THE *IN-VITRO* EFFECT OF *LACHESIS MUTAS* 6CH, 9CH AND 12CH ON THE COAGULATION OF BLOOD

A Mini Dissertation presented to the Faculty of Health Sciences, Technikon Witwatersrand, in partial fulfilment of the requirement for the degree of Master of Technology: Homoeopathy

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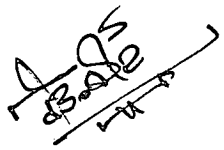
DECLARATION

I declare that this Mini Dissertation is my own, unaided work. It is being submitted for the Master of Technology at the Technikon Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other Technikon or University.



(Signature of Candidate)

9th day of September 2002

ABSTRACT

Haemostasis refers to the prevention of spontaneous bleeding and control of traumatic haemorrhage. The haemostatic mechanism consists essentially of two proteolytic reactions. The first is the clotting mechanism, the end product of which is thrombin. The second is the fibrinolytic mechanism, the end product of which is plasmin.

Snake venoms are rich sources of enzymes, which induce a wide range of pharmacological activities, including anticoagulant and haemorrhagic effects. It has been established that *Lachesis mutas*, a remedy prepared from the venom of a pit viper snake of South America, has an effect on the haemostatic mechanism. *Lachesis mutas* is used extensively in homoeopathy as a blood coagulator. The toxicological symptoms of *Lachesis mutas* occur from the disorganisation of the blood.

This *in-vitro* study aimed to determine the effect of homoeopathically prepared *Lachesis mutas* 6CH, 9CH, 12CH on the coagulation of blood. Twenty volunteers, fulfilling specified criteria were recruited. Twenty millilitres of blood was drawn from each volunteer. The blood was then centrifuged and the plasma separated into five tubes. Tube one served as the plasma control. The second tube contained saline, the third tube *Lachesis mutas* 6CH, the fourth tube *Lachesis mutas* 9CH, and *Lachesis mutas* 12CH was added into the last tube. After an incubation period of one hour at room temperature, using the Automated Coagulation Laboratory (ACL) analyzer, the following tests were run on each tube: Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT).

Statistical analysis revealed that the different homoeopathic potencies of *Lachesis mutas* did not have an effect on the coagulation of blood when tested *in-vitro*, specifically regarding the PT and APTT tests. This study did not yield substantial results. Further *in-vitro*, as well as *in-vivo*, studies need to be conducted to determine the potential therapeutic use of homoeopathic remedies on blood coagulation.

To my late dad, mother and brothers,
for being there when I needed them the most.

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CHAPTER ONE

INTRODUCTION

1.1 Statement of the problem

The normal haemostatic mechanism consists of a balance between haemorrhage and thrombosis. This balance is achieved through the interaction of blood vessels, platelets, coagulation and anticoagulation factors, and the fibrinolytic factors (Rice, 1996:1). When this balance is disrupted haemostatic abnormalities occur.

There are many specific problems which can be isolated that can cause blood to loose its ability to coagulate. This haemorrhagic diathesis is seen in the toxicological picture of many homoeopathic remedies.

The venom of snakes has the ability to alter the haemostatic mechanism of blood. The remedy *Lachesis mutas* is prepared from the venom of *Lachesis mutas*, a pit viper snake of South America (Reckeweg; 1991: 244).

The toxicological picture of this remedy is similar to that of haemorrhagic tendencies experienced in patients. It is for this reason that *Lachesis mutas* can be used to treat haemorrhagic tendencies.

1.2 Objective of the study

The objective of this study was to treat human plasma with *Lachesis mutas* in centesimal potencies (6CH; 9CH and 12CH) and to assess the *in-vitro* effects that this remedy in the various potencies may have on the coagulation of blood, specifically on the PT and APTT.

CHAPTER TWO

LITERATURE REVIEW

2.1 Homoeopathy

Homoeopathic medicine is a clinico-pharmaceutical system, which uses microdoses of substances derived from plants, minerals or animals for the purposes of stimulating the natural healing response (Bellavite and Signorini, 1995:8). The word 'homoeopathy' was coined from two ancient Greek terms. These are *homoeo- or omio-* meaning "similar", and *pathos* which means "hardship or suffering". Homoeopathy therefore means "similar suffering" (Hayfield, 1995:11). The founder of homoeopathy was the German physician and chemist Samuel Hahnemann (Koehler, 1989:18).

There are four principles on which homoeopathy is based:

1) The medicine is similar to the disease.
2) The doses used are minute and potentised.
3) The treatment is individually chosen for each person.
4) The treatment stimulates the body's natural healing power called the vital force.

2.1.1 The law of similars

The most important principle of homoeopathy is the law of similars (Adams, 1996:4). Hahnemann popularised the phrase "*similia similibus curentur*" which means "like is cured by like" (Ullman, 1981:66). This means that a substance that produces symptoms in a healthy person cures those symptoms in a sick person (Vithoulkas, 1985:19).

2.1.2 The law of minimum dose and potentisation

Homoeopathic medicines are produced by means of a process of serial dilution and succussion aimed at endowing the solutions with a greater therapeutic effect.

The process of succussion was initially used for the diluted product but was later observed that this procedure was necessary to increase the therapeutic effect of the dilutions. Thus the progressively increasing dilutions were called potencies, and the dilution and succussion process termed potentisation or dynamisation (Bellavite and Signorini, 1995:10).

There are two ranges of potencies namely, the decimal scale and the centesimal scale. The decimal scale is a 1 in 10 dilution and is designated 'X' or 'D' potencies, and the centesimal scale is a 1 in 100 dilution and is called 'C' potencies (Boyd, 1989:53).

2.1.3 Treatment based on individuality

Homoeopathic medicine is taken by healthy volunteers in repeatedly, very small doses to discover the set of symptoms it creates. These are called the provings, which are double-blind. As soon as changes in the provers' state of health begin, the medicine is stopped before any permanent changes are produced. Each prover's symptoms are recorded, and these records are a description of what that medicine can cause. This is called the remedy picture of that medicine (Bernard, 1999:59).

All the symptoms experienced by the sick person are recorded and compared with the remedy pictures until a similar drug picture or proving picture is matched. When this remedy is taken in a potentised dose it stimulates the body to heal itself (Adams, 1996:4).

2.1.4 The body's natural healing power

Homoeopathic medicine does not treat the illness directly, but enables the human body to do the necessary healing for itself (Adams, 1996:7). The action of this medicine is on the vital force. The vital force is the sprit, or "invisible driver" that, amongst other things, controls and regulates the body's healing mechanisms. The

vital force is highly efficient and intelligent, and tries to keep the body as healthy as possible (Hayfield, 1995:15). Any derangement to the vital force will bring about disease and is rectified by the aid of homoeopathic medicines.

2.2. Homoeopathic remedies

Homoeopathic remedies are primarily extracted from the three kingdoms of nature, namely the vegetable kingdom, animal kingdom and mineral kingdom. If the source of the remedies is physiological with active principles, the remedies are called sarcodes, and if the sources of the remedies are pathological secretions or products, these remedies are called nosodes (Eizayaga, 1991:162).

Some of the remedies are derived from biologically highly toxic compounds, such as certain mineral elements, organic and inorganic chemical poisons, and animal or plant poisons. The symptoms they caused were deduced from accidental intoxication, but obviously they cannot be used as such in human experimentation and is thus taken in potency (Bellavite and Signorini, 1995:11).

