable interactions from molecular docking outputs after which inference was made of potential interactions between venom toxins and their human target proteins as well as venom proteins and phytocompounds from the plant with antivenin activity.

CHAPTER FOUR
RESULTS

4.1. Selection of plants

A total of twenty-two plants were reported by the four herbalists as being used for management of snake bite (Table 4.1). The familiarity index resulted in eight plants that could have been included in the study, based on a score of 50% and above (Table 4.2). Two of the plants; mukuutu and mukenia were excluded due to uncertainties on verification of their botanical names. Mukuutu is easily confused with another plant 'mukutu' while mukenia was referred to as 'muchomoro' by a herbalist and the two are different plants. Mukengeta was also excluded because it was not readily available for collection. The remaining five plants; *Zanthoxylum chalybeum*, *Solanum incanum*, *Tagetes minuta*, *Tithonia diversifolia* and *Sansevieria suffruticosa* were included in the study.

Table 4.1: Plants reported to have anti-snake venom activity as reported by herbalists in Machakos, Kitui and Makueni Counties, Kenya.

S. No	***Local name***	***Botanical name***	***Family***
1.	Munoamathoka	*Dicostachys cinerea*	Fabaceae
2.	Mukenea	*Zanthoxylum chalybeum*	Rutaceae
3.	Muthaa	*Senna didymobotrya*	Fabaceae
4.	Mukenia	*Lantana camara*	Verbenaceae
5.	Muuku/Kiuuku	*Terminalia brownii*	Combretaceae
6.	Mukilyulu	*Bersama abyssinica*	Rutaceae
7.	Mukuswi	*Acacia brevispica*	Fabaceae
8.	Kivuti/Muvuti	*Erythrina abyssinica*	Fabaceae
9.	Mulaa	*Tithonia diversifolia*	Asteraceae
10.	Mukuutu/Kikuutu	*Microglossa pyrifolia*	Boraginaeae
11.	-	*Sansevieria suffruticosa*	Asparagaceae
12.	Muvemba	*Garcinia volkensii*	Clusiaceae
13.	Mutongatongu	*Solanum renschii*	Solanaceae
14.	Musuusuu	*Indigofera spicata*	Fabaceae
15.	Muvangi	*Tagetes Minuta*	Asteraceae
16.	Mulalambila	*Hibiscus Spp.*	Malvaceae
17.	Musilingu	*Grewia fallax*	Tilaceae
18.	Muveta	*Combretum collinum*	Combretaceae
19.	Mukengenta	*Cassia sengueana*	Fabaceae
20.	Mutonga	*Opilia amentacea*	Opiliceae
21.	Muchomoro	*Lantana camara*	Verbenaceae
22.	Mukondu/Kikondu	*Solanum incanum*	Solanaceae

Table 4.2: Familiarity index of plants commonly used in the management of snake bite in Machakos, Kitui and Makueni Counties, Kenya

S no.	***Plant***	***Frequency***	***Familiarity index***
1.	Munoamathoka	1	25
2.	Muthaa	1	25
3.	Muuku	1	25
4.	Mukilyulu	1	25
5.	Mukuswi	1	25
6.	Kivuti	1	25
7.	Muvemba	1	25
8.	Mutongatongu/mutongu	1	25
9.	Musuusuu	1	25
10.	Muliambela	1	25
11.	Musilungu	1	25
12.	Muveta	1	25
13.	Mukige	1	25
14.	Muchomoro	1	25
15.	Mukenia	2	50
16.	Mukuutu	2	50
17.	Mukengeta	2	50
18.	Muvangi	2	50
19.	*S. Suffruticosa*	2	50
20.	*T. diversifolia*/mulaa	2	50
21.	Mukenea/kikenea	3	75
22.	Mukondu/kikondu	3	75

4.2. Dry weight of crude extract

The dry weight of crude aqueous extracts after lyophilization is shown in Table 4.3 below.

Table 4.3: Percentage dry weight of plant extracts

Plant species	***Plant part***	***Weight (g) of plant powder***	***Weight (g) of aqueous product***	***Percentage dry weight (% w/w)***
Sansevieria suffruticosa	Leaves	200	3.4	1.7
Solanum incanum	Fruits	200	9.82	4.91
Tagetes minuta	Leaves	200	9.36	4.68
Tithonia diversifolia	Leaves	200	2.47	1.23
Zanthoxylum chalybeum	Root bark	150	5.14	3.43

4.3. Median lethal dose of aqueous extracts of plants

Four out of the five plants; *S. suffruticosa, T. minuta, T. diversifolia* and *Z. chalybeum;* had a median lethal dose (LD_{50}) of 2085mg/Kg while *S. incanum* had a much lower median lethal dose (LD_{50}) of 100mg/Kg (Table 4.4a-f). To establish the doses of the aqueous fractions of the herbal extracts that would be used for the study, the highest dose that did not cause mortality (0% mortality) after 48 hours of observation was taken. For this study therefore, the working dose for *S. suffruticosa, T. minuta, T. diversifolia* and *Z. chalybeum* herbal extracts was taken as 1500mg/Kg and that of *S. incanum* 316 mg/Kg.

Table 4.4a: Median lethal dose (LD_{50}) of aqueous fraction of *T. minuta* leaves in mice species *M. musculus*

Group	***Dose of extract (mg/kg)***	***No. of deaths***	***% of deaths***	***LD_{50} (mg/kg)***
1	0	0/3	0	2085.665
2	10	0/3	0	
3	100	0/3	0	
4	1000	0/3	0	
5	1500	0/1	0	
6	2900	1/1	100	
7	5000	0/1	0	

Table 4.4b: Median lethal dose (LD_{50}) of aqueous fraction of *S. incanum* fruits in mice species *M. musculus*

Group	***Dose of extract (mg/kg)***	***No. of deaths***	***% of deaths***	***LD_{50} (mg/kg)***
1	0	0/3	0	316.2278
2	10	0/3	0	
3	100	0/3	0	
4	1000	3/3	100	
5	1500	1/1	100	
6	2900	1/1	100	

Table 4.4c. Median lethal dose (LD_{50}) of aqueous fraction of *Z. chalybeum* root bark in mice species *M. musculus*

Group	***Dose of extract (mg/kg)***	***No. of deaths***	***% of deaths***	***LD_{50} (mg/kg)***
1	0	0/3	0	2085.665
2	10	0/3	0	
3	100	0/3	0	
4	1000	3/3	100	
5	1500	1/1	100	
6	2900	1/1	100	
7	5000	1/1	100	

Table 4.4d: Median lethal dose (LD_{50}) of aqueous fraction of *T. diversifolia* leaves in mice species *M. musculus*

Group	***Dose of extract (mg/kg)***	***No. of deaths***	***% of deaths***	***LD_{50} (mg/kg)***
1	0	0/3	0	2085.665
2	10	0/3	0	
3	100	0/3	0	
4	1000	0/3	0	
5	1500	1/1	100	
6	2900	1/1	100	
7	5000	0/1	0	

Table 4.4e: Median lethal dose (LD_{50}) of aqueous fraction of S. *suffruticosa* leaves in mice species *M. musculus*

Group	***Dose of extract (mg/kg)***	***No. of deaths***	***% of deaths***	***LD_{50} (mg/kg)***
1	0	0/3	0	2085.665
2	10	0/3	0	
3	100	0/3	0	
4	1000	0/3	0	
5	1500	0/1	0	
6	2900	1/1	100	
7	5000	1/1	100	

Table 4.4f: Median lethal doses of aqueous fractions of herbs commonly used in *D. polylepis* snake bite in mice species *M. musculus.*

Plant extract	****Highest dose (mg/kg) with 0% mortality***	***Highest dose (mg/kg) with 100% mortality***	***LD_{50} (mg/kg) $LD_{50}=\sqrt{(d_0 x d_{100})}$***
Sansevieria suffruticosa leaves	1500	2900	2085.665
Solanum incanum Fruits	100	1000	316.2278
Tagetes minuta Leaves	1500	2900	2085.665
Tithonia diversifolia Leaves	1500	2900	2085.665
Zanthoxylum halybeum Root bark	1500	2900	2085.665

**Taken as the working dose for use in this study.*

4.4. *In vitro* and *in vivo* effect of aqueous fractions on *D. polylepis* venom in laboratory mice

The antivenin effect of aqueous fractions from the five plants was carried out by initially determining the median lethal dose (LD_{50}), the venom challenge dose and the effective dose (ED_{50}) of commercially obtained anti-snake venom (ASV).

4.4.1. Medium lethal dose (LD_{50}) of *D. polylepis* crude venom

Mice (in six groups of five mice each) were injected with increasing doses of crude venom as shown in Table 4.5a, until 100% mortality was observed in a 24-hour period. After dose and response adjustment using the corrected formula, probits of the percentage responses were plotted against their corresponding Log_{10} doses (Figure 4.1). The LD_{50} read at probit 5 was 2.5 mL/Kg**,** the antilog of which was 316.22 mL/Kg (3.1622mL/Kg given the corrected formula 'Dose x 100'). This translated to 0.0240 mg/Kg given that 1mL of venom working solution contained 0.0076mg of crude venom.

Table 4.5a: Survival data set in determining the median (LD_{50}) dose of *D. polylepis* venom in albino mice

Group N=5 mice per group	***Volume of crude venom injected (ml/kg of 0.0076mg/ml)***	***Adjusted dose (x100)***	***Log dose***	***Response (dead/total)***	***Percentage response***	***Corrected formula***	***Probit of corrected value of % response***
1	1.25	125	2.1	0/5	0	5	3.36
2	2.5	250	2.4	2/5	40	40	4.75
3	5.0	500	2.7	4/5	80	80	5.84
4	7.5	750	2.9	4/5	80	80	5.84
5	10	1000	3	5/5	100	95	6.64
6	20	2000	3.3	5/5	100	95	6.64

Key: A; Alive D; Dead

Corrected formula:

For the 0% dead (100% survival), 100(0.25/n)

For the 100% Dead (0% survival), 100[(5-0.25)/n]

Where n=the number of animals in the group

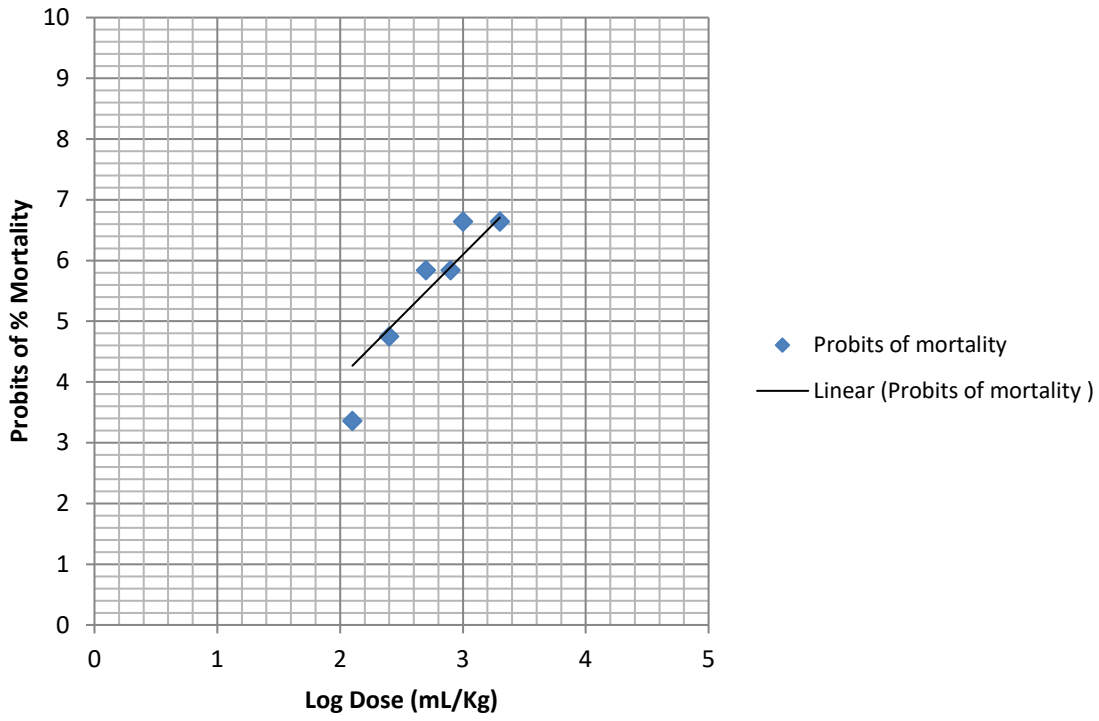


Figure 4.1: Probits of percentage mortality against Log_{10} dose of crude venom

4.4.2. Venom challenge dose

The challenge dose for this study was established by injecting multiples of the LD_{50} (0.0240mg/Kg) to groups of mice, one at a time, until 100% mortality was observed over a period of 24 hours. A multiple of four (4) of the LD_{50} (0.0960mg/Kg) was established as the venom challenge dose resulting in 100% case fatality synonymous to untreated envenomation due to *D. polylepis* (Table 4.5b).

Table 4.5b: Multiple of LD_{50} of *D. polylepis* crude venom resulting in 100% mortality

Group N=5	***Multiple of LD_{50} injected (mL/Kg of 0.0240mg/Kg crude venom)***	***Percentage Survival***
1	1 (0.0240 mg/Kg)	60
2	2 (0.0480 mg/Kg)	40
3	3 (0.0720 mg/Kg)	20
4	4 (0.0960 mg/Kg)	0
5	5 (0.1200 mg/Kg)	0
6	6 (0.1440 mg/Kg)	0

4.4.3. Effective dose (ED_{99}) of anti-snake venom (ASV)

The effective dose of commercially available ASV with a label claim of 100mg of ASV protein per mL was determined by intraperitoneal injection of increasing doses (1000, 2000, 3000, 4000, 5000 and 6000mg/Kg) of the ASV into mice immediately after injecting them with the venom challenge dose (0.0960mg/Kg). The mice were observed for a 24hour period until 100% survival was recorded. All the mice in the group that received 3000mg/Kg and above of ASV survived (Table 4.5c); thus, the effective dose in laboratory mice of ASV for this study was taken to be 3000mg/Kg.

Tabel 4.5c: Effective anti-venom dose (ED_{99}) for neutralization of *D. polylepis* venom challenge dose in laboratory mice *Mus musculus*

Group	***Vol of anti-snake venom (asv) injected (ml/kg)***	***Total asv protein (mg/kg)***	***Percentage Survival***
1	10	1000	0
2	20	2000	0
3	30	3000	100
4	40	4000	100
5	50	5000	100

4.4.4. *In vitro* activity of aqueous herbal extracts against *D. polylepis* venom

A mixture of the venom challenge dose (0.0960mg/Kg) and the working doses of herbal extracts as established for each plant was incubated for one hour at 37^0C. The effect of venom extract interaction after the incubation period was observed in mice after intraperitoneal injection of the mixture; the end point being alive or dead. The highest survival rate observed was for *S. suffruticosa* (40%) followed by *S. incanum*, *T. minuta* and *T. diversifolia* which recorded a survival rate of 20%. *Z. chalybeum* did not save any mice as shown in Table 4.5d. The results however indicated that none of the five herbal extracts had a significant *in vitro* effect on the venom when compared to the negative control.

Table 4.5d: *In vitro* effect of aqueous herbal extracts on *D. polylepis* venom in mice

Treatment	***Percentage survival***	***Calculated χ^2 against the vehicle***
SSL	40	2.5
SIF	20	1.111
ZCB	0	0
TML	20	1.111
TDL	20	1.111
Positive control	100	10**
Negative control	0	0

** and ** indicate a value of ($p < 0.05$) and ($p < 0.001$) relative to the vehicle respectively*
Note: If calculated χ^2 value is less than tabulated critical at P<0.05 (3.814) and p<0.001 (6.635) there is no significant difference between the treatment and the vehicle.

KEY

SSL - *Sansevieria suffruti*cosa leaves
SIF - *Solanum incanum* fruits
TML - *Tagetes Minuta* leaves
TDL - *Tithonia diversifolia* leaves
ZCB - *Zanthoxylum chalybeum* root bark

4.4.5. *In vivo* activity of aqueous herbal extracts against *D. polylepis* venom

The venom challenge dose (0.0960mg/Kg) was injected intraperitoneally into mice immediately prior to intraperitoneal injection of working doses of herbal extracts. In this test, *T. diversifolia* recorded the highest survival rate of 60% followed by *T. minuta* (40%) with *S. suffruticosa* and *S. incanum* saving 20% of the mice population each. *Z. chalybeum* recorded 0% survival of the mice population as illustrated in Table 4.5e. Out of the five herbal extracts, only that of *T. diversifolia* showed a significant *in vivo* effect against the venom with a higher critical value of 4.286 against the tabulated (3.814) at P<0.005 but lower than tabulated (6.635) at P<0.01.

Table 4.5e: *In vivo* effect of aqueous herbal extracts on *D. polylepis* venom in mice.

Treatment	***Percentage survival***	***Calculated χ^2 against the vehicle***
SSL	20	1.111
SIF	20	1.111
ZCB	0	/0
TML	40	2.5
TDL	60	4.286*
Positive control	100	10**
Negative control	0	0

** and ** indicate a value of ($p < 0.05$) and ($p < 0.001$) relative to the vehicle respectively. Note: If calculated χ^2 value is less than tabulated critical at $p<0.05$ (3.814) and $p<0.001$ (6.635) there is no significant difference between the treatment and the vehicle.*

KEY

SSL - *Sansevieria suffruti*cosa leaves
SIF - *Solanum incanum* fruits
TML - *Tagetes Minuta* leaves
TDL - *Tithonia diversifolia* leaves
ZCB - *Zanthoxylum chalybeum* root bark

4.5. Pharmacological effects of aqueous leaf extract of *T. diversifolia* on *D. polylepis* snake bite wound

Antivenin studies carried out on the five herbal extracts revealed that *T. diversifolia* had significant ($p < 0.05$) *in vivo* antivenin effect against *D. polylepis* venom. Additional pharmacological tests on *T. diversifolia* aqueous extract to establish other benefits in *D. polylepis* snake bite namely; anti-nociceptive, anti-pyretic, anti-inflammatory and antimicrobial effects were carried out.

4.5.1. Anti-nociceptive activity of orally and intraperitoneally administered aqueous leaf extract of *T. diversifolia* in mice

The aqueous extract of *T. diversifolia* at 100, 200 and 300 mg/Kg was administered orally into mice using a drenching syringe and the activity of extract on pain was tested using the Hotplate method and compared to diclofenac sodium (positive control) as shown in Table 4.6a. The extract at a dose of 300mg/Kg had a significant ($p < 0.05$) effect on pain (reduced nociception) when compared to the negative control. The anti-nociceptive activity of the extract at 300mg/Kg was comparable to that of 50mg/Kg of diclofenac sodium throughout the observation period.

Table 4.6a Anti-nociceptive effect after oral administration of aqueous leaf extract of *T. diversifolia* in mice

Treatment	***Pain tolerance in seconds***			
	0 min	**15 min**	**30 min**	**60min**
N. Saline	6.18±0.35	7.7±0.92	6.85±0.85	9.52±1.08
Extract 100mg/kg	9.12±0.62	11±1.05	9.88±2.09	13.57±1.44
Extract 200mg/kg	9.42±0.97	9.93±1.31	10.6±2.11	12.28±1.86
Extract 300mg/kg	11.25±1.14 *	14.98±2.4 *	14.17±1.59 *	15.28±1.16 *
Diclofenac 50mg/kg	14.22±1.43 **	14.18±2.01 *	14.7±1.72 *	14.1±1.85 *

**and ** indicate a value of ($p < 0.05$) and ($p < 0.001$) relative to the vehicle respectively.*

The experiment was repeated using the intraperitoneal route of administration where a dose of 300mg/Kg of the extract showed significant anti-nociceptive effect in mice at 95% confidence interval similar to the positive control (Table 4.6b).

Table 4.6b: Anti-nociceptive effect after intra-peritoneal treatment of aqueous leaf extract of *T. diversifolia* in mice

Treatment	***Pain tolerance time in seconds***			
	0 min	**15 min**	**30 min**	**60min**
N. Saline	8.32±0.32	8.08±0.44	8±0.67	10.02±1.11
Extract 100mg/kg	7.47±0.83	10.07±1.58	12.27±1.48	12.73±2.03
Extract 200mg/kg	7.6±0.95	13.43±1.5 *	12.97±1.5	15.22±1.6
Extract 300mg/kg	14.22±1.81 *	15.41±1.26 *	14.58±0.43 *	19.14±1.55 *
Diclofenac 50mg/kg	15.65±1.8 *	17.16±0.99 **	16.57±1.36 **	20.9±2.93 *

and ** indicate a value of ($p < 0.05$) and ($p < 0.001$) relative to the vehicle respectively*.**

4.5.2. Anti-pyretic activity of orally and intraperitoneally administered aqueous leaf extract of *T. diversifolia* in rats

Pyrexia was induced in rats by injecting turpentine into the groin area followed by a one hour waiting period. Baseline temperature was recorded before the administration of aqueous extract (100, 200 and 300mg/Kg) orally using a drenching syringe. A digital thermometer was used to take anal temperatures at 0, 1, 2 and 3 hours after the turpentine injections. It was observed that a dose of 300mg/Kg of the extract had significant antipyretic effect at 99% confidence level similar to the positive control (paracetamol 100mg/Kg) as indicated in Table 4.6c.

Table 4.6c: Anti-pyretic effect after oral administration of aqueous leaf extract of *T. diversifolia* in rats.

Treatments	***Rectal temperature (^{0}C)***			
	0hr	**1hr**	**2hrs**	**3hrs**
N. Saline	39.08±0.14	39.25±0.19	39.95±0.21	39.8±0.22
100mg/kg	38.54±0.16	38.76±0.17	39.88±0.12	39.46±0.12
200mg/kg	38.78±0.21	38.96±0.17	39.4±0.16	39.26±0.21
300mg/kg	38.2±0.08	38±0.07 **	37.76±0.24 **	37.72±0.15 **
Paracetamol 100mg/kg	38.3±0.09	37.84±0.09 **	37.68±0.12 **	37.18±0.31 **

** and ** indicate a value of ($p < 0.05$) and ($p < 0.001$) relative to the vehicle respectively.*

When the procedure above was repeated with a change in route of administration to intraperitoneal, the same observation was made as with the oral route where the extract at 300mg/Kg had significant antipyretic effect ($p < 0.001$) confidence interval and was comparable to the positive control.

Table 4.6d: Anti-pyretic effect after intra-peritoneal administration of aqueous extracts of *T. diversifolia* in rats

Treatments	***Rectal temperature (^{0}C)***			
	0hr	**1hr**	**2hrs**	**3hrs**
N. Saline	39.9±0.3	40.8±0.2	39.8±0.1	39.4±0.2
Extract 200mg/kg	39.9±0.2	39.7±0.4 *	39.8±0.5	38.4±0 *
Extract 300mg/kg	39.7±0.2	38.9±0.2 **	37.7±0.2 **	38±0.1 **
Paracetamol 100mg/kg	39.8±0.1	38.9±0.2 **	38.4±0.1 **	38±0.1 **

** and ** indicate a value of ($p < 0.05$) and ($p < 0.001$) relative to the vehicle respectively.*

4.5.3. Anti-inflammatory activity of orally and intraperitoneally administered aqueous leaf extract of *T. diversifolia* in mice

Mice (in five groups of five mice each) were treated orally with extract at 100, 200 and 300mg/Kg, positive control (diclofenac sodium) and negative control (normal saline). Prior to this treatment, inflammation had been induced in the left hind paw of mice by injecting0.1 mL of λ-carrageenan (1% in NaCl 0.9%). The paw diameter was measured using a digital caliper prior to treatments and at 1.5, 3 and 6 hours after the treatments as shown on Table 4.6e. The results showed that the extract at 300mg/Kg had significant effect at 95% confidence level and was comparable to 50mg/Kg of diclofenac sodium throughout the observation period.

Table 4.6e: Anti-inflammatory effect after oral administration of aqueous leaf extracts of *T. diversifolia* in mice

Treatment arms	***% change in paw diameter***			
	0hr	1.5hr	3hr	6hr
N. Saline	0±0	45.81±3.86	60.19±5.45	60.16±6.59
Extract 100mg/kg	0±0	29.05±4.98	37.83±8.44	28.29±4.54
Extract 200mg/kg	0±0	27.06±4.11	36.69±3.17	37.76±6.06
Extract 300mg/kg	0±0	21.32±2.42 *	29.25±4.98 *	26.61±5.6 *
Diclofenac 50mg/kg	0±0	25.46±4.9 *	24.15±5.44 *	23.52±5.48 *

**and ** indicate a value of ($p < 0.05$) and ($p < 0.001$) relative to the vehicle respectively.*

The activity of the extract was established using the same procedure as above but with a change to intra-peritoneal route. Similar observations were made where 300mg/Kg of extract was found to be as effective as the positive control (diclofenac sodium) at 95% interval at the third and sixth hour of observation.

Table 4.6f: Anti-inflammatory effect in mice after intra-peritoneal administration of aqueous leaf extract of *T. diversifolia*

Treatment arms	***% change in paw diameter***			
	0hr	1.5hr	3hr	6hr
N. Saline	0±0	45.11±4.49	60.58±9.55	52.91±7.93
100mg/kg	0±0	48.09±7.99	53.52±10.91	38.3±8.03
200mg/kg	0±0	34.99±8.27	34.68±7.08	38.06±7.9
300mg/kg	0±0	37.69±3.13	29.76±6.74 *	29.62±4.37 *
Diclofenac 50mg/kg	0±0	22.76±1.31 *	29.61±3.23 *	24.88±4.12 *

**and ** indicate a value of ($p < 0.05$) and ($p < 0.001$) relative to the vehicle respectively.*

4.5.4. Antimicrobial effects of *T. diversifolia* aqueous extract

Six microbial organisms; *Staphylococus* aureus, *Streptococus* pyogenes, *Streptococus agalactiae, Pseudomonas aeruginosa, Escherihia* coli and *Candida albians* were subjected to various concentrations of the aqueous extract of *T. diversifolia.* Antimicrobial activity of this extract was investigated to establish additional benefits when it is applied to snake bite wounds which are prone to microbial infections. Two of these organisms; *S. pyogenes* and *S. aureus* are of particular interest in snake bite cases where the site of punctured skin is at risk of developing cellulitis. The results showed that *E. coli* and *C. albicans* were not susceptible to *T. diversifolia* extract while *S. agalactiae* showed susceptibility to the extract with zones of inhibition exceeding those of the positive control at increasing doses of the extract from 200mg/mL to 1000mg/Kg. *P. aeruginosa* showed increasing susceptibility to increasing doses of extract from 50mg/mL but the zones of inhibition were lower than those of the positive control. *S. aureus* and *S. pyogenes* showed increasing susceptibility to extract from 25mg/mL. For *S. aureus*, doses above 200mg/mL of the extract were statistically similar in their inhibition of the organism (Table 4.6g).

Table 4.6g: Inhibition zones (mm) at different concentrations of aqueous leaf extract of *T. diversifolia*

	Inhibition zones (diameter in mm)								
Test Organisms	**Positive Control**	**25mg/kg**	**50 mg/ml**	**100 mg/ml**	**200mg/ml**	**400 mg/ml**	**800 mg/ml**	**1000 mg/ml**	**Negative control**
S. aureus	41±0.58a	10±0b	12.5±0.29c	15.5±0.29d	19.5±0.29ej	20±0fij	21.5±0.29ghi	22.5±0.29h	0±0b
S. agalactiae	18.5±0.29a	0±0b	9.5±0.29c	13.5±0.29d	20±0ei	21±0fi	26±0g	27.5±0.29h	0±0b
S. pyogenes	35±0.58a	12.5±0.87b	16.5±0.29cl	18.5±0.29djkl	20.5±0.29ejk	21±0.58fij	23.5±0.29ghi	26±0.58h	0±0b
P. aeruginosa	29±1.15a	0±0b	9.5±0.29c	13.5±0.29d	16.5±0.29ei	17.5±0.29fhi	19.5±0.29gh	21±0g	0±0b
E. coli	23.5±0.29a	0±0b	0±0b	0±0b	0±0b	0±0b	0±0b	0±0b	0±0b
C. albicans	15.5±0.29a	0±0b	0±0b	0±0b	0±0b	0±0b	0±0b	0±0b	0±0b

Similar letters indicate statistical significance within rows ($p<0.05$)

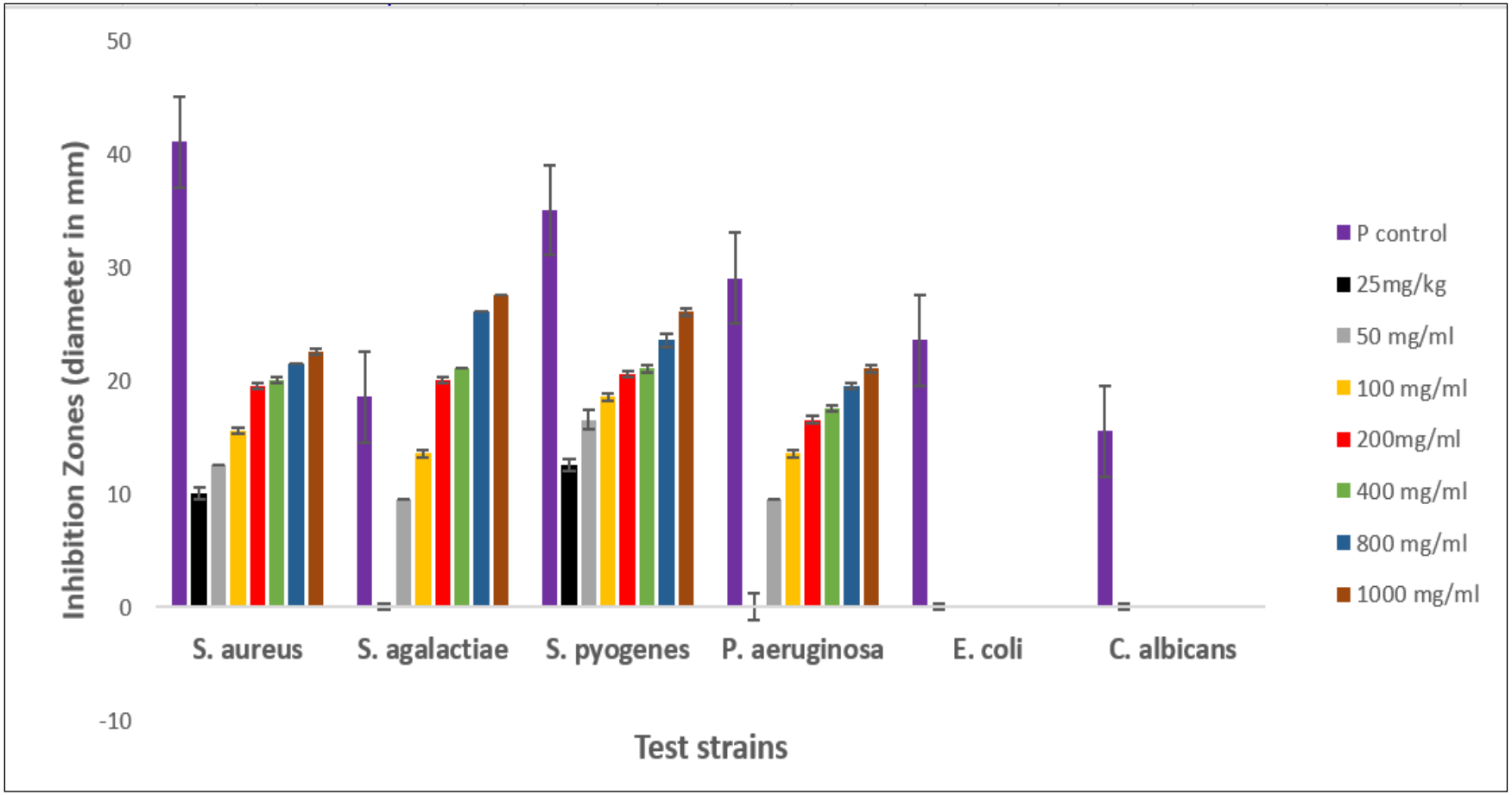


Figure 4.2: Inhibition zones at different concentrations of aqueous leaf extract of *T. diversifolia* for different organisms.

Susceptible micro organism at minimum concentration of extract with least zone of inhibition	Serial dilutions of *T. diversifolia* extract starting with concentration with least zone of inhibition for each susceptible micro organism											50uL DMSO
		1	2	3	4	5	6	7	8	9	10	11	12
S. aureus (25mg/ml)	1	NG	NG	NG	G	G	G	G	G	G	G	G	G
Control drug-Benzyl penicillin	2	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	G
S. pyogenes (25mg/ml).	3	NG	G	G	G	G	G	G	G	G	G	G	G
Control drug-Benzyl penicillin	4	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	G
S. agalactiae (50mg/ml)	5	NG	NG	NG	NG	G	G	G	G	G	G	G	G
Control drug-Benzyl penicillin	6	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	G
P. aeruginosa (50mg/ml)	7	NG	NG	G	G	G	G	G	G	G	G	G	G
Control drug-Gentamicin	8	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	G

Figure 4.3: Illustration of 96 well plate showing growth (G) in yellow and No Growth (NG) in red for various concentrations of extracts in determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extracts.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the four organisms that were susceptible to *T. diversifolia* extract at various concentrations showed that *S. aureus,* and *P. aeruginosa* had the least MIC and MBC (1.56 and 3.125mg/mL) compared to *S. pyogenes* and *S. agalactiae* (6.25 and 12.5 mg/mL) respectively.

Table 4.6h: Minimum Inhibitory and Minimum Bactericidal Concentration for organisms susceptible to aqueous extract of *T. diversifolia*

Susceptible organism	***MIC***	***MBC***
S. aureus	1.56 mg/ml	3.125mg/ml
S. pyogenes	6.25mg/ml	12.5mg/ml
S. agalactiae	1.56 mg/ml	3.125mg/ml
P. aeroginosa	6.25mg/ml	12.5mg/ml

4.6. Phytochemical analysis of methanol extracts of herbal preparations

Analysis of phytochemicals present in herbal extracts used in this study included qualitative analysis of the five herbal extracts that were subjected to *in vitro* and *in vivo* antivenin studies. Additionally, for *T. diversifolia* aqueous extract which showed significant *in vivo* antivenin activity, mineral composition of dry herbal powder of using x-ray fluorescence (XRF), Liquid Chromatography-Mass Spectroscopy (LC-MS/MS) and Gas Chromatography-Mass spectroscopy (GC-MS) analysis of the extracts was done.

4.6.1. Qualitative phytochemical analysis of crude methanol extracts of plants under study

Phytochemical analysis of methanol extracts from the five plants was done to determine presence or absence of flavonoids, cardiac glycosides, terpenes, tannins, saponins and alkaloids with their colour intensity visually inspected to approximate their abundance. All the plants studied were found to contain the various phytochemicals tested. There was indication that cardiac glycosides and terpenes

were abundant in four out of the five plants; *Sansevieria suffruticosa, Solanum incanum, Tithonia diversifolia, Zanthoxylum chalybeum*. Saponins were not observed in *T. minuta* extract while alkaloids were abundant in *Zanthoxylum chalybeum* compared to the other four extracts (Table 4.7a).

Table 4.7a: Qualitative phytochemical analysis of crude methanol extracts

Phytochemical	***Sansevieria suffruticosa* Leaves**	***Solanum incanum* fruits**	***Tagetes minuta* leaves**	***Tithonia diversifolia* Leaves**	***Zanthoxylum chalybeum* Root bark**
Flavonoids	+	++	+++	+	+++
Cardiac glycosides	+++	+++	++	+++	+++
Terpenes	+++	+++	+++	++	+++
Tannins	+	+++	++	++	+++
Saponins	++	+++	-	+	+++
Alkaloids	+	++	++	+	+++

KEY: (-): Absent, (+): Weakly Present, (++): Moderately present, (+++): Strongly present

4.6.2. Mineral composition of dry leaf powder of *T. diversifolia* using x-ray fluorescence (XRF)

Semi-quantitative analysis of the dry leaf powder of *T. diversifolia* using the S1 Titan XRF spectrometer revealed that the powder contained detectable amounts of potassium (K), calcium (Ca), chromium (Cr), manganese (Mn), iron (Fe), nickel (Ni), copper (Cu) and zinc (Zn). The levels of K were highest, followed by Fe and Ca respectively.

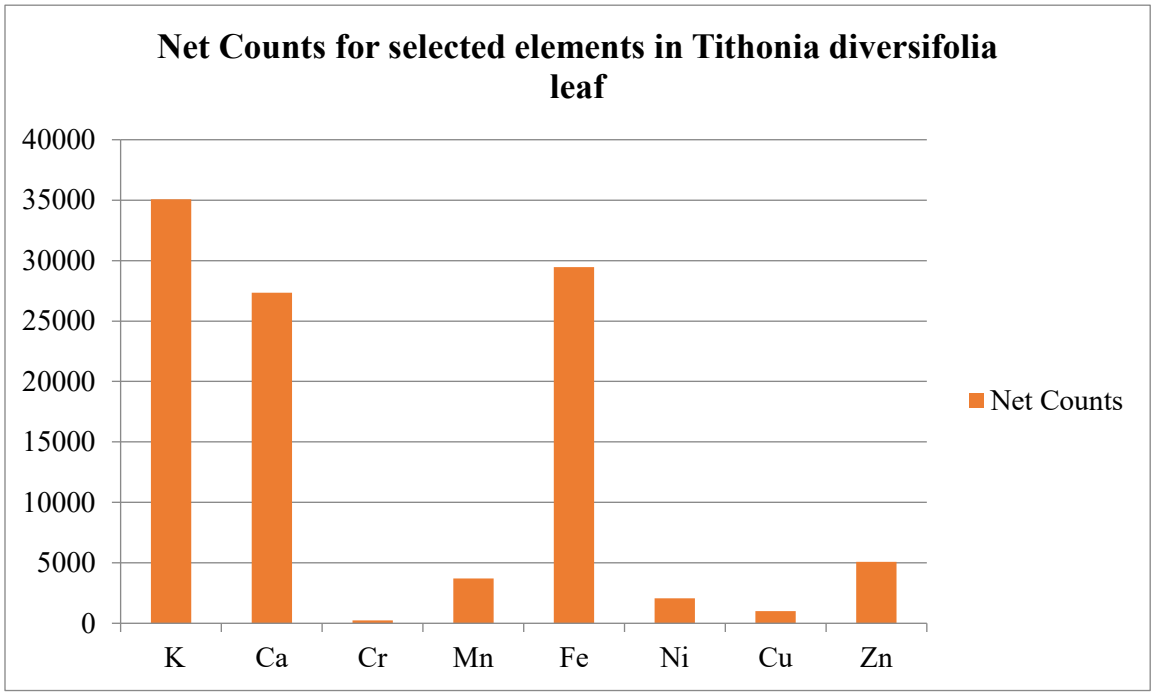


Figure 4.4: Elemental composition of the dried leaf powder of *Tithonia diversifolia*

4.6.3. Liquid Chromatography- Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS) of aqueous fraction of *T. diversifolia*

Qualitative phytochemical analysis was done on the aqueous fraction of *T. diversifolia* which was used in the antivenin and pharmacological studies. The analysis was targeted using multiple reaction monitoring (MRM) in which the Shimadzu method package for primary metabolites was used to screen the sample for primary metabolites. Thirty-eight primary metabolites were identified in the sample most of which were amino acids (Table 4.7b).