2.3. Classification of Serpentes

There are approximately 2927 species of snakes in eighteen families. Snakes are easily recognizable in that all snakes are limbless, although boas have vestiges of limbs. Their eyes are lidless and their tongue is forked. Many species are poisonous, but only few can actually kill man. There are two groups of snakes divided largely on their hunting skills: the constrictors (boas, anacondas, pythons) who strangulate their prey, and the venomous (cobras, rattlesnakes) who poison their prey. The sea snakes are the most venomous of all the snakes (Visser and Chapman, 1978:13). The eighteen families are illustrated in the following table.

Species	Common Name	Number in species
Anomalopidae	Dawn blind snakes	16
Typhlopidae	Blind snakes	217
Leptotyphlopidae	Slender blind snakes	90
Aniliidae	Pipe snakes	1
Anomochilidae	Dwarf pipe snakes	2
Boidae	Boas, pythons	64
Bolyeridae	Round Island boas	2
Cylindrophiidae	Asian pipe Snakes	10
Loxocemidae	Mexican burrowing pythons	1
Tropidophiidae	Wood snakes	23
Uropeltidae	Short-tail snakes	47
Xenopeltidae	Sunbeam snakes	2
Acrochordidae	File snakes	3
Atractaspididae	Mole vipers	62
Colubridae	Colubrids	1847
Elapidae	Cobras, kraits, coral snakes	239
Hydrophidae	Sea snakes	61
Viperidae	Vipers, pit vipers	240

Table 2.1 Classification of Serpentes (Visser and Chapman, 1978:67)

2.3.1. Venomous snakes

There are three types of venomous snakes. These are classified as the Opisthoglyph, Proteroglyphs and Solenoglyph groups.

2.3.1.1 Opisthoglyph group

These are the rear-fanged snakes. Their fangs are enlarged rear teeth with a 'groove' that venom flows down while they are swallowing the prey. They are mostly harmless or mildly venomous but there are two major exceptions. The

Boomslang (*Dispholidus typus*) and the Twig snake (*Thelotornis kirtlandi*) have killed humans before. Other good examples of this type of snake are the Mangrove (*Bitis dendrophila*) and Hognose snakes (Heterodon species) (Phelps, 1981:15).

2.3.1.2 Proteroglyphs group

These are the fixed front fang snakes. These snakes have small non-movable front fangs. When they bite they hang on and 'chew' to envenomate the prey. Obvious examples of this type of snake are the cobras (Naja), kraits (Bungarus), mambas (Dendroaspis), and coral (Micrurus) snakes. These are considered as some of the deadliest snakes in the world (Phelps, 1981:15).

2.3.1.3 Solenoglyph group

These snakes have movable front fangs. The fangs fold back until they are needed. This is what makes these snakes more dangerous to work with. They can grab on to the hand like a cobra would, but they can also open their mouth to almost 180 degrees with the fangs extended straight out. This enables them to strike at any portion of the body because it is more of a 'stab' than a bite. Among them are the representatives from the families of the vipers (Viperidae) rank and Grubenottern (Crotalidae), of which the rattling type occurring in north and Central America is the most well-known kind. Examples include rattlesnakes (Crotalus), eyelash vipers (Bothriechis), gaboon vipers (Bitis), cottonmouths and copperheads (Agkistrodon) (Phelps, 1981:16).

2.3.2. Pit vipers vs. vipers

The difference between the pit vipers and true vipers is a small pit in front and just below the eye of the pit vipers. This heat-sensing organ enables the 'pit' vipers to hunt and strike with great accuracy even in total darkness (Department of the Navy Bureau of Medicine and Surgery, 1991:76).

2.3.3. Venom Properties

Venom is modified saliva. Its primary function is to kill the prey and then it also helps to digest it.

Some venom are referred to as haematoxic, which means that they primarily affect the blood. Almost all American pit vipers fall into this category. Haematoxic venom destroys tissue and is very painful (Gopalakrihnakone and Chou, 1990:37).

Neurotoxic venoms attack the nervous system and brain. These may cause almost no pain, but shuts down the respiratory system and interfere with heart functions. Good examples of these types of snakes are the cobra and coral snakes (Gopalakrihnakone and Chou, 1990:37).

Snake venom is made up of about twenty different enzymes. Species usually have six to twelve of these enzymes. These enzymes determine the toxicity of the snake and whether it is haemotoxic or neurotoxic (Department of the Navy Bureau of Medicine and Surgery, 1991:98).

2.4 Haemostasis

Haemostasis is the arrest of bleeding or haemorrhage at the site of vascular injury (Guyton and Hall, 1996:463). The system of checks and balances responsible for preventing exsanguinations while preserving the blood's liquidity is very complex, involving a interplay between vascular endothelium, platelets, coagulation, complement and fibinolysis (Beck, 1985:415).

When a blood vessel is severed, an immediate response is achieved by reflex vasoconstriction and formation of a platelet plug (primary haemostasis). This buys time for the slower formation of a tough fibrin meshwork (thrombus) that seals off the defect (secondary haemostasis) and provides a scaffold for angiogenic repair of the vascular wall (Kay, 1988:45).

The haemostatic sequence is orchestrated and confined to the area of vascular damage by secretions elaborated by the vascular endothelium. Failure of the haemostatic mechanism may lead to bleeding disorders or thrombotic disorders. There are also several processes that occur to limit the extension of the blood clot and to resolve the obstruction to blood flow. The fibrinolytic system removes the fibrin clot once it has achieved its haemostatic function (Rice, 1996:2).

2.4.1 The physiological cascade

The physiological coagulation cascade occurs *in-vivo*. This pathway is somewhat different from the traditional "waterfall" hypothesis. Factor XII is not included in this scheme because patients with isolated deficient Factor XII activity do not have bleeding problems (Rice, 1996:2). Therefore Factor XII probably does not play the role originally hypothesized in the classic "waterfall" hypothesis. In the physiological cascade, the extrinsic pathway is the initiator of events. Normal non-vascular cells have a protein on their cell membrane called tissue factor; endothelial cells, however do not. Under normal circumstances, blood and its constituents are not exposed to tissue factor (Rice, 1996:3).

When either blood vessels or tissue injury occurs, the plasma becomes exposed to tissue factor, allowing a complex between Factor VII and tissue factor to form. Factor VII is then activated to VIIa. The tissue factor-Factor VIIa complex subsequently catalyses the initial activation of Factor IX (to IXa) and Factor X (to Xa). When the concentration of tissue factor is high, preferential activation of Factor X occurs (Rice, 1996:3).

However, when the concentration of tissue factor is low, activation of Factor IX is predominant. When formed, Factor IXa participates in further activation of X to Xa. Once Xa is formed, in the presence of activated Factor V, it can catalyse the conversion of prothrombin to thrombin. This in turn catalyses the conversion of fibrinogen to fibrin, the main constituent of a clot. This mechanism is felt to generate only limited amounts of thrombin. When small quantities of thrombin

have been generated, thrombin may bind and activate a protein called Tissue Factor Pathway Inhibitor (TFPI). The thrombin-TFPI complex then inactivates the earlier formed Factor VII-tissue factor complexes, thus inhibiting further synthesis of Factor IXa and Xa through that mechanism. This represents a negative feedback system controlling haemostasis (Rice, 1996:7).

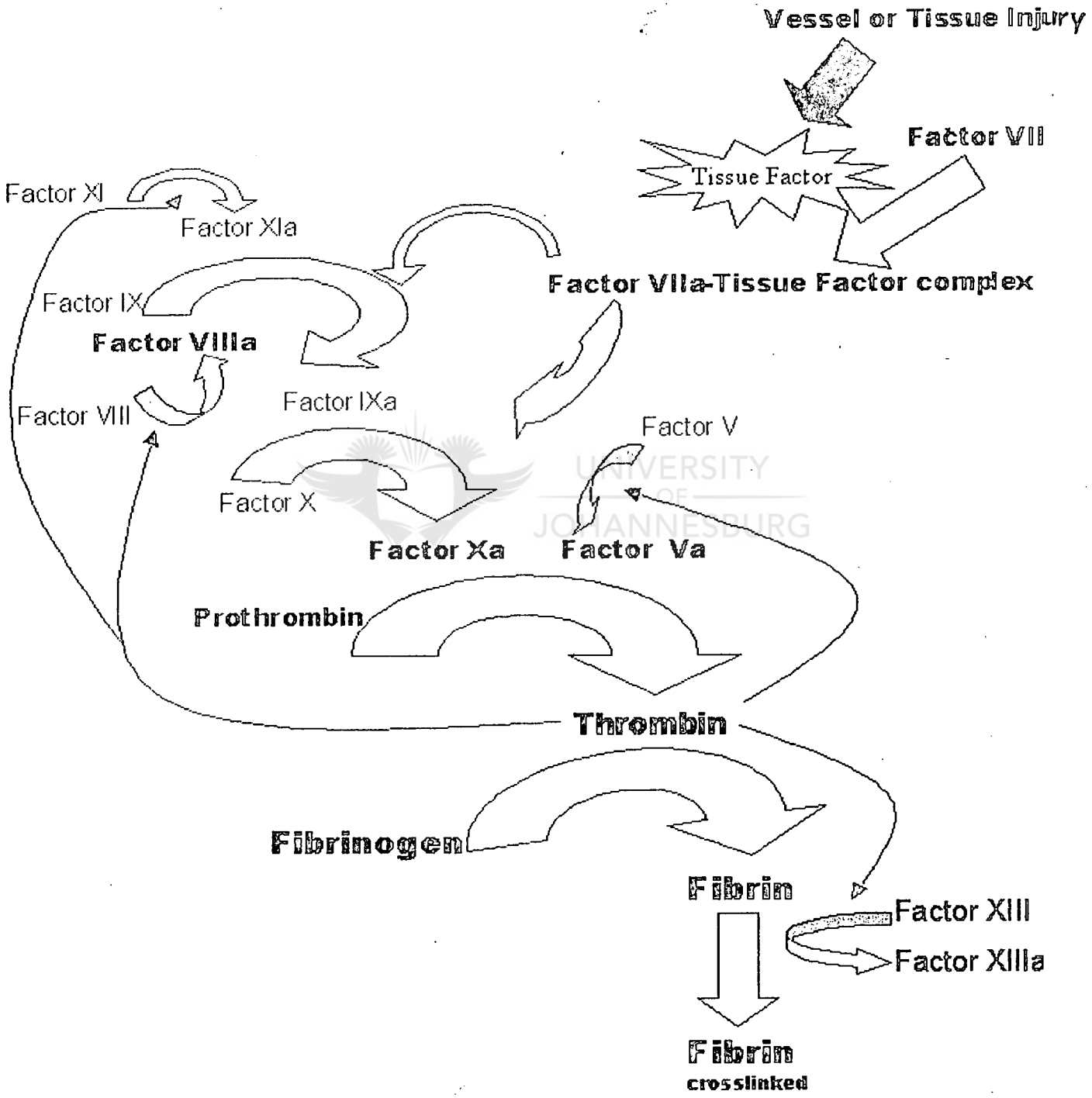

Figure 2.1 The physiological cascade (Rice, 1996:7)

2.4.2 Prothrombin and the conversion of prothrombin to thrombin

2.4.2.1 Prothrombin

Prothrombin or Factor II is a single chain glycoprotein. Its domain structure differs from that of most of the other vitamin K dependent (VKD) plasma proteins with the NH_2-terminal half consisting of the gla and two-kringle domains, while the C-terminal half constitutes the SP domain. There are three N-linked sugar chains, two in the first kringle and one in the SP domain. Like other VKD proteins, prothrombin binds to anionic phospholipid membranes that is Ca^{2+} dependent and mediated by its gla domain. The second kringle domain mediates prothrombin binding to Factor Va and to Factor Xa thus promoting prothrombin activation by prothrombinase (Beutler *et al.*, 1995:1269). This structure can be seen in **Figure 2.2**.

2.4.2.2 Conversion of prothrombin to thrombin

Prothrombinase converts prothrombin to thrombin by sequential cleavage of two bonds. Initially Factor Xa cleaves prothrombin resulting in the transient disulfide linked intermediate meizothrombin. This cleavage is associated with extensive conformational changes in the SP domain of prothrombin that are associated with generation of an enzyme active site that hydrolyses small synthetic substances and formation of its fibrinogen recognition anion binding site. Subsequent proteolysis releases the NH_2-terminal half of the molecule consisting of the gla and two-kringle domain from α-thrombin consisting of an NH_2-terminal residue A chain disulphide linked to a C-terminal B chain. Prothrombin cleavage is required for generation of the full spectrum of thrombin activity on macromolecular substrates. The prothrombin activation pathway described is complicated by the fact that both meizothrombin and α-thrombin can cleave between the two-kringle domains, forming fragment 1 and fragment 2 (Beutler *et al.*, 1995:1270). This is illustrated in the following diagram: (**Figure 2.2**).

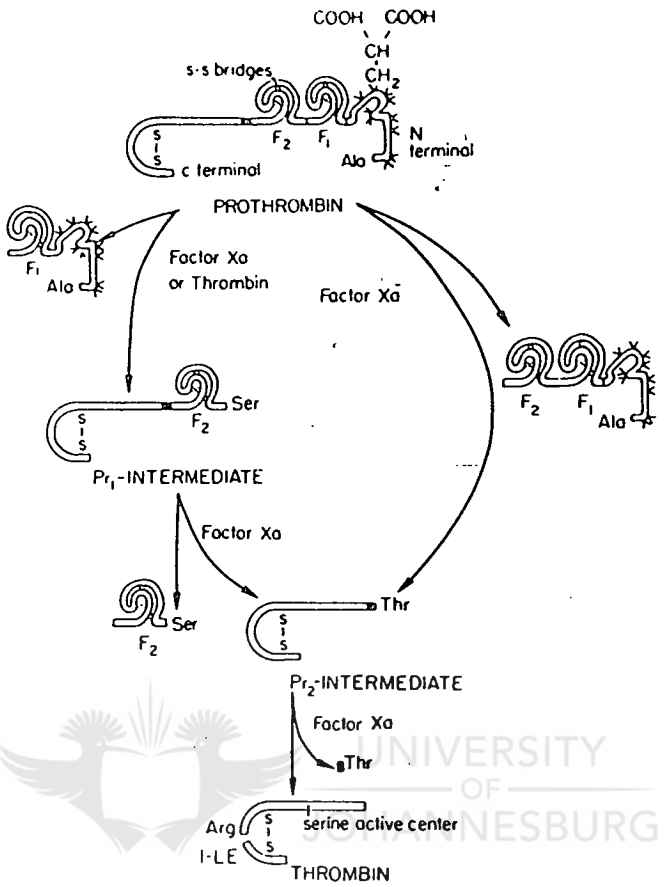

Figure 2.2 The common pathway of the conversion of prothrombin to thrombin (Beck, 1991:519)

2.4.3 Snake venom containing prothrombin activators

There are several different types of activators of prothrombin in snake venoms. According to Markland (1998: 1753) these activators have been divided into four types or areas of activation:

- The first type converts prothrombin directly into meizothrombin, which then is auto catalytically converted to thrombin. The activation of this type

is not influenced by components of the prothrombin activator complex i.e. Factor Va, $CaCl_2$ and phospholipid.