Table 4.7b: Liquid Chromatography- Mass Spectroscopy (LCMS/MS) analysis of aqueous fraction of *T. diversifolia*

S. No	***Name***	***m/z (1)***	***m/z (2)***	***Retention time (min)***	***Start Time***	***End Time***	***Peak Area***	***Ref. (1) m/z (1)***	***Ref. (1) m/z (2)***	***Acquisition mode***	***Esi polarity***	***Target collision energy***	***Ref. (1) collision energy***
1	Asparagine	133.1	87.15	2.608	0	3.953	20090546	133.1	28.05	MRM	+ve	-12	-29
2	Aspartic acid	134	74.05	2.664	0	3.953	2163858	134	88.1	MRM	+ve	-15	-13
3	Serine	105.9	60.1	2.585	0	3.96	965281			MRM	+ve	-12	
4	Alanine	89.9	44.1	2.621	0	3.972	593005			MRM	+ve	-12	
5	4-Hydroxyproline	132.1	86.05	2.7	0	3.991	63083	132.1	68.05	MRM	+ve	-15	-22
6	Glycine	75.9	30.15	2.584	0.029	4.029	226909			MRM	+ve	-11	
7	Glutamine	147.1	84.15	2.649	0.073	4.073	9874258	147.1	130.1	MRM	+ve	-18	-16
8	Threonine	120.1	74.15	2.666	0.133	4.133	347879	120.1	56.05	MRM	+ve	-13	-17
9	Dimethylglycine	104.1	58.05	2.547	0.189	4.189	170193	104.1	44.05	MRM	+ve	-16	-38
10	Methionine sulfoxide	166	74.1	2.787	0.206	4.206	22164	166	55.95	MRM	+ve	-14	-25
11	Glutamic acid	147.9	84.1	2.723	0.253	4.253	5796089	147.9	56.1	MRM	+ve	-17	-30
12	Cytidine monophosphate	324	112.05	3.317	0.26	4.26	1519	324	95	MRM	+ve	-14	-54
13	Citrulline	176.1	70.05	2.704	0.321	4.321	46387	176.1	159.05	MRM	+ve	-25	-14
14	Proline	116.1	70.15	2.986	0.609	4.609	153815169	116.1	28.05	MRM	+ve	-18	-35
15	Ornitine	133.1	70.1	2.607	0.679	4.679	6491418	133.1	116.05	MRM	+ve	-18	-15
16	2-Aminobutyric acid	104.1	58.05	2.541	0.831	4.831	2261065	104.1	41.05	MRM	+ve	-12	-26

S. No	*Name*	*m/z (1)*	*m/z (2)*	*Retention time (min)*	*Start Time*	*End Time*	*Peak Area*	*Ref. (1) m/z (1)*	*Ref. (1) m/z (2)*	*Acquisition mode*	*Esi polarity*	*Target collision energy*	*Ref. (1) collision energy*
17	Lysine	147.1	84.1	2.646	0.894	4.894	6156395			MRM	+ve	-18	
18	Histidine	155.9	110.1	2.392	0.901	4.901	1533429	155.9	56.1	MRM	+ve	-15	-35
19	Adenosine monophosphate	348	136.05	2.99	0.969	4.969	2231	348	97.1	MRM	+ve	-20	-31
20	Uracil	113	70	3.718	0.986	4.986	7206			MRM	+ve	-17	
21	Arginine	175.1	70.1	3.191	1.365	5.365	206609	175.1	60.1	MRM	+ve	-23	-16
22	4-Aminobutyric acid	104.1	87.05	2.539	1.69	5.69	15699512	104.1	45.1	MRM	+ve	-14	-22
23	Cytidine 3',5'-cyclic monophosphate	306	112.1	3.48	2.093	6.093	2727	-	-	MRM	+ve	-22	
24	Glutathione	308	179.1	3.339	2.543	6.543	1141			MRM	+ve	-13	
25	Valine	118.1	72.15	3.455	2.761	6.761	539138	118.1	55.05	MRM	+ve	-13	-24
26	Methionine	149.9	56.1	5.404	3.304	7.304	7035	149.9	104.1	MRM	+ve	-18	-14
27	Thymine	127.1	54.05	5.838	3.448	7.448	4792	127.1	110.05	MRM	+ve	-29	-8
28	Adenosine 3',5'-cyclic monophosphate	330	136.05	4.691	4.179	8.179	1433	330	119.1	MRM	+ve	-26	-54
29	Isoleucine	132.1	86.2	5.897	5.241	9.241	62442	132.1	69.15	MRM	+ve	-12	-19
30	Leucine	132.1	86.05	5.898	5.52	9.52	60946	132.1	30.05	MRM	+ve	-12	-18
31	Phenylalanine	166.1	120.1	8.931	6.068	10.068	511992	166.1	103.1	MRM	+ve	-15	-29
32	Acetylcholine	147.1	87.05	8.388	7.165	11.165	19958	147.1	88.05	MRM	+ve	-16	-16

S. No	*Name*	*m/z (1)*	*m/z (2)*	*Retention time (min)*	*Start Time*	*End Time*	*Peak Area*	*Ref. (1) m/z (1)*	*Ref. (1) m/z (2)*	*Acquisition mode*	*Esi polarity*	*Target collision energy*	*Ref. (1) collision energy*
33	Tryptophan	205.1	188.15	9.17	8.092	12.092	117169	205.1	146.1	MRM	+ve	-12	-18
34	Malic acid	133.1	114.95	3.636	0.358	4.358	150755	133.1	71.15	MRM	-ve	17	17
35	Isocitric acid	191.2	111.1	2.634	0.358	4.358	29459	191.2	73	MRM	-ve	15	24
36	Citric acid	191.2	111.1	2.638	1.209	5.209	30722	191.2	87.05	MRM	-ve	13	20
37	Xanthine	151	108	2.735	2.093	6.093	726	151	42	MRM	-ve	20	21
38	Fumaric acid	115	71.1	5.857	2.571	6.571	24603	115	26.95	MRM	-ve	11	14

4.6.4. Gas chromatography-Mass spectroscopy (GC-MS) analysis of crude methanol extract of T. diversifolia leaves

The crude methanol extract of *T. diversifolia* leaves was analyzed using GC-MS and the compounds detected were identified using the National Institute of standards and Technology (NIST) library.

4.6.4.1. Compound Identification

Fourteen compounds were identified from the analysis accounting for fifty one percent of the total extract. The most abundant compound was Camphene (7.6%) followed by Menthol (7.5%), αLongipinene (5.5%), pthalic acid (5.1%) while each of the other compounds had a peak abundance of less than 5% as shown in Table 4.7c below. The least abundant compounds were Dibutyl phthalate (0.2%) and Napthalene which at 0.02% peak abundance was present in very minute quantities.

Table 4.7c: GC-MS Identification of phytocompounds in crude methanol leaf extract of *T. diversifolia* order of retention time and as derived from the National Institute of Sciences and Technology (NIST) Library

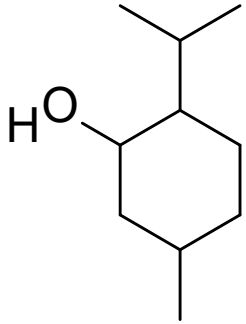
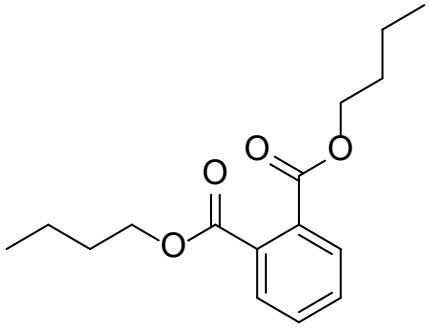
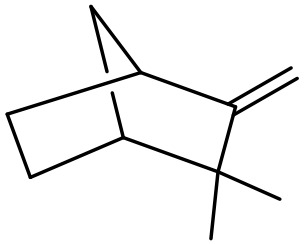
S. No.	***Retention time (minutes)***	***Chemical name***	***Molecular formula***	***Molecular weight (g/mol)***	***Nist library identification number***	***% peak area abundance***
1.	5.8589	Camphene, *2,4*-Di-tert-*butylphenol*	$C_{14}H_{22}O$	206.3239	79-92-5	7.5768
2.	13. 9370	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{17}H_{30}OSi$	278.5	96-76-4	4.4563
3.	14.5143	Alloaromadendrene, 1H-Cycloprop[e]azulene, decahydro-1, 1, 7-trimethyl-4-methylene-, [1aR-(1a.alpha.)	$C_{15}H_{24}$	204.35	25246-27-9	4.2132
4.	14.5225	α-Longipinene, Tricyclo[5.4.0.0(2.8)]undec-9-ene, 2,6,6,9-tetramethyl	$C_{15}H_{24}$	204.35	5989-08-2	5.5128
5.	15.8180	Kuromatsuen, Longifolene, 1,4-methanoazulene, decahydro-4,8,8-trmethyl-9-methylene-,[1S-(1.alpha.,3a.be)	$C_{15}H_{24}$	204.35	475-20-7	2.2314
6.	15.8180	Napthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,(1.alpha.)	$C_{10}H_{8}$	128.17	5951-61-1	0.0231
7.	17.4854	Citronellyl propionate, 6-Octen-1-ol, 3,7-dimethyl-, propanoate	$C_{13}H_{24}O_{2}$	212.3285	141-14-0	2.3421

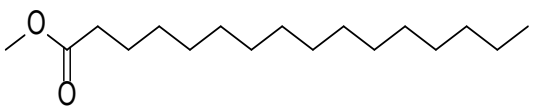
S. No.	*Retention time (minutes)*	*Chemical name*	*Molecular formula*	*Molecular weight (g/mol)*	*Nist library identification number*	*% peak area abundance*
8.	17.8191	Diisobutyl phthalate, 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl)ester	$C_{16}H_{22}O_4$	278.3435	84-69-5	5.1213
9.	18.4191	Methyl palmitate, Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.4507	112-39-0	4.5476
10.	18.7922	Dibutyl phthalate, 1,2-Benzenedicarboxylic acid *dibutyl* ester	$C_{16}H_{22}O_4$	278.3435	84-74-2	0.2135
11.	20.2088	Menthol, Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1α,2β,5α)	$C_{10}H_{20}O$	156.2652	1490-04-6	6.4536
12.	20.2088	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1α,2β,5α)-(.+/-.)	$C_{10}H_{20}O$	156.2652	15356-70-4	1.2316
13.	20.2088	Butanoic acid, 3,7-dimethyl-6-octenyl ester	$C_{14}H_{26}O_2$	226.3550	141-16-2	6.0934
14.	28.7004	Ethanedinitrile	C_2N_2	52.0348	460-19-5	1.3426

4.6.4.2. Chemical structures of identified compounds using GC-MS

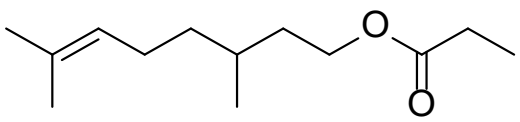
The chemical structures of the fourteen identified compounds were drawn using Chemdraw software.

Table 4.7d: Two-dimensional chemical representations, IUPAC and common names (in brackets) of the identified compounds.

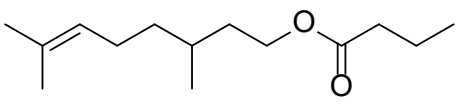
		
(1) CID_1254 5-methyl-2-propan-2-ylcyclohexan-1-ol (Menthol)	(2) CID_3026 dibutyl benzene-1,2-dicarboxylate (Dibutyl phthalate)	(3) CID_6616 2,2-dimethyl-3-methylidenebicyclo [2.2.1] heptane (Camphene)



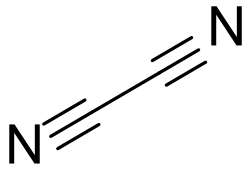
(4)
CID_8181
methyl hexadecanoate
(Methyl palmitate)



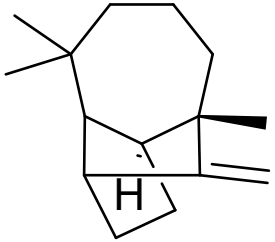
(5)
CID_8834
3,7-dimethyloct-6-enyl
propanoate (Citronellyl propionate)



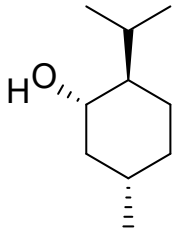
(6)
CID_8835
3,7-dimethyloct-6-enyl butanoate
(Citronellyl butyrate)



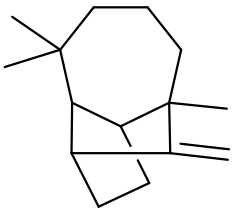
(7)
CID_9999
oxalonitrile
(Ethane dinitrile)



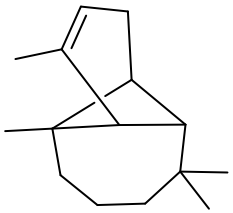
(8)
CID_10137
(1S,7S)-3,3,7-trimethyl-8-methylidenetricyclo
[5.4.0.02,9] undecane
(Longifolene)



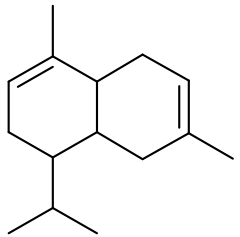
(9)
CID_165675
(1S,2R,5S)-5-methyl-2-propan-2-
ylcyclohexan-1-ol
(d-menthol)



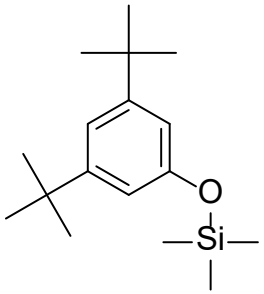
(10)
CID_289151
3,3,7-trimethyl-8-methylidenetricyclo [5.4.0.02,9] undecane
(D-Longifolene)



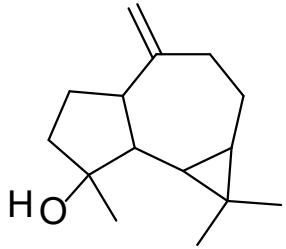
(11)
CID_520957
2,6,6,9-tetramethyltricyclo [5.4.0.02,8] undec-9-ene (Alpha-Longipinene)



(12)
CID_521380
4,7-dimethyl-1-propan-2-yl-1,2,4a,5,8,8a-hexahydronaphthalene
(Naphthalene)



(13)
CID_528937
(3,5-ditert-butylphenoxy)-trimethylsilane
Phenol, 2,4-bis-(1,1-dimethylethyl) TMS



(14)
CID_6432640 (7S)-1,1,7-trimethyl-4-methylidene-1a,2,3,4a,5,6,7a,7b-octahydrocyclopropa[h]azulen-7-ol
(Spathulenol)

4.7. Molecular interactions of phytocompounds from methanol extract of *T. diversifolia* with venom proteins from *D. polylepis* and its mammalian protein targets

4.7.1. Molecular interactions of phytocompounds from methanol extract of *T. diversifolia* with venom proteins from *D. polylepis*

The fourteen compounds identified using GC-MS techniques were docked against venom proteins involved in envenomation. The venom proteins (toxins) used were dendrotoxins (1DTK) and α-neurotoxins (1NTX) which target the nicotinic acetylcholine receptor; fasciculins (1FSC) which target the enzyme acetylcholinesterase (5HQ3) and calciseptine (1TFS) which targets L-Type calcium channels (5GJV).

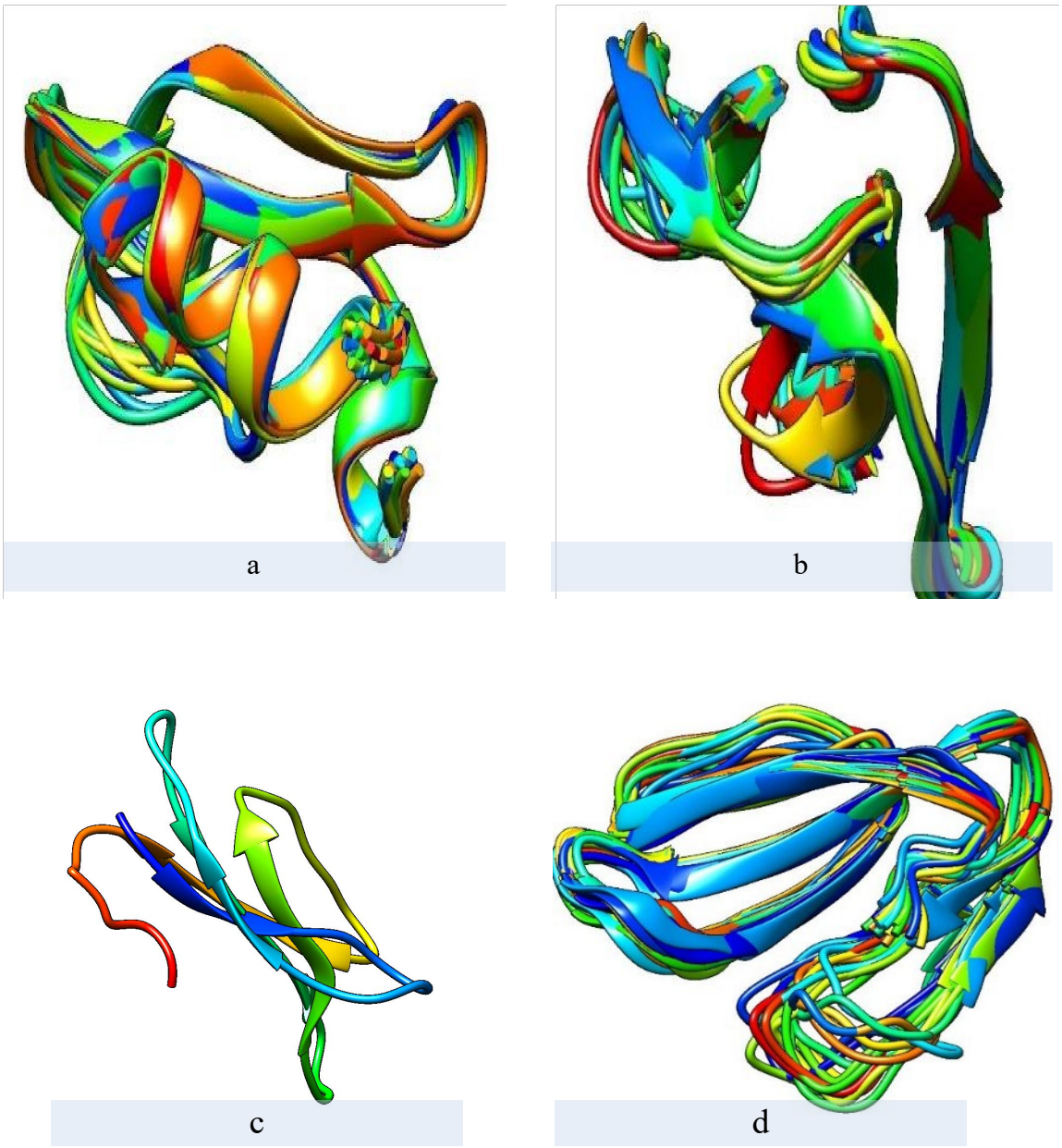


Figure 4.5: Three-dimensional representation of *D. polylepis* venom proteins used for molecular docking study. Generated by Chimera software

[a] Dendrotoxin PDB ID 1DTK; [b] α-Neurotoxin PDB ID 1NTX; [c] Fasciculin PDB ID 1FSC; [d] Calciseptine PDB ID 1TFS.

Molecular docking computer simulation methods using AutoDockVina® resulted in findings of binding activity of some phytocompounds with venom proteins (Figure 4.5). An interaction was categorized as 'Bad' if the energy required for bonding of chemical compound and target protein was below -4.9 kcal/mol, 'Fair' if it was between -5 and -6.9 kcal/mol and 'Good' if it was equal to and above -7 Kcal/mol.

Overall, the results were 'Bad' for each of the fourteen compounds when docked with neurotoxin (1NTX), fasciculins (1TFS) and calcispetine (1TFS). Five compounds namely CID_10137, CID_289151, CID_520957, CID_521380 and CID_6432640 showed 'Fair' results when docked with dendrotoxin (1DTK) as shown in Table 4.8a.

Table 4.8a: Binding affinity energy (kcal/mol) of phytocompounds in *T. diversifolia* methanol leaf extract with *D. polylepis* venom proteins

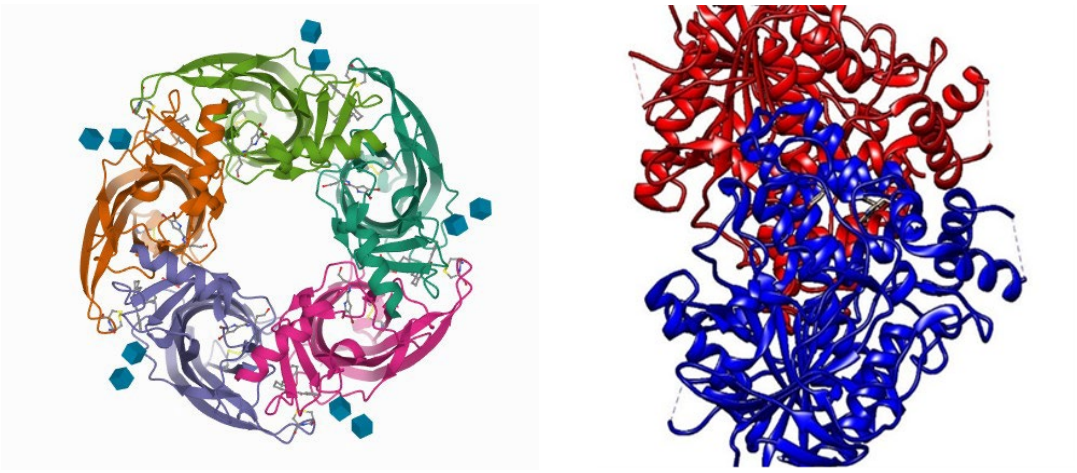
Name of protein (PDB code)	***Chemical name of phytocompound***	***Compound identification code***	***Binding affinity (kcal/mol)***	***Inference***
ẞ-dendrotoxin (1DTK)	Menthol	CID_1254	-4.4	Bad
	Dibutyl phthalate	CID_3026	-4.7	Bad
	Camphene	CID_6616	-4.0	Bad
	Hexadecanoic acid, methyl ester	CID_8181	-3.6	Bad
	6-Octen-1-ol, 3,7-dimethyl-, propanoate (Citronellyl propionate)	CID_8834	-4.0	Bad
	Butanoic acid, 3,7-dimethyl-6-octenyl ester	CID_8835	-4.0	Bad
	Ethanedinitrile	CID_9999	-2.8	Bad
	1,4-Methanol Azulene	CID_10137	-5.2	Fair
	d Menthol	CID_165675	-4.2	Bad
	1,4-Methanol Azulene	CID_289151	-5.1	Fair
	2,6,6,9-tetramethyltricyclo [5.4.0.02,8] undec-9-ene	CID_520957	-5.4	Fair
	Napthalene	CID_521380	-5.4	Fair
	(3,5-ditert-butylphenoxy)-trimethylsilane	CID_528937	-4.9	Bad
	1H-Cycloprop[e]azulene	CID_6432640	-5.7	Fair
α-neurotoxin (1NTX)	Menthol	CID_1254	-4.3	Bad
	Dibutyl phthalate	CID_3026	-4.3	Bad
	Camphene	CID_6616	-3.8	Bad
	Hexadecanoic acid, methyl ester	CID_8181	-3.8	Bad

Name of protein (PDB code)	*Chemical name of phytocompound*	*Compound identification code*	*Binding affinity (kcal/mol)*	*Inference*
	6-Octen-1-ol, 3,7-dimethyl-, propanoate (Citronellyl propionate)	CID_8834	-4.1	Bad
	Butanoic acid, 3,7-dimethyl-6-octenyl ester	CID_8835	-4.0	Bad
	Ethanedinitrile	CID_9999	-2.2	Bad
	1,4-Methanol Azulene	CID_10137	-4.9	Bad
	d Menthol	CID_165675	-4.4	Bad
	1,4-Methanol Azulene	CID_289151	-4.7	Bad
	Tricyclo[5.4.0.9(2.8)]undec-9-ene, 2,6.6,9-tetramethyl-	CID_520957	-4.8	Bad
	Napthalene	CID_521380	-5.0	Bad
	Phenol, 2,4-bis(1.1-demethylethyl)	CID_528937	-4.8	Bad
	1H-Cycloprop[e]azulene	CID_6432640	-5.0	Bad
Fasciculins (1FSC)	Menthol	CID_1254	-3.6	Bad
	Dibutyl phthalate	CID_3026	-4.6	Bad
	Camphene	CID_6616	-3.7	Bad
	Hexadecanoic acid, methyl ester	CID_8181	-2.9	Bad
	6-Octen-1-ol, 3,7-dimethyl-, propanoate (Citronellyl propionate)	CID_8834	-3.8	Bad
	Butanoic acid, 3,7-dimethyl-6-octenyl ester	CID_8835	-3.3	Bad
	Ethanedinitrile	CID_9999	-2.2	Bad
	1,4-Methanol Azulene	CID_10137	-4.3	Bad
	d Menthol	CID_165675	-3.8	Bad

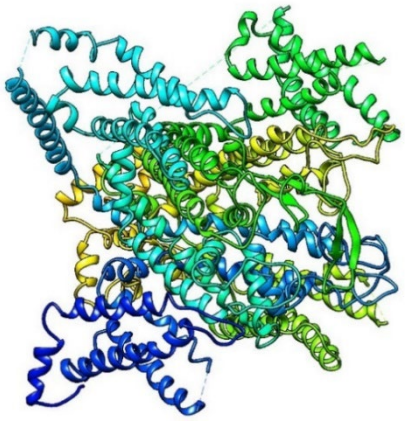
Name of protein (PDB code)	*Chemical name of phytocompound*	*Compound identification code*	*Binding affinity (kcal/mol)*	*Inference*
Calciseptine (1TFS)	1,4-Methanol Azulene	CID_289151	-4.5	Bad
	Tricyclo[5.4.0.9(2.8)]undec-9-ene, 2,6.6,9-tetramethyl-	CID_520957	-4.8	Bad
	Napthalene	CID_521380	-4.7	Bad
	Phenol, 2,4-bis(1.1-demethylethyl)	CID_528937	-4.3	Bad
	1H-Cycloprop[e]azulene	CID_6432640	-4.7	Bad
	Menthol	CID_1254	-4.2	Bad
	Dibutyl phthalate	CID_3026	-4.4	Bad
	Camphene	CID_6616	-3.7	Bad
	Hexadecanoic acid, methyl ester	CID_8181	-3.4	Bad
	6-Octen-1-ol, 3,7-dimethyl-, propanoate (Citronellyl propionate)	CID_8834	-3.7	Bad
	Butanoic acid, 3,7-dimethyl-6-octenyl ester	CID_8835	-3.5	Bad
	Ethanedinitrile	CID_9999	-2.2	Bad
	1,4-Methanol Azulene	CID_10137	-4.7	Bad
	d Menthol	CID_165675	-3.9	Bad
	1,4-Methanol Azulene	CID_289151	-4.5	Bad
	Tricyclo [5.4.0.9(2.8)] undec-9-ene, 2,6.6,9-tetramethyl-	CID_520957	-4.8	Bad
	Napthalene	CID_521380	-4.5	Bad
	Phenol, 2,4-bis (1.1-demethylethyl)	CID_528937	-4.9	Bad
	1H-Cycloprop[e]azulene	CID_6432640	-4.9	Bad

Molecular interactions of phytocompounds from methanol extract of *T. diversifolia* with mammalian targets of venom proteins from *D. polylepis*

Mammalian targets of *D. polylepis* toxins (Figure 4.6), were docked with each of the fourteen compounds. There were five, twelve and six compounds that showed 'Good' results when docked with nicotinic acetylcholine receptor (5AFM), acetylcholinesterase enzyme (1FSC) and L-type calcium channel (5GJV) respectively as shown in Table 4.7b. Notably, compound CID_9999 (Ethanedinitrile) showed 'Bad' results for all three mammalian targets of the venom.



a b



c

Figure 4.6: Three-dimensional representation of mammalian proteins targeted by *D. polylepis* venom proteins; [a] Nicotinic acetylcholine receptor PDB ID 5AFM and [b] Human acetylcholinesterase enzyme PDB ID 5HQ3. [c] L-Type calcium channel PDB ID 5GJV. *Generated by Chimera software*

Table 4.8b: Binding affinity energy (kcal/mol) of phytocompounds in *T. diversifolia* methanol leaf extract with mammalian targets of *D. polylepis* venom proteins

Name of Protein (PDB Code)	***Chemical Name of phytocompound***	***Compound identification Code***	***Binding affinity (Kcal/Mol)***	***Inference***
Nicotinic acetylcholine receptor (5AFM)	Menthol	CID_1254	-5.4	Fair
	Dibutyl phthalate	CID_3026	-5.7	Fair
	Camphene	CID_6616	-5.7	Fair
	Hexadecanoic acid, methyl ester	CID_8181	-4.3	Bad
	6-Octen-1-ol, 3,7-dimethyl-, propanoate (Citronellyl propionate)	CID_8834	-5.2	Fair
	Butanoic acid, 3,7-dimethyl-6-octenyl ester	CID_8835	-5.6	Fair
	Ethanedinitrile	CID_9999	-2.5	Bad
	1,4-Methanol Azulene	CID_10137	-6.9	Good
	dL Menthol	CID_165675	-5.9	Fair
	1,4-Methanol Azulene	CID_289151	-6.3	Good
	Tricyclo [5.4.0.9(2.8)] undec-9-ene, 2,6.6,9-tetramethyl-	CID_520957	-6.8	Good
	Napthalene	CID_521380	-6.8	Good
	Phenol, 2,4-bis (1.1-demethylethyl)	CID_528937	-5.9	Fair
	1H-Cycloprop[e]azulene	CID_6432640	-6.5	Good
	Menthol	CID_1254	-7.0	Good
	Dibutyl phthalate	CID_3026	-7.0	Good

Name of Protein (PDB Code)	*Chemical Name of phytocompound*	*Compound identification Code*	*Binding affinity (Kcal/Mol)*	*Inference*
Acetylcholinesterase enzyme (5HQ3)	Camphene	CID_6616	-5.9	Fair
	Hexadecanoic acid, methyl ester	CID_8181	-6.6	Good
	6-Octen-1-ol, 3,7-dimethyl-, propanoate (Citronellyl propionate)	CID_8834	-6.0	Good
	Butanoic acid, 3,7-dimethyl-6-octenyl ester	CID_8835	-6.4	Good
	Ethanedinitrile	CID_9999	-2.9	Bad
	1,4-Methanol Azulene	CID_10137	-6.4	Good
	d Menthol	CID_165675	-6.8	Good
	1,4-Methanol Azulene	CID_289151	-6.2	Good
	Tricyclo[5.4.0.9(2.8)]undec-9-ene, 2,6.6,9-tetramethyl-	CID_520957	-6.6	Good
	Napthalene	CID_521380	-7.5	Good
	Phenol, 2,4-bis(1.1-demethylethyl)	CID_528937	-7.4	Good
	1H-Cycloprop[e]azulene	CID_6432640	-7.3	Good
L-Type calcium channel (5GJV)	Menthol	CID_1254	-5.9	Fair
	Dibutyl phthalate	CID_3026	-6.5	Fair
	Camphene	CID_6616	-5.7	Fair
	Hexadecanoic acid, methyl ester	CID_8181	-5.4	Fair
	6-Octen-1-ol, 3,7-dimethyl-, propanoate (Citronellyl propionate)	CID_8834	-5.8	Fair

Name of Protein (PDB Code)	*Chemical Name of phytocompound*	*Compound identification Code*	*Binding affinity (Kcal/Mol)*	*Inference*
	Butanoic acid, 3,7-dimethyl-6-octenyl ester	CID_8835	-5.3	Fair
	Ethanedinitrile	CID_9999	-2.9	Bad
	1,4-Methanol Azulene	CID_10137	-7.5	Good
	d Menthol	CID_165675	-5.9	Fair
	1,4-Methanol Azulene	CID_289151	-7.5	Good
	Tricyclo [5.4.0.9(2.8)] undec-9-ene, 2,6.6,9-tetramethyl-	CID_520957	-7.4	Good
	Napthalene	CID_521380	-7.6	Good
	Phenol, 2,4-bis (1.1-demethylethyl)	CID_528937	-7.1	Good
	1H-Cycloprop[e]azulene	CID_6432640	-7.5	Good

4.7.2. Phytocompounds with reliable binding affinity energy (kcal/mol) with target molecules involved in snake bite envenomation.

Further analysis of hydrophobic and hydrophilic bonds involved in 'Good' molecular interactions resulted in eleven best docking results. Three compounds, CID_1254 (menthol), CID_165675 (d-menthol) and CID_6432640 (spathulenol) presented the best interactions at the active binding site of 5AFM target protein. Six compounds; CID_1254 (menthol), CID_8181 (methyl palmitate), CID_8834 (citronellyl propionate), CID_8835 (citronellyl butyrate), CID_165675 (d-menthol) and CID_6432640 (spathulenol) presented best docking results at the active binding site of 5HQ3 target protein and two compounds; CID_1254 (menthol) and CID_8181 (methyl palmitate) had the best affinity bonds with 5GJV (L-type calcium channel) binding site. Table 4.8c captures residues involved in the hydrophobic interactions and hydrogen bonding interactions.

Table 4.8c: Summary docking results of molecular interactions between *T. diversifolia* phytocompounds and target proteins in *D. polylepis* envenomation

Name of complex	***Functional residues in active site of protein involved in hydrophobic binding interactions***	***Functional residues involved in hydrogen bonding and bond distance within 4Å***
5AFM_CID_1254	Trp145, Tyr184, Cys186, Cys187, Tyr191	[1] O -- OH-Tyr91 Bond distance = 3.01 Å
5AFM_CID_165675	Tyr91, Tyr184, Cys186, Tyr191	[1] O -- O-Ser144 Bond distance = 3.08 Å [2] O -- O-Trp145 Bond distance =2.97 Å
5AFM_CID_6432640	Tyr91, Tyr184, Cys186, Cys187,Tyr191	[1] O -- O-Trp145 Bond distance = 2.69Å

Name of complex	*Functional residues in active site of protein involved in hydrophobic binding interactions*	*Functional residues involved in hydrogen bonding and bond distance within 4Å*
5HQ3_CID_1254	Tyr124, Phe338, Trp286, Ser293, Val294, Phe295, Phe297, Tyr341	[1] O -- O- Arg296 Bond distance = 2.94 Å [2] O -- N-Arg296 Bond distance = 3.16 Å
5HQ3_CID_8181	Trp86, Gly121, Gly122, Ser203, Trp286, Phe297, Phe338, Tyr341, His447	[1] O2 -- OH-Tyr124 Bond distance = 2.91 Å [2] O2 -- O-Ser125 Bond distnace = 3.19 Å [3] O1-- OH-Tyr337 Bond distance 2.85 Å
5HQ3_CID_8834	Tyr124, Trp286, Ser293, Val294, Phe295, Phe297,Phe338, Tyr341	[1] O1 -- OH-Tyr72 Bond distance = 3.19 Å
5HQ3_CID_8835	Asp74, Leu76, Tyr124, Tyr337, Phe338, Tyr341, Trp286	[1] O1-- OH-Tyr72 Bond distance =2.82 Å [2] O2 -- OG1-Thr75 Bond distance = 2.92 Å
5HQ3_CID_165675	Tyr124, Trp286, Ser293, Val294, Phe297, Tyr337, Phe338, Tyr341	[1] O -- N-Phe295 Bond distance = 3.14 Å
5HQ3_CID_6432640	Tyr72, Tyr124, Trp286, Ser293, Val294, Phe295, Arg296	[1] O -- O-Tyr341 Bond distance =3.01 Å
5GJV_CID_1254	Leu202, Phe205, Met206, Ile209, Ile1244, Leu1247, Leu1257	O -- O-Leu202 Bond distance =3.04 Å
5GJV_CID_8181	Tyr210, Ile213, Leu217, Phe218, Trp309, Phe310, Ile312, Tyr313, Thr316, Phe1234, Ala1233, Ile305, Leu1237	O -- O-Thr316 Bond distance = 2.89 Å

Illustrations of the eleven interactions in Figure 4.7a-i indicate where the carbon residues are represented as red spikes and the green colored residues are involved in hydrogen bonding representing the hydrogen molecule interacting with the next higher affinity of electrons or highly electronegative atoms found in the vicinity of 4Å.