- The second type can cleave both bonds in prothrombin leading to active two-chain thrombin. The activation of this type is strongly stimulated by phospholipids and Factor Va in the presence of $CaCl_2$, but is inactive against prothrombin in the absence of cofactors.
- The third type also cleaves the prothrombin bonds leading to a two-chain thrombin. The activation of prothrombin in this type differs from the second type in that it only requires phospholipid and $CaCl_2$. They do not require Factor Va.
- The fourth type is not able to generate active thrombin, but convert prothrombin into modified forms which are enzymatically inactive. There are uncertainties whether these are specific proteinases or broad-acting venom proteinases (Markland, 1998:1753).

2.4.4 Fibrinogen and the conversion of fibrinogen to fibrin

2.4.4.1 Fibrinogen

The fibrinogen molecule is a large trinodular disulfide bonded glycoprotein composed of two symmetric half molecules. Each half molecule contains three distinct polypeptide chains called the Aα, Bβ, and γ-chains. The central nodule or E-domain contains the NH_2-termini of all six polypeptide chains forming the NH_2-terminal disulfide knot. The two outer D-domain nodules are composed of the C-terminal two-thirds of both the Bβ and γ chains. These two sub-domains are located diagonally along the long axis of the molecule. Between the E- and D-domains, there is a stretch of approximately 120 amino acids from each of the three chains that forms an α-helical structure known as the coiled-coil domain. This region of the molecule is supported on both sides by a set of disulfide bonds called disulfide rings (Lee *et al.*, 1999:701). The diagram is illustrated below.

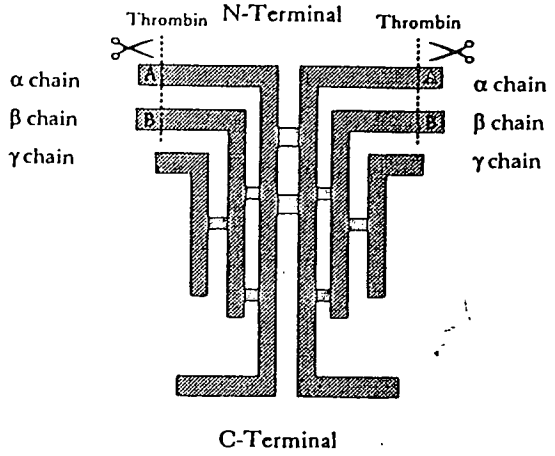

Figure 2.3 Structure of the fibrinogen molecule (Pallister, 1994:458)

2.4.4.2 Conversion of fibrin to fibrinogen

The first phase in converting soluble fibrinogen into an insoluble fibrin clot requires the release of fibrinopeptides A and B by thrombin. Thrombin cleaves two specific arginine-glycine bonds in Aα and then the Bβ-chains. This action releases fibrinopeptides A and B, respectively from the NH_2-terminal disulfide knot. The release of fibrinopeptide A from the Aα chain initiates a process in which fibrin monomers begin to gel (Lee *et al.*, 1999:703).

The second phase of fibrin formation is the polymerisation of fibrin monomer molecules. When fibrinopeptide A and B are released, two sets of binding in the E-domain and D-domain are exposed which cooperate in the assembly of fibrin polymers. Binding sites on the Aα-chain in the E-domain of one fibrin monomer interact with complementary sites on the γ-chain of the D-domain of a second fibrin monomer. The noncovalent interactions between the contact sites of half-staggered overlapping monomers are called D-E contact sites (Lee *et al.*, 1999:703).

After formation of the initial D-E contact, additional fibrin molecules are added and stimulate D-D contact as well. As this process continues, a 2-stranded protofibril is formed. Once a critical mass of long protofibrils is established, the protofibrils form lateral contacts with other protofibrils to form thicker fibres,

which eventually form a three-dimensional gel (Beutler *et al.*, 1995:1270). This second phase of fibrin formation is illustrated below.

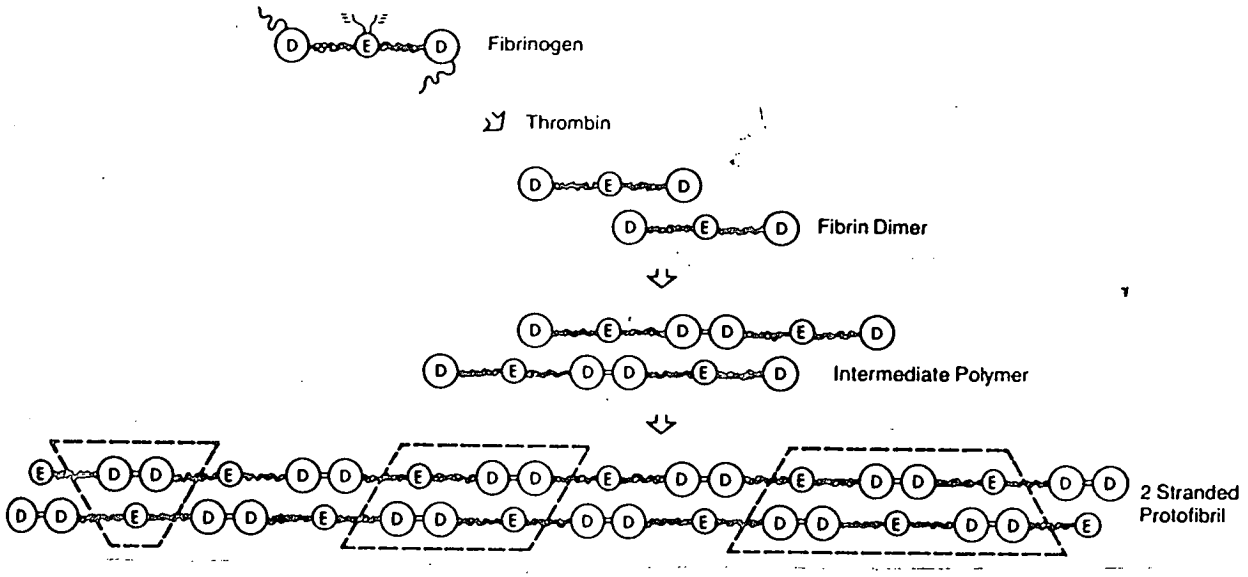

Figure 2.4 Fibrinogen conversion to fibrin clots (Beutler *et al.*, 1995:1319).

2.4.5 Snake venoms able to clot fibrinogen

The ability of a group of snake venom enzymes to clot fibrinogen has resulted in these enzymes being called thrombin-like. These enzymes are mainly in the venom of snakes from true vipers and pit vipers. There are several groups of snake venom fibrinogen clotting enzymes based on the rates of release of fibrinopeptides A and B from fibrinogen (Markland, 1998:1754).

2.4.5.1 Venombin A group

The first class of thrombin-like enzymes releasing fibrinopeptide A is represented by three, well characterised proteins with similar properties: ancrod from the Malayan pit viper (*Callosclasma rhodostoma*) venoms, batroxobin from *Bothrops atrox* and crotalase from the venom of the eastern diamondback rattlesnake (*Crotalus adamanteus*). *In-vivo* studies show these enzymes act as defibrinogenating agents to remove fibrinogen from blood. In the test tube they act as procoagulants and form a fibrin clot. All three enzymes of the venombin A

group are able to cleave the Arg16-Gly17 (arginine-glycine) peptide bond in the Aα-chain of the fibrinogen, leading to the release of fibrinopeptide A and the conversion of fibrinogen to a fibrin clot (Markland, 1998:1755).