5AFM_CID_1254

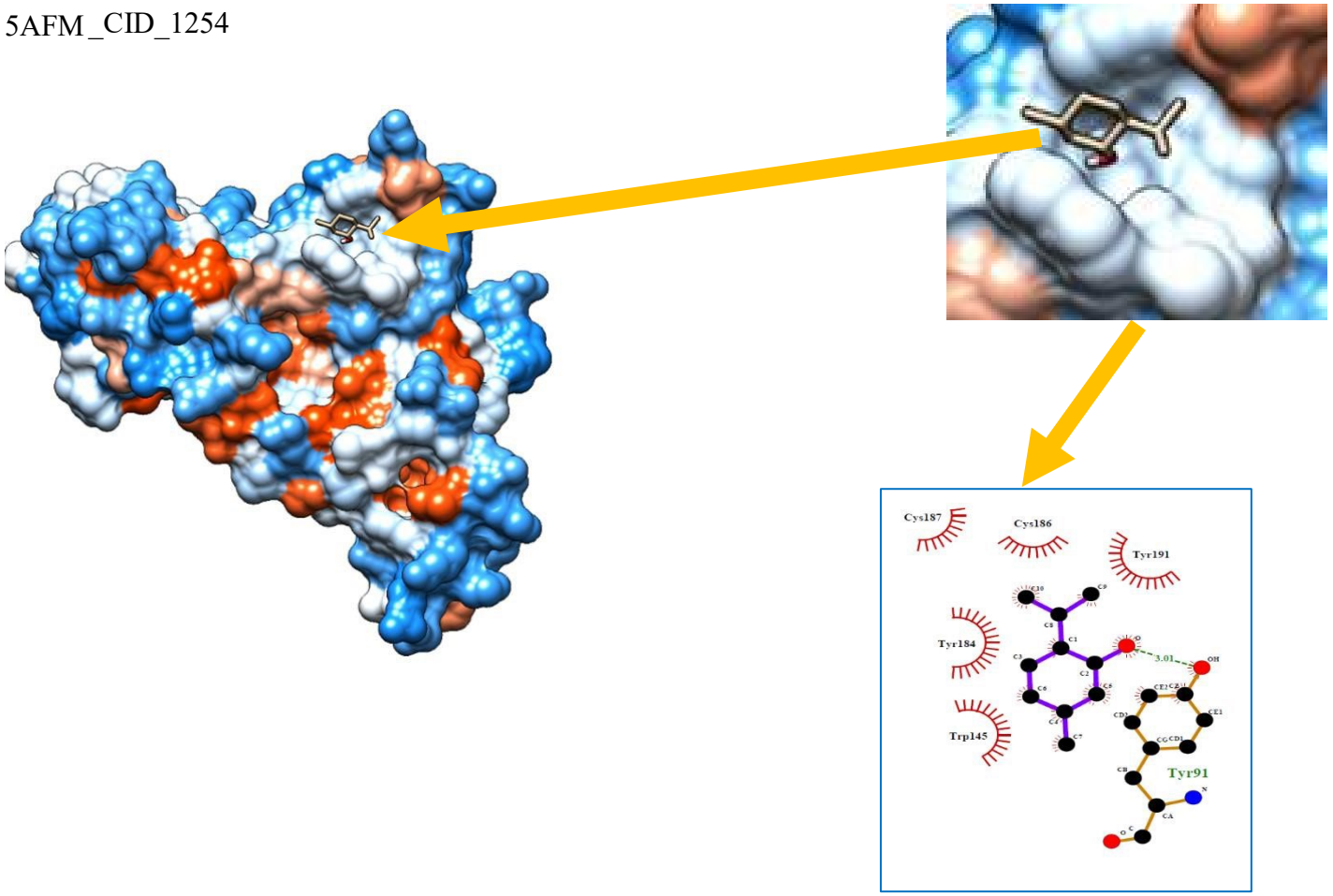


Figure 4.7a: Molecular interaction between 5AFM and CID_1254 (nicotinic acetylcholine receptor and menthol) showing hydrophobic binding with Trp145, Tyr184, Cys186, Cys187 and Tyr191. One oxygen-hydroxyl bond is seen on Tyr91 with a bond distance of 3.01 Å. © *Gladys Mwangi*

5AFM_CID_165675

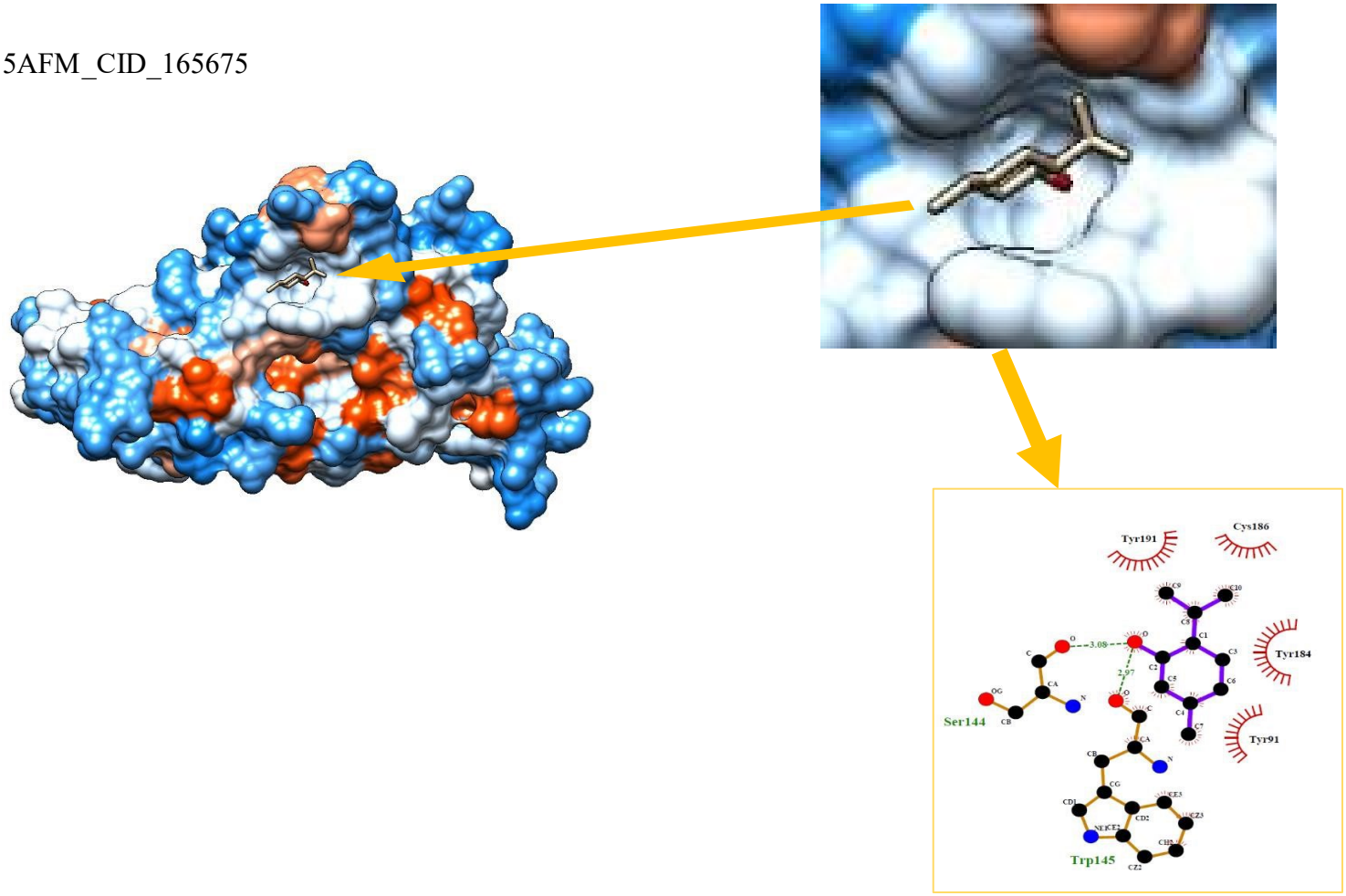


Figure 4.7b: Molecular interaction between 5AFM and CID_165675 (nicotinic acetylcholine receptor and d-menthol) showing hydrophobic binding with Tyr91, Tyr184, Cys186, Tyr191. Two oxygen bonds are seen on Ser144 with a bond distance of 3.08 Å and on Trp145 with a bond distance of 2.97 Å. © *Gladys Mwangi*

5AFM_CID_6432640

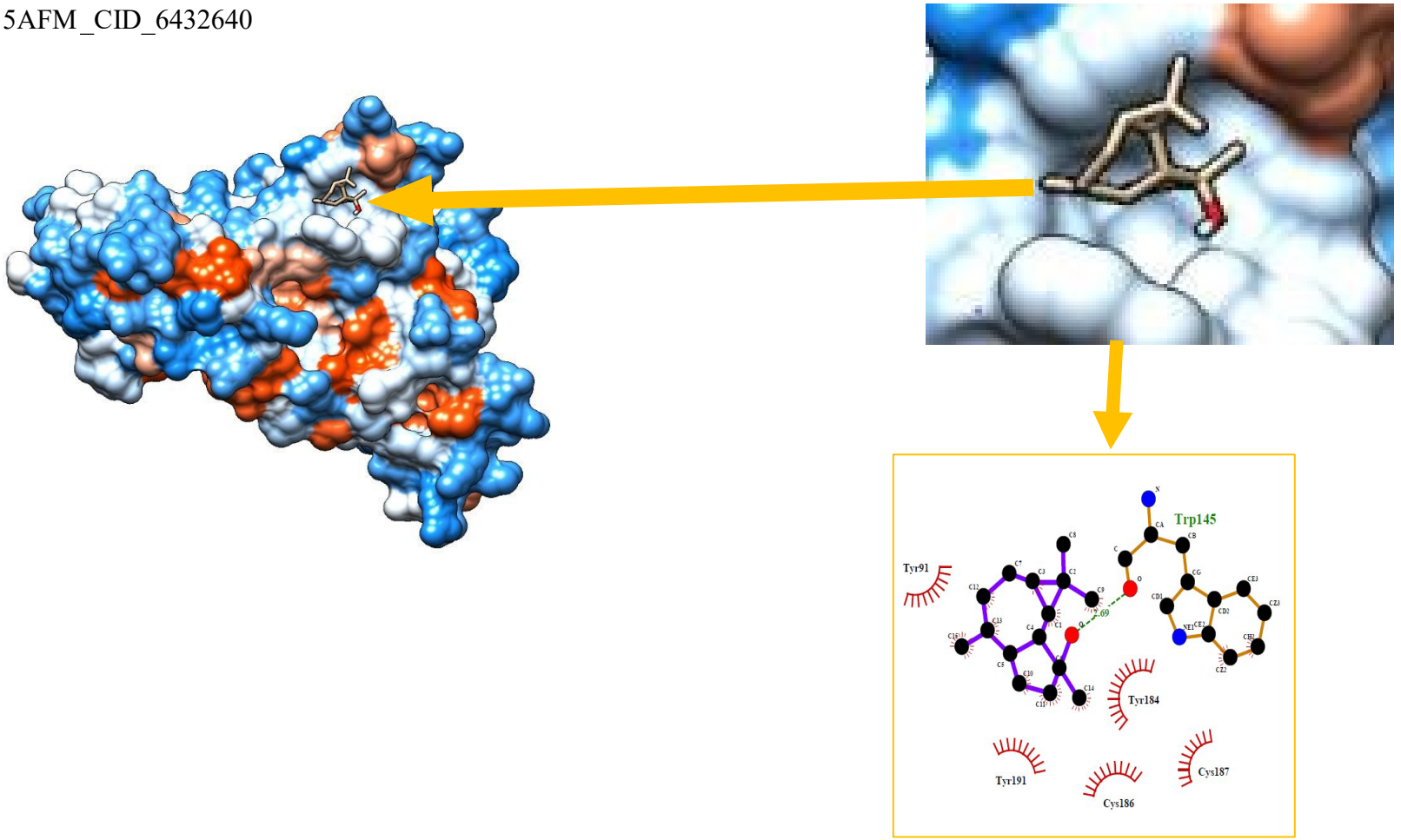


Figure 4.7c: Molecular interaction between 5AFM and CID_6432640 (nicotinic acetylcholine receptor and spathulenol) showing hydrophobic binding with Tyr91, Tyr184, Cys186, Cys187, Tyr191. One oxygen-oxygen bond is seen on Trp145 with a bond distance of 2.69Å. © *Gladys Mwangi*

5HQ3_CID_1254

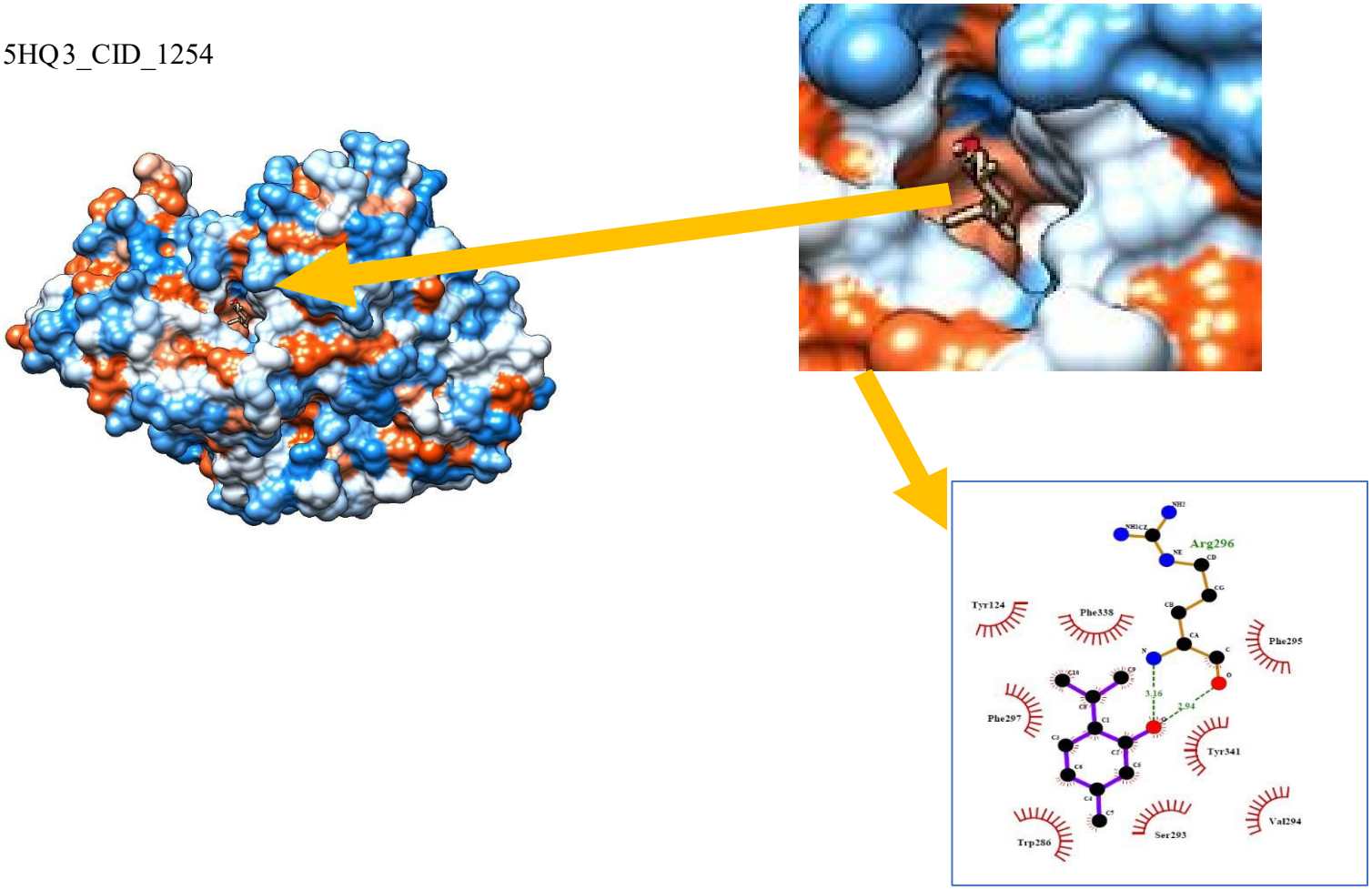


Figure 4.7d: Molecular interaction between 5HQ3 and CID_1254 (acetylcholinesterase enzyme and menthol) showing hydrophobic binding with Tyr124, Phe338, Trp286, Ser293, Val294, Phe295, Phe297, Tyr34. One oxygen-oxygen bond and an oxygen-nitrogen bond are seen on Arg296 with a bond distance of 2.94 Å and 3.16 Å respectively. © *Gladys Mwangi*

5HQ3_CID_8181

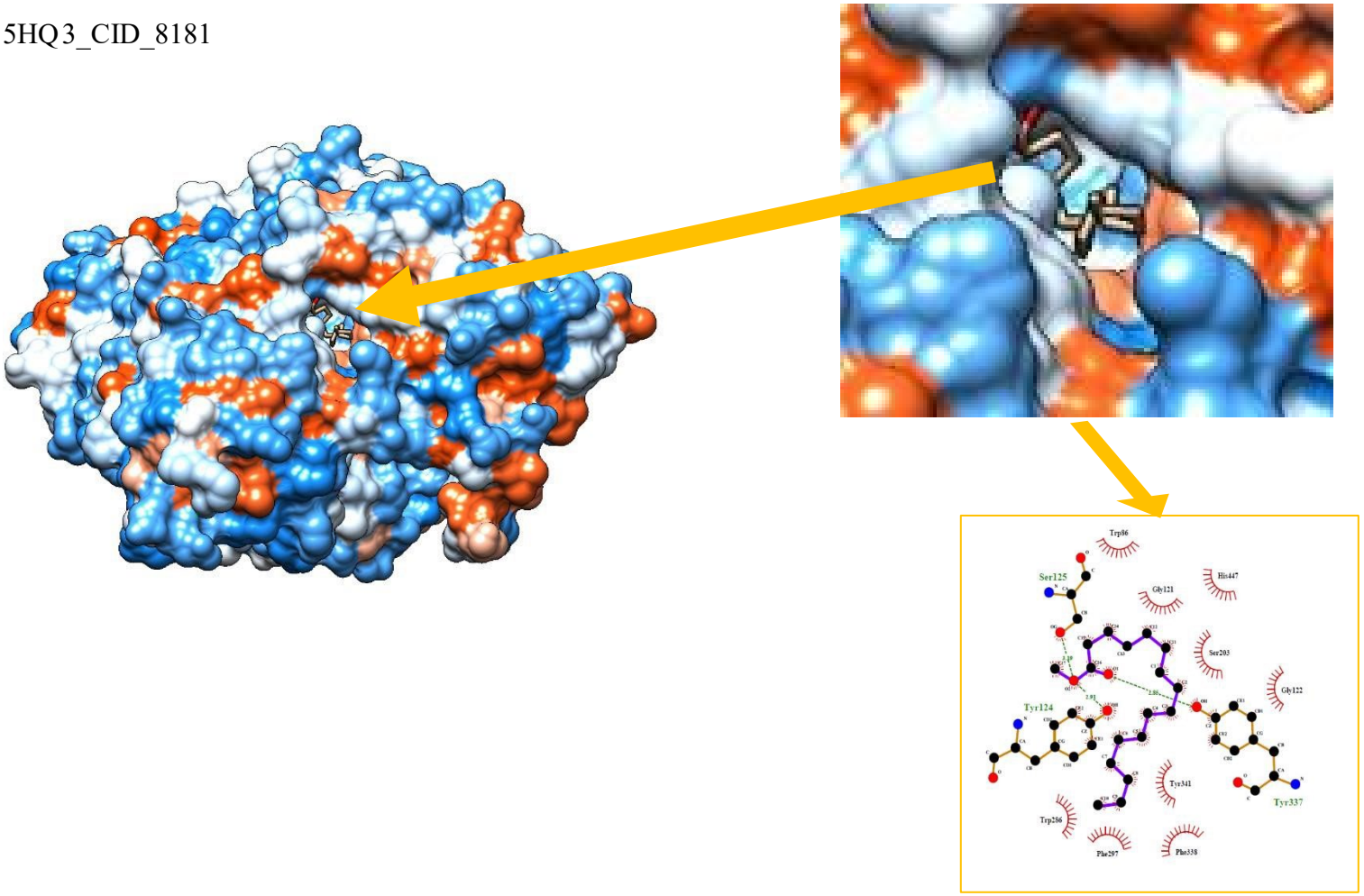


Figure 4.7e: Molecular interaction between 5HQ3 and CID_8181 (acetylcholinesterase enzyme and methyl palmitate) showing hydrophobic binding with Trp86, Gly121, Gly122, Ser203, Trp286, Phe297, Phe338, Tyr341, His447. One oxygen-hydroxyl bond with Tyr 124, an oxygen-hydroxyl bond with Ser 125 and an oxygen -hydroxyl bond at Tyr 337 are seen with 2.91 Å, 3.19 Å and 2.85 Å bond distance respectively. © *Gladys Mwangi*

5HQ3_CID_8834

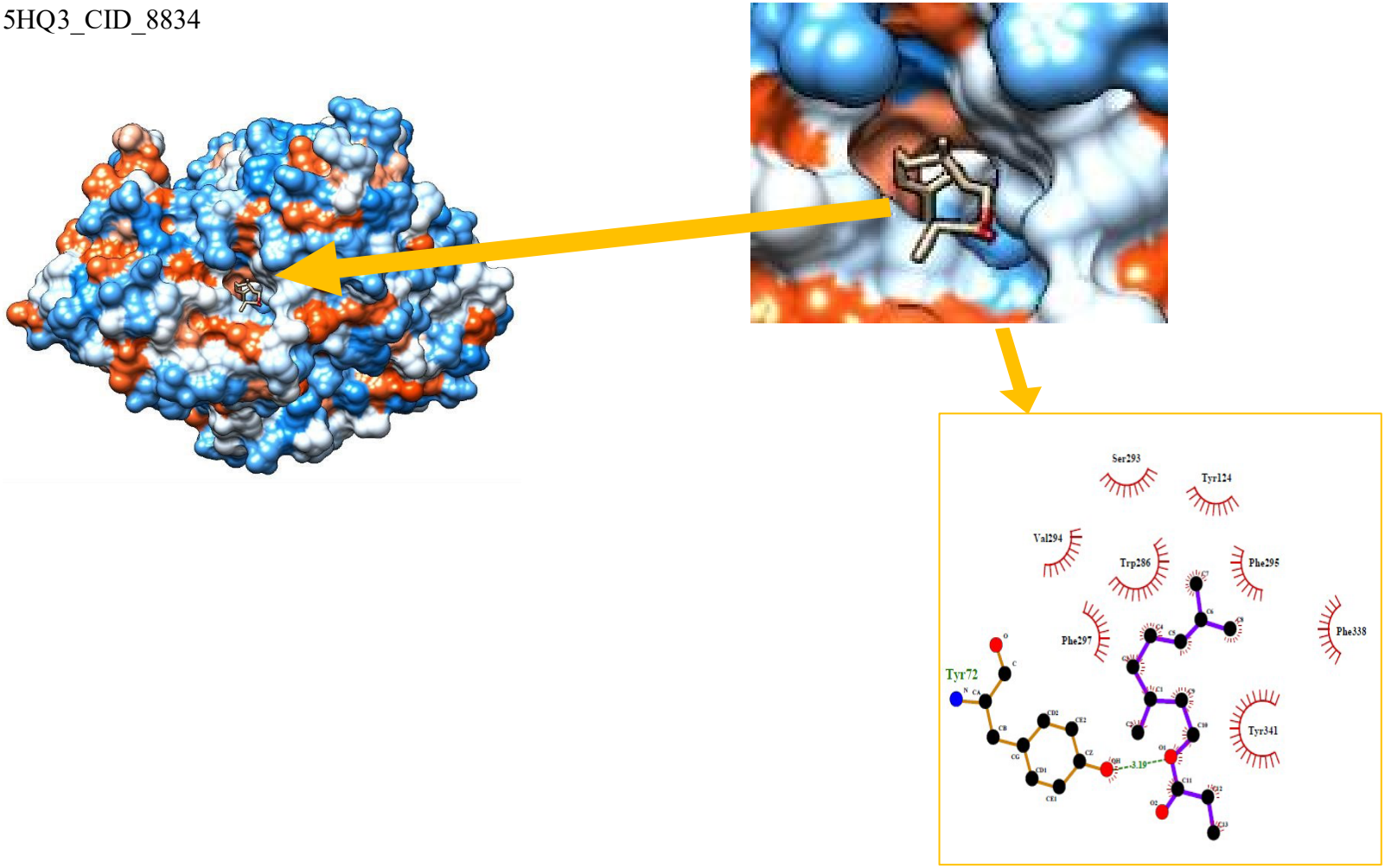


Figure 4.7f: Molecular interaction between 5HQ3 and CID_8834 (acetylcholinesterase enzyme and citronellyl propionate) showing hydrophobic binding with Tyr124, Trp286, Ser293, Val294, Phe295, Phe297, Phe338, Tyr341. One oxygen-hydroxyl bond with Tyr723 with a bond distance of 3.19 Å is seen. © *Gladys Mwangi*

5HQ3_CID_8835

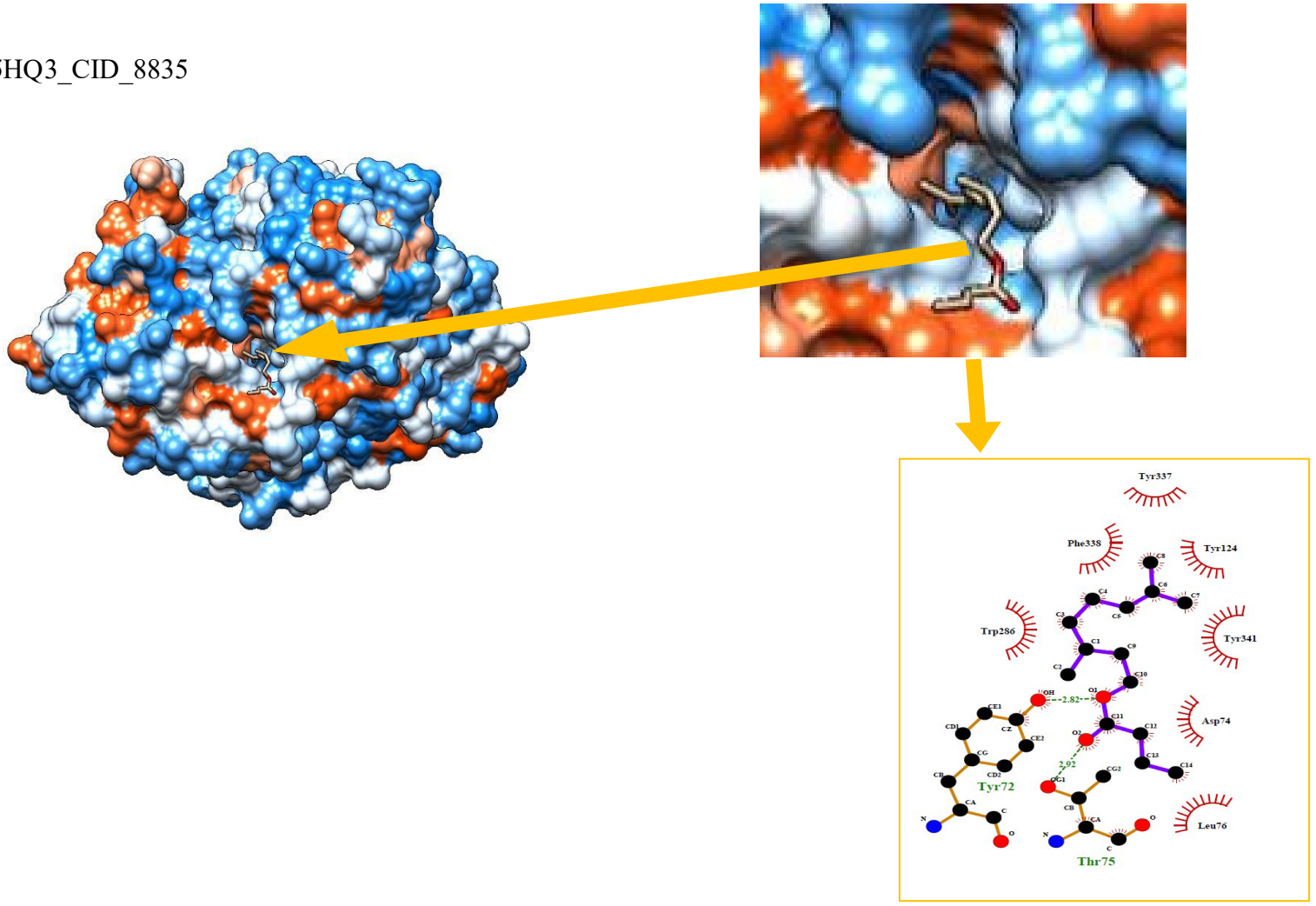


Figure 4.7g: Molecular interaction between 5HQ3 and CID_8835 (acetylcholinesterase enzyme and citronellyl butyrate) showing hydrophobic binding with Asp74, Leu76, Tyr124, Tyr337, Phe338, Tyr341, Trp286. One oxygen-hydroxyl bond at Tyr72 with a bond distance of 2.82 Å and an oxygen-oxygen bond with Thr75 with bond distance of 2.92 Å are seen. © *Gladys Mwangi*

5HQ3_CID_165675

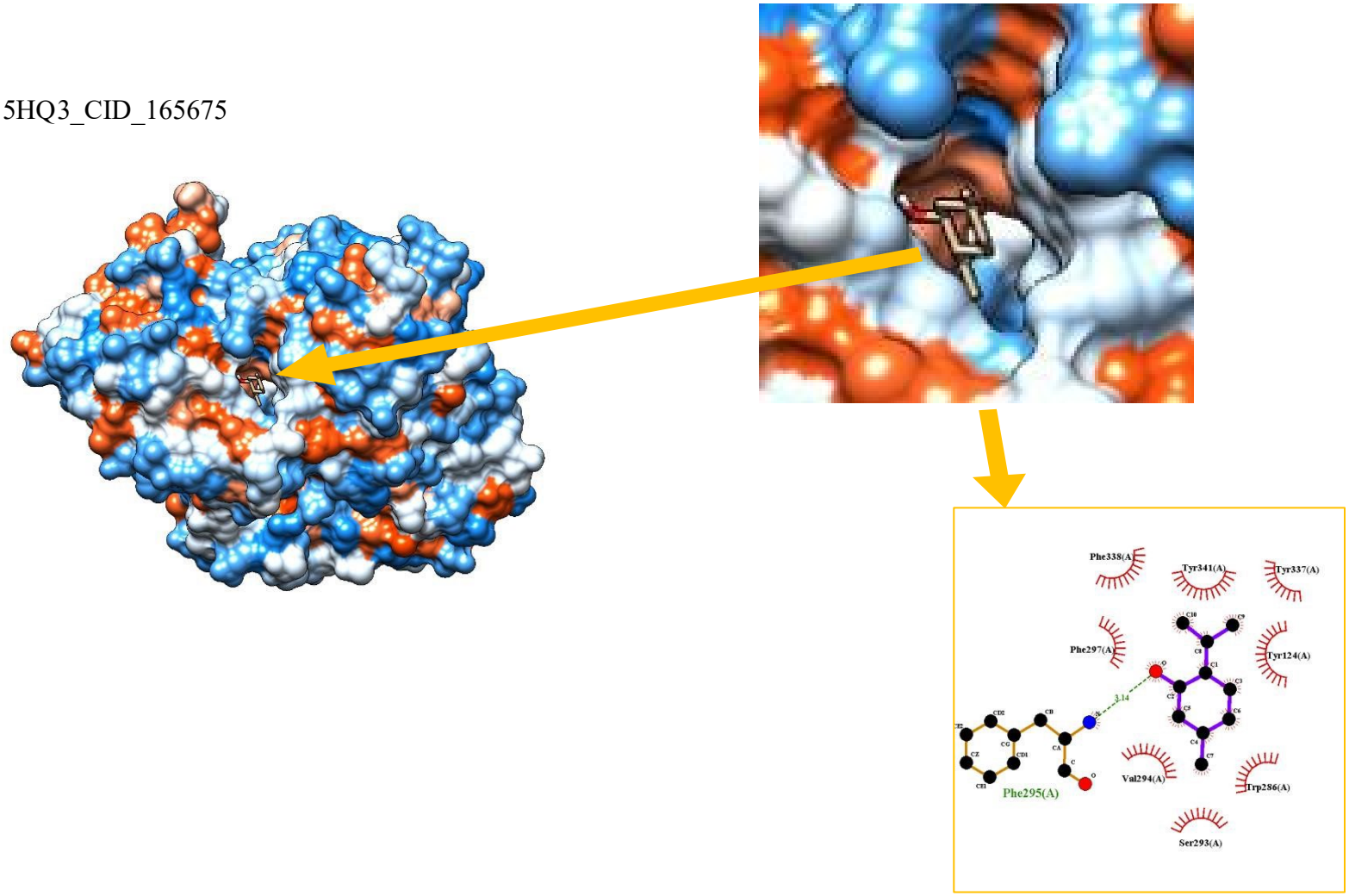


Figure 4.7h: Molecular interaction between 5HQ3 and CID_165675 showing hydrophobic binding with Tyr124, Trp286, Ser293, Val294, Phe297, Tyr337, Phe338 and Tyr341. One oxygen-nitrogen bond with Phe295 is seen with a bond distance of 3.14 Å.

5HQ3_CID_6432640

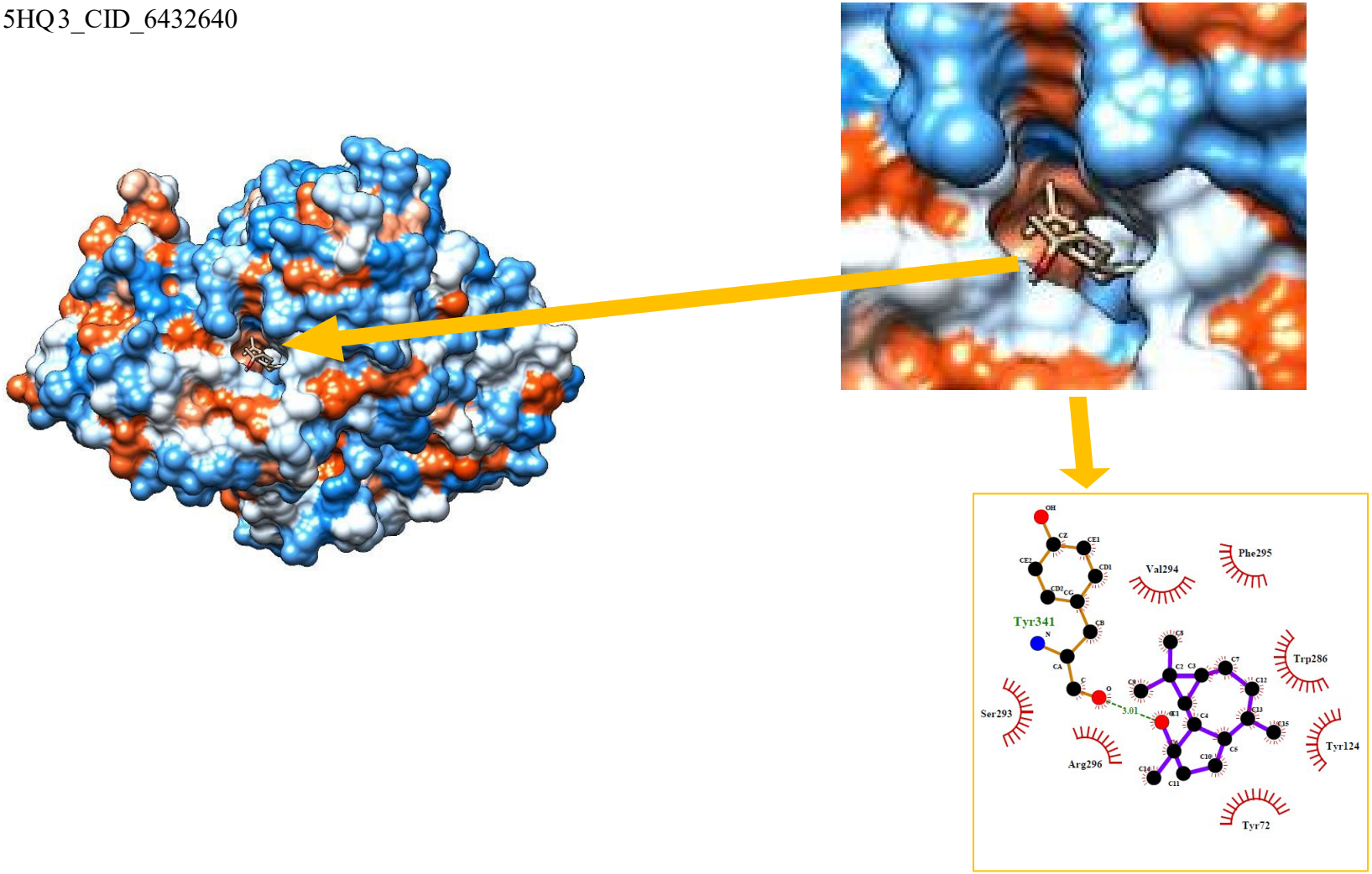


Figure 4.7i: Molecular interaction between 5HQ3 and CID_6432640 (acetylcholinesterase enzyme and spathulenol) showing hydrophobic binding with Tyr72, Tyr124, Trp286, Ser293, Val294, Phe295, Arg296. One oxygen-oxygen bond with Tyr341 is seen with a bond distance of 3.01Å. © *Gladys Mwangi*

5GJV _CID_1254

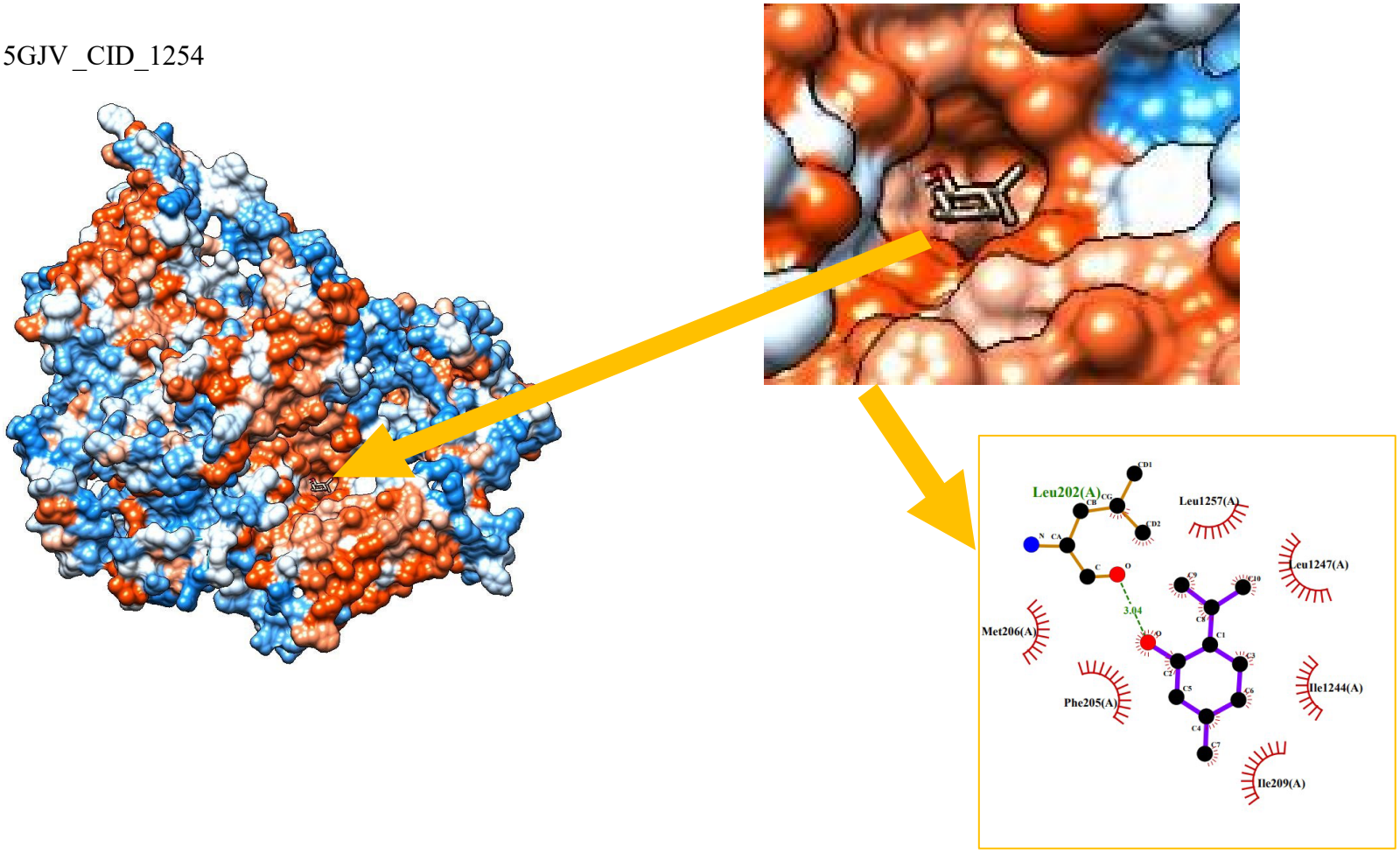


Figure 4.7j: Molecular interaction between 5GJV and CID_1254 (L-type calcium channel and menthol) showing hydrophobic binding with Leu202, Phe205, Met206, Ile209, Ile1244, Leu1247, Leu1257. One oxygen-oxygen bond with Leu202 with a bond distance of 3.04 Å is seen. © *Gladys Mwangi*

5GJV _CID_8181

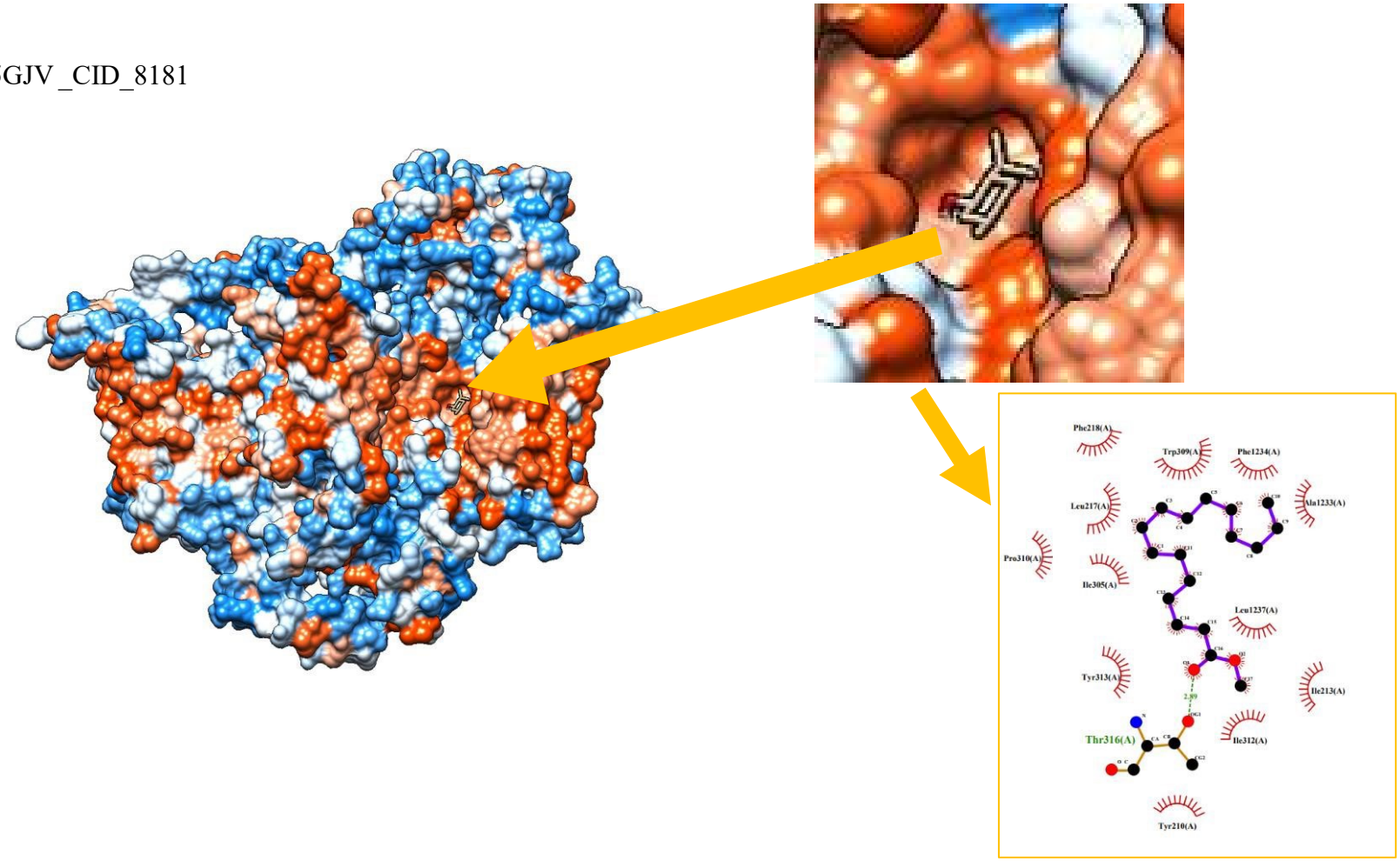


Figure 4.7k: Molecular interaction between 5GJV and CID_8181 (L-type calcium channel and methyl palmitate) showing hydrophobic binding with Tyr210, Ile213, Leu217, Phe218, Trp309, Phe310, Ile312, Tyr313, Thr316, Phe1234, Ala1233, Ile305, Leu1237.One oxygen-oxygen bond with Thr316 with a bond distance of 2.89Å is seen. © *Gladys Mwangi*

The eleven interactions were with six phytocompounds namely; CID_1254 (menthol), CID_165675 (d-menthol), CID_6432640 (spathulenol), CID_8181 (methyl palmitate), CID_8834 (Citronellyl propionate) and CID_8835 (Citronellyl butyrate). The three dimensional chemical structures of these compounds are as shown in Figure 4.7l.