2.4.5.2 Venombin AB group

The second class of thrombin-like enzymes releases both fibrinopeptide A and B. The enzyme that represents this comes from the venom of the Gaboon viper (*Bitis gabonica*). Gabonase is a serine proteinase and glycoprotein. The coagulant action of gabonase is primarily on the cleavage of the Arg16-Gly17 bond nearest to the amino terminus of the Aα-chain of fibrinogen, releasing fibrinopeptide A. Fibrinopeptide B is released more slowly by cleavage of the Arg15-Gly16 bond nearest to the amino terminus of the Bβ-chain (Markland, 1998:1758).

2.4.5.3 Venombin B group

The third class of thrombin-like enzymes releases fibrinopeptide B and is represented by enzymes from the southern copperhead (*Agkistrodon contortrix contortrix*) venom. The enzyme, called venzyme, releases fibrinopeptide B much more rapidly then fibrinopeptide A and clots fibrinogen only after a prolonged incubation (Markland, 1998:1758).

2.4.6 Activation of thrombin

Thrombin or Factor IIa is a multifunctional enzyme generated from prothrombin at the sites of vascular injury. It is the ultimate product of the clotting cascade, being directly or indirectly responsible for the formation and stabilization of fibrin clots (Kay, 1988:58).

Thrombin sequentially cleaves in the Aα and Bβ-chains of fibrinogen, releasing the small fibrin peptides A and B from the fibrin monomer. These exposed regions of the monomer are necessary for the spontaneous association to form the meshwork of a clot. Concurrently thrombin cleaves a single bond in the α subunit of Factor XIII, which activates this transglutaminase. Thrombin catalyses several

reactions that amplify the clotting cascade including the feedback activation of the cofactor proteins, Factor VIII and V, and platelet activation. Thrombin is one of the most potent platelet agonists, which leads to the platelet secretary response that ultimately results in platelet aggregation, release of granular contents, and translocation of anionic phospholipids to the platelet surface, and the generation of platelet derived micro vesicles (Lee *et al.*, 1999:708).

2.4.7 Thrombin-like snake venom enzymes

Thrombocytin is a venom proteinase. This proteinase exerts a thrombin-like action on platelets and thrombin-sensitive plasma clotting Factors V, VIII, and XIII. But unlike thrombin, neither converts fibrinogen into fibrin nor activates protein C.

2.4.7.1 Action on Factor V

The venom of *Vipera russelli*, besides being a highly potent activator of Factor X (RVV-X), contains a serine proteinase, which by cleavage of a single peptide bond, converts Factor V into Factor Va. In contrast to thrombin and thrombocytin (which also activates factor V), RVV-X is inhibited by antitrombin III-heprin complex. Factor V after activation by thrombin, RVV-V or thrombocytin, loses its activity faster than does the precusor molecule (Meier and Stocker, 1996:70).

2.4.7.2 Action on Factor VIII

Thrombocytin from *Bothrops atrox* venom causes a significant increase in Factor VIII clotting activity (Meier and Stocker, 1996:71).

2.4.7.3 Action on Factor XIII

Treatment of Factor XIII with thrombin or thrombocytin causes proteolytic cleavage of the α-chain and release of a peptide. The resulting activated Factor XIII causes fibrin cross-linkage. A fibrin clot formed by batroxobin, in presence of Factor XIII and either thrombocytin or thrombin, is rendered insoluble (Meier and Stocker, 1996:71).

2.4.8 The fibrinolytic system

Fibrinolysis regulates the degradation and dissolution of fibrin. It uses elements from plasma, platelets, tissue, and other blood cells to regulate the degradation of fibrin. This is brought about by the conversion of plasminogen, a plasma protein that circulates as a zymogen to the serine proteinase, plasmin. The physiological function of plasmin is limited to the degradation of the fibrin clot and extra cellular matrix molecule (Kay, 1988:98).

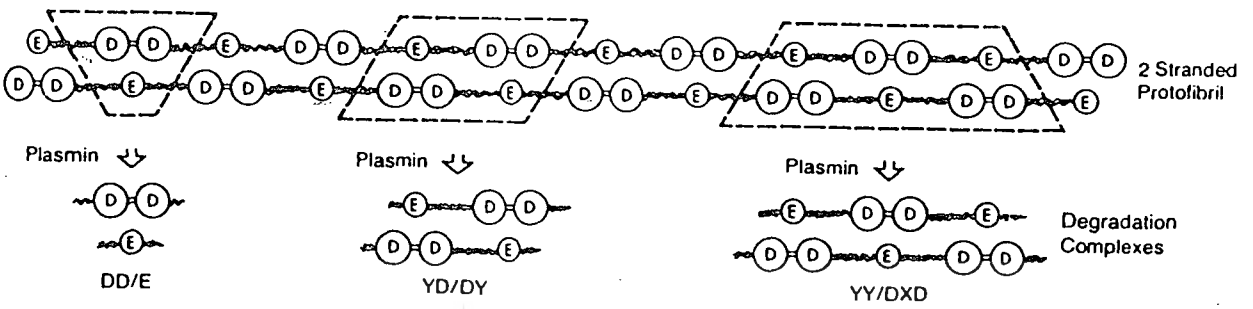

Figure 2.5 Plasmic degradation of the cross-linked fibrin (Beutler *et al.*, 1995:1319).

Plasmin is converted from its proenzyme form, plasminogen, by enzymes. Excited activator molecules some how seek out plasminogen molecules trapped within the clot and convert them to plasmin by specifically cleaving the bonds that hold plasminogen together. Plasmin is also capable of digesting not only fibrin but also fibrinogen and Factor V and VIII (Jandl, 1991:430).

Fibrinogen is represented as a trinodular structure. Plasmin initially removes the C-terminal portion of Aα-chain and the first 42 amino acids of the Bβ-chain. This action produces a clotable form of fibrinogen called fragment X. Fragment X can than be cleaved in the two coiled-coil regions producing fragment D and Y. Fragment Y can be further degraded along the remaining coiled-coil region to produce fragment E and a second fragment D (Lee *et al.*, 1999:718).

When the fibrin clot is degraded, plasmin activity is targeted between the D and E domain within the coiled-coil region. This action releases different sized fragments and the structure and integrity of the fibrin polymer is lost. Complete degradation of the clot yields a dimeric form of fragment D. This fragment known as D-dimer, is the smallest degradation product containing the intermolecular γ-chain cross link (Lee *et al.*, 1999:718).

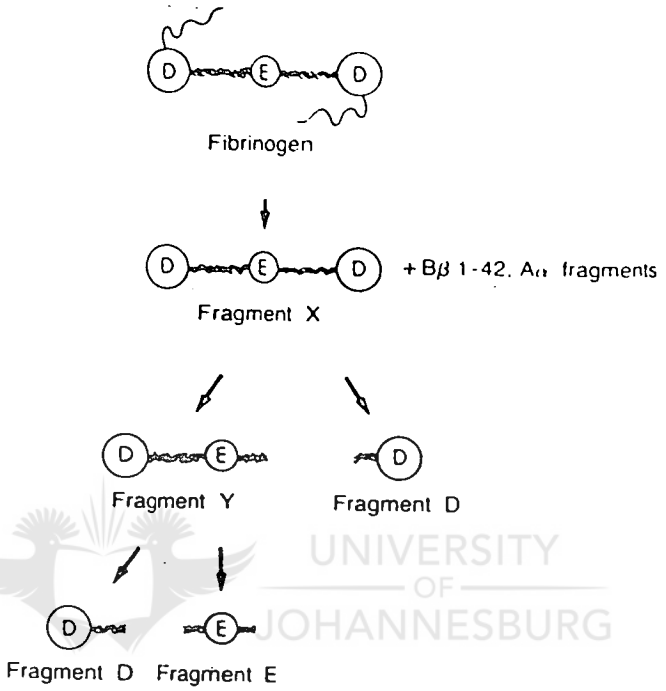

Figure 2.6 Degradation of fibrinogen by plasmin (Beutler *et al.*, 1995:1318).