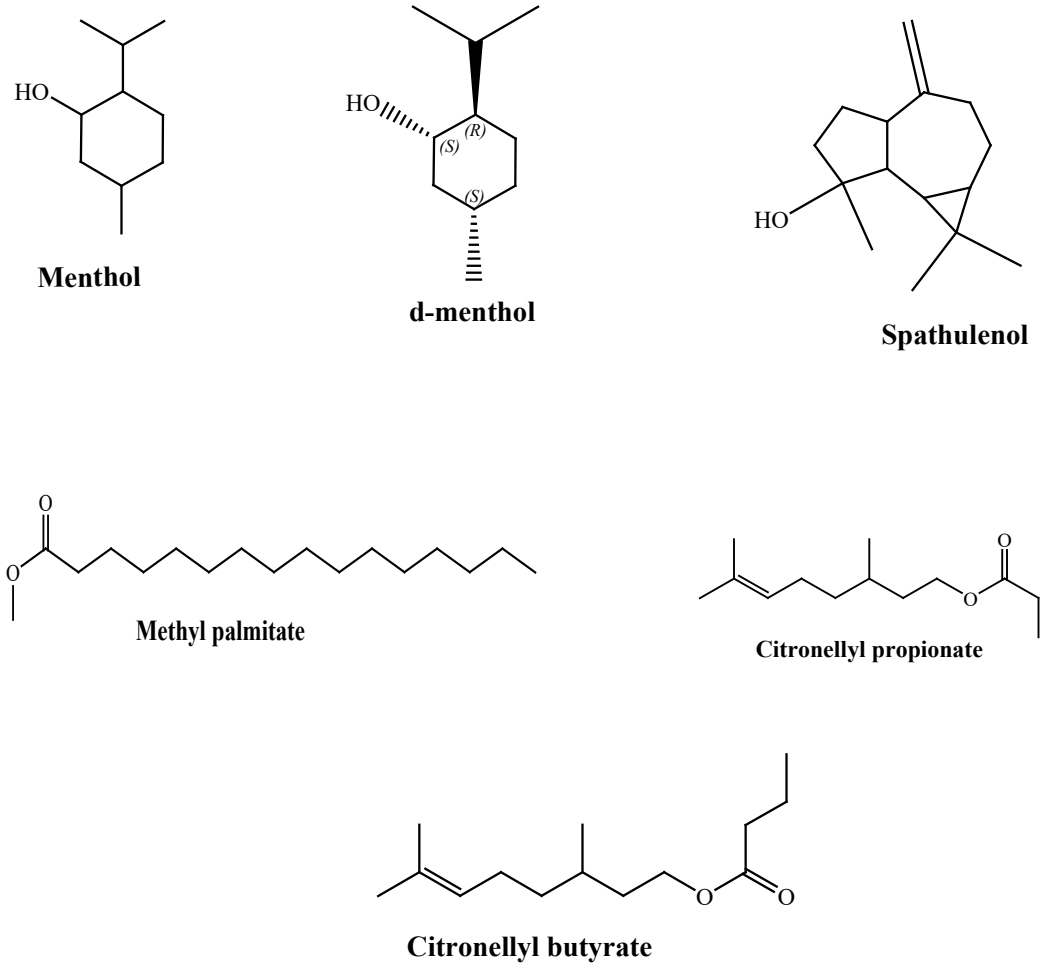


Figure 4.7l: Three-dimensional chemical structures of seven phytocompounds from aqueous extract of *T. diversifolia* with best docking results in *D. polylepis* envenomation.

CHAPTER FIVE
DISCUSSION

5.1. Median lethal doses of herbs used for medicinal purposes

Medicinal plants have for years been known to have activity against venom from animal bites, hence the interest in them as alternative therapies which is also supported by their availability, affordability and perceived safety profile. Appropriate doses for use are recommended by herbalists and there is little evidence of objectively determined doses using well designed scientific studies. The concern would be on negative effects of administering excessive amounts of herbal products and sub optimal quantities which may fail to achieve desired results. Thus, the highest doses that do not cause harm to the patient would be desirable.

The determination of median lethal dose levels of selected plants for this study was therefore critical in establishing the appropriate doses of the extracts to be used for the antivenin and selected pharmacological studies. This was done by first determining the median lethal dose (LD_{50}) of the aqueous extracts which for four out of the five plants studied (*S. suffruticosa*, *T. minuta*, *T. diversifolia* and *Z. chalybeum)* was 2085mg/Kg body weight in mice using the intra-peritoneal route. *S. incanum* extract had a much lower median lethal dose of 316mg/Kg. Mortality was used as the end point for this study which sought to establish survival rate of mice after intraperitoneal injection with *D. polylepis* venom, which is known to kill within minutes. The highest dose with a hundred percent survival for extracts of *S. suffruticosa*, *T. minuta*, *T. diversifolia* and *Z. chalybeum* was 1500mg/Kg and 100 mg/Kg for *S. incanum,* were used for subsequent studies. This large

difference in median lethal dose levels observed indicates that plant extracts should not be assumed to be similar in quantites recommended for use, emphasizing the need to control doses of herbal products. The results are similar to those of Mirajkar (2006) who reported that the use of 1 to 2 g/Kg of plant extracts is considered safe for most plants, noting that this range is not applicable to all plants and while adverse effects occurs with increasing doses of extracts, they can occur in relatively lower doses for some plants, hence the need to determine median lethal dose levels.

Aydin *et al.,* (2016) in a guidance manual for the toxicity assessment of traditional herbal medicines submit that *in vivo* experiments should be designed to determine dose-response relationships and that these should be compared to concentration-toxicity relationships. Additionally, long term use of herbal medicines warrants toxicity studies focused on carcinogenic, hepatotoxic, teratogenic and genotoxicity. Other concerns of safety of herbal medicines include potential presence of heavy metals as reported by (Deswal *et al.,* 2019) who assessed ten herbal medicines for presence of lead, copper, cadmium, iron, chromium, manganese, nickel and zinc. The results showed presence of heavy metals in all ten herbal medicines.

These scenarios reveal the need to conduct safety and toxicity studies on herbal medicines as exemplified in data collected from randomized control trials on herbal medicine-drug interactions which identified 23% incidences of pharmacodynamic interactions and 77% pharmacokinetic interactions all associated with modification of herbal prescriptions, coexistence of inducers and inhibitors, pharmacogenomics, dose of active ingredients in

herbs, time course of interaction, routes of administration and pharmacokinetic profiles (Zhang *et al.,* 2017).

5.2. *In vitro* and *in vivo* activity of herbal extracts against *D. polylepis* snake venom

5.2.1. LD_{50} and venom challenge dose of *D. polylepis* venom

The establishment of LD_{50} for the venom in use was important since venom from snakes even from the same species differ. This is exhibited in various LD_{50} of *D. polylepis* crude venom in literature compared to 0.024/Kg determined in this study (Table 4.4a). This LD_{50} differs from 0.341mg/Kg (Ochola *et al.,* 2019) but is close to the one listed in the database, 'most venomous snakes in the world' which indicates an LD_{50} of 0.05mgKg (Sharma *et al*., 2019). The value established in this study suggests a more potent venom from the one in literature, however this can be attributed to certain factors such as differences between crude and aqueous venoms and storage conditions of the venom prior to use. Studies show that whereas there is little effect on quantity of venom proteins due to storage conditions, their potency and synergistic and/or additive capability may be affected. LD_{50} differences as much as four factors have been observed particularly in mice models. It is also known that snake venom differs from snake to snake depending on age, diet and region, hence the need to determine these parameters for a given venom sample (Cantú *et al.,* 2017; Salazar *et al.,* 2007; Tan, 2016).

The venom challenge dose established in this study, ($4xLD_{50}$), however was consistent with the WHO guidelines (WHO, 2010c) on the determination of venom challenge dose which guide that the venom challenge dose in most studies is 3-6 times the established LD_{50} of venom.

5.2.2. *In vitro* activity of herbal extracts against *D. polylepis* snake venom

Studies done on many plants to establish various activities in biological models have revealed that crude and fractions of some herbal extracts can antagonize the activity of various snake venoms and purified toxins (Amui *et al.,* 2011). This evidence is critical in the pursuit of alternative or complementary management approaches to snake bite. Notably, nearly all of these studies have been done in South East Asia and South America, with few if any originating from the Sub-Saharan Africa region, where Kenya lies. Due to the specificity of snake venom from different regions, evidence of efficacy of medicinal plants used in Kenya would be a good beginning to the establishment of local solutions for effective management of envenomation following snake bite and the basis for this study which sought to investigate the activity of herbal extracts from selected Kenyan plants (*S. incanum*, *S. suffruticosa*, *T. minuta*, *T. diversifolia* and *Z. chalybeum*) in management of snake bite.

During the *in vitro* study, comparison of the percentage survival after peritoneal injection of mixture of venom and extracts after incubation at 37^0C for 1 hour showed that there was no statistical difference in survival rates between mice in the negative control group and those subjected to aqueous extracts of all five plants (Table 4.5d). *Z. chalybeum* root bark in particular showed no protective effect from the venom, leading to 100% mortality. The other four plants showed varying survival rates; *T. minuta* leaves (25%) and *T. diversifolia* leaves (25%), *S. incanum* fruit (33.3%) and *S. suffruticosa* leaves (50%). There is a possibility that at higher doses, both *S. incanum* fruit (33.3%) and *S. suffruticosa* leaves (50%) may achieve better results. However, higher doses were found to be toxic to the mice and thus are not recommended. These results reveal that there is neutralization

activity of four out of the five selected plant extracts and although not statistically significant, can be considered as one of the mechanisms of action of the extracts on *D. polylepis* venom components, particularly dendrotoxins, as was later established using molecular docking techniques (Table 4.5b and c).

5.2.3. *In vivo* activity of herbal extracts against *D. polylepis* snake venom

T. diversifolia extract was the only extract that had significant *in vivo* effect against the venom with a higher calculated χ^2 value of 4.286 against the tabulated at P<0.005 (3.814) at 95% confidence interval but lower than the critical value of 6.635 at P<0.01 at 99% confidence interval (Table 4.5e).

A comparison of the *in vitro* and *in vivo* results revealed that the survival rate of S. *suffruticosa* leaves (50%) and *S. incanum* fruit (33.3%) was higher in the *in vitro* study than in the *in vivo* study in which 20% of mice survived for each of the two extracts. This implies that the two plant extracts are more effective in neutralizing the venom than in their interactions with venom mammalian targets. *T. minuta* leaves (25%) and *Z. chalybeum* root bark (0%) had the same survival rates in both the *in vivo* and *in vitro* study.

These results may be explained by the theory that plant remedies work in a multiplicity of ways in a biological system with actions such as improvement of the victim's immune system, competing for venom targets in the body, neutralization of venom components and digestion of venom protein (Gomes *et al*., 2010). The *in vitro* results simulate the neutralization activity of the extracts on venom constituents while the *in vivo* results indicate lower neutralization capacity of the venom within a biological system, but also

suggests that there are additional mechanisms of action within a biological system, that may have led to a higher survival rate for *T. diversifolia* herbal extract.

Overall, the leaf extract of *T. diversifolia* was the second most active in the *in vitro* and the most active in the *in vivo* study. Aside from the antivenin activity, *T. diversifolia* is known for other medical uses notably in wound management which would be a desirable benefit of the extract in management of *D. polylepis* and other snake bites which are associated with inflammation and cellulitis.

5.3. Pharmacological activities of herbal extracts on snake bite wound

A bite from a snake is extremely painful with inflammatory reaction and skin exposure expected at the site of the bite. This may explain the application of medicaments on the site of the bite in traditional medicine practice although the priority in management of snake bite is to prevent or save victims from envenomation. *T. diversifolia* emerged as a plant of interest in this study, having shown significant protective activity against venom from *D. polylepis.*

5.3.1. Anti-nociceptive effects of *T. diversifolia* aqueous extract

Prior to the anti-nociceptive assay all the animals in this study were subjected to sensory motor test before the hot plate test to rule out sensory and motor impairments. The results obtained from both the oral and intraperitoneal routes of drug administration for the 300mg/kg dose were comparable to those of the reference drug (diclofenac sodium) at 95% confidence interval ($P<0.05$). This activity is characterized by a prolonged response latency after the animal is placed on the hotplate. The hotplate anti-nociceptive model produces

quantifiable behavior of the mice in terms of time taken for the animal to respond to the noxious stimuli.

Nonsteroidal anti-inflammatory drugs as well as opioids have been observed to inhibit paw licking and jumping behavior while opioids only inhibit paw licking (de Araujo Oliveira *et al*., 2020). Other studies on nociceptive activities of *T. diversifolia* have shown that it has a highly comparable analgesic activity with 5mg/Kg dose of indomethacin with the highest activity being observed only 30 minutes after treatment with a dose of 300mg/kg methanol extracts (Sijuade *et al.,* 2016). It is therefore likely that aqueous extract of *T. diversifolia* contains phytochemicals that act at supra-spinal level as well as at the central nervous level. This mechanism can be investigated further by use of different antinociceptive assays such as formalin tail flick test which is spinally mediated. Similar activity has been observed in dichloromethane extracts of *S. incanum* extracts (Mwonjoria & Kariuki, 2011) and aqueous extracts of *S. nigrum* (Zakaria *et al.,* 2009).

Presence of phytocompounds that are known to possess antinociceptive activity in methanol extracts of plants such as alkaloids, terpenes and flavonoids such as luteloin and nepetin (Tagne *et al.,* 2018) may be responsible for the significant pain inhibition activity observed in this study. The citrus flavonoid eriocitrin for eample has been found to have remarkable antinoiceptive effect in postoperative pain conditions (Alghamdi, 2020) as is the finding for flavonoids in *Leea indica* leaves for orofacial pain (Daya *et al*., 2021). This clearly suggests that phytocompounds present in *T. diversifolia* (Table 4.6a) could be investigated further for use as anti nociceptive agents and potential alternatives tso NSAIDs.

5.3.2. Anti-pyretic effects of *T. diversifolia* aqueous extract

Evaluation of antipyretic potential of aqueous methanol extracts of *T. diversifolia* at 300mg/kg, revealed that treatment via both the oral and intraperitoneal route resulted in significant antipyretic activities against turpentine induced fever that were comparable to the reference drug at the 1st, 2nd and 3rd hour after drug administration. Turpentine is known to induce fever by acting as an endogenous pyrogen that interacts with interleukins, IL-1β and IL-6, resulting in synthesis and release of prostaglandin E2 (Horai *et al*., 1998; Kozak *et al.,* 1998). Prostaglandin E_2 causes the body's thermostat, the hypothalamus, to be set at higher level by binding to PGE receptors on the neurons in the preoptic area that innervates the dorsomedial hypothalamus causing an increase in body temperature (Dinarello, 2015). Inhibition of synthesis and release of PGE_2 by blockage of the activity of cyclooxygenase enzymes activity has been widely utilized in management of fever. Phytochemicals such as quercetin, flavones, morins, and alkaloids have been shown to possess this pharmacological activity through arachidonic acid peroxidation (Baumann *et al.,* 1980).

Conventional antipyretic drugs, analgesics as well as anti-inflammatory drugs such as NSAIDs interact with membrane lipids responsible for physiochemical properties of the cell membrane such as permeability, elasticity, fluidity and micro-viscosity (Tsuchiya & Mizogami, 2012). Such interactions have been observed in compounds of plant origin such as alkaloids, flavonoids, terpenes, and fatty acids etc. which are believed to give them their therapeutic activities (Tsuchiya, 2015). Besides inhibition of cyclooxygenase enzymes, alteration of the cellular membrane's physiochemical properties is essential in management of fever as a cyclooxygenase independent activity since cyclooxygenases are integral membrane proteins (Doyen *et al.,* 2008). A similar mechanism of action involving

functional and structural disruption of cellular membranes has been observed in aspirin which interferes with cholesterol raft formation through disturbances of membrane organization (Alsop *et al.,* 2015). It is therefore possible that the antipyretic activity of *T. diversifolia* observed in this assay could be as a result of either inhibition of cyclooxygenase enyzmes or interference with functional and structural membrane disruptions.

5.3.3. Anti-inflammatory activity of aqueous extract of *T. diversifolia*

The study on anti inflammatory activity of aqueous extract of *T. diversifolia* used five groups of five mice each which were treated with extract at 100, 200 and 300mg/Kg, positive control (diclofenac) and negative control (normal saline). Prior to this treatment, inflammation had been induced in the left hind paw of mice by injecting 0.1 mL of λcarrageenan (1% in NaCl 0.9%). The group that showed the least increase in paw diameter (least inflammation) for both oral and intraperitoneal routes of administration were those treated with the extract at 300mg/Kg whose effect was comparable to the positive control at 95% confidence level.

A key event in the process of inflammation involves the release of arachidonic acid from cellular membrane lipids, a reaction mainly catalysed by the enzyme Phospholipase A_2 (PLA_2) alongside phospholipase C and diacylglycerol lipase (Ito *et al.,* 2002). The acyl group on the phosphoglycerides attached on the intracellular membrane is hydrolyzed by the enzyme phospholipase A_2 releasing arachidonic acid from the membrane phophoglycerides. Some steroidal plant and triterpenoids phytochemicals have been shown to block this reaction by their role in regulation of synthesis of the protein lipocortine.

Other phytochemicals such triterpenes and betulinic acid act by binding directly to the enzyme phospholipase A_2 (Wiart, 2006). Flavonoids with a C-ring-2, 3-double bond, such myricetin, quercetin and kaempferol remarkably inhibit PLA_2 in snake venom while flavanones such as naringenin and hesperetin and flavanone itself carry similar effects but in less potentials (Welton *et al.,* 1986). Several plant extracts such as *Trichilia catigua* (Barbosa *et al.*, 2004), oleanolic triterpenoids, pectolinaringenin flavonoids and ursolic acid of *Baccharin uncenella,* (Zalewski *et al.,* 2011), *Aloe vera* (Kammoun *et al.,* 2011) and *Cochinchina momordica* seeds (J. M. Kang *et al.,* 2009) have been found to actively exhibit antiinflammatory effects by inhibiting the phospholipase A_2 enzymes.

Various mechanisms have been attributed to the antiinflammatory effects of plant phytochemicals. These include inhibition of lipooxygenase exzymes (LOX). These group of enzymes and especially arachidonate 5-lipoxygenase are associated with inflammatory processes whereby they play a role in synthesis and release of leukotrienes from arachidonic acids. Leukotrienes acts as mediators of allergic and inflammatory reactions and therefore inhibition of lipooxygenases will result in inhibition of several inflammatory pathways (Sanda & Jisak, 2011). Flavonoids from *P. granatum* (Schubert *et al*., 1999), methanol leaf extracts of *Solanum xanthocarpum* and methanol seed extracts of *Longifolia nees* have been reported to treat inflammation by inhibiting the 5-lipoxygenase enzyme. Studies indicate that fatty acid-like phytochemicals such as phenols (excluding triterpenes and polyacetylenes that have reducing activities) inhibit the activity of 5-LOX. Similar activities are observed in hydroxylated coumarins and polyphenols while flavonoids and quinones first require to be hydroxylated to hydroquinones (Nworu & Akah, 2015).

Previous studies indicate that polar extracts of *T. diversifolia* showed a faster and better antiinflammatory activity than a 10mg/kg indomethacin dose in rats which was attributed to the synergetic interaction of the chlorogenic acids in the polar extracts with other constituents present (Santos *et al*., 2006). At low doses, different extracts were observed to inhibit neutrophil recruitment when applied topically to the inflamed ears of rats (Chagas-Paula *et al*., 2015). In the light of this understanding, the antiinflammatory effects of *T. diversifolia* may be attributed to three main classes of secondary metabolites namely flavonoids, chlorogenic acids and sesquiterpene lactones. Indeed, the qualitative analysis of the aqueous extract of *T. diversifolia* showed presence of flavonoids and terpenes which may be responsible for its anti-inflammatory effects.

5.3.4. Antimicrobial activity of aqueous leaf extract of *T. diversifolia*

The puncture wound of snake bite is prone to infection by *Staphylococus aureus* (32%) followed by *Escherihia coli* (15%) according to (Garg *et al*., 2009) while other studies find that monomicrobial infections with entero-bacteriacea are more common. This study assessed the antimicrobial activity of *T. diversifolia* on six common microoganisms in wounds; *S. aureus, S. pyogenes, S. agalactiae, P. aeruginosa,* and C. *albicans* as an additional pharmacological benefit in management of snake bite. The six microorganisms were subjected to various concentrations of the aqueous extract of *T. diversifolia*. Cellulitis due to *D. polylepis* snake bite has been documente and is attributed to *S. pyogenes* and *S. aureus.* The results reveal that *S. aureus* and *S. pyogenes* showed statistically significant ($P<0.005$) susceptibility to the extract from 50mg/mL and above with zones of inhibition of 12.5mm and 16mm respectively at this concentration. For *S. aureus*, subsequent doses after 200mg/mL were statistically similar in their inhibition of the organism suggesting

that there may be added benefit in increasing the dose, toxicity notwithstanding. *S. aureus* recorded an MIC of 1.56mg/ml and *S. pyogenes* 6.25mg/ml while the MBC for the two was 3.125 and 12.5mg/ml respectively. Overall, it was interesting to note that the extract was effective in inhibiting and killing these two microorganisms of interest in snake bite. A study by Ogundare, (2007) agrees with the susceptibility of *S. aureus* to methanol extract of *T. diversifolia* (12 mm zone of inhibition and MIC of 6.25mg/ml). Molecular docking of the phytochemicals of *T. diversifolia* carried out using levofloxacin as template resulted in the presence of compounds more effective in inhibiting DNA gyrase enzyme f microorganisms. These findings offer an opportunity for further studies into the potential of active phytoconstituents in the plant as lead compounds for antimicrobial formulations (Okiti & Osuntokun, 2020a).

5.4. Phytochemical analysis of aqueous extract of *T. diversifolia*

Phytochemical constituents found in herbal extracts are known to be the active principles in any plant, either singly or in unison and knowledge of each plant's phytochemical composition may be beneficial in understanding their effect on snake venom as observed with other pharmacological effects above. The five plants selected for this study had varying phytochemical constituents and showed differences in their safety and efficacy profiles against venom from *D. polylepis*.

5.4.1. Qualitative phytochemical analysis of herbal extracts commonly used in snake bite

Qualitative tests for phytocompounds carried out in the five herbal extracts detected flavonoids, cardiac glycosides, terpenes, tannins and alkaloids in all the plants (Table 4.4a).

Saponins were detected in all the extracts except that of *T. minuta.* The plant extract with the strongest presence of all phyto-constituents tested was *Z. chalybeum* while *T. diversifolia* showed strong presence of cardiac glycosides and weak presence of flavonoids. (Kumar & Kumari, 2015) reported that *S. suffruticosa* leaf methanolic extracts were high in alkaloids unlike the findings of this study as well as phenols. Steroids and tannins were present in moderate amounts while in this study tannins were present in low amounts. Saponins and quinones were present in mild amounts while flavonoids and terpenes were absent, unlike the findings in this study in which flavonoids were present in low quantities and terpenes in high quantities.

Other studies corroborate findings on S. *incanum* which contains saponin steroids, in particular glycoalkaloids, which are found in all parts of the plant, but in highest concentrations in the fruit, the main glycoalkaloid being solasonine. Other compounds isolated from the fruits include the alkaloids solasodine and solamargine, and the steroidal sapogenins diosgenin and yamogenin. The fruits also contain dimethylnitrosamine, flavonoids and chlorogenic acid, a phenolic derivative (Matu, 2008).

Opinde & GW, (2016) reported presence of tannins, alkaloids and flavonoids in the methanol leaf extract of *T. minuta* similar to this study. The absence of saponins in this study however was unlike that of other researchers like (Opinde & Gatheri, 2016) and (Rachuonyo, 2016) both of whom detected saponins in methanol extract of the plant.

Methanol extracts of *T. diversifolia* leaves showed presence of saponins and tannins (Ogundare, 2007) while qualitative phytochemical observation by Omolola, (2019) revealed the presence of tannins, saponins, flavonoids and terpenoids similar to this study.

Additionally, Okiti & Osuntokun (2020) did qualitative and quantitative phytochemical analysis that showed the presence of alkaloids, anthraquinone, cardiac glycosides, flavonoids, phlobotannins, reducing sugars, saponins, steroids and tannins similar to this study.

Zanthoxylum chalybeum root bark extract was found to be rich in alkaloids and in a study conducted by Tabuti (2011), its root bark, collected from Kenya and Zimbabwe, yielded the fluroquinolone alkaloid skimmianine, the benzophenanthidine alkaloids chelerythrine and nitidine, the aporphine alkaloids tembetarine, magnoflorine, N-methylcorydine, N-methylisocorydine (menisperine) and berberine and the phenylethyamine candicine. This study found the root bark was abundant in flavonoids, terpenes, saponins, tannins, alkaloids and cardiac glycosides.

5.4.2. Mineral composition of *T. diversifolia* leaf powder

The mineral composition of *T. diversifolia* leaf powder analyzed using X-ray flouresence showed high net counts of potassium, iron and calcium. Other minerals detected included manganese, nickel, copper, zinc and chromium. This semi-quantitative analysis with the counts obtained only indicative of relative amounts of the selected essential elements in the plant powder sample is an important indicator of amounts of heavy metals since these should be controlled due to their toxic nature in amounts above those recommended for human consumption. Alternatively, presence of recommended minerals and in the recommended quantities may be advantageous as supplementary to dietary sources. The actual quantities of minerals in the leaf powder can be determined using a calibration curve

of counts versus concentartion in a similar powder of a selected internal standard mineral. Moreover, other techniques such as atomic absorption spectroscopy (AAS) may be used.

5.4.3. Liquid Chromatography-Tandem Mass spectrometry analysis of aqueous fraction of *T. diversifolia*

LC-MS/MS analysis done on the aqueous extract of *T. diverfolia* leaf extract revealed thirty-eight compounds, most of them amino acids (Table 4.7b). Amino acids such as Asp, Glu, Ser, Gly, Ala and Leu are involved in alkaloid and osmolyte synthesis, cell metabolism and ammnia detoxification (Moran-Palacio *et al*., 2014) suggesting that their presence contributes to therapeutic benefits of plant extracts.

5.4.4. Gas chromatography-mass spectrometry analysis on *T. diversifolia* leaf extract

This study identified fourteen compounds in the crude methanol extract of *T. diversifolia* using GC-MS techniques whose two-dimensional structures as drawn by ChemDraw Ultra 10.0 and their IUPAC names are shown in Table 4.7c. This technique is valuable in detecting unknown compounds in a mixture of constituents (Janani & Singaravadivel, 2014b; Sisodia, 2020) and was therefore useful in identifying compounds with potential activity against *D. polylepis*.

Studies show that secondary metabolites such as alkaloids, steroids, quinonoids, phenols, terpenoids, saponins, xanthenes, tannins are reported to bind proteins in snake venom leading to their inactivation. They may also competitively block receptors of venom proteins, inhibit or inactivate venom enzymes. Flavonoids, terpenoids, tannins, polyphenols, and some minerals such as selenium from plants have the ability to neutralize

free radicals and stabilize cell membranes thereby making them less destructible by venom enzymes such as phospholipase A_2 (Gómez-Betancur *et al*., 2019).

Some of the compounds identified in *T. diversifolia* extract, which included terpenes (camphene, Longifolene, menthol, cyclohexanol, 1H-Cycloprop[e]azulene, α-Longipinene), (phenols (2,4-bis(1,1-dimethylethyl), napthalene) would be responsible for the antivenin activity of the extract against *D. polylepis*. Pinostrobin, an isolated flavanone found in the leaves of Renealmia alpinia has been reported to neutralize *in vitro* activity of venom from *B. asper* in addtion to its antihemorrhagic and antimyotoxic effects. Due to the interaction between the enzymes of the venom and the hydroxyl groups (through hydrogen bonds) in polyphenolic compounds found in plant extracts, stable complexes are formed inactivating the enzymes.

5.5. Molecular docking analysis of methanol extract of *T. diversifolia* against *D. polylepis* venom

The understanding of the mechanism of envenomation by toxins and their interaction with protein targets in the human body is important in determining molecular interactions with phytocompounds (ligands) since they would be expected to counter these mechanisms. Molecular docking is a structure-based approach that has been used in drug development to predict binding mode and affinity of two molecules, a protein and a ligand, and their preferred orientation as well as the strength of the bond formed (Lengauer & Rarey, 1996). This computer aided simulation technique helps us to understand the best orientation fit in which a ligand binds a protein of interest in a key-and-lock or hand-inglove analogy

(Jorgensen, 1991). Three dimensional structures of the molecules of interest are simulated by use of molecular graphic software tools and has previously been used to understand the mechanism of action of apigenin based molecule against the protein 3DSLBothropasin, the main haemorraghic factor obtained from *Bothrosp jararaca* venom (Srinivasa *et al*., 2014).

5.5.1. Molecular interactions between phytocompounds and *D. polylepis* venom components and mammalian targets

The fourteen compounds identified using GC-MS were docked with venom and human target proteins as involved in *D. polylepis* envenomation. The *D. polylepis* venom proteins used in this study included 1DTK-dendrotoxin, 1NTX-neurotoxin, 1FSC-fasciculin, 1TFS-calciseptine and mammalian targets of the venom were 5AFM-human nACh receptor, 5HQ3-human acetylcholinesterase and 5GJV-L-Type calcium channel. The results of protein-ligand affinity in this study reveal eleven docking affinities that were considered 'Good' owing to the low energy (=/> -7 kcal/mol) required and therefore ease of protein-ligand docking as well as presence of hydrogen and hydrophobic bonds with protein residues (Table 4.7b and c).

Three compounds, CID_1254 (menthol), CID_165675 (d-menthol) and CID_6432640 (spathulenol) gave the best docked results in the active binding site of 5AFM (human acetylcholine receptor) target protein, while six compounds, CID_1254 (menthol), CID_8181 (methyl palmitate), CID_8834 (citronellyl propionate), CID_8835 (citronellyl butyrate), CID_165675 (d-menthol) and CID_6432640 (spathulenol) presented best docking results in the active binding site of 5HQ3 (human acetylcholinesterase enzyme) target protein. CID_1254 (menthol) and CID_8181 (methyl palmitate) showed the best docking results with 5GJV (L-type calcium channel).

Compounds showing best binding interaction with protein residues in the vicinity of 4Å as visualized by Chimera and LigPlot+ softwares and presented as red spikes indicate the residues involved in the hydrophobic interactions, usually among the carbon residues. The green colored residues are involved in hydrogen bonding representing the hydrogen molecule interacting with the next higher affinity of electrons or highly electronegative atoms found in the vicinity of 4Å as shown in Figure 4.7a-k. Overall, CID_165675 showed the best docking results with 5AFM, forming two hydrogen bonds with Ser144 and Trp145, and CID_8181 showed the best results with 5HQ3 forming three hydrogen bonds with Tyr124, Ser125 and Tyr337.

5.5.2. Possible mechanisms of action of best docking interactions

1DTK-dendrotoxin is known to block voltage gated potassium ions channels thus facilitating acetylcholine release at the presynaptic terminal (Lancelin *et al.,* 1994) while 1NTX-neurotoxin disrupts transmission of nerve signal at the neural muscular junction by blocking acetylcholine from binding to muscle nicotinic acetylcholine receptor (nAChR) (Teixeira-Clerc *et al*., 2002). Results from this study indicated only 'Fair' interactions with 1DTK for five phytocompounds; CID_10137, CID_289151, CID_520957, CID_521380 and CID_6432640. Whereas these results were not 'Good' they may explain the *in vitro* activity observed against *D. polylepis* venom (Table 4.7a), but may not be relied on as the main interactions conferring protection against the venom.

For CID_1254 (menthol), CID_165675 (d-menthol) and CID_6432640 (spathulenol) which interacted strongly with 5AFM human nicotinic acetylcholine receptor (nAChR), the target for 1DTK and 1NTX, it can be postulated that they counter the activity of 1DTK at the pre-synaptic membrane and that of 1NTX at the post synaptic membrane possibly

by blocking their interaction with the receptor at these sites. A closer look at the residues involved in ligand binding at 5AFM reveals that three segments of the α-subunit containing key residues around Tyr-93, between Trp-149 and Asp-152 and between Val-188 and Asp-200 are the main determinants for ligand binding. In addition, four discontinuous segments of the non αsubunits located opposite the α-subunit habor major determinants for ligand selectivity.

The γ subunit has key residues Lys-34, between Trp-55 and Gln-59, between Ser-111 and Tyr-117 and between Phe-172 and Asp-174 (Osaka *et al*., 1999). This study could however not substantively establish if the residues involved in interactions between CID_1254, CID_165675 and CID_6432640 and 5AFM are among those indicated above which warrants further studies due to the 'Good' docking results obtained.

CID_1254 (menthol), CID_8181 (methyl palmitate), CID_8834 (citronellyl propionate), CID_8835 (citronellyl butyrate), CID_165675 (d-menthol) and CID_6432640 (spathulenol) which presented best docking results in the active binding site of 5HQ3 (human acetylcholinesterase enzyme) possibly counter the interaction between fasciculins (1FSC) and 5HQ3-human acetylcholinesterase enzyme thus preventing their potent inhibitory activity on 5HQ3, ensuring breakdown of acetylcholine at the synapse is uninhibited allowing for termination of synaptic transmission. Further interrogation of docking data for 5HQ3 revealed that all six phytocompounds interacted with aromatic residues, Trp-286, Tyr-72 and Tyr-124 which have the most marked influence on fasciculin binding (Riva, 2013).

CID_1254 (menthol) and CID_8181 (methyl palmitate) showed the best docking results with 5GJV (L-type calcium channel) that is blocked by 1TFS-calciseptine found in *D. polylepis* venom interfering with the excitation-contraction coupling of skeletal, smooth and cardiac muscle (Yasuda *et al*., 1994). It can be postulated therefore that these six phytocompounds conferred protection to mice by countering the interaction between 1TFS and its mammalian target 5GJV. Residues involved in ligand binding are in three transmembrane segments of 5GJV; Tyr III56, Tyr IV56 and Gln IIIS5. The residues Thr-1066, Gln-1070 and Asp-955 are located in the transmembrane segment IIIS5 and are involved in binding of 1,4-dihydropyridine calcium blockers (Mitterdorfert *et al*., 1996; Sinnegger *et al*., 1997) and may suggest possible sites of action of the six compounds which counter calciseptine, a potent calcium channel blocker.

These results indicate that some phytocompounds in the aqueous extract of *T. diversifolia* have little direct effect on *D. polylepis* venom proteins; α-neurotoxins (1-NTX), dendrotoxins (1-DTK), fasciculins (1-FSC) and calciseptine (1-TFS). A number of phytocompounds however interact with venom protein targets in the human body; nicotinic acetylcholine receptor (5 AFM), acetylcholinesterase enzyme (5HQ3) and L-type calcium channel (5GJV). This finding is consistent with the results of the *in vitro* study where the extract did not have significant neutralization activity of venom components when the two were mixed together and incubated at 37^0C for one hour but showed significant effects in the *in vivo* study. The main mechanisms of action of the phytocompounds is therefore proposed to be at the three targets of venom proteins;

1. At nicotinic acetylcholine receptor (5AFM), CID_1254 (menthol), CID_165675 (d-menthol) and CID_6432640 (spathulenol) antagonize dendrotoxins (1-DTK) at the

presynaptic membrane preventing the initial excess release of acetylcholine and thus flaccid paralysis; and/or antagonizing α-neurotoxins (1-NTX) at the post synaptic membrane, enhancing interaction of acetylcholine with the post synaptic membrane thus preventing muscle weakness and paralysis. Notably, α-neurotoxins (1-NTX) are more potent toxins compared to dendrotoxins (1-DTK), however, the study did not establish the level of antagonism of phytocompounds with each of the toxins.

2. At acetylcholinesterase enzyme (5HQ3), CID_1254 (menthol), CID_8181 (methyl palmitate), CID_8834 (citronellyl propionate), CID_8835 (citronellyl butyrate), CID_165675 (d-menthol) and CID_6432640 (spathulenol) antagonize fasciculins (1-FSC) preventing inhibition of the enzyme which is an active metobilizer of acetylcholine at the synapse, thus enhancing metabolism of acetylcholine preventing its accumulation at the synapse which would cause muscle fasciculations and flaccid paralysis.
3. At L-type calcium channel (5GJV), CID_1254 (menthol) and CID_8181 (methyl palmitate) antagonize the interaction between calciseptine (1-TFS) in *D. polylepis* venom, preventing myotoxicity.

These mechanisms of action have been simulated using bioinformatics following significant results in mice model and can be further substantiated using bioassays. With properly designed clinical trials, the aqueos extract of *Tithonia diversifolia* has potential for use as an anti-venin product in *D. polylepis* snake bite.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

This study had five objectives with the overall being to establish the anti-venin and anti-snake bite effects of aqueous extacts of *Sansevieria suffruticosa* leaves*, Solanum incanum* fruits*, Tithonia diversifolia* leaves*, Tagetes Minuta* leaves and *Zanthoxylum chalybeum* root bark against venom from *Dendroaspis polylepis*.

The null hypothesis (H_0) for this study which was; Herbal extracts from *S. suffruticosa* leaves*, S. incanum* fruits*, T. diversifolia* leaves*, T. minuta* leaves and *Z. chalybeum* stem bark do not have activity against *D. polylepis* venom and snake bite effects in mice; is therefore rejected for aqueous extract of *T. diversifolia* leaves. The null hypothesis is accepted for *S. suffruticosa* leaves*, S. incanum* fruits*, T. minuta* leaves and *Z. chalybeum* stem bark for the antivenin activity, however anti- snake bite effects were not investigated for these four extracts.

The findings of this study imply potential use of aqueous extracts of *T. diversifolia* leaves in management of *D. polylepis* snake bite but would require properly designed clinical trials and inference to human use noting that the study was done in mice model. Additionally, the extract has potential use in management of pain, pyrexia, inflammation and anti-microbial agent.

6.2. Recommendations

1. The recommended doses of aqueous extracts of *Sansevieria suffruticosa* leaves, *Tithonia diversifolia* leaves, *Tagetes Minuta* leaves and *Zanthoxylum chalybeum* root bark as determined in mice model are 1500mg/Kg and that of *Solanum incanum* fruits is 100mg/Kg. Inference should be done for doses applicable to human beings.
2. Aqueous extracts of *Zanthoxylum chalybeum* root bark should not be used as an antivenin as it recorded hundred percent mortality in both the *in vitro* and *in vivo* antivenin study.

6.3. Suggestions for further research

1. Bioassays of the eleven molecular interactions identified using molecular docking should be carried out with an aim of substantiating the results and proposing them as lead compounds for pharmaceutical formulation.
2. *T. diversifolia* by virtue of its activity against *D. polylepis* venom should be tested on other venomous species of snakes found in Kenya, particularly the vipers, adders and cobras since these contribute heavily to the snake bite burden in Kenya.
3. Studies on combination of extracts from several plants with significant antivenin effect can be done since these may yield better results and is the practice with herbalists.

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APPENDICES

Appendix I: Ethical Approval Letter

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

P.O. Box 30197,
00100 Nairobi,
Kenya.

Tel: 4449004/4442014/ 6
Ext. 2300
Direct Line. 4448648

REF: FVM BAUEC/2018/296

Dr. Gladys Mwangi,
Kenyatta University
19/02/2018

Dear Mwangi,

RE: Approval of proposal by Faculty Biosafety, Animal use and Ethics committee

Antivenom activity of herbs commonly used in Kenya against black mamba (*dendroaspis polylepis*) snake bite.

Gladys Wangechi Mwangi P97/27235/2011

We refer to your PhD. proposal submitted to our committee for review and your application letter dated 5th February 2018. We have reviewed your application for ethical clearance for the study.

The number of mice and the protocol used to determine crude extract LD50; ED50 for anti-snake venom, neutralization activity of the extract, anti-nociceptive, anti-inflammatory and antipyretic effect using mice meets the minimum standards of the Faculty of Veterinary medicine ethical regulation guidelines.

We also note that a KVB registered veterinary surgeon will supervise the study.

We hereby give approval for you to proceed with the project as outlined in the submitted proposal.

Yours sincerely,

Kaluwa

Dr. Catherine Kaluwa, Ph.D
Chairperson, Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine,
University of Nairobi

Appendix II: NACOSTI Permit for Research

THIS IS TO CERTIFY THAT:
DR. GLADYS WANGECHI MWANGI
of KENYATTA UNIVERSITY, 1060-100
NAIROBI,has been permitted to conduct
research in *Nairobi County*

on the topic: *ANTIVENOM ACTIVITY OF HERBS COMMONLY USED IN KENYA AGAINST BLACK MAMBA(DENDROAPSIS POLYLEPIS)SNAKE BITE*

for the period ending:
9th March,2018

Permit No : NACOSTI/P/17/32416/16118
Date Of Issue : 9th March,2017
Fee Recieved :Ksh 2000

.....
Applicant's Signature

.....
Director General
National Commission for Science, Technology & Innovation

CONDITIONS

1. You must report to the County Commissioner and the County Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit.
2. Government Officer will not be interviewed without prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two(2) hard copies and one (1) soft copy of your final report.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice

REPUBLIC OF KENYA

National Commission for Science, Technology and Innovation

RESEACH CLEARANCE PERMIT

Serial No.A 13106

CONDITIONS: see back page

Appendix III: Data tool for interviews with herbalists

NAME: ..... DATE: .....

COUNTY: .....

CONTACT: .....

QUESTION	ANSWER	REMARKS
Years of experience		
Types of snakes in the region		
No. Of patients managed		
Frequency of cases		
Presentation of patients		
Outcome of treatment		
Remedies (concotions)		
Preparation of plant		
Administration		

Appendix IV: Data sets for study on median lethal dose levels of herbal extracts

Table IVa: Survival data set of mice injected with *T. minuta* extract

Wt. (Kg) of mouse	Dose of extract (mg/Kg)	Wt. (mg) of extract	Volume of extract (mL)	Time of injection	Response	Time of Response
			Phase 1			
			Group 1			
1 -0.0078	10	0.078	0.0078	12.31PM	A	>24HRS
2-0.0077	10	0.077	0.0077	12.32PM	A	>24HRS
3-0.0097	10	0.097	0.0097	12.33PM	A	>24HRS
			Group 2			
1-0.0096	100	0.96	0.096	12.35PM	A	>24HRS
2-0.0128	100	1.28	0.128	12.36PM	A	>24HRS
3-0.0081	100	0.81	0.081	12.37PM	A	>24HRS
			Group 3			
1-0.0097	1000	9.7	0.97	12.40PM	A	>24HRS
2-0.0151	1000	15.1	1.51	12.42PM	A	>24HRS
3-0.0103	1000	10.3	1.03	12.44PM	A	>24HRS
			Phase 2			
1-0.0085	1500	12.75	1.275	12.47PM	A	>24HRS
2-0.0135	2900	39.15	3.915	12.53PM	D	1.30PM
3-0.0116	5000	58.00	5.800	11.45AM	A	>24HRS

Table IVb*:* Survival data set of mice injected with *Solanum incanum* halved fruit extract

Wt. (Kg) of mouse	**Dose of extract (mg/Kg)**	**Wt. (mg) of extract**	**Volume of extract (mL)**	**Time of injection**	**Response**	**Time of Response**
			Phase 1			
			Group 1			
1-0.0080	10	0.080	0.0080	1.14PM	A	>24HRS
2-0.0096	10	0.096	0.0096	1.15PM	A	>24HRS
3-0.0116	10	0.116	0.0116	1.16PM	A	>24HRS
			Group 2			
1-0.0112	100	1.12	0.112	1.18PM	A	>24HRS
2-0.0081	100	0.81	0.081	1.19PM	A	>24HRS
30.0079	100	0.79	0.079	1.20PM	A	>24HRS
			Group 3			
1-0.0087	1000	8.7	0.87	1.22PM	D	1.49PM
2-0.0110	1000	11	1.10	1.24PM	D	1.48PM
3-0.0065	1000	6.5	0.65	1.25PM	D	1.55PM
			Phase 2			
1-0.0106	1500	15.9	1.59	1.29PM	D	1.51PM
1-0.0119	2900	34.51	3.45	1.35PM	D	2.35PM
1-0.0090	5000	45	4.5	1.43PM	D	1.55PM

Table IVc: Survival data set of mice injected with *Zanthoxylum chalybeum* stem bark

Wt. (Kg) of mouse	Dose of extract (mg/Kg)	Wt. (mg) of extract	Volume of extract (mL)	Time of injection	Response	Time of Response
			Phase 1			
			Group 1			
1 -0.0156	10	0.156	0.0156	2.11PM	A	>24HRS
2-0.0167	10	0.167	0.0167	2.12PM	A	>24HRS
3-0.0116	10	0.116	0.0116	2.13PM	A	>24HRS
			Group 2			
1-0.0163	100	1.63	0.096	2.07PM	A	>24HRS
2-0.0112	100	1.12	0.128	2.09PM	A	>24HRS
3-0.0170	100	1.70	0.081	2.09PM	A	>24HRS
			Group 3			
1-0.0186	1000	18.6	1.86	2.03PM	A	>24HRS
2-0.0123	1000	12.3	1.23	2.03PM	A	>24HRS
3-0.0124	1000	12.4	1.24	2.04PM	A	>24HRS
			Phase 2			
1-0.0148	1500	22.2	2.22	2.01PM	A	>24HRS
2-0.0133	2900	38.5	3.85	2.00PM	D	1.30PM
3-0.0124	5000	62	6.2	1.59PM	A	>24HRS

Table IVd: Survival data set of mice injected with *Tithonia diversifolia* leaf extract

Wt. (Kg) of mouse	Dose of extract (mg/Kg)	Wt. (mg) of extract	Volume of extract (mL)	Time of injection	Response	Time of Response
			Phase 1			
			Group 1			
1 -0.0078	10	0.078	0.0078	12.31PM	A	>24HRS
2-0.0077	10	0.077	0.0077	12.32PM	A	>24HRS
3-0.0097	10	0.097	0.0097	12.33PM	A	>24HRS
			Group 2			
1-0.0096	100	0.96	0.096	12.35PM	A	>24HRS
2-0.0128	100	1.28	0.128	12.36PM	A	>24HRS
3-0.0081	100	0.81	0.081	12.37PM	A	>24HRS
			Group 3			
1-0.0097	1000	9.7	0.97	12.40PM	A	>24HRS
2-0.0151	1000	15.1	1.51	12.42PM	A	>24HRS
3-0.0103	1000	10.3	1.03	12.44PM	A	>24HRS
			Phase 2			
1-0.0085	1500	12.75	1.275	12.47PM	A	>24HRS
2-0.0135	2900	39.15	3.915	12.53PM	D	1.30PM
3-0.0116	5000	58.00	5.800	11.45AM	A	>24HRS

Table IVe: Survival data set of mice injected with *Sanseviera suffruticosa* leaf extract

Wt. (Kg) of mouse	Dose of extract (mg/Kg)	Wt. (mg) of extract	Volume of extract (mL)	Time of injection	Response	Time of Response
			Phase 1			
			Group 1			
1 -0.0078	10	0.078	0.0078	12.31PM	A	>24HRS
2-0.0077	10	0.077	0.0077	12.32PM	A	>24HRS
3-0.0097	10	0.097	0.0097	12.33PM	A	>24HRS
			Group 2			
1-0.0096	100	0.96	0.096	12.35PM	A	>24HRS
2-0.0128	100	1.28	0.128	12.36PM	A	>24HRS
3-0.0081	100	0.81	0.081	12.37PM	A	>24HRS
			Group 3			
1-0.0097	1000	9.7	0.97	12.40PM	A	>24HRS
2-0.0151	1000	15.1	1.51	12.42PM	A	>24HRS
3-0.0103	1000	10.3	1.03	12.44PM	A	>24HRS
			Phase 2			
1-0.0085	1500	12.75	1.275	12.47PM	A	>24HRS
2-0.0135	2900	39.15	3.915	12.53PM	D	1.30PM
3-0.0116	5000	58.00	5.800	11.45AM	A	>24HRS

Appendix V: Data sets for *in vitro* and *in vivo* study

Table Va: Parameters in determining the median lethal (LD_{50}) dose of venom in laboratory mice

Group	Weight of mice (Kg)	Dose of venom injected (mls/Kg of 1%v/v crude venom)	Volume of venom injected (mls)	Percentage response
1	0.0158	1.25	0.019	0
	0.0238	1.25	0.029	
	0.0201	1.25	0.025	
	0.0146	1.25	0.018	
	0.0173	1.25	0.021	
2	0.0235	2.5	0.058	40
	0.0233	2.5	0.058	
	0.0168	2.5	0.042	
	0.0196	2.5	0.049	
	0.0832	2.5	0.208	
3	0.0198	5.0	0.099	80
	0.0154	5.0	0.077	
	0.0151	5.0	0.075	
	0.0231	5.0	0.115	
	0.0154	5.0	0.077	
4	0.0153	7.5	0.114	80
	0.0194	7.5	0.145	
	0.0183	7.5	0.137	
	0.0179	7.5	0.134	
	0.0170	7.5	0.127	
5	0.0205	10.0	0.205	100
	0.0165	10.0	0.165	
	0.0236	10.0	0.236	
	0.0166	10.0	0.166	
	0.0270	10.0	0.270	
6	0.0221	20.0	0.442	100
	0.0190	20.0	0.380	
	0.0212	20.0	0.424	
	0.0213	20.0	0.426	
	0.0176	20.0	0.352	

Corrected formula:

For the 0% dead (100% survival), 100(0.25/n)

For the 100% Dead (0% survival), 100[(5-0.25)/n]

Where n=the number of animals in the group.

Table Vb: Parameters in determining *D. polylepis* venom challenge dose in laboratory mice

Multiple of LD_{50} (2.5mls/Kg of 1% v/v crude venom)	**Weight of mice (Kg)**	**Dose of venom injected (ml of 1% v/v crude venom)**
1	0.0257 0.0167 0.0147 0.0162	2.5
2	0.0144 0.0154 0.0200 0.0182	5.0
3	0.0161 0.0194 0.0245 0.0154	7.5
4	0.0188 0.0205 0.0150 0.0196	10
5	0.0221 0.0164 0.0185 0.0186	12.5

Table Vc: Parameters in determining the effective dose (ED99) of anti-snake venom (ASV) in laboratory mice

GROUP	Weight of mice (Kg)	Dose of ASV injected (mg/Kg)	Volume of venom injected (Venom challenge dose; 10ml/Kg)
1	0.0249	1000	0.249
	0.0285		0.285
	0.0221		0.221
	0.0230		0.230
2	0.0200	2000	0.200
	0.0210		0.210
	0.0263		0.263
	0.0244		0.244
3	0.0270	3000	0.270
	0.0277		0.277
	0.0218		0.218
	0.0212		0.212

Table Vd: Response of mice injected intraperitoneally with mixture of *T. diversifolia* plant extract and *D. polylepis* venom after incubation at 37^0C for 1 hour (*in vitro* sudy)

Mice sets	Wt. of mouse (Kg)	Median lethal dose of extract(mg/Kg)	Vol. of extract injected at 10mg/ml	Vol. of 1%v/v venom injected at 10mls/Kg	Time of injection	Response	Time of response	Survival rate	Percentage survival
				SIF					
Mouse 1	0.0157	100	0.157	0.157	0601	D		2/5	40
Mouse 2	0.0105	100	0.105	0.105	0602	D			
Mouse 3	0.0114	100	0.114	0.114	0604	A			
Mouse 4	0.0121	100	0.121	0.121	0605	A			
Mouse 5	0.0124	100	0.124	0.124	0605	D			
				SSL					
Mouse 1	0.0089	1000	0.89	0.089	0612	D		2/5	40
Mouse 2	0.0120	1000	12.0	0.120	0613	D			
Mouse 3	0.0113	1000	11.3	0.113	0614	A			
Mouse 4	0.0144	1000	14.4	0.144	0616	A			
Mouse 5	0.01165	1000	11.7	0.117	0617	D			
				ZCB					
Mouse 1	0.0099	100	0.099	0.099	0608	D		0/5	0
Mouse 2	0.0104	100	0.104	0.104	0609	D			
Mouse 3	0.0098	100	0.098	0.098	0609	D			
Mouse 4	0.0096	100	0.096	0.096	0610	D			
Mouse 5	0.009925	100	0.099	0.099	0611	D			
				TML					
Mouse 1	0.0116	1000	1.16	0.116	0549	A		2/5	40
Mouse 2	0.0109	1000	1.09	0.109	0552	D			
Mouse 3	0.0147	1000	1.47	0.147	0554	D			
Mouse 4	0.0112	1000	1.12	0.112	0558	D			

Mouse 5	0.0121	1000	1.21	0.121	0559	A		
				TDL				
Mouse 1	0.0094	1000	0.94	0.094	0610	A	1/5	20
Mouse 2	0.0118	1000	1.18	0.118	0612	D		
Mouse 3	0.0115	1000	1.15	0.115	0615	D		
Mouse 4	0.0094	1000	0.94	0.094	0617	D		
Mouse 5	0.0105	1000	1.05	0.105	0618	D		
NEGATIVE CONTROL (1%v/v Venom10mls/Kg + Water for injection 10mls/Kg)								
			Vol of water injected	**Vol of venom injected**			**Survival rate**	**Percentage survival**
Mouse 1	0.0188	-	0.188	0.188	0620	D	0/5	0
Mouse 2	0.0205	-	0.205	0.205	0621	D		
Mouse 3	0.0150	-	0.150	0.150	0622	D		
Mouse 4	0.0196	-	0.196	0.196	0623	D		
Mouse 5	0.0185	-	0.185	0.185	0624	D		
POSITIVE CONTROL (1%v/v Venom 10mls/Kg + Anti-snake venom 30mls/Kg)								
Mice sets			**Vol. of ASV injected at**	**Vol. of venom injected**				
Mouse 1	0.019	-	0.57	0.19	0626	A	5/5	100
Mouse 2	0.018	-	0.54	0.18	0627	A		
Mouse 3	0.020	-	0.60	0.20	0627	A		
Mouse 4	0.021	-	0.63	0.20	0628	A		
Mouse 5	0.023	-	0.69	0.23	0629	A		

Table Ve: Response of mice injected intraperitoneally with *D. polylepis* venom followed by *T. diversifolia* leaf extract

Mice sets	Wt. of mouse(Kg)	Dose of extract(mg/Kg)	Vol. of extract injected at 10mg/Kg	Vol. of 1%v/v venom injected at 10mls/Kg	Time of injection	Response	Time of response	Survival rate	Percentage survival
				SIF-GROUP 1					
Mouse 1	0.0281	100	0.281	0.281	1454	D		2/5	40
Mouse 2	0.0262	100	0.262	0.262	1456	A			
Mouse 3	0.0306	100	0.306	0.306	1458	D			
Mouse 4	0.0238	100	0.238	0.238	1459	D			
Mouse 5	0.0271	100	0.271	0.271	1500	D			
				SSL-GROUP 2					
Mouse 1	0.0299	1000	2.99	0.299	1503	D		2/5	40
Mouse 2	0.0381	1000	3.81	0.381	1505	D			
Mouse 3	0.0228	1000	2.28	0.228	1508	D			
Mouse 4	0.0279	1000	2.79	0.279	1511	A			
Mouse 5	0.0260	1000	2.60	0.260	1511	D			
				ZCB-GROUP 3					
Mouse 1	0.0308	100	0.308	0.308	1549	D		0/5	0
Mouse 2	0.0360	100	0.360	0.360	1551	D			
Mouse 3	0.0329	100	0.329	0.329	1553	D			
Mouse 4	0.0258	100	0.258	0.258	1555	D			
Mouse 5	0.0321	100	0.321	0.321	1556	D			
				TML-GROUP 4					
Mouse 1	0.0275	1000	2.75	0.275	1521	D		2/5	40
Mouse 2	0.0276	1000	2.76	0.276	1523	A			

Mouse 3	0.0295	1000	2.95	0.295	1525	D		
Mouse 4	0.0254	1000	2.54	0.254	1528	D		
Mouse 5	0.0283	1000	2.83	0.283	1529	D		
				TDL-GROUP 5				
Mouse 1	0.0275	1000	2.75	0.275	1558	D	4/5	80
Mouse 2	0.0193	1000	1.93	1.193	1559	A		
Mouse 3	0.0235	1000	2.35	1.235	1600	A		
Mouse 4	0.0198	1000	1.98	0.198	1601	A		
Mouse 5	0.0233	1000	2.33	0.233	1602	A		
			NEGATIVE CONTROL (1%v/v Venom + Water for injection 10mls/Kg)-GROUP 6					
			Vol of Water injected	**Vol of venom injected**				
Mouse 1	0.0277	-	0.831	0.188	1427	D	0/5	0
Mouse 2	0.0189	-	0.567	0.205	1428	D		
Mouse 3	0.0250	-	0.750	0.150	1428	D		
Mouse 4	0.0166	-	0.498	0.196	1429	D		
Mouse 5	0.0222	-	0.666	0.222	1430	D		
			POSITIVE CONTROL (1%v/v Venom+ Anti-snake venom 30mls/Kg)-GROUP 7					
			Vol of water injected	**Vol of venom injected**				
Mouse 1	0.0215	-	0.645		1420	A	5/5	100
Mouse 2	0.0318	-	0.954		1421	A		
Mouse 3	0.0287	-	0.861		1422	A		
Mouse 4	0.0182	-	0.546		1423	A		
Mouse 5	0.0234	-	0.702		1425	A		

Appendix VI: Data sets for pharmacological studies

Table VIa: Anti-nociceptive activity of orally administered aqueous extract of *T. diversifolia* leaves on laboratory mice using the hot plate method

Group	**Weight of mice (Kg)**	**Volume of Treatment administered**	**Time to reaction**			
			0 min	**15 min**	**30 min**	**60min**
Normal Saline	0.025		6.2	4.7	8.9	10.6
	0.0245		7.1	9.3	5.7	11.4
	0.028		6.4	4.9	8.4	11.7
	0.0232		6.9	4.7	8.8	11.1
	0.022		5.8	8.4	4.2	6
	0.2217		4.7		5.1	6.3
100mg/kg			0 min	15 min	30 min	60min
	0.0282		9.7	10.4	10.4	10.8
	0.0217		8.6	8.5	8.2	19.4
	0.0278		9.6	15.2	14.9	11.8
	0.0255		7	9.3	5.2	15.2
	0.0230		8.3	9.6	10.7	9.8
	0.0275		11.5	13		14.4
200mg/kg			0 min	15 min	30 min	60min
	0.0249		7	15.6	11.6	6.4
	0.0224		13.5	10.1	5.4	19.5
	0.0318		8	6.5	6.4	8.6
	0.0266		10.4	8.2	17.9	13.8
	0.0234		7.8	8.1	7	12
	0.0258		9.8	11.1	15.3	13.4
300mg/kg			0 min	15 min	30 min	60min

	0.0238		7		8.8	12.9
	0.0197		10.6	17.9	19.3	15.1
	0.0280		14.6	9.1	18.5	18.3
	0.0245		9.4	19	17.7	18.4
	0.02414		8.6	9.6	8.8	15.7
	60.0233		17.3	19.3	11.9	21.3
Diclofenac		0.251	0 min	15 min	30 min	60min
50mg/Kg	0.0251		19	22	19.9	17
Of 5mg/ml	0.0255		13.9	20.4	19.6	17.3
	0.0257		12.2	10.4	9.3	19.2
	0.0216		10.4	13	13	13.7
	0.0215		18	11.39	13.8	18.8
	0.0188		11.8	7.9	12.6	8.6

Table VIb: Anti-nociceptive activity of intraperitoneally administered aqueous extract of *T.*
diversifolia leaves on laboratory mice using the hot plate method

Group	**Weight of mice (Kg)**	**Volume of treatment injected**	**Time to reaction**			
			0 min	**15 min**	**30 min**	**60min**
Negative Control	0.243		7.6	8.4	7.3	10.5
Normal Saline	0.020		7.5	12.5	9	15.5
10mls/Kg	0.0244		10.6	10.1	8.4	6.3
	0.225		8.8	8.3	5	11.1
	0.247		19.1	8.8	9.1	13
	0.022		13.6	6.8	9.2	9.2
Extract	0.0246		4.7	7.8	14.3	10.2
100mg/kg **of**	0.0223		4.6	7.1	12.3	6.8
(20mg/ml	0.0247		5.6	16.1	19.6	21.1
solution)	0.0236		7.3	13.5	18.4	15.5
	0.0247		8.4	6.5	8.8	10.7
	0.0239		9.2	9.4	9.2	12.1
Extract	0.0221		6.5	8.7	6.7	9.8
200mg/kg	0.0246		4.1	11.6	13.3	17.8
of	0.022		5.3	14.5	16.8	14.4
(20mg/ml	0.0237		6.1	17	16.5	19.7
solution)	0.0252		11.6	18	12.1	17.9
	0.0233		7	10.8	12.4	11.7
Extract	10.0207		11.1	16.1	14.6	13.5
300mg/kg	0.0227		13.5	18.9	15.7	18.1
of	0.0214		10.5	7.9	13.5	12.7
(20mg/ml	0.0211		9.7	11.7	13.4	20.3
solution)	0.0231		12.3	13.5	15.9	21.21
	0.0221		18.7	16.8	14.4	24

Diclofenac	0.0263	0.016	19.3	15.4	16.7	25.3
Sodium	0.0275		9.6	16.9	16.9	27.6
15mg/Kg	0.0204		10.8	21	11.6	28.9
(25mg/ml	0.0264		17.3	16.4	21.2	13.5
solution)	0.0247		20.1	16.1	18.6	13.2
	0.0247		16.8	10	14.4	16.9

Table VIc: Anti-pyretic activity of orally administered aqueous extract of *T. diversifolia* leaves in laboratory rats

Group	**Weight of rat (Kg)**	**Volume (ml) of treatment administered**	**0hr**	**1hr**	**2hrs**	**3hrs**
Normal Saline	0.126	1.26	39.1	39.5	39.7	39.6
10ml/Kg	0.212	2.12	38.6	38.8	39.5	39.4
	0.236	2.36	38.8	38.5	39.5	39.1
	0.157	1.57	39.1	39.6	39.8	39.9
	0.227	2.27	39.4	39.5	40.6	40.4
	0.148	1.48	39.5	39.6	40.6	40.4
100mg/Kg	0.169	0.845	38.9	38.5	39.9	39.5
of	0.265	1.325	38.8	38.8	40	39.2
(20mg/ml	0.128	0.64	38	38.3	39.5	39.4
solution)	0.165	0.825	-	-	-	-
	0.203	1.015	38.6	38.9	39.8	39.3
	0.143	0.715	38.4	39.3	40.2	39.9
200mg/kg	0.266	2.66	39.5	39.3	39.1	39.7
of	0.260	2.60	38.5	38.9	39.2	38.5
(20mg/ml	0.154	1.54	38.5	39.3	39.3	39.2
solution)	0.230	2.30	38.4	38.4	39.4	39.4
	0.160	1.60	39	38.9	40	39.5
	0.169	1.69	-	-	-	-
300mg/kg	0.189	2.835	38.1	38	37.7	37.8
of	0.139	2.085	38.2	38.1	38	37.9
20mg/ml solution	0.180	2.7	38.2	37.8	38.5	37.2
	0.177	2.655	38.5	38.2	37.5	38.1
	0.142	2.13	38	37.9	37.1	37.6

	0.130	1.95	-	-	-	-
Paracetamol	0.110	0.22	38.4	37.5	37.6	37.3
100mg/Kg	0.136	0.272	38.4	38	37.3	37.4
of 50mg/ml	0.132	0.264	38	37.8	37.9	37.8
solution	0.163	0.326	38.5	38	37.6	37.4
	0.152	0.304	38.2	37.9	38	36
	0.145	0.29	-	-	-	-

Table VId: pyretic activity of intraperitoneally administered aqueous extract of *T. diversifolia* leaves in laboratory rats

Group	**Weight of mice (Kg)**	**Volume of treatment injected (ml)**	**0hr**	**1hr**	**2hrs**	**3hrs**
Normal Saline	0.155	1.55	40.2	41.3	39.5	39.8
10ml/Kg	0.145	1.45	40.4	40.8	39.9	39.5
	0.151	1.151	40.3	40.4	39.9	38.8
	0.160	1.60	38.5	-	-	-
	0.175	1.75	39.5	40.8	39.8	39.6
	0.181	1.181	40.5	40.5	39.8	39.3
Extract	0.191	0.955	38.8	41.5	-	-
100mg/kg	0.202	1.01	40.4	40.7	38.5	39.6
(20mg/ml	0.234	1.17	-	-	-	-
solution)	0.222	1.11	-	-	-	-
	0.184	0.92	-	-	-	-
	0. 156	0.0312	-	-	-	-
Extract	0.230	2.3	39.4	39.1	38.5	38.3
200mg/kg	0.175	1.75	39.7	39.3	40.4	39.2
(20mg/ml	0.156	1.56	39.8	39.5	39.2	39.2
solution)	0.154	1.54	40.2	40.5	40.2	-
	0.236	2.36	39.8	39.4	38.8	38.4
	0.187	1.87	-	-	-	-
Extract	0.176	2.64	39.4	38.6	37.8	37.7
300mg/kg	0.164	2.46	40.3	39.1	38.6	38.2
(20mg/ml	0.258	3.87	40.2	38.6	37.8	38
solution)	0.189	2.835	38.9	38.8	37.5	37.9

	0.234	3.51	39.6	38.6	37	38
	0.195	2.925	39.5	39.7	37.6	37.9
Paracetamol	0.167	0.334	39.8	39	38.3	37.8
100mg/Kg	0.186	0.372	39.8	39.2	38.5	38.3
(50mg/ml	0.159	0.318	39.7	38.83	38.6	38.2
preparation)	0.247	0.494	40	39.5	38.6	38.3
	0.245	0.49	39.4	38.4	38.4	37.9
	0.221	0.442	39.8	38.6	37.9	37.6

Table VIe: inflammatory activity of orally administered aqueous extract of *T. diversifolia* leaves in laboratory mice

Group	**Weight of mice (Kg)**	**Volume of treatment (ml)**	**Response (Paw diameter in mm)**			
			0hr	**1.5hr**	**3hr**	**6hr**
Negative Control Normal Saline 10mls/Kg	1		2.52	3.31	3.62	3.4
	2		2.14	3.09	3.42	3.57
	3		2.17	3.1	3.33	3.27
	4		1.94	2.99	3.57	3.54
	5		2.06	3.26	3.33	3.3
	6		2.05	2.95	3.25	3.4
Extract 100mg/kg (20mg/ml solution)	1		2.03	2.87	3.09	2.8
	2		1.82	2.53	2.94	2.37
	3		2.26	2.55	3.17	2.55
	4		2.12	2.52	2.42	2.6
	5		2.22	2.74	2.47	2.73
	6		1.83	2.54	2.7	2.62
Extract 200mg/kg (20mg/ml solution)	1		2.09	2.7	2.92	3.04
	2		2.42	2.95	3.2	2.91
	3		2.23	3.17	3.07	2.83
	4		2.34	2.39	2.95	2.44
	5		2.1	2.64	2.84	3.02
	6		1.89	2.67	2.82	2.88
Extract 300mg/kg (20mg/ml solution)	1		2.24	2.83	3.26	3.42
	2		2.45	3.04	3.36	2.97
	3		2.31	2.95	3.04	3.01

	4	2.38	2.86	3	2.89
	5	2.38	2.8	2.99	2.83
	6	2.51	2.81	2.75	2.89
Diclofenac Sodium 15mg/Kg **(25mg/ml solution)**	1	1.99	2.62	2.89	2.85
	2	2.04	2.68	2.47	2.39
	3	2.18	2.78	2.77	2.74
	4	2.17	2.72	2.69	2.71
	5	2.57	2.62	2.66	2.63
	6	2.12	2.86	2.63	2.71

Table VIf: inflammatory activity of orally administered aqueous extract of *T. diversifolia* leaves in laboratory mice

Group	**Weight of mice (Kg)**	**Volume of treatment (ml)**	**Response (Paw diameter in mm)**			
			0hr	**1hr**	**2hr**	**3hr**
Negative Control	**1**		2.40	4.26	4.10	3.62
Normal Saline 10mls/Kg	**2**		2.17	3.21	4.15	3.94
	3		2.44	3.67	3.77	3.68
	4		2.39	3.28	3.63	3.66
	5		2.39	3.17	3.22	3.19
	6		2.46	3.87	3.58	3.58
Extract 100mg/kg Of (20mg/ml solution)						
			0hr	**1hr**	**2hr**	**3hr**
100mg/kg	**1**		2.98	4.00	4.16	3.50
Extract 200mg/kg	**2**		2.32	2.98	4.05	3.49
Of (20mg/ml solution)	**3**		2.54	3.93	4.10	3.54
	4		2.07	3.59	3.70	3.32
	5		2.86	3.63	3.23	3.07
	6		2.53	3.97	3.54	3.39
			0hr	**1hr**	**2hr**	**3hr**
200mg/kg	**1**		1.98	2.92	3.09	2.96
Extract 300mg/kg	**2**		2.01	2.84	3.05	2.66
of	**3**		2.25	3.39	3.09	3.20
(20mg/ml solution)	**4**		2.44	2.64	2.82	3.42
	5		2.32	2.88	3.42	2.63
	6		2.25	3.39	2.73	3.65

Diclofenac Sodium 15mg/Kg **(25mg/ml solution)**		**0hr**	**1hr**	**2hr**	**3hr**
300mg/kg	**1**	2.06	3.03	2.99	2.76
	2	2.03	2.97	3.21	2.78
	3	2.42	3.07	2.82	3.02
	4	2.15	3.00	2.91	2.51
	5	2.35	3.23	3.05	2.75
Negative Control Normal Saline 10mls/Kg	**6**	2.22	3.07	3.05	2.75
		0hr	**1hr**	**2hr**	**3hr**
Diclofenac	**1**	2.44	3.30	3.03	3.01
	2	2.45	3.06	3.00	3.29
	3	2.46	3.08	3.10	2.74
	4	2.26	2.79	3.18	3.00
	5	2.29	2.80	2.89	2.81
	6	2.45	2.89	3.25	3.02

Table VIg: Inhibition zones at different concentrations of *T. diversifolia* aqueous extract on selected microorganisms

	INHIBITION ZONES (diameter in mm)							
	P control	**25 mg/kg**	**50 mg/ml**	**100 mg/ml**	**200 mg/ml**	**400 mg/ml**	**800 mg/ml**	**1000 mg/ml**
S. aureus	41	10	12.5	15.5	19.5	20	21.5	22.5
S. agalactiae	18.5	0	9.5	13.5	20	21	26	27.5
S. pyogenes	35	12.5	16.5	18.5	20.5	21	23.5	26
P. aeruginosa	29	0	9.5	13.5	16.5	17.5	19.5	21
E. coli	23.5	0	0	0	0	0	0	0
C. albicans	15.5	0	0	0	0	0	0	0

Table VIh: Minimum Inhibitory and minimum bactericidal concentrations of aqueous extract of *T. diversifolia* on susceptible microorganisms

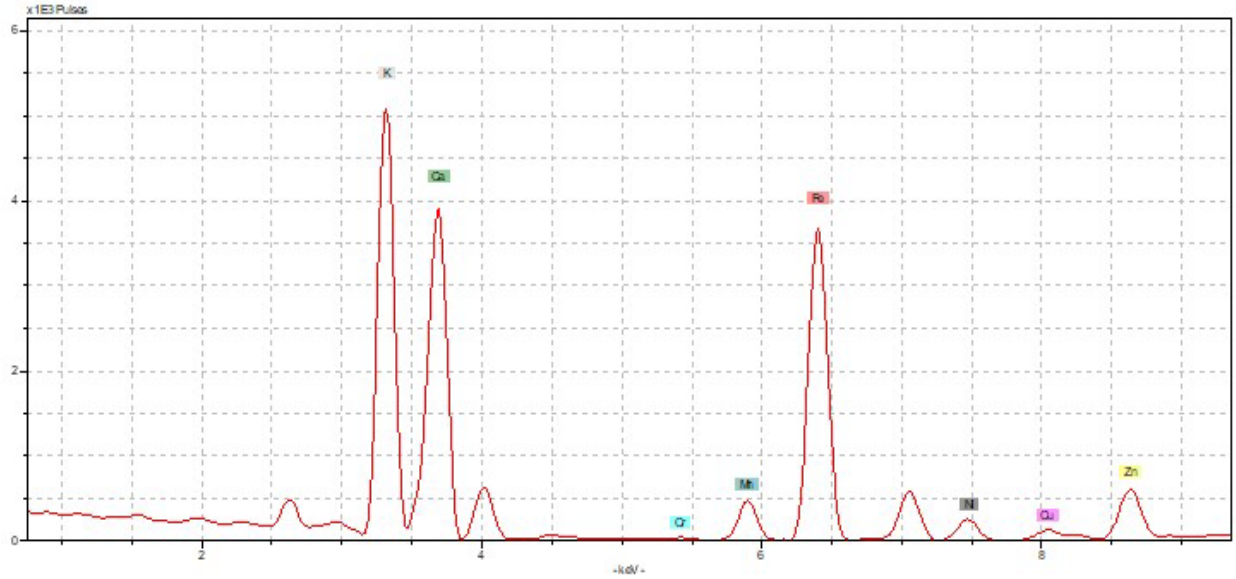
	1	2	3	4	5	6	7	8	9	10	11	12
S. aureus (25mg/ml)	NG	**NG**	**NG**	**G**	G	G	G	G	G	G	G	G
Control drug- Benzyl penicillin	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	G
S. pyogenes (25mg/ml)	**NG**	**G**	G	G	G	G	G	G	G	G	G	G
Control drug- Benzyl penicillin	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	G
S. agalactiae (50mg/ml)	NG	NG	**NG**	**NG**	**G**	G	G	G	G	G	G	G
Control drug- Benzyl penicillin	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	G
P. aeruginosa (50mg/ml)	**NG**	**NG**	**G**	G	G	G	G	G	G	G	G	G
Control drug- Gentamicin	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	G