2.4.9 Snake venom acting on fibrinolysis

In general, it would appear that venoms from the different families of snakes contain predominantly two different fibrinolytic activities, one specific for the Aα-chain and another for the Bβ-chain (Markland, 1998:1761).

The Aα-chain-specific fibrinogenases have been isolated. The Aα-chain fibrinogenases are zinc-containing metalloendoproteinases. The enzymes appear to have disulphide bonds that are critical to their structure or function. They apparently degrade the Aα-chain of fibrinogen first, and then the Bβ-chain. These enzymes are found in different genera of the family Crotalidae, including

Agkistrodon, Crotalus, and *Trimeresurus*. The Aα-chain fibrinogenases are inhibited in serum, probably by α2-macroglobulin (Markland, 1998:1762).

The second group of venom fibrinogenase are those with specificity for the Bβ-chain of fibrinogen. These proteinases, in contrast to the metalloproteinases that cleave specifically the Aα-chain of fibrinogen, are serine proteinases. The Bβ-chain fibrinogenases appear to be more heat stable than the Aα-chain fibrinogenases. Although they are serine proteinases, the Bβ-chain fibrinogenases do not resemble venom fibrinogen-clotting serine proteases, which cleave either fibrinopeptide A or B or both from fibrinogen (Markland, 1998:1762).

2.5. *Lachesis mutas*

Lachesis mutas (bushmaster) is a venomous snake of the viper family. This snake is found in the forests of Costa Rica, south of the Amazon River basin (Melgarejo *et al.*, 1997: 55). It belongs to the Solenoglyph group of poisonous snakes and is classified as:

- Phylum Chordata
- Subphylum Vertebrata
- Class Reptilia
- Order Suamata
- Family Crotalidae
- Subfamily Viperidae (Furtado *et al.*, 1997:32).

The snake is normally about 1.8 metres long but may attain twice this length. It is pinkish or tan in colour, marked with large, dark, diamond shaped blotches. Although seldom encountered, the bushmaster is dangerous, with potentially lethal venom. It is the only American pit viper that lays eggs rather than bearing live young (Fuly *et al.*, 1999:14).

2.5.1 Toxicological action

Even though this snake has large fangs, delivers large amounts of venom, and has an aggressive nature, few human bites have been recorded because of its nocturnal nature. Envenomation presents predominately with oedema, pain, haematologic, abdominal and cardiovascular manifestations. Dizziness and blistering wounds can also be present. In severe envenomations, peripheral circulatory collapse and bloody diarrhoea may manifest (Minton, 1971:90).

2.5.2 Signs and symptoms of envenomation

These signs and symptoms will usually manifest early, though their development will vary considerably from case to case. Pain and oedema usually begins within the first few minutes after the bite. The symptoms can be divided into four categories:

a. Local: Oedema, pain, and blister formation around the bite site.
b. Cardiovascular: Diastolic and systolic hypotension, bradycardia, diminished heart sounds, brief loss of consciousness, blurry vision, and dizziness.
c. Abdominal: Intense colic, vomiting, and diarrhoea.
d. Haematology: Haemorrhage (wound, rectal, gingival) (Davidson, 2001:4-5).

In severe envenomation one or more of the following clinical pictures can occur:

- Hypotension and decreased heart rate secondary to peripheral circulatory collapse.
- Intense colic with bloody diarrhoea (Davidson, 2001:5).

2.5.3 The Materia Medica picture

The Materia Medica clinical picture is seen as haemorrhages, bruises, purpura and a characteristic congestive, blue-violet, cyanosed aspect of the mucosa and the

skin (Boerike, 1997:389). These haemorrhages are seen as epistaxis, bleeding gums, haematemesis, maelena, haematuria, meno-metrorrhagia, and haemoptysis (Jouanny; 1984: 215). A similar symptom picture is seen in different coagulation disorders.

This striking resemblance in the change of the blood after poisoning with *Lachesis mutas* shows why it is of such efficacy in the cure of haemorrhages (Gibson; 1996: 292).

2.5.4 Haematological action

The venom of *Lachesis mutas* contains a full range of potent enzymes, proteolytic, cytolytic, neurotoxic and coagulant. The approximate dry yield of *Lachesis* is 280mg to 400mg and the lethal dose of 50 (LD50) is 5.93mg per kilogram (Pinney; 1981: 81). The blood is attacked primarily, while the nervous system is at first aroused and excited. The main affinity is with the blood, causing disintegration of red cells, lowered coaguability after initial tendency to thrombosis, and impaired resistance to infection with associated liability to gangrene and necrosis. In the actual region of the bite the effects are extreme swelling of the part, purple or black discolouration, formation of blood blisters and terrible pain (Gibson; 1996: 292).

Generally *Lachesis mutas* venom consumes prothrombin and fibrinogen. This results in a DIC (Disseminated Intravascular Coagulopathy) type coagulopathy with an increased coagulation and bleeding times. Fibrinogen levels and sedimentation rates are generally decreased. White blood cell counts may be elevated and the haematocrit decreased, but cell morphology is usually within normal limits (Davidson, 2000:8).

According to Sano-Martins (1997:17), *Lachesis mutas* accidents do not present significant consumption of coagulation factors other than fibrinogen. However, definitive conclusion cannot be drawn for *Lachesis mutas* envenomation, since

only few cases of coagulation disturbance have been reported. Nonetheless, disturbances of blood coagulation and secondary activation of the fibrinolytic system are common findings in envenomed patients. Salvereira *et al* (1989:865) found that the venom contains serine proteases similar to gyroxin. The enzyme cleaves only fibrinopepide A from fibrinogen, it does not activate Factor XIII and is devoid of kallikrein-activity. Costa *et al* (1997:45) isolated two haemorrhagic toxins LHF-I and LHF-II from the venom of *Lachesis mutas.* These toxins show high proteolytic activity and were charaterised as metalloproteinases.

2.6 Haematological disorders

Haematological disorders comprise of acquired coagulation disorders, hereditary coagulation disorders and drug induced coagulation disorders.

2.6.1 Acquired coagulation disorders

2.6.1.1 Disseminated Intravascular Coagulopathy (DIC)

DIC usually results from entrance into or generation within the material of blood with tissue factor activity, which initiates coagulation. The laboratory findings vary with the intensity of the DIC. In the chronic, slow form of DIC, the PT is normal to minimally prolonged and the APTT is short (Berkow *et al.*, 1992:1221).

2.6.1.2 Vitamin K deficiency

Factors II, VII, IX and X depend on vitamin K for their synthesis and are produced in the liver. Therefore vitamin K deficiency or liver dysfunction may produce coagulation disorders (Brown, 1993:340). Laboratory findings shows a lengthened PT because of depression of Factor VII and the APTT will be normal because prothrombin, Factor IX and Factor X levels fall slower than Factor VII levels (Berkow *et al.*, 1992:967).