1 Yellow-growth (G).

2 Red-no growth (NG).

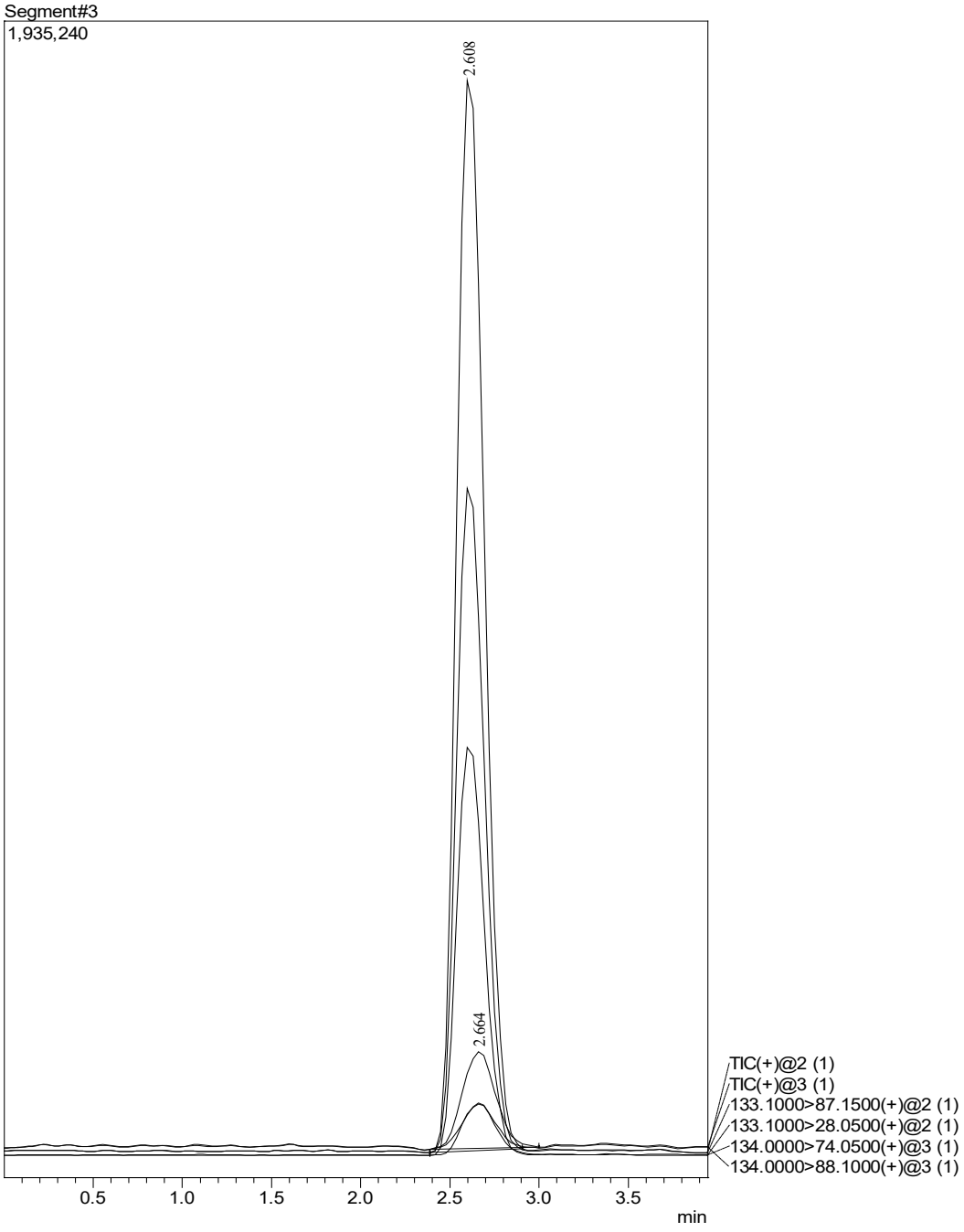
Appendix VII: Spectra for phytochemical analyses

a) XRF spectrum of the dry leaf powder of *Tithonia diversifolia*

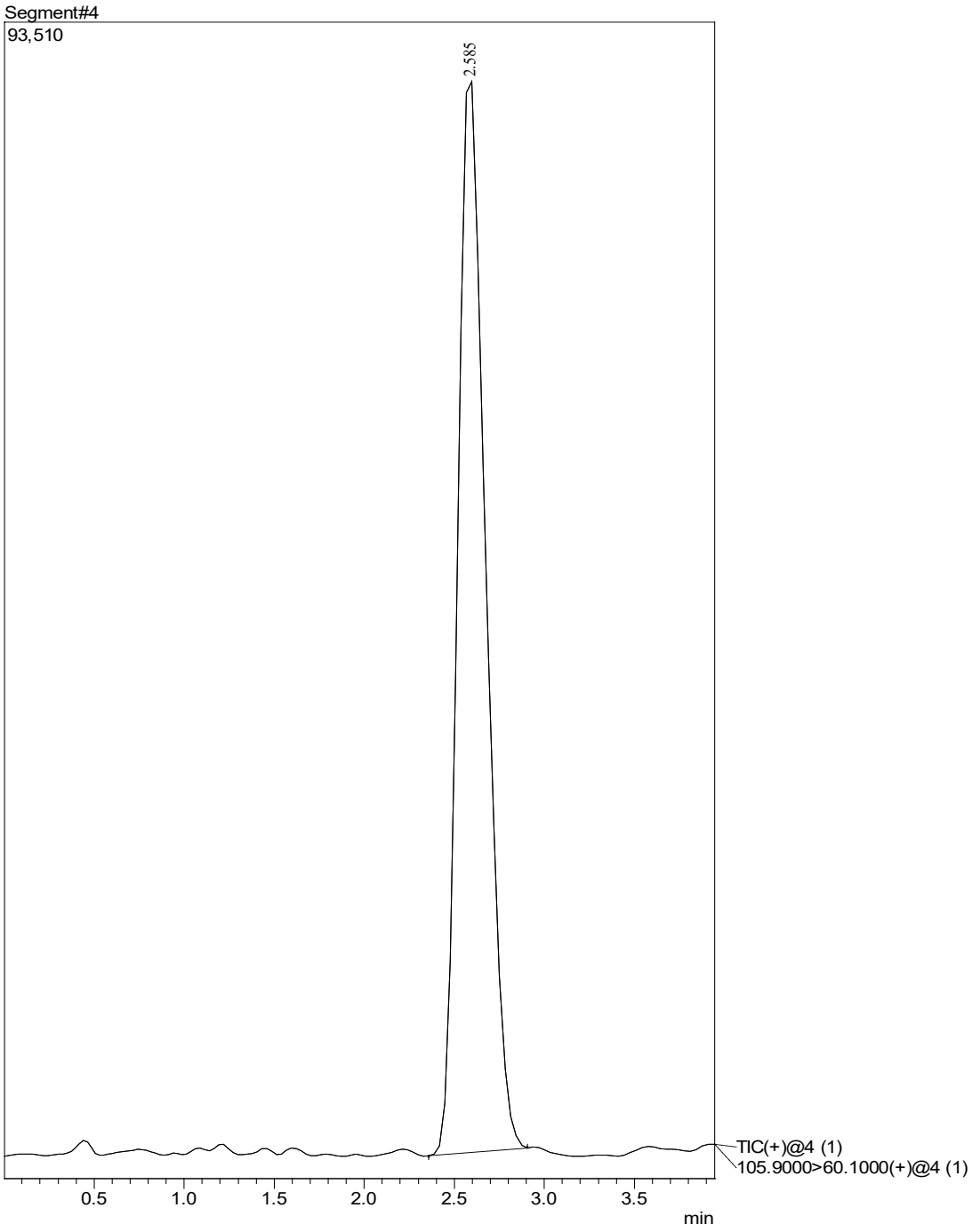


b) **LCMS/MS spectra of compounds detected in aqueous fraction of *T. diversifolia***

Asparagine and Aspartic acid



i. Serine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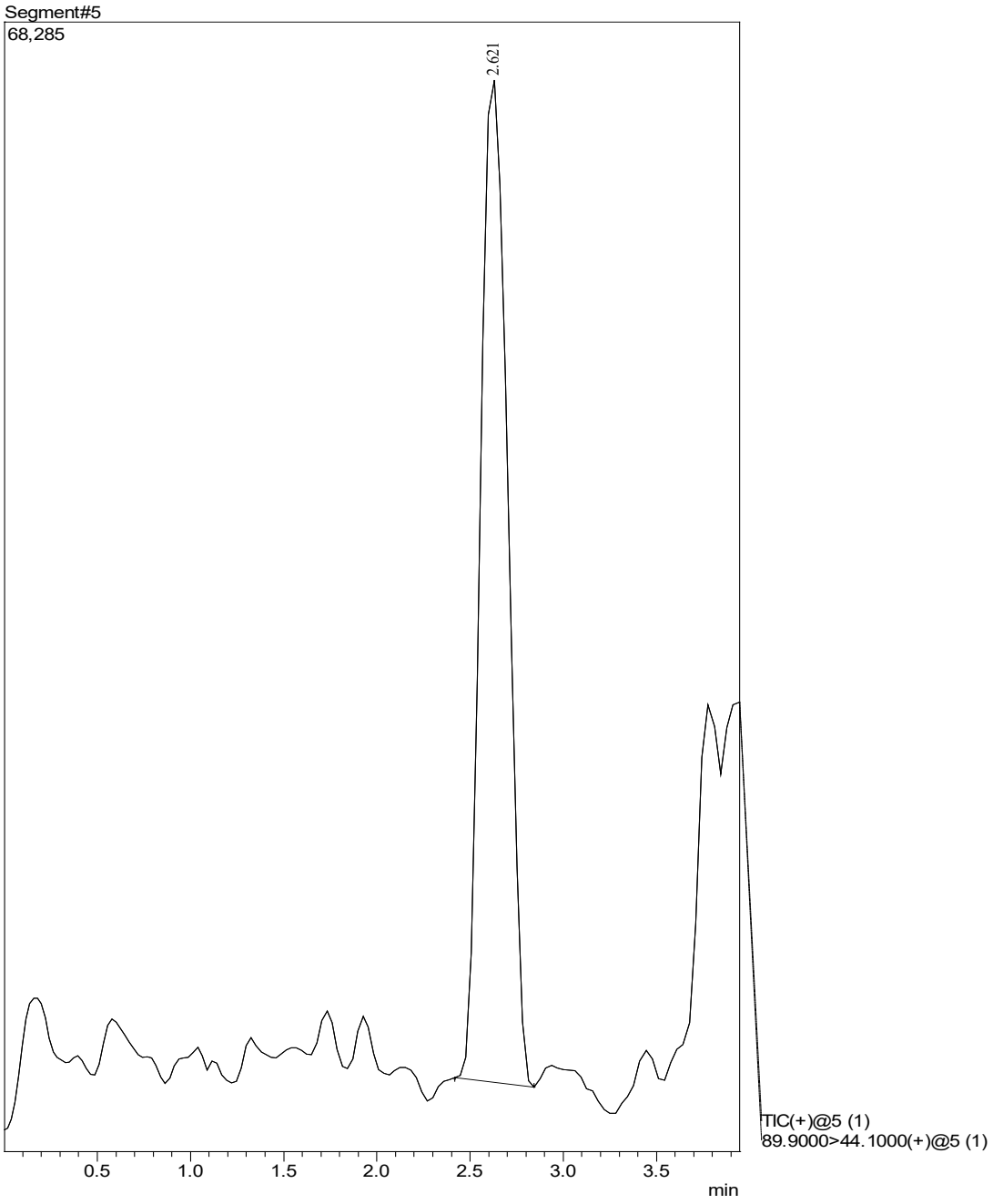
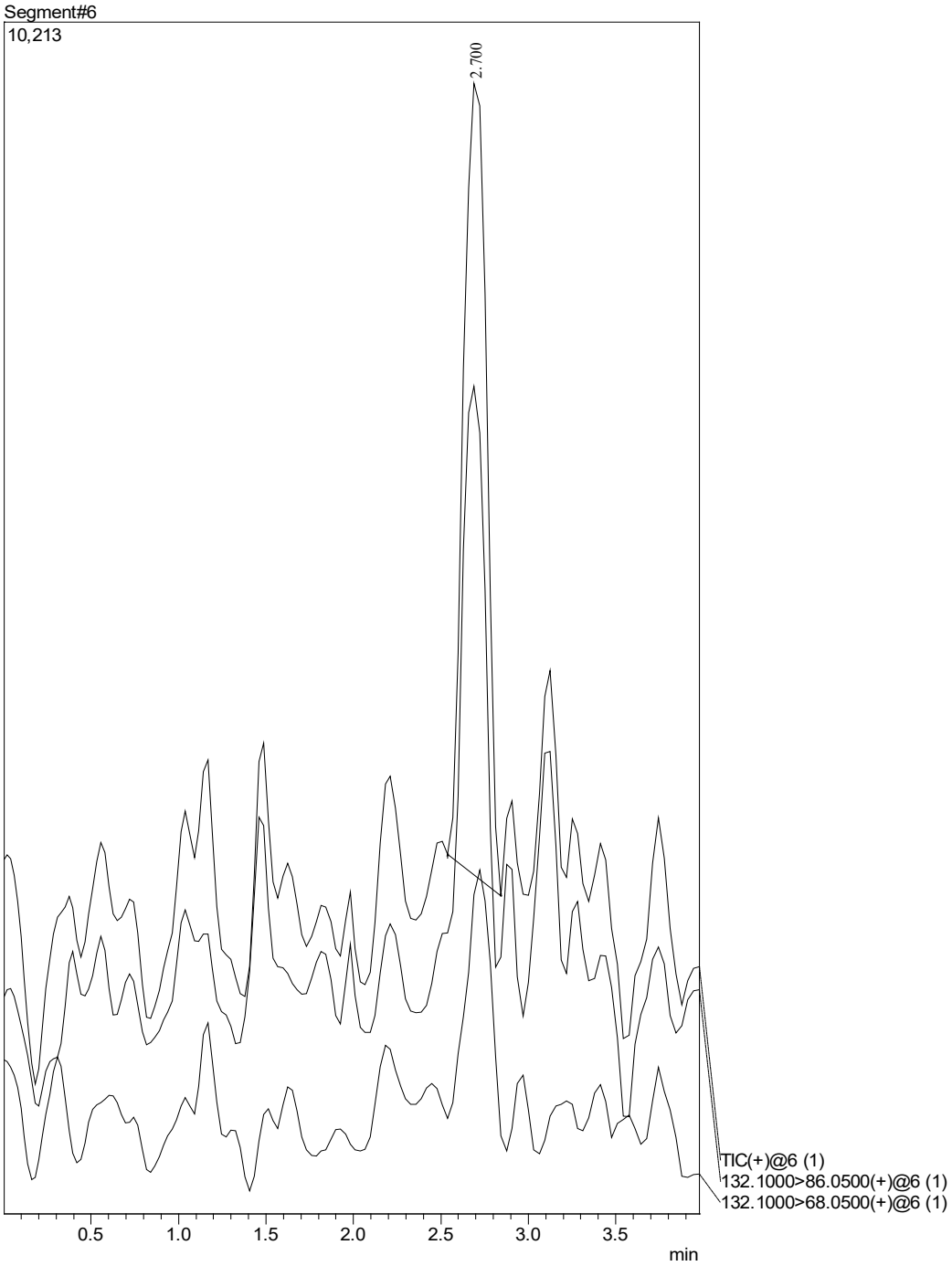
93,510
2.585
TIC(+)@4 (1)
105.9000>60.1000(+)@4 (1)
0.5
1.0
1.5
2.0
2.5
3.0
3.5
min

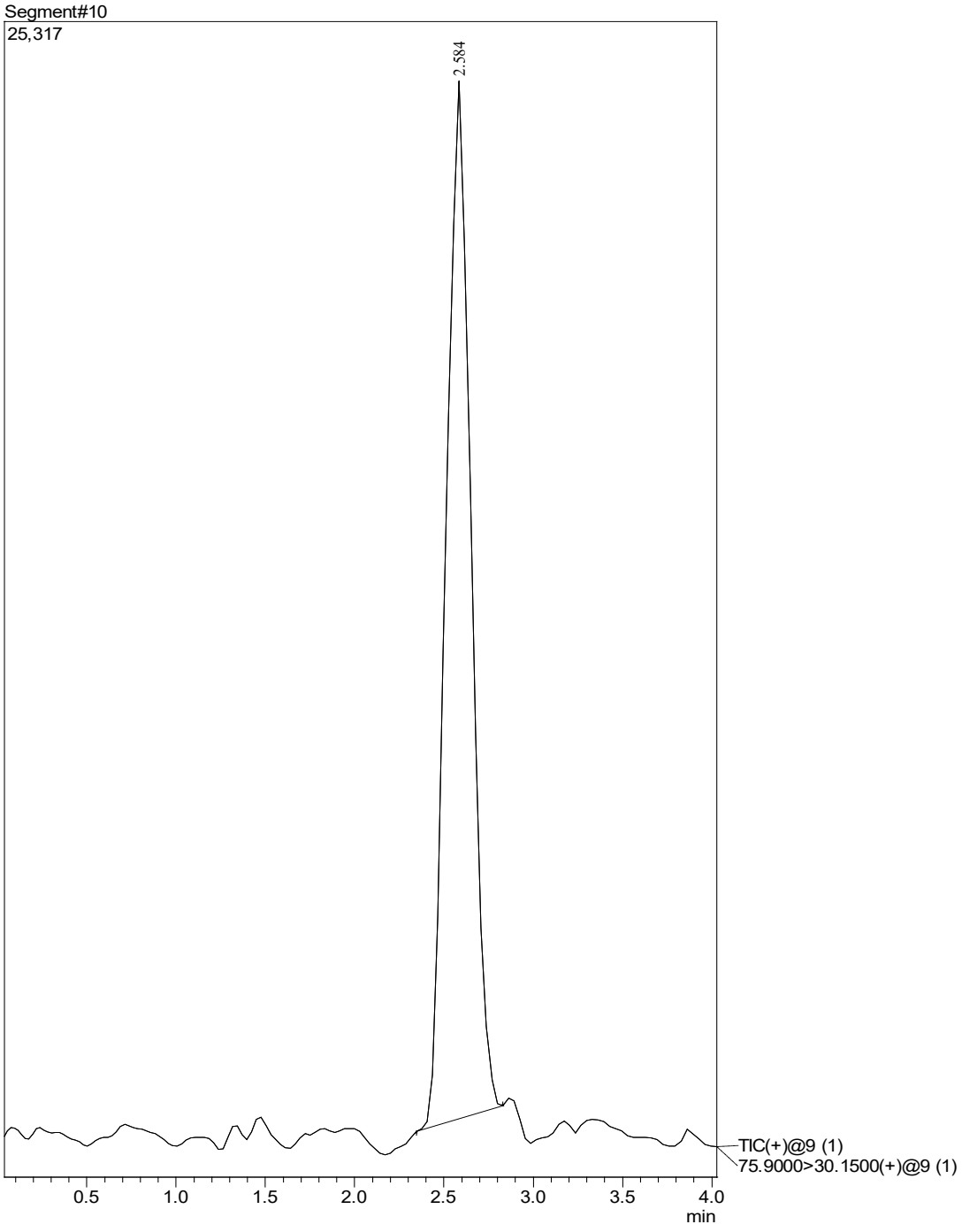
ii. Alanine


Segment#5
68,285
2.621
TIC(+)@5 (1)
89.9000>44.1000(+)@5 (1)
0.5
1.0
1.5
2.0
2.5
3.0
3.5
min

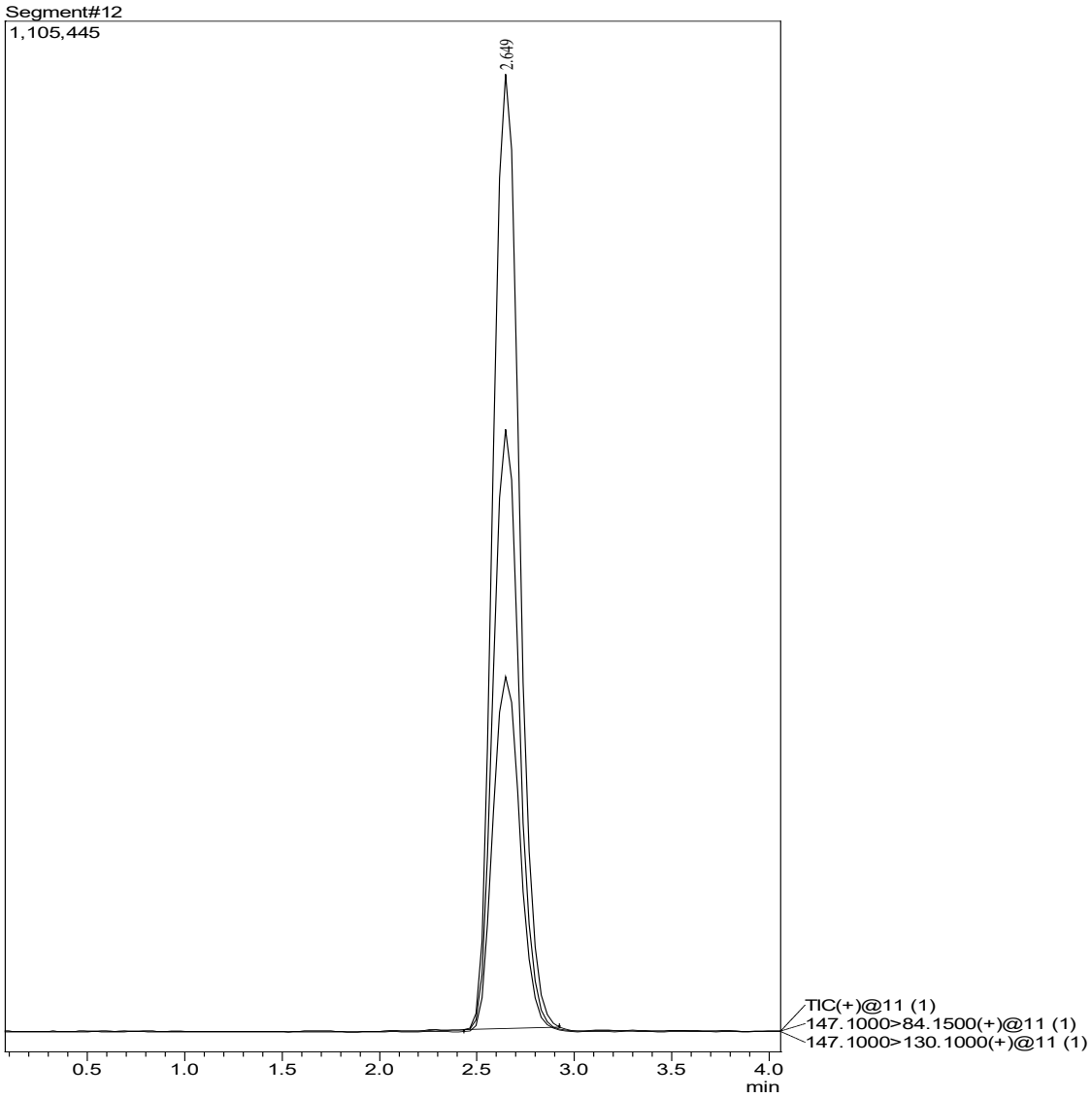
iii. 4-Hydroxyproline



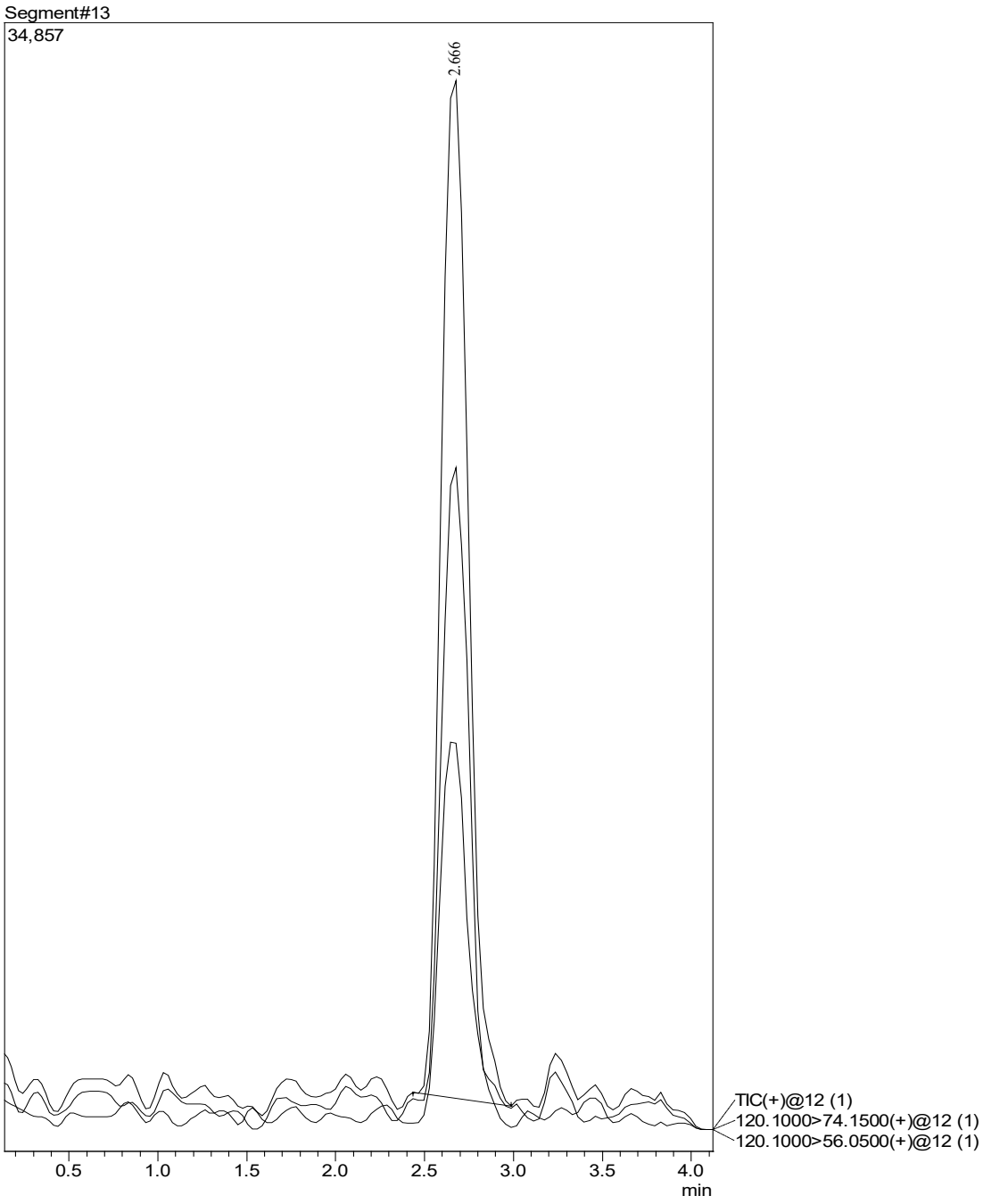
iv. Glycine


Segment#10
25,317
2.584
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
min
TIC(+)@9 (1)
75.9000>30.1500(+)@9 (1)

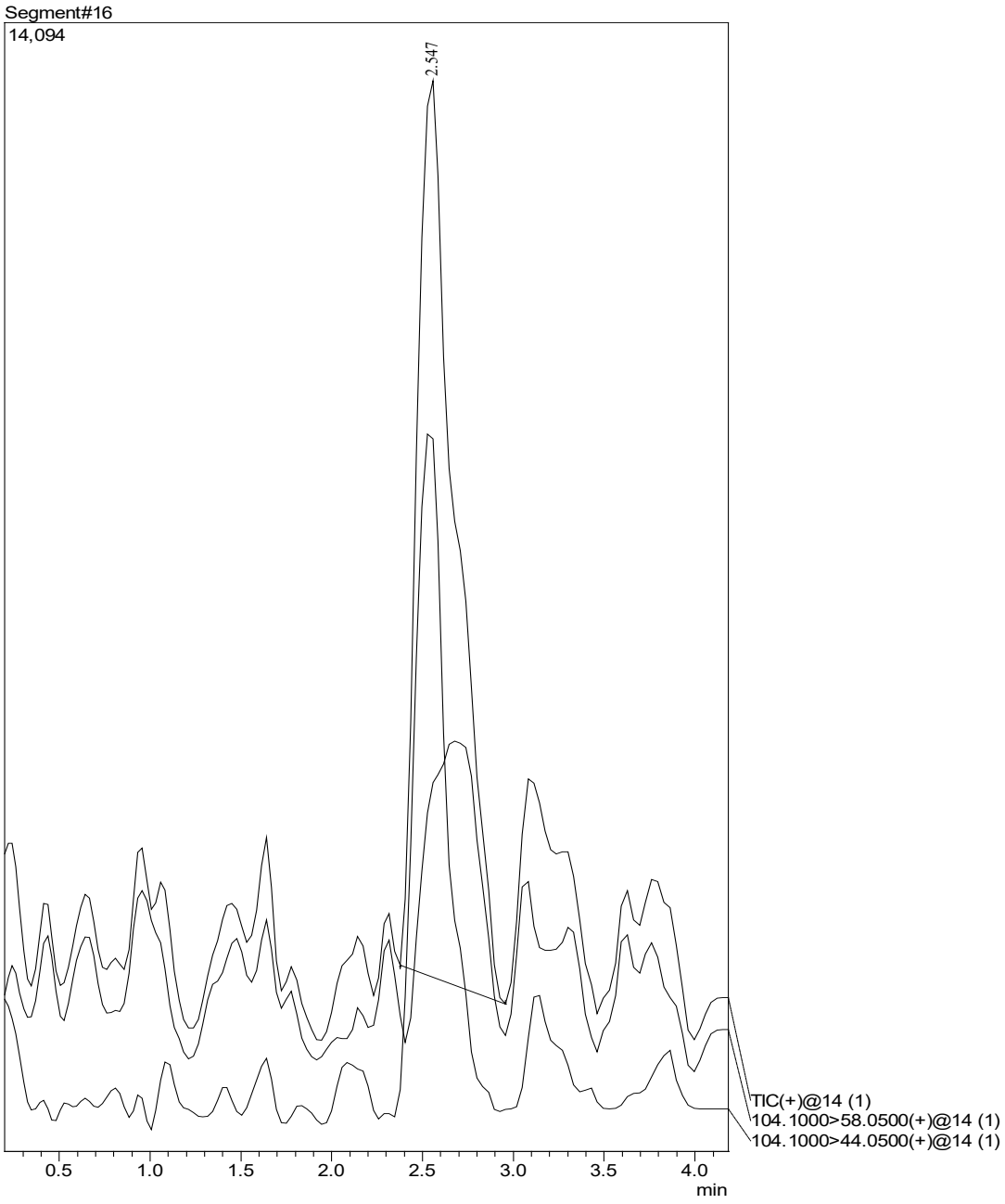
v. Glutamine


Segment#12
1,105,445
2.649
TIC(+)@11 (1)
147.1000>84.1500(+)@11 (1)
147.1000>130.1000(+)@11 (1)
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
min

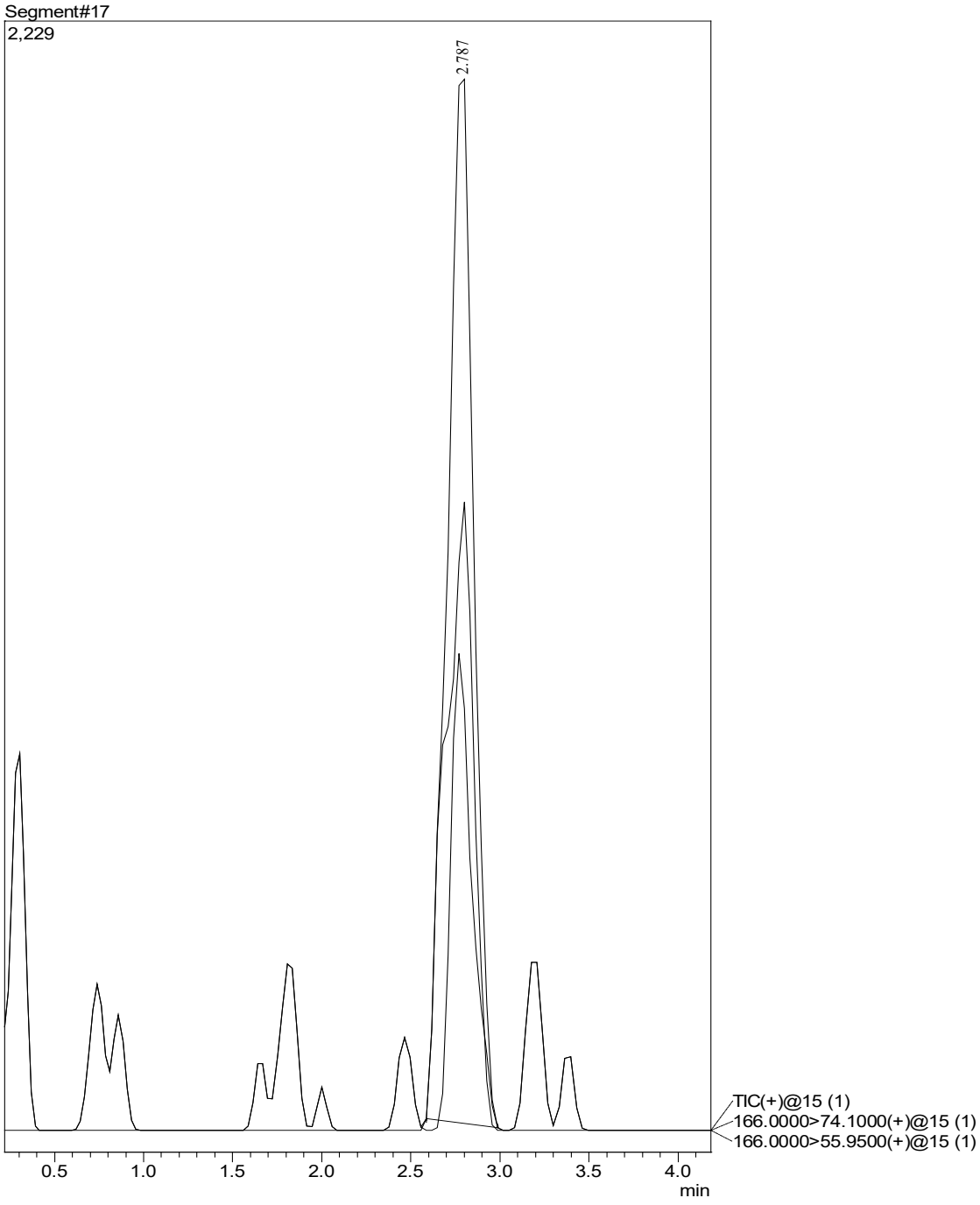
vi. Threonine



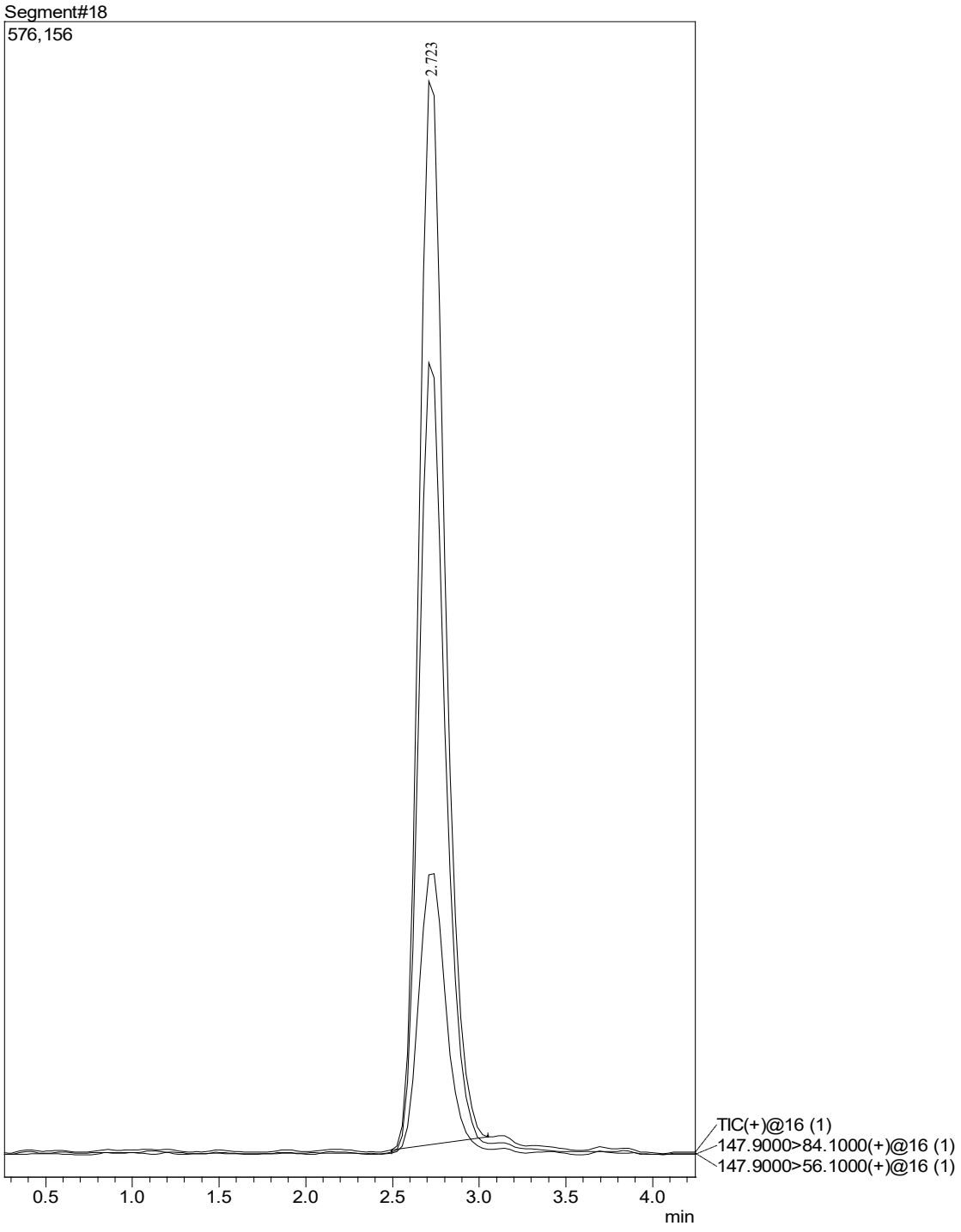
vi. Dimethylglycine



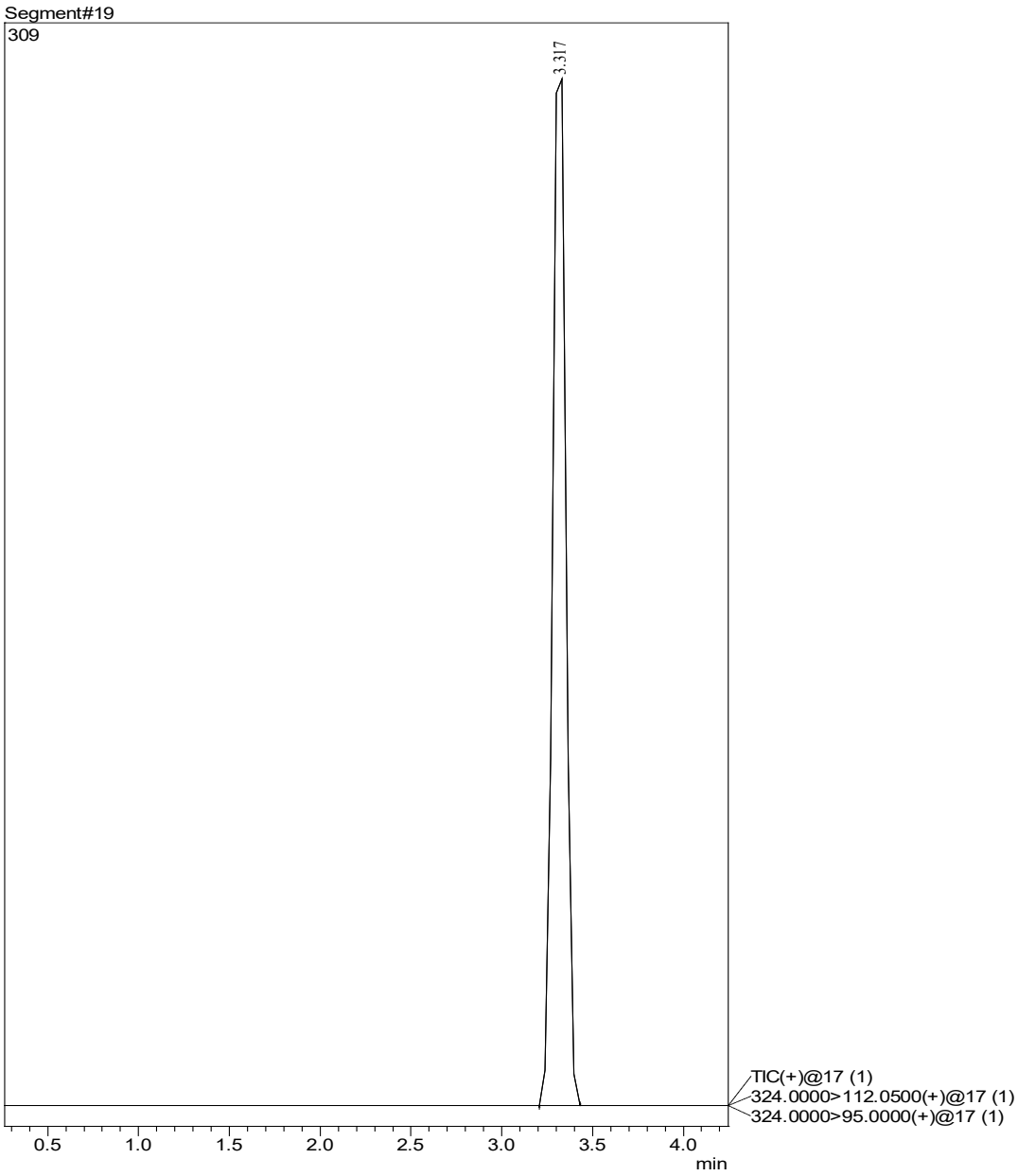
vii. Methionine sulphoxide



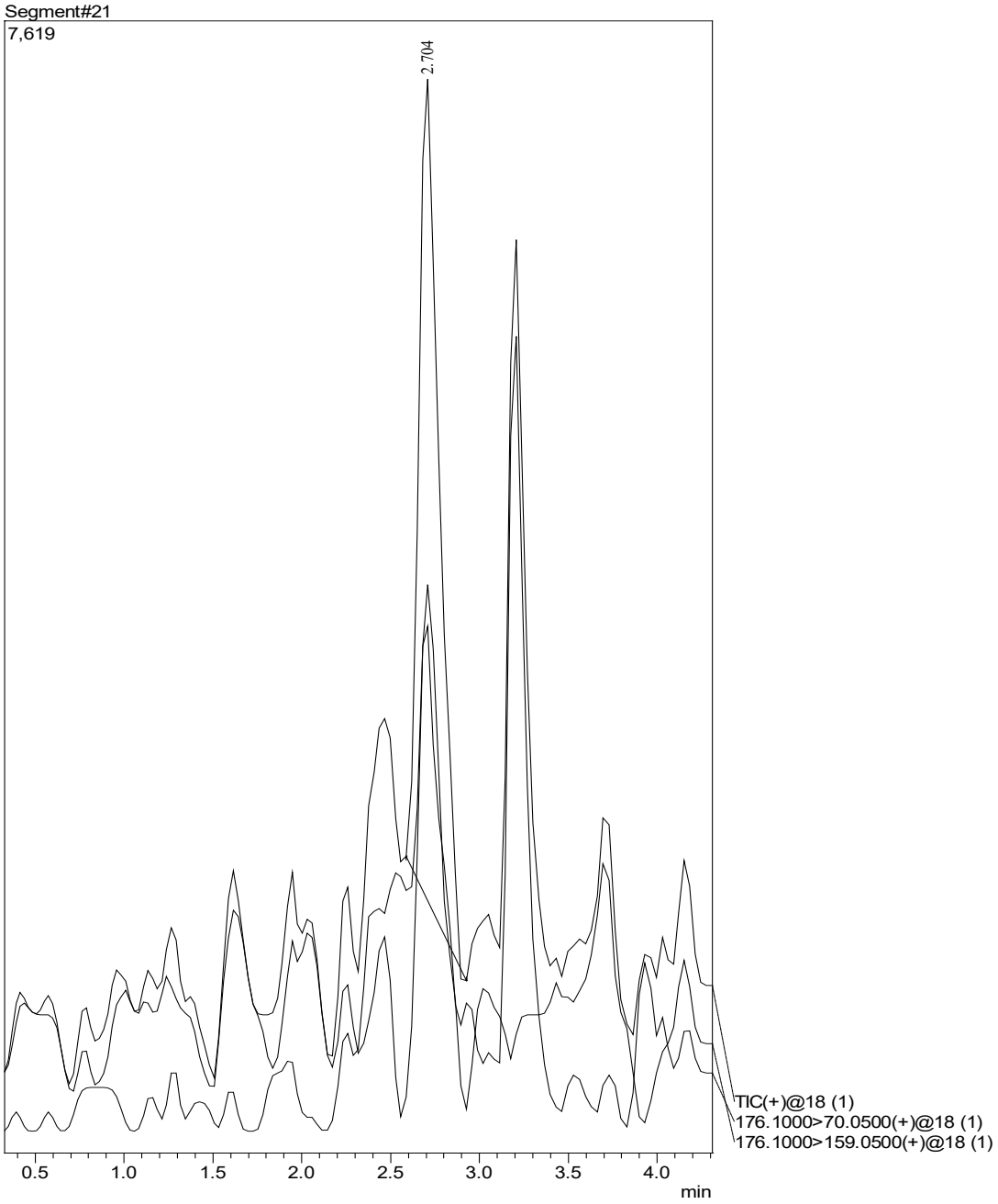
viii. Glutamic acid



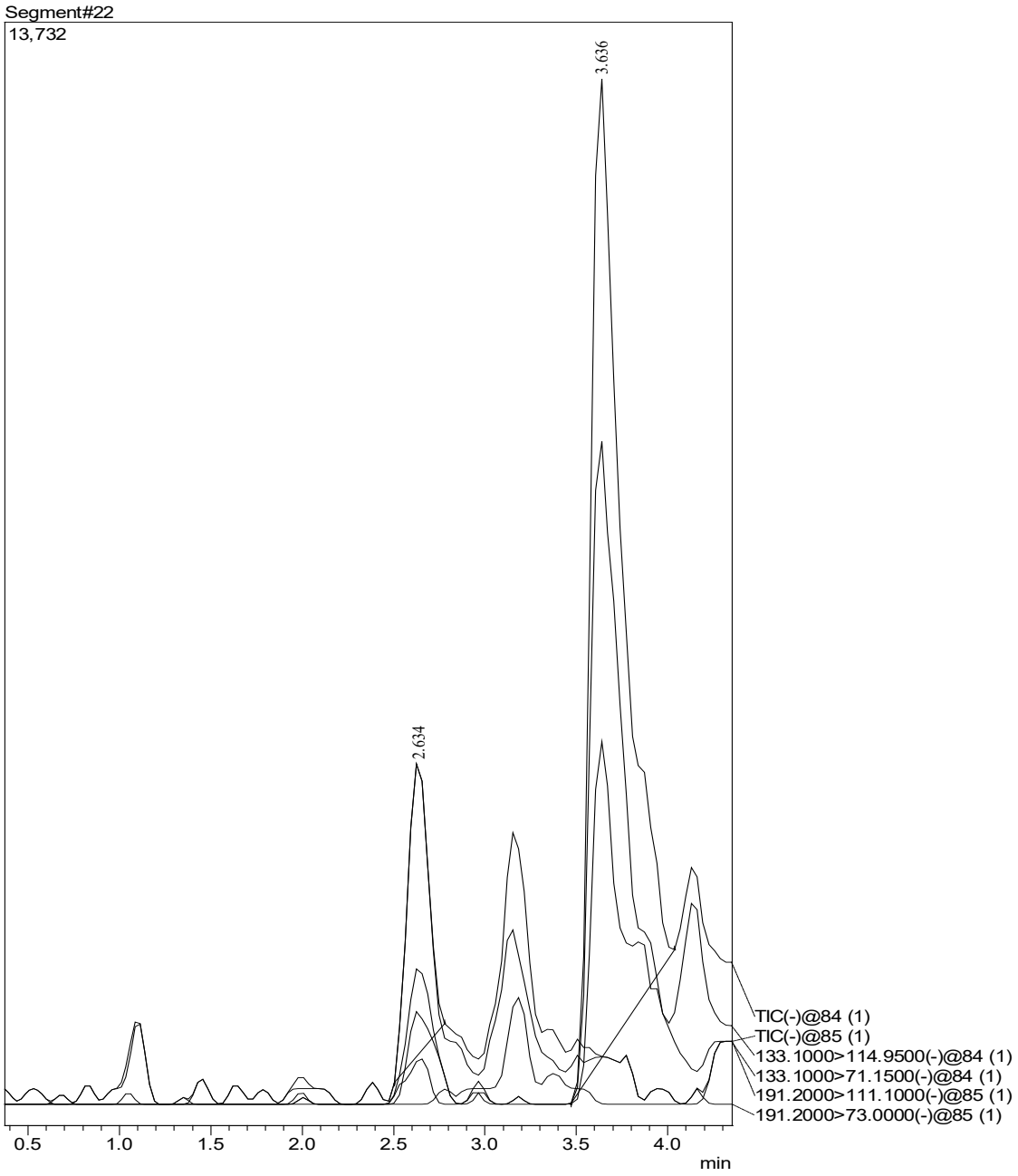
ix. Cytidine monophosphate



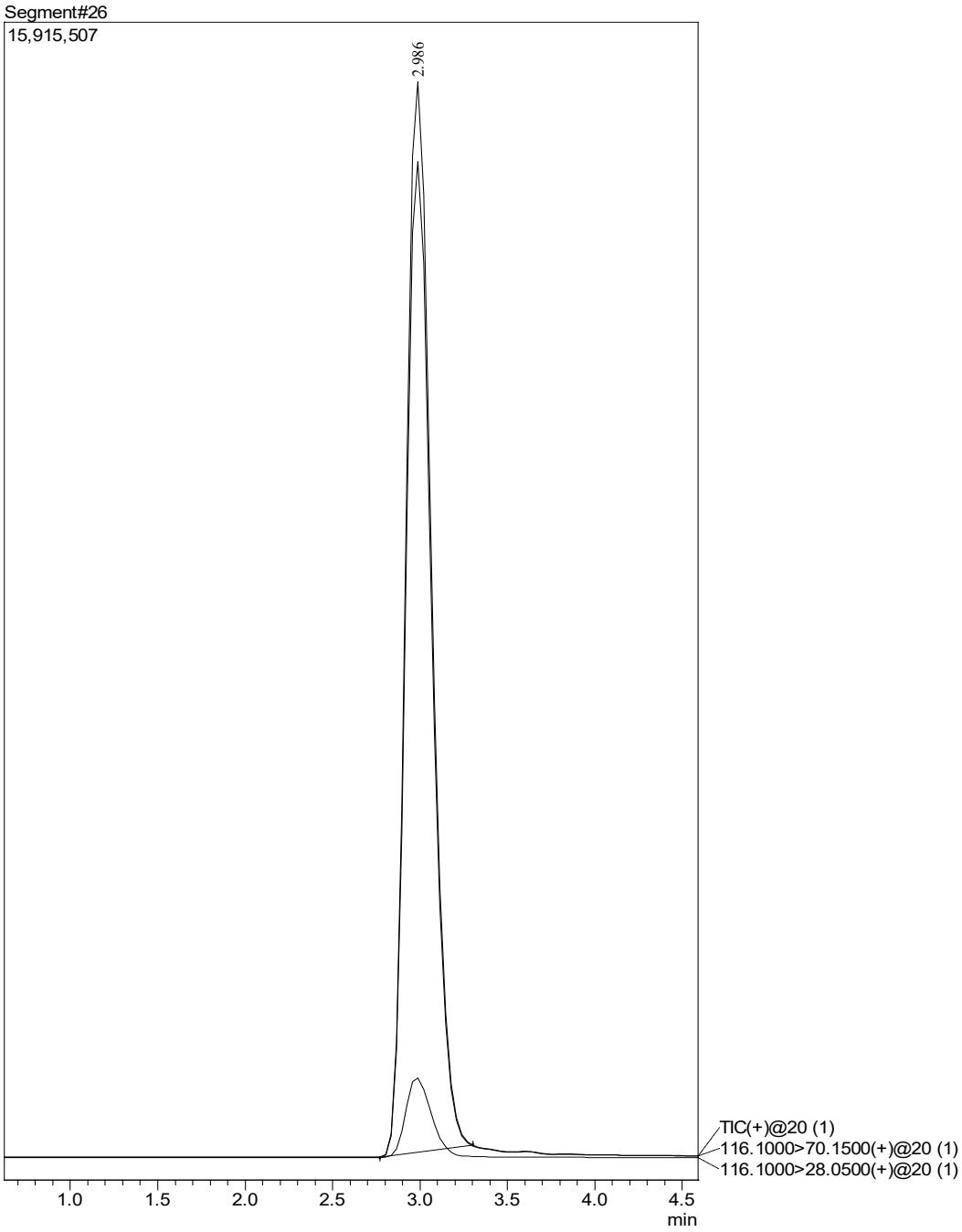
x. Citrulline



xi. Malic acid and Isocitric acid



xii. Proline


Segment#26
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116.1000>70.1500(+)@20 (1)
116.1000>28.0500(+)@20 (1)
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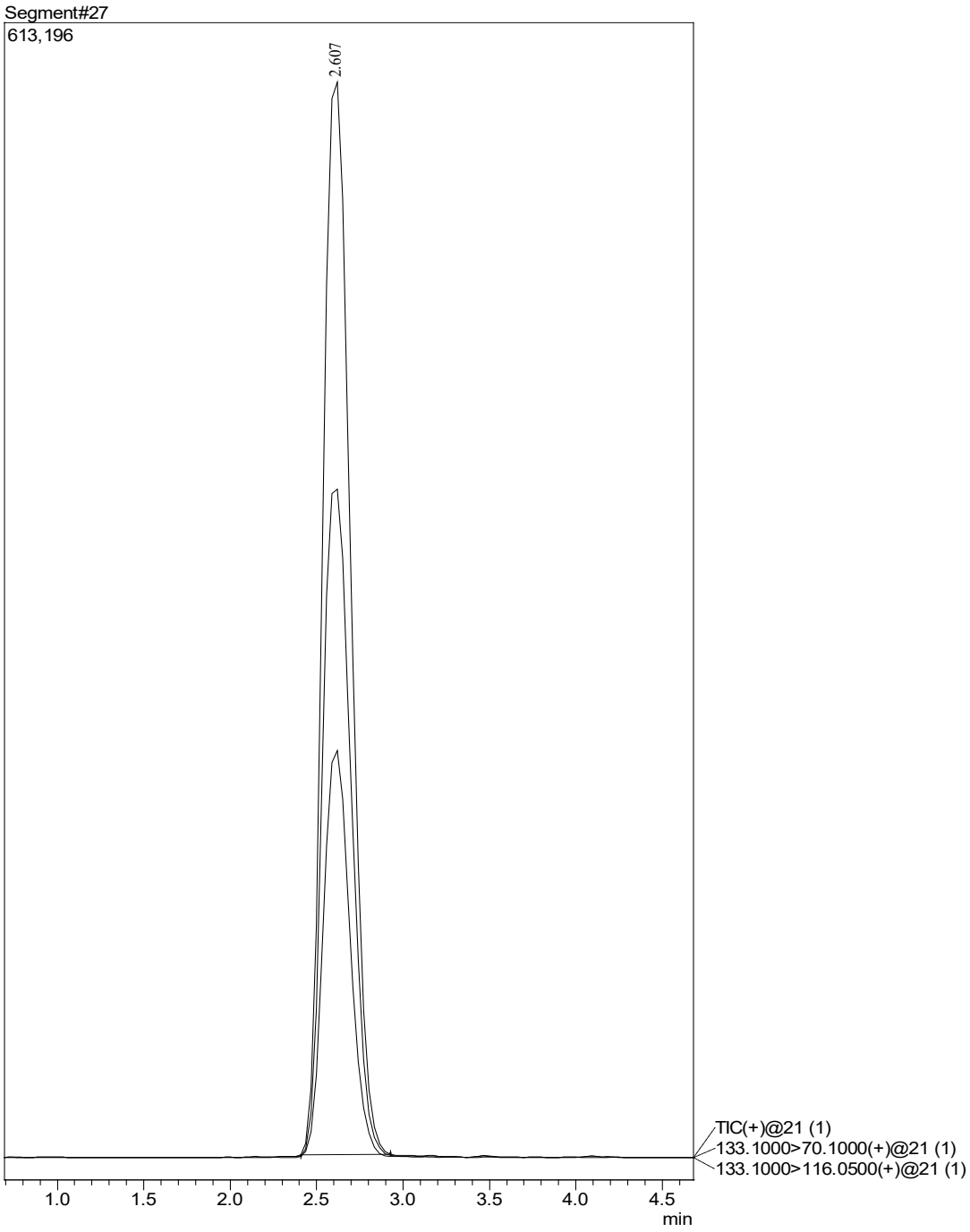
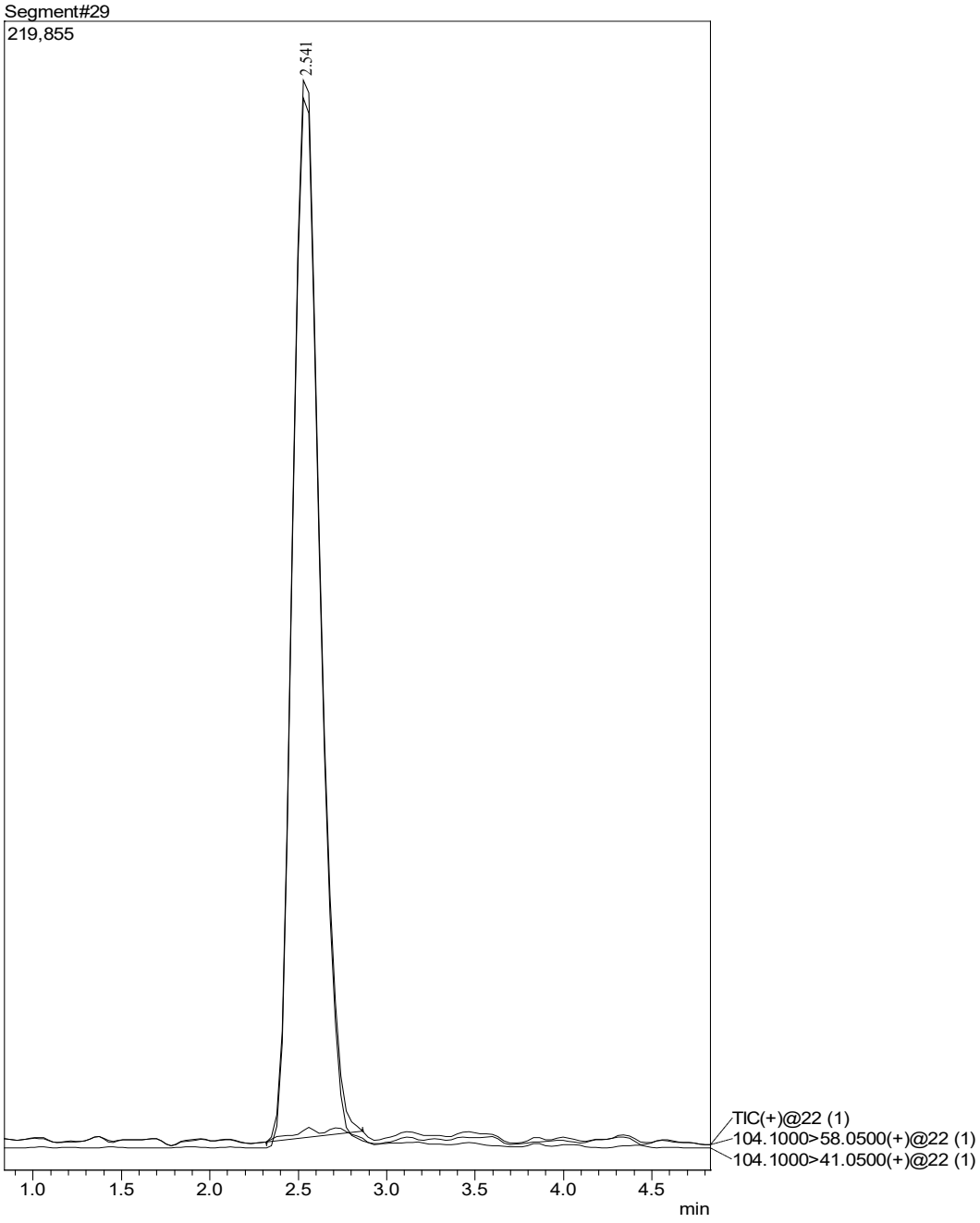
1.5
2.0
2.5
3.0
3.5
4.0
4.5
min

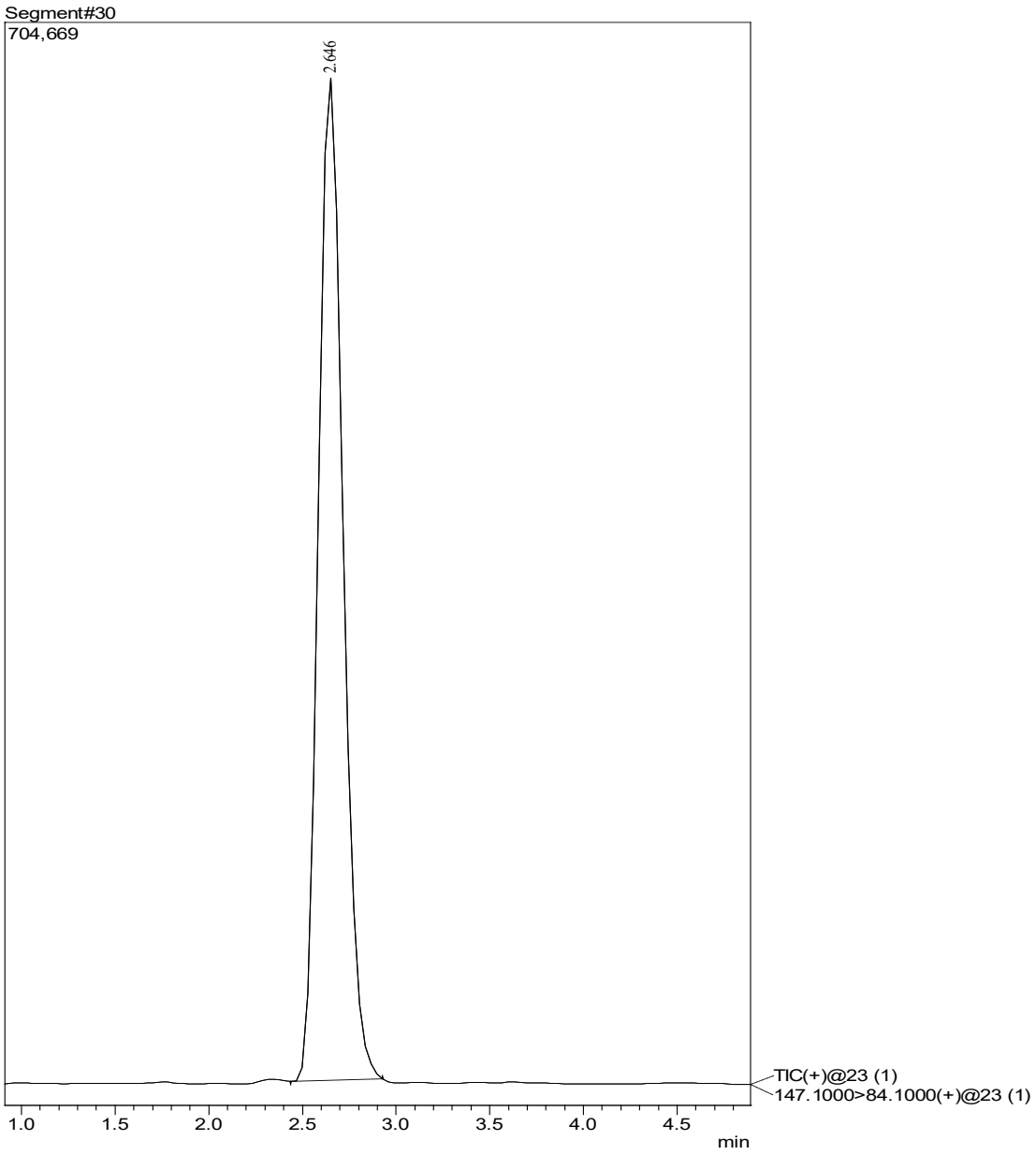
Ornitine


Segment#27
613,196
2.607
TIC(+)@21 (1)
133.1000>70.1000(+)@21 (1)
133.1000>116.0500(+)@21 (1)
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
min

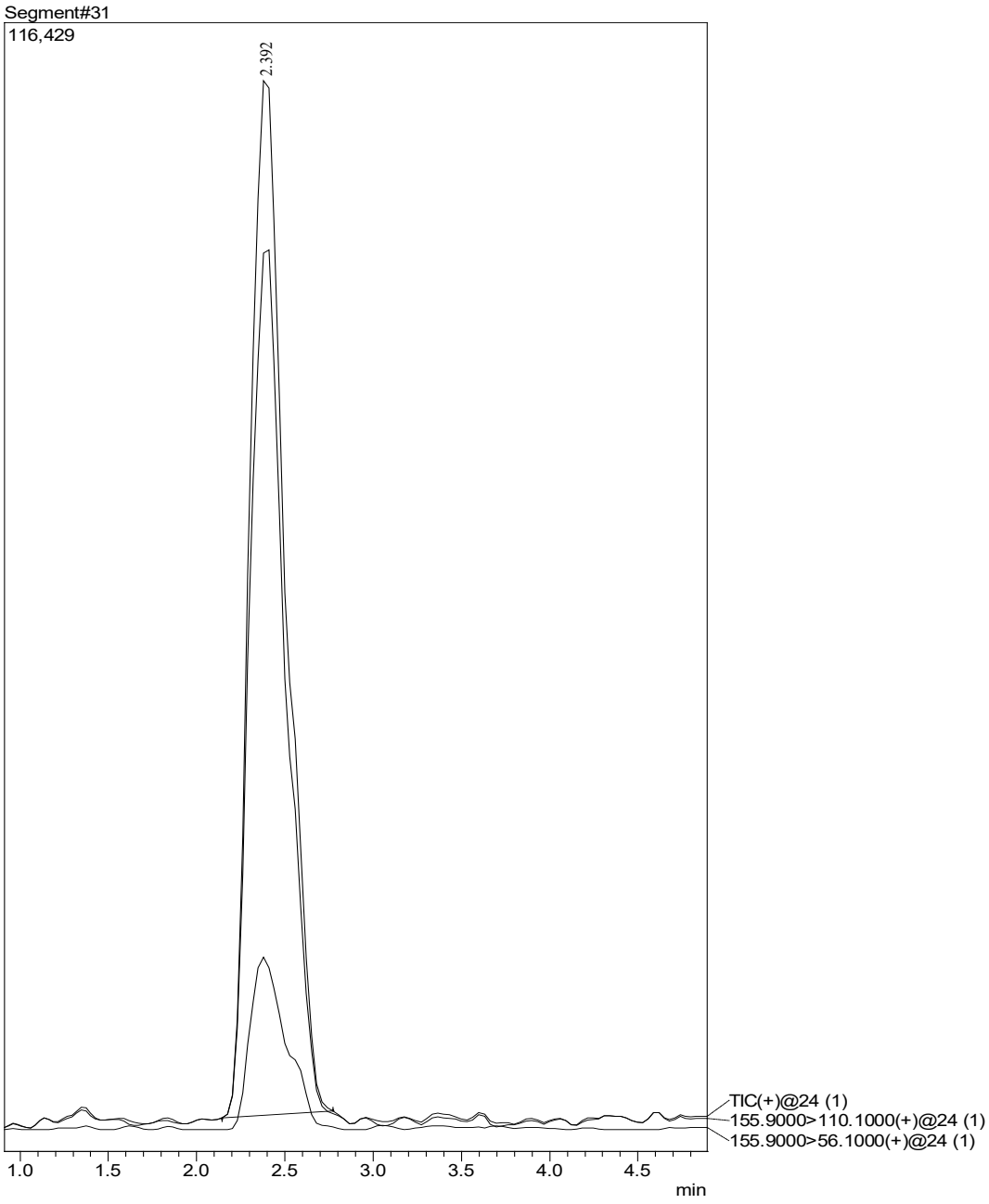
xiii. 2-Aminobutyric acid



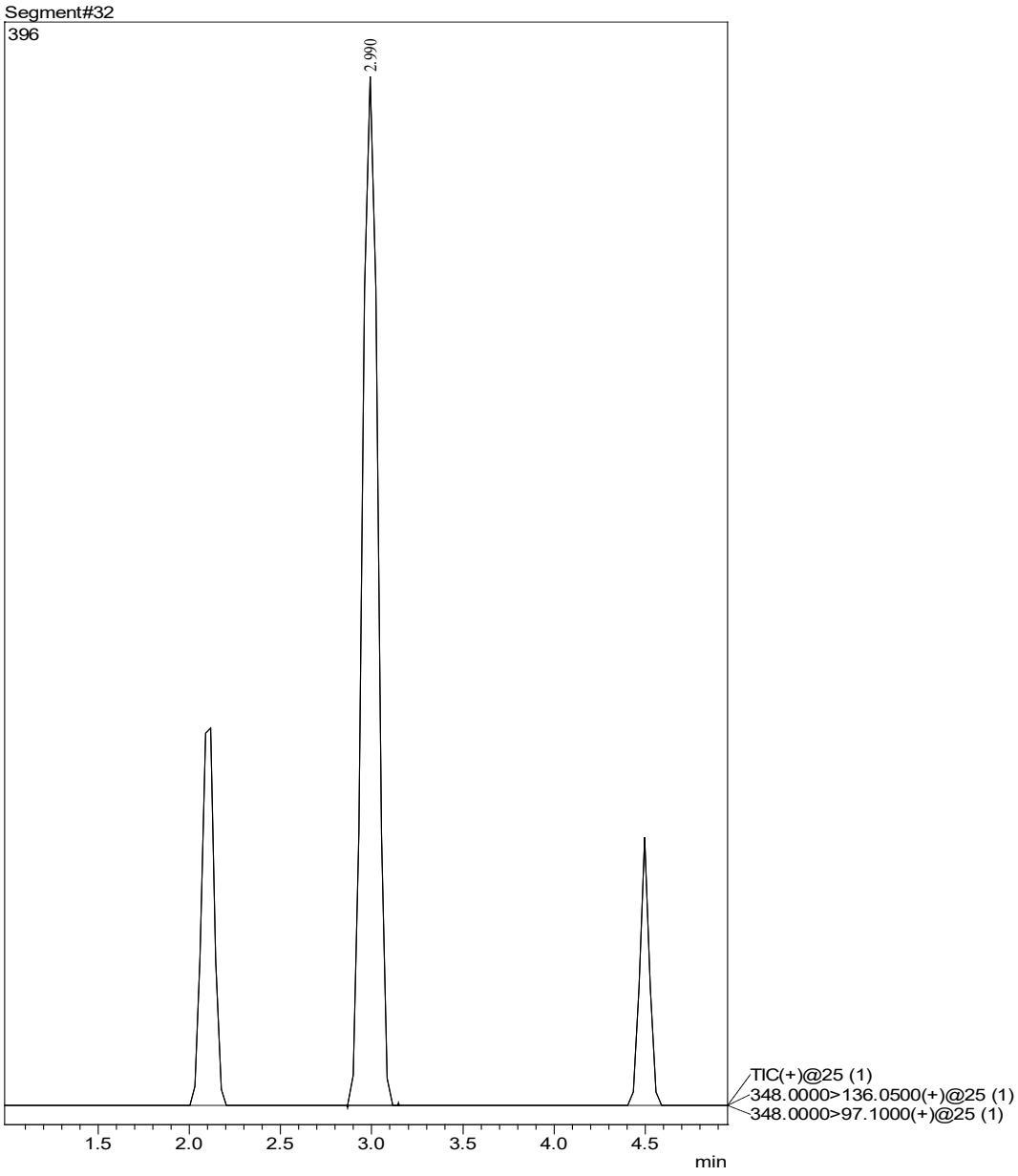
xiv. Lysine


Segment#30
704,669
2.646
TIC(+)@23 (1)
147.1000>84.1000(+)@23 (1)
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
min