2.6.1.3 Liver disease

A variety of haemostatic defects may be found in liver disease. These include multiple factor deficiencies, abnormalities of fibrinolysis, thrombocytopaenia and qualitative platelet abnormalities. All of the coagulation factors, except factor VIII, are synthesized in the liver. Patients with liver disease may have severe haemorrhaging (Brown, 1993:341-342)

2.6.2 Hereditary coagulation disorders

The most common of bleeding disorders due to hereditary clotting factor deficiencies are the haemophilias. Haemophilia A has a Factor VIII deficiency and haemophilia B has a Factor IX deficiency. Haemophilics display a problem coagulating. Laboratory findings shows a prolonged APTT and normal a PT (Berkow *et al.*, 1992:1218).

2.6.3 Drug induced coagulation disorders

Coumarin drugs act as anticoagulant drugs by opposing the action of vitamin K and preventing the process of γ-carboxylation from taking place. Warfarin is a coumarin drug (Beck, 1985:517).

Heparin is another drug responsible for anticoagulation. It activates antithrombin III sufficiently to inhibit Factor X, which is required to convert prothrombin to thrombin (Beck, 1985:604)

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials

See Appendix A

3.2 Principles of laboratory tests for coagulation system

There are a number of laboratory tests available to evaluate the coagulation system. These tests include the prothrombin time (PI or PT), activated partial thromboplastin time (PTT or APTT), thrombin time (TT) and assays for fibrinogen concentration. The activated partial thromboplastin time (APTT) and the prothrombin time (PT) tests were used for this study.

3.2.1 Activated Partial Thromboplastin Time (APTT)

APTT screens for abnormalities of the blood coagulation reactions, triggered by the exposure of plasma to a negatively charged surface. Plasma is incubated for three minutes with a reagent supplying procoagulant phospholipid. Calcium is then added and the clotting time recorded, with the normal ranging from twenty-eight to thirty-four seconds (Dacie and Lewis; 1996: 300). The APTT is sensitive to deficiencies of all the clotting factors except factors VII and XIII. A prolonged test time can stem from a deficiency of one or more coagulation factors or from presence of an inhibitor of a plasma-clotting factor, or an inhibitor of procoagulant phospholipid (Berkow; 1992: 1205).

3.2.2 Prothrombin Time (PT)

In the PT test, plasma is recalcified in the presence of a high concentration of a tissue factor reagent. The test screen for abnormalities of factors V, VII, X, prothrombin and fibrinogen. The normal PT range is between ten and twelve

seconds. A PT that is greater than or equal to two seconds longer than a laboratory normal control value should be considered as prolonged (Dacie and Lewis; 1996: 299). PT is a valuable screening test for disordered coagulation in a variety of acquired conditions such as vitamin K deficiency, liver disease, disseminated intra vascular coagulation (Berkow; 1992: 1205).

3.3 Methods to measure the coagulation system

3.3.1 The Manual Coagulation Test

The PT test is carried out as follows: In a thirty seven degrees centigrade waterbath, a commercial preparation of phospholipid and tissue factor (thromboplastin) is added to an aliquot of the subject's citrated plasma. The mixture is recalcified and the stopwatch is started.

The APTT test is carried out as follows: In a thirty seven degrees centigrade waterbath, Kaolin or celite is first incubated with an aliquot of subject's citrated plasma for three to five minutes to activate Factor XII. Phospholipid is then added and the mixture is recalcified at time zero, when the stopwatch is started.

The clotting time in both the PT and the APTT is determined by stopping the stopwatch when the clot is visible. The process between the stopwatch being started and stopped involves dipping the test tube into a waterbath and removing it to check for a clot in the mixture. The waterbath keeps the mixture at body temperature.

There are various short-comings in the manual method. These include not being able to spot the clot in the clear plasma mixture, the clot forming while the test tube is submerged in the waterbath, the time delay in operating the stopwatch and the clot not forming in both the test tubes at the same time. Thus the Automated Coagulation Laboratory (ACL) analyser was preferred.

3.3.2 The Automated Coagulation Laboratory (ACL) analyser

The ACL system is designed for laboratory use to provide direct haemostatic measurements.

The ACL is a fully automatic microcomputer with a controlled microcentrifugal analyser. The ACL system incorporates a video display unit (VDU) that continually displays the status of the instrument and gives instructions on how to proceed.

When a sampling cycle is initiated, the samples and the reagents are sequentially pipetted into a twenty place acrylic rotor. Sample and the reagents are mixed via a centrifugal force and the clotting times recorded.

The optical measurements take place in one of two channels, either by a nephelometric channel or by a chromogenic channel. Coagulometric analysis in the extrinsic and intrinsic pathways is performed in the nephelometric channel. Here, the light ray is directed to the measuring cuvette of the rotor by means of an optic fibre system and the scattered light is read by means of a solid-state detector.

3.4 Homoeopathically prepared *Lachesis mutas*

3.4.1 Potencies utilised

The remedy, *Lachesis mutas,* was prepared in centesimal potencies for the purpose of this study. The potencies used were the 6CH, 9CH, and 12CH. The remedies in their respected potencies were obtained from Natura (Pretoria, Gauteng, South Africa), a manufacturer of homoeopathic remedies.

3.4.2 Preparation of potencies

The remedy, *Lachesis mutas* is prepared from the venom of the snake *Lachesis mutas*. The venom is milked from the snake and then dried into crystals at low temperatures. These crystals are then called the stock. The centesimal potencies are prepared from this stock. The centesimal dilution method is prepared using the process of serial dilution and succussion aimed at endowing the solutions with a greater therapeutic effect, while simultaneously nullifying the toxic effect. This process is called potentisation or dynamisation. The process involves adding two drop of the stock to ninety-eight drops of the diluent being normal saline. The solution resulting from the add-mixture of the two substances is subject to vigorous shaking with an impact known as succussion. The succussion is carried out a hundred times. The resultant dilution is the first centesimal potency or 1CH. The number followed by the letters 'CH' (Centesimal Hahnemannian) designates the potencies. The process of adding one drop of the previous potency to the diluent and succussion is repeated for subsequent potencies.

3.4.3 Avogadro's constant

Avogadro's constant is the number of molecules in one mole of any substance and is denoted by the number $6.02554X10^{23}$. This constant suggests that if a dilution goes beyond this number, there would be no molecule of the original solute in the solution. This constant more or less corresponds to the homoeopathic potency of 12CH. Once the 12CH potency is exceeded, there would be no molecules of the original substance left in solution. Potencies from 1CH to 12CH are frequently used, and at this level there are still molecules present in solution.

3.5 Methodology

3.5.1 Sample group

The study was conducted with the recruitment of twenty subjects, ten males and ten females between the ages of twenty and fifty years. The subjects participated on a voluntary basis and were recruited from verbal advertisement. The suitable volunteers were issued with an information sheet, explaining the purpose of the study and their role as participants in this research. They were also required to fill out a questionnaire (Appendix C) and sign a consent form (Appendix B). Once the blood had been collected, no further participation was necessary.

3.5.2 Selection criteria

Subjects were excluded if they were:

- using anti-coagulant therapy such as Warfarin, Heparin and Disprin.
- diagnosed with a haematological or coagulation disorder or a family history thereof.
- diagnosed as having malaria or have visited malaria stricken areas in the last year.
- having iron deficiency, anaemia, hypotension, hypertension, heart disease or jaundice.