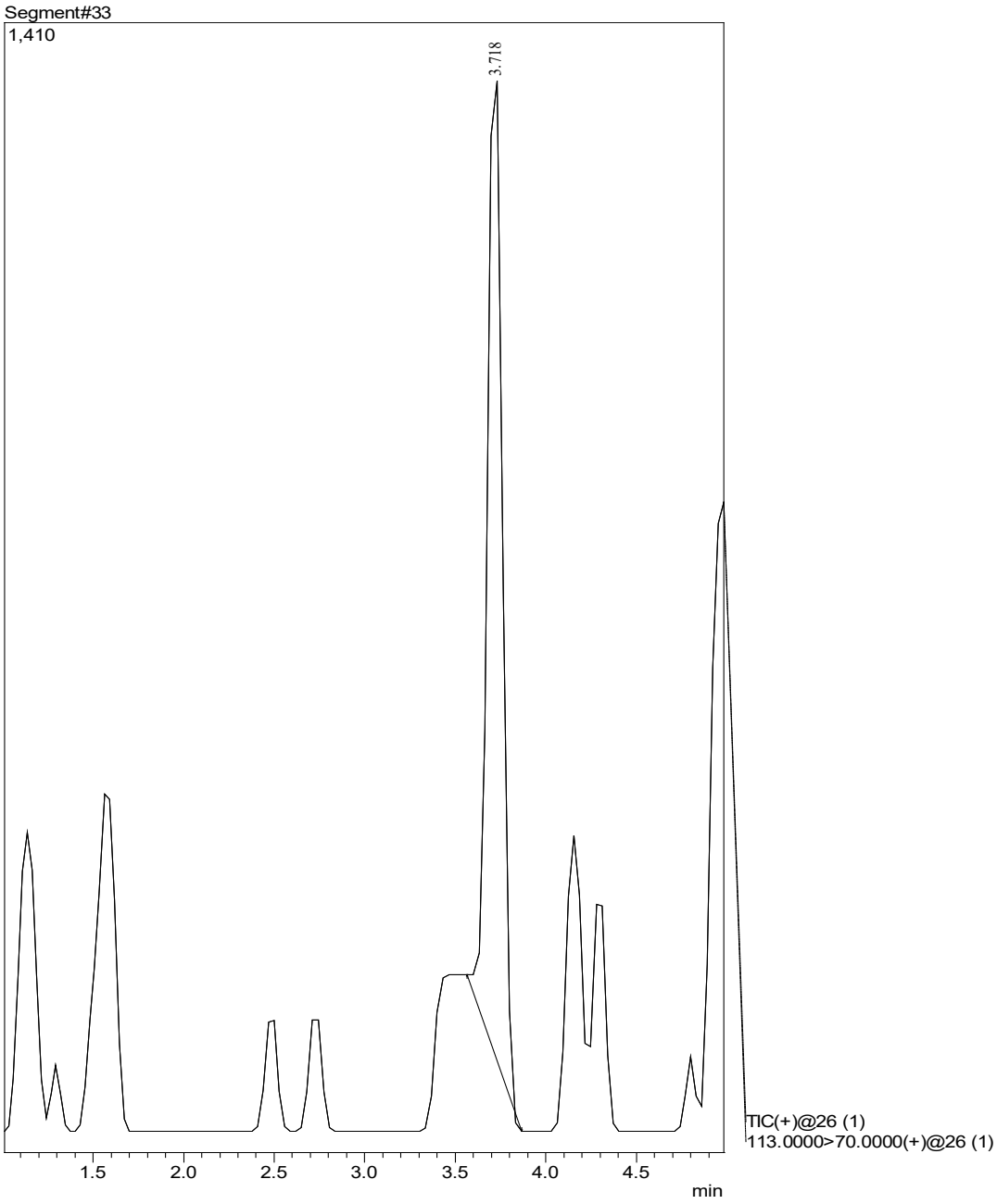
xv. Histidine



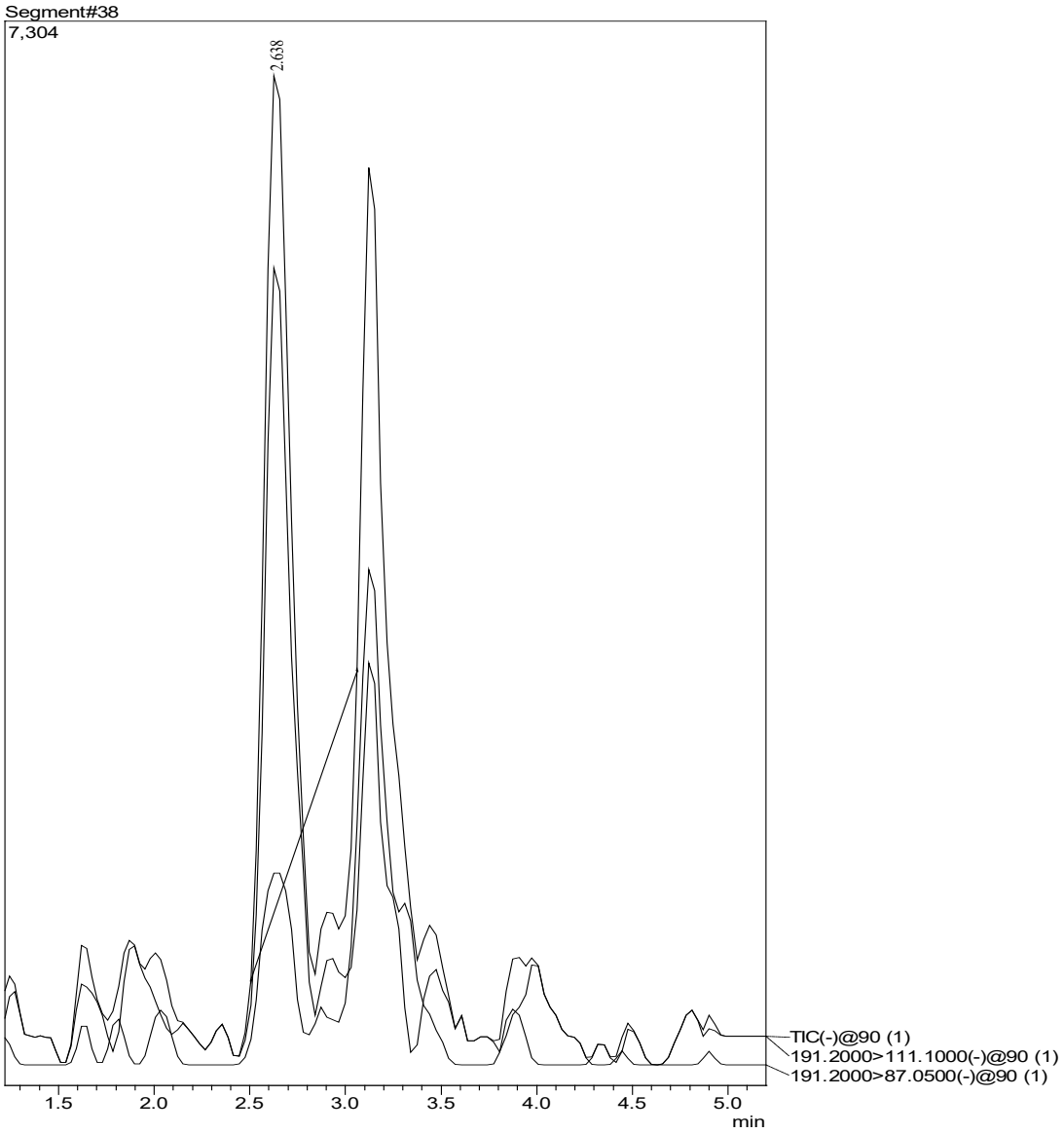
xvi. Adenosine monophosphate



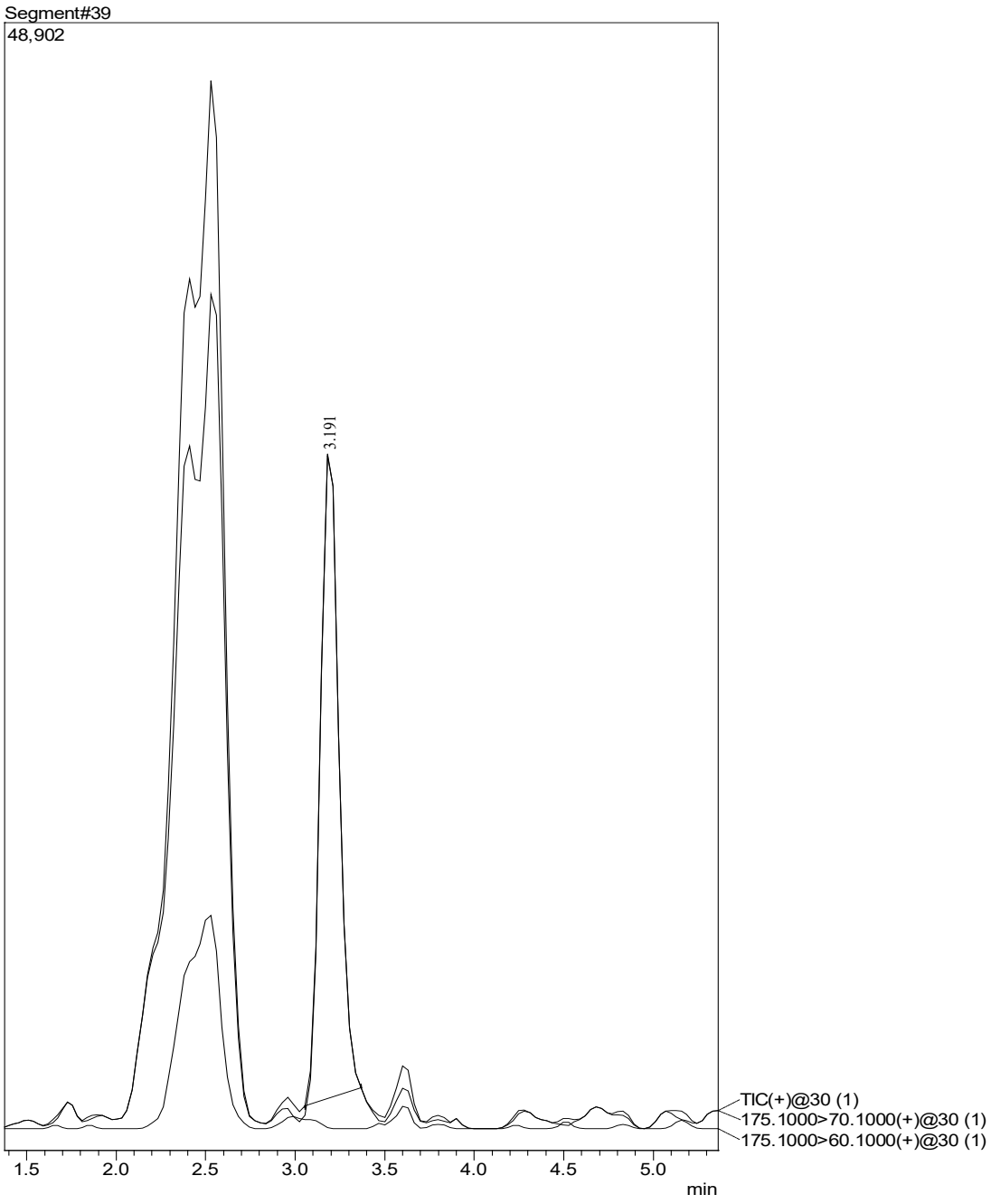
xvii. Uracil


Segment#33
1,410
3.718
1.5
2.0
2.5
3.0
3.5
4.0
4.5
min
TIC(+)@26 (1)
113.0000>70.0000(+)@26 (1)

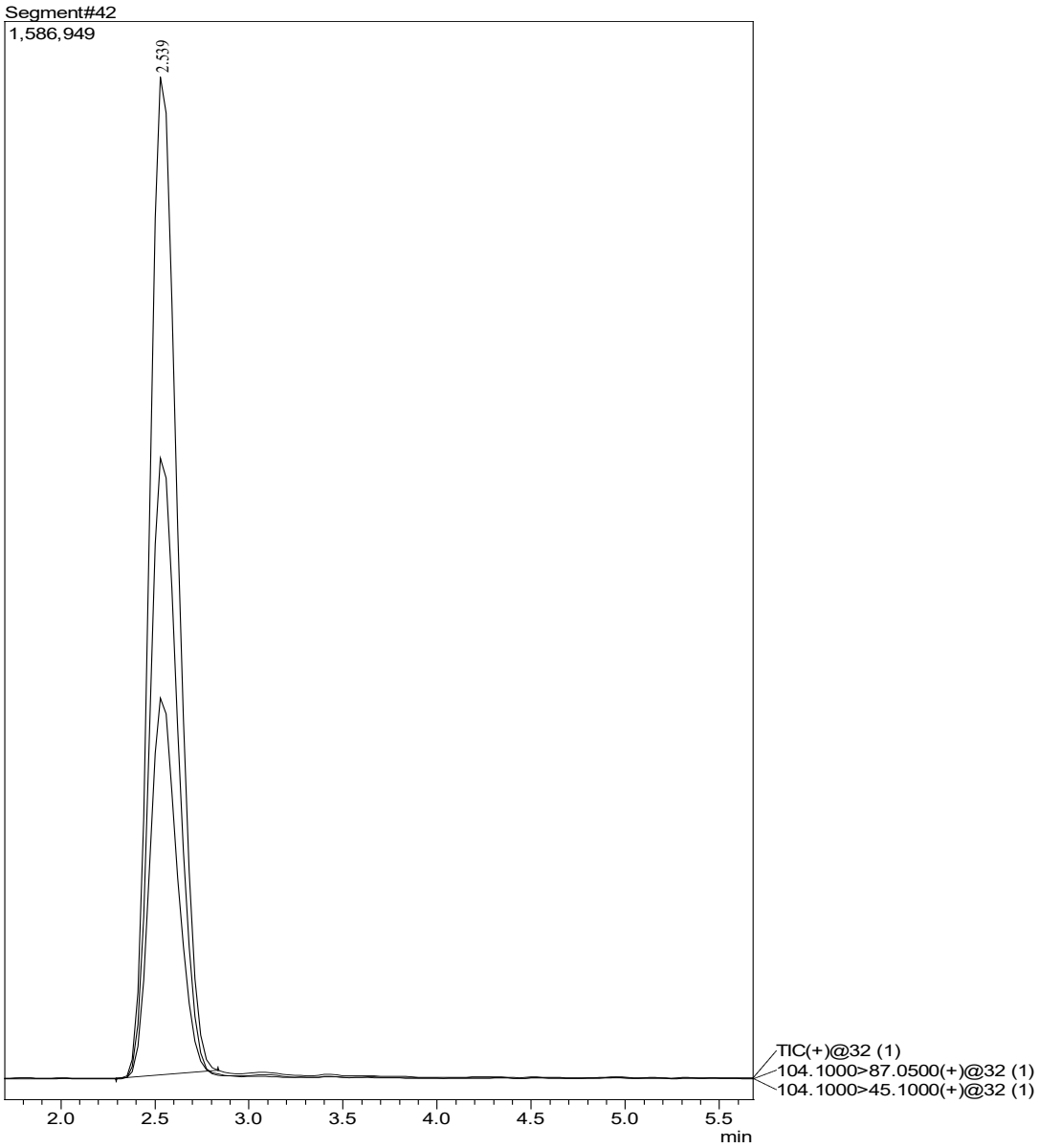
xviii. Citric acid



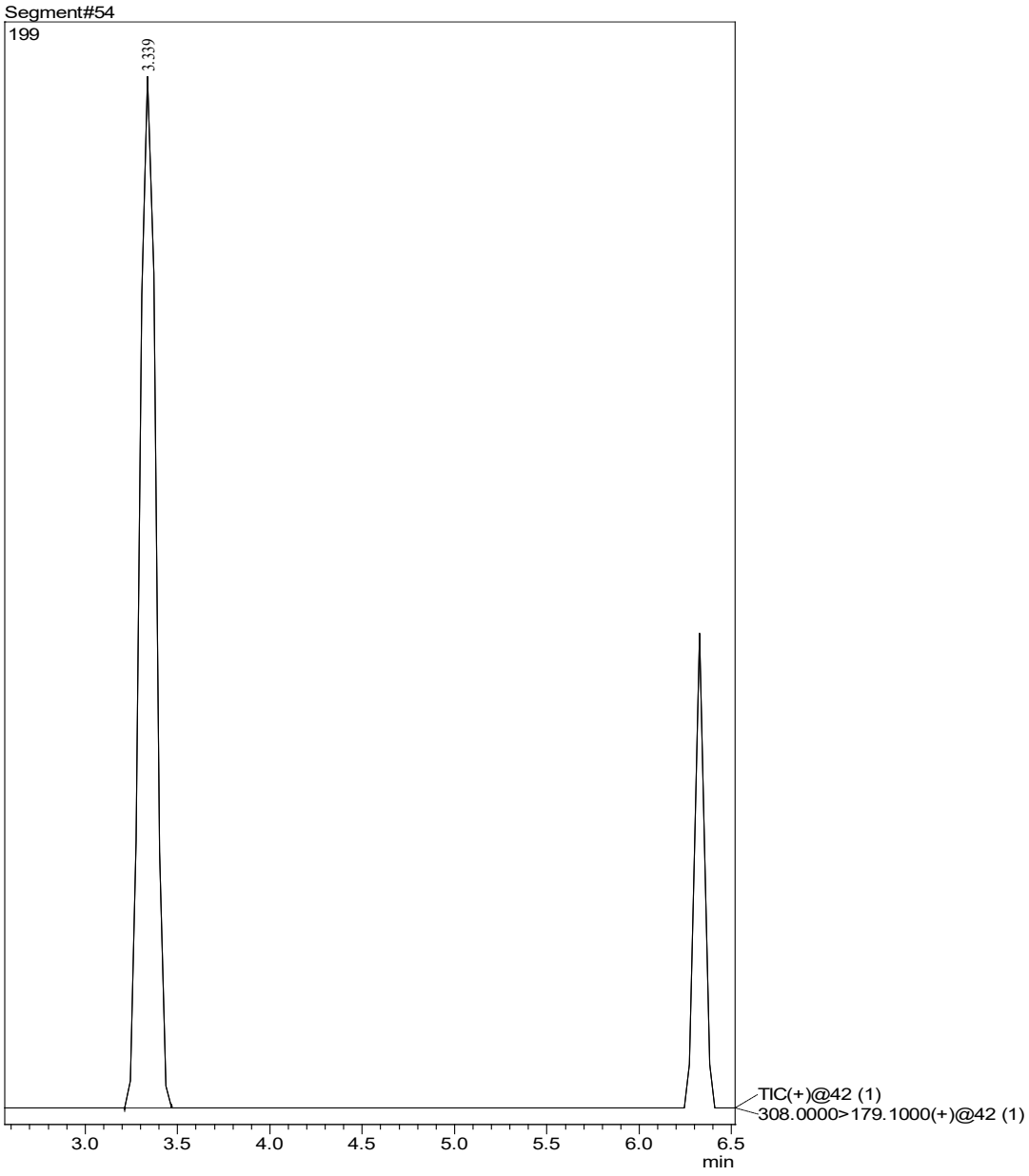
xix. Arginine



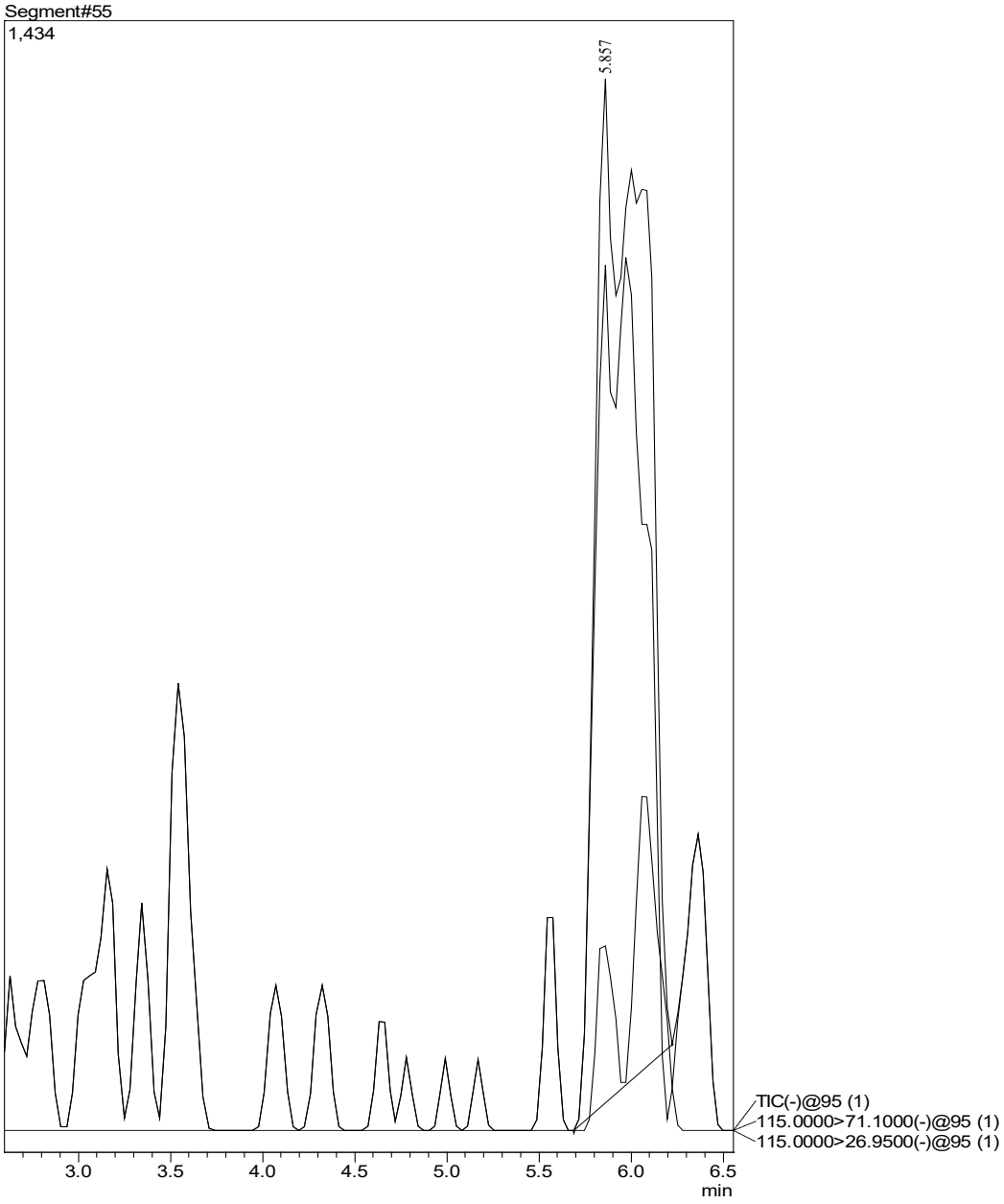
xx. 4-Aminobutyric acid



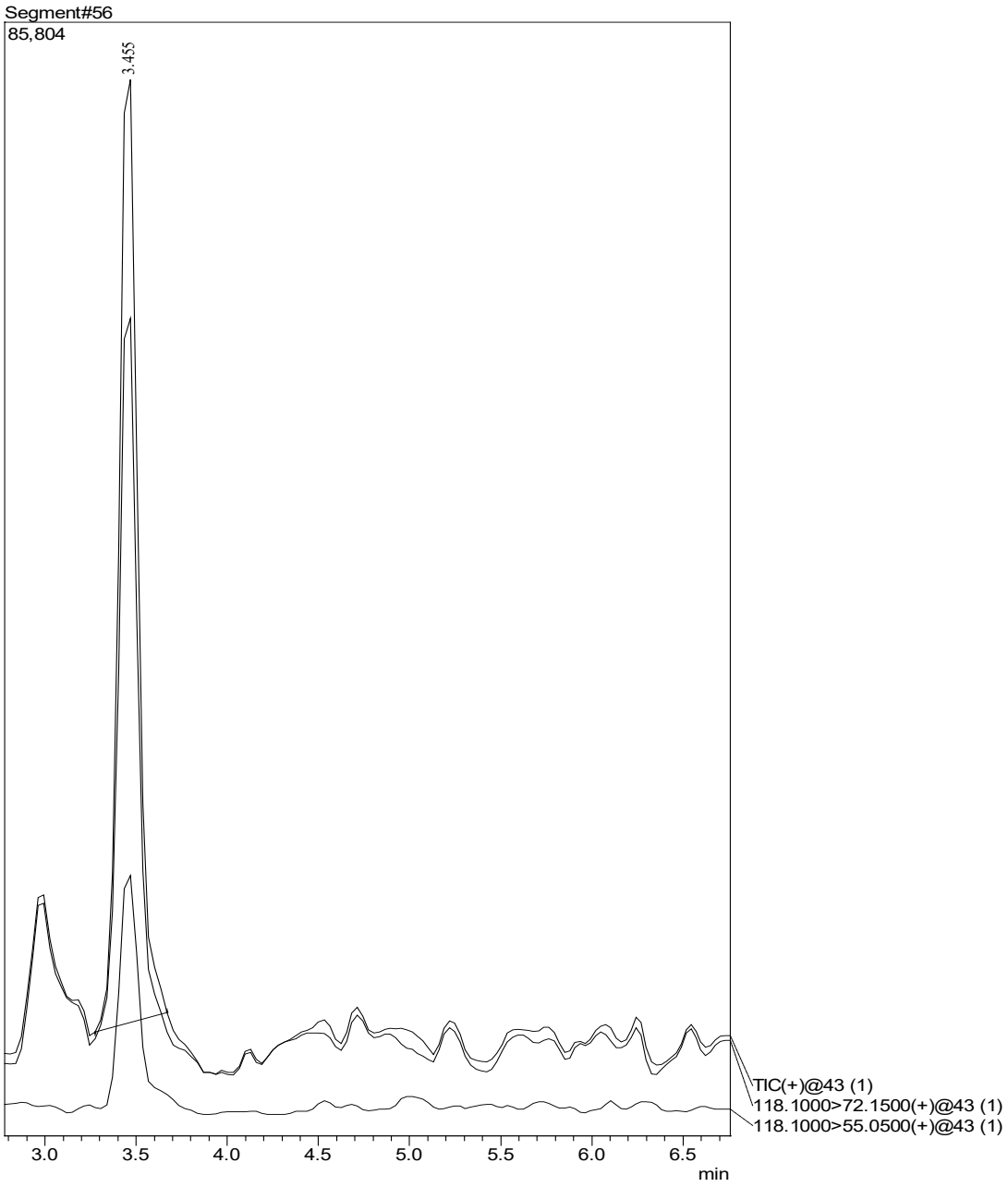
xxi. Glutathione



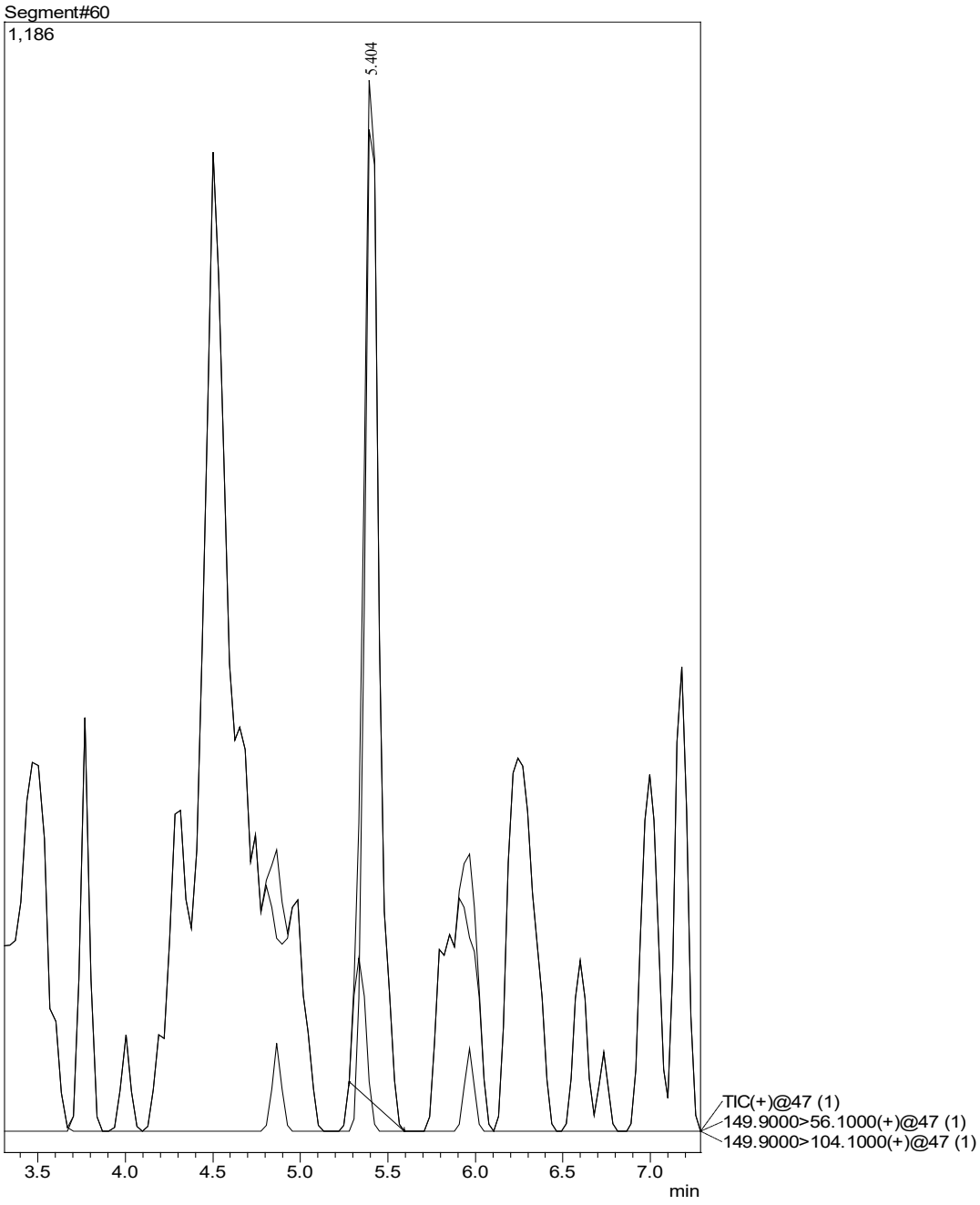
xxii. Fumaric acid



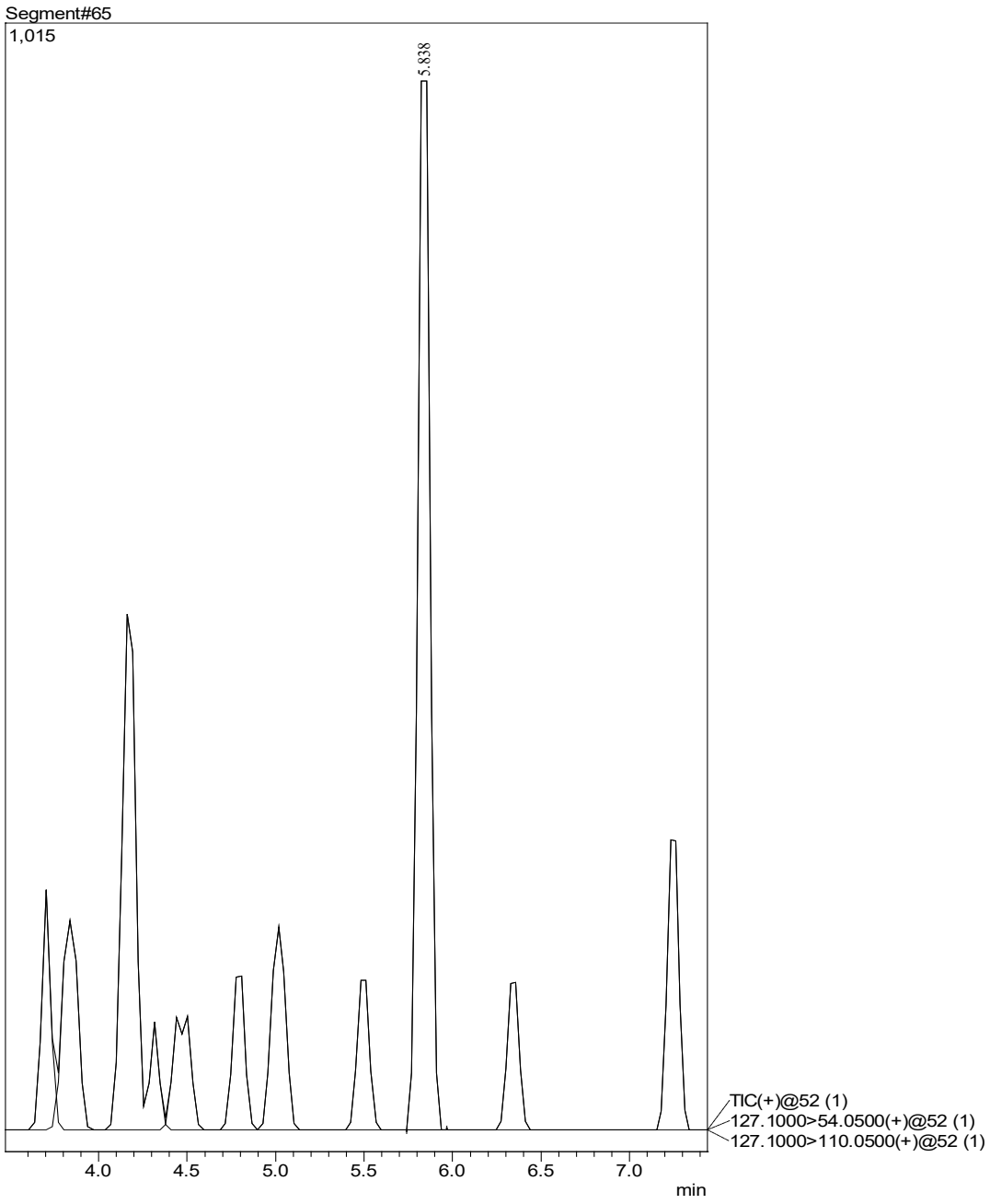
xxiii. Valine



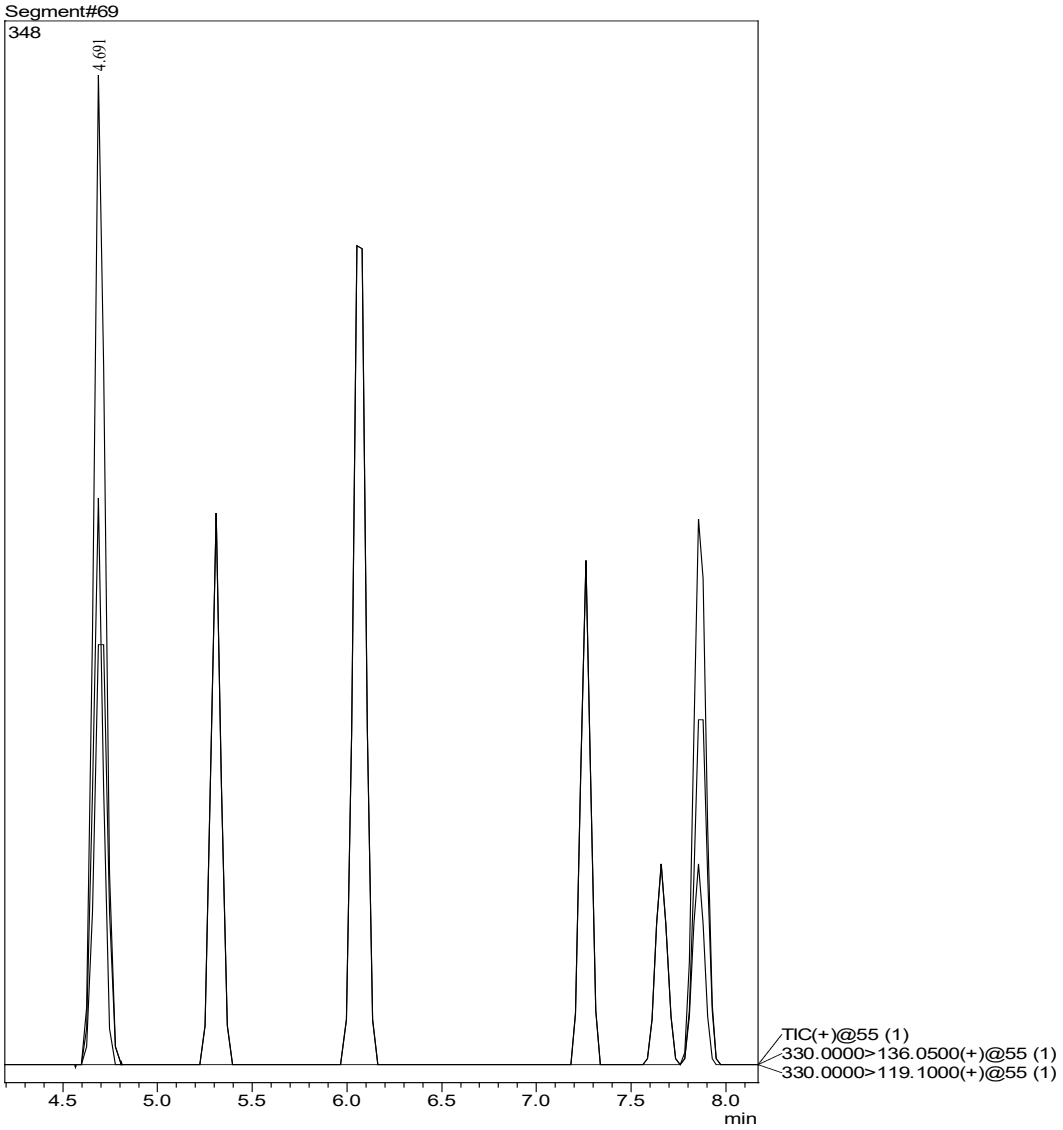
xxiv. Methionine



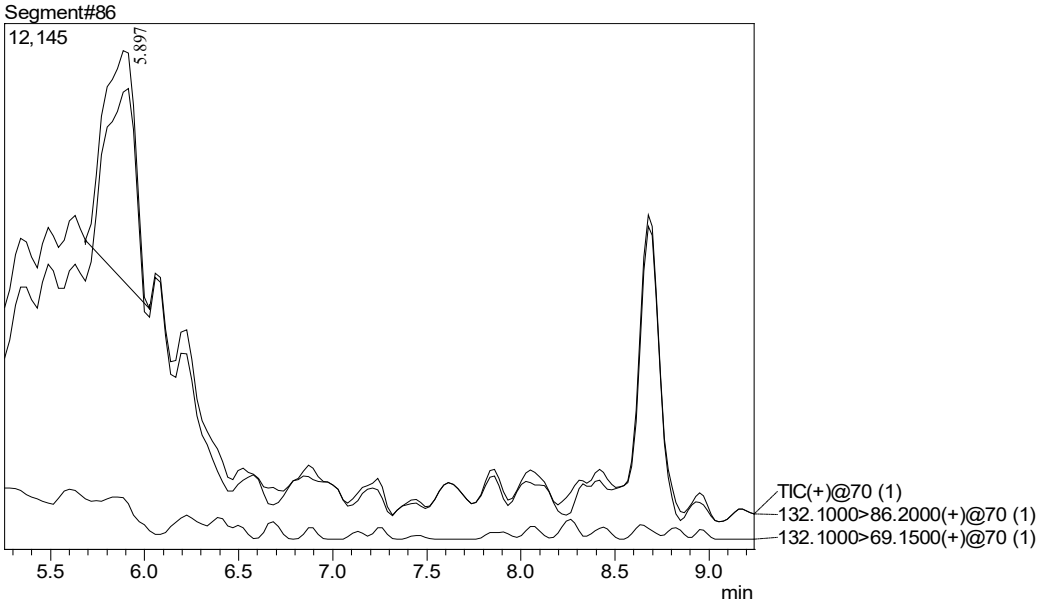
xxv. Thymine


Segment#65
1,015
5.838
TIC(+)@52 (1)
127.1000>54.0500(+)@52 (1)
127.1000>110.0500(+)@52 (1)
4.0
4.5
5.0
5.5
6.0
6.5
7.0
min

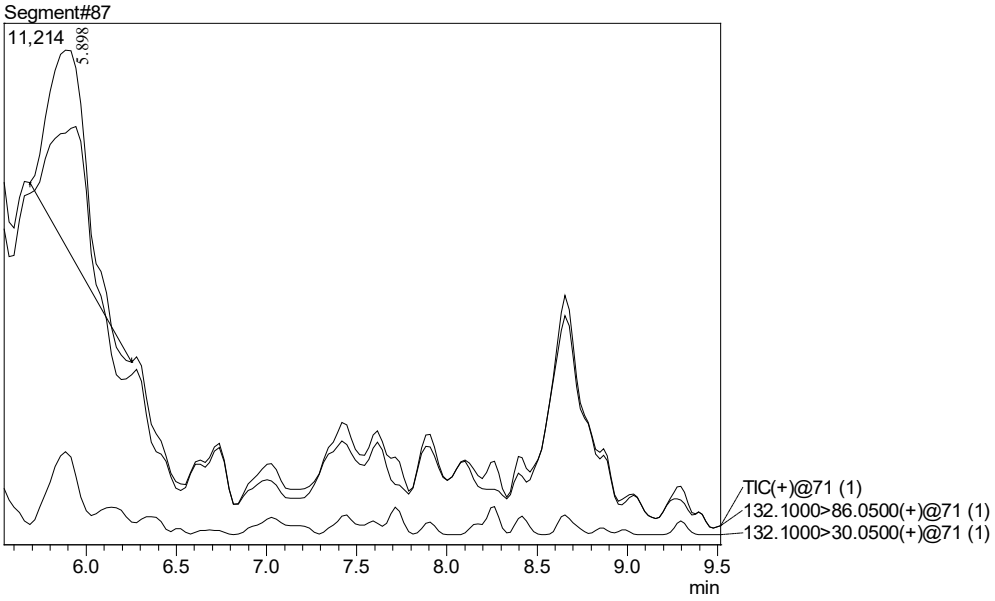
xxvi. Adenosine 3',5'-cyclic monophosphate



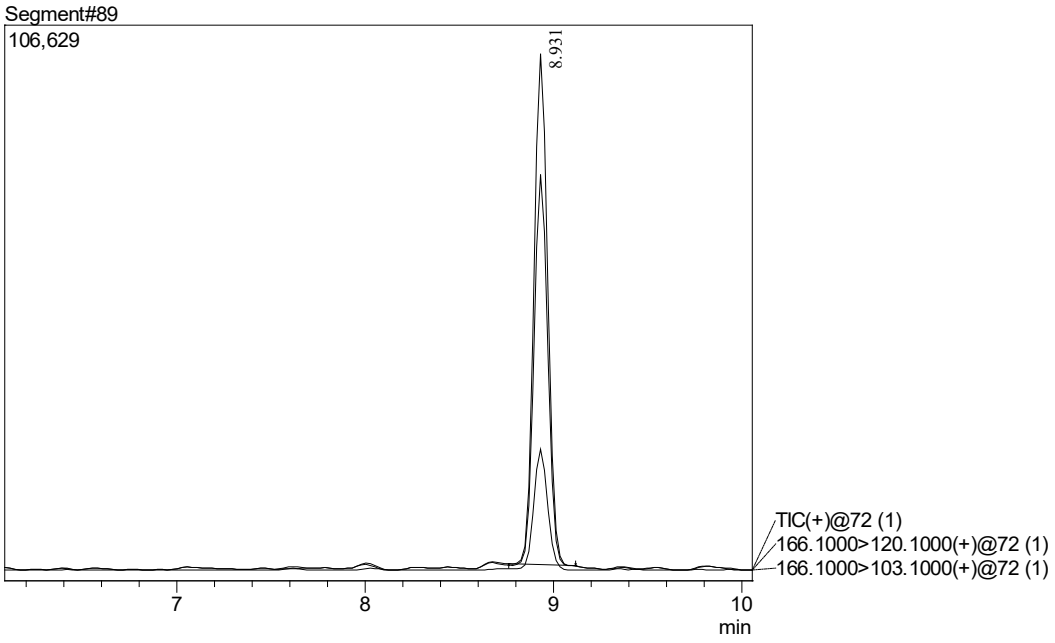
xxvii. Isoleucine



xxviii. Leucine



xxix. Phenylalanine



xxx. Tryptophan