3.5.3 Sample collection

Twenty millilitres of blood was drawn from each volunteer. Phlebotomists at the WITS Medical School Day Care Ward collected the samples. The standard venipuncture technique was used to draw the blood. Venous blood was drawn with minimum stasis into sodium citrate (3.2%) tubes. A total of four sodium citrate tubes were used per subject. The tubes were inverted to allow the sodium citrate to mix with the blood, thereby preventing coagulation. During drawing of

blood samples precautions were taken to avoid haemolysis as damaged red blood cells affect coagulation.

3.5.4 Sample preparation

The samples collected were immediately centrifuged, at 3000rpm for 10 minutes at 4°C. The resultant supernatant (plasma) was alloquated into 1ml volumes in plastic tubes. Plastic tubes as well as plastic pipettes were used to avoid the activation of the contact phase of blood coagulation.

3.5.5 Sample storage

The samples were stored in a freezer at a temperature of –20°C.

3.5.6 Sample usage

The samples were thawed in a water bath at 37°C and aliquots of 400 µl were placed into five cuvettes:

- the first cuvette contained only plasma
- the second was incubated with 40 µl of saline (saline control)
- the third with 40 µl of *Lachesis mutas* 6CH
- the fourth with 40 µl of *Lachesis mutas* 9CH and
- the fifth with 40 µl of *Lachesis mutas* 12CH.

Calibrated pipettes were used for both alloquating the plasma into the cuvettes, as well as for adding and mixing the potencies. The samples were then incubated for a period of one hour at room temperature.

3.5.7 The use of the ACL Machine

Following the incubation period, the samples were placed into the loading disk of the ACL analyser. Numbers 1-6 were used on the loading disk, No.1 being the

plasma pool or NCP (normal control plasma), No.2 the baseline sample, No.3 the saline control, No.4 the 6CH potency, No.5 the 9CH potency, and No.6 the 12CH potency. The ACL operative conditions required a pool of NCP or calibration plasma. The NCP consisted of lyophilised normal human plasma, which has the same characteristics of normal human fresh plasma. The main purpose of this NCP was to check the whole system namely, the instrument and reagents.

The calibration plasma was used on the ACL in the following way. Firstly, it was used to outline the calibration curve for PT-FIB. Secondly, during analysis a position on the rotor was reserved for the calibration plasma for the purpose of checking and following any eventual change of the system. The normal values for the calibration plasma was a PT between 9 and 11 seconds and APTT between 28 and 33 seconds. This calibration plasma or NCP (supplied by ILEX) was refilled for every run on the ACL machine.

The sample disk was placed into the ACL analyser and the reference emulsion checked. For this study the coagulimetric test PT-FIB/APTT was used. The screen was checked and set for the PT-FIB/APTT test. The reagents were filled into their respective cups. In the first cup thromborel was added, in the second cup APTT-Sp was added and in the third cup APTT-$CaCl_2$ was added. The thermal paper was checked and the machine was programmed to start. A new rotor was loaded and the test proceeded. The sample numbers were keyed in and each test run took an approximate of 15 minutes. The results were printed out on the thermal paper. The NCP, PT and APTT for each of the samples were recorded.

The ACL displayed and printed the results in:

- s (time)
- R (ratio)
- INR (International Normalised Ratio)
- % (Activity)
- U/ml
- Mg/dl or g/l

This procedure was repeated for each subsequent run. The time results were statistically analysed using the independent 2-sample T-test on the SPSS statistics programme. A p-value of less than 0,05 was considered significant.

CHAPTER FOUR

RESULTS

4.1 Introduction

Snake venoms have been shown to contain proteins that are active in the haemostatic mechanism. These proteins may either have a procoagulant or an anticoagulant activity. Snake venoms from genera *Lachesis mutas* have *in-vitro* proteolytic and indirect haemolytic activities (Otero, 1998:1226). Numerous researchers, Sano-Martins(1997:17), Salvereira *et al*(1989:865), Costa *et al*(1997:45), among others have found some change in the haemolytic activity of blood when exposed to the venom of *Lachesis mutas*.

Since this snake has been known to cause a change in the coagulation of blood, it was decided to determine what action the diluted venom of this snake has on the *in-vitro* coagulation of blood. To assess any changes, the prothrombin time (PT) and the activated partial thromboplastin time (APPT) were run on the ACL analyser.

4.2 Group analysis

The results were recorded for the total number of samples as well as for each group of samples:

- Group 1 represented the baseline
- Group 2 represented the saline
- Group 3 represented the 6CH potency
- Group 4 represented the 9CH potency, and
- Group 5 represented the 12CH potency

The baseline served as a comparison for the results of the different potencies of the remedy. The saline served as a control for the experiment, to show that the

diluent that was used in the preparation of the different potencies, did not effect the coagulation of the blood.

4.3 Result analysis

The statistical analysis was conducted by taking into account the mean, standard deviation and 2-tailed significance values of both the prothrombin time (PT) and partial thromboplastin time (APTT). The mean and standard deviation of the PT and APTT tests of each group were compared to the baseline results.

The PT normal values depend on the thromboplastin used, and the APPT depends on the reagents used and the pre-incubation period. The normal values for the PT and APTT tests differ and thus each laboratory should thus establish its own normal range (Dacie and Lewis, 1995:308).

A comparison was also drawn between the three potencies that were utilised in this study. This was used to assess whether there were any significant differences between the different potencies.

4.3.1 Descriptive results

4.3.1.1 Comparison of the baseline and saline control

The group results of saline control were compared to the baseline results. The PT results are tabulated in Table 4.1 and the APTT results in Table 4.2. The PT and APTT appeared to be prolonged in the saline control but these results were not significantly different from the baseline value.

Table 4.1 The results of the PT test of baseline and saline groups with mean and standard deviation.

No	Baseline	Saline
1	10.60	11.10
2	12.60	11.10
3	11.10	10.90
4	10.50	10.80
5	11.70	12.30
6	10.50	11.50
7	11.10	12.60
8	10.90	12.00
9	12.30	13.30
10	12.00	17.10
11	12.30	13.20
12	11.40	12.00
13	15.00	12.80
14	10.60	11.10
15	12.10	11.80
16	13.20	10.50
17	11.30	12.80
18	11.30	12.60
Mean	11.6944	12.1944
Std. Deviation	1.1373	1.4984
p-value	Not significant (0.268)	

Table 4.2 The results of the APTT test of baseline and saline groups with mean and standard deviation.

No.	Baseline	Saline
1	39.80	39.10
2	60.90	51.60
3	42.10	39.10
4	39.60	39.90
5	45.60	47.40
6	38.80	43.00
7	33.80	38.80
8	37.30	41.70
9	49.60	47.30
10	43.20	55.60
11	37.60	45.10
12	41.50	43.20
13	50.10	43.50
14	39.10	40.30
15	37.00	37.90
16	45.40	36.90
17	35.70	37.90
18	42.80	47.80
Mean	42.2167	43.1167
Std. Deviation	6.4590	5.1675
p-value	Not significant (0.647)	

4.3.1.2 Comparison of the baseline and *Lachesis mutas* 6CH

The group results of *Lachesis mutas* 6CH, the first centesimal potency used, were compared to the baseline results. The PT results are tabulated in Table 4.3 and the APTT results in Table 4.4. With the addition of the 6CH potency, the PT and APTT appeared to be prolonged but these results were not significantly different from the baseline value.

Table 4.3 The results of the PT test of baseline group and *Lachesis mutas* 6CH with mean and standard deviation.

No	Baseline	*Lachesis mutas* 6CH
1	10.60	15.30
2	12.60	11.70
3	11.10	11.30
4	10.50	11.70
5	11.70	12.10
6	10.50	12.00
7	11.10	12.10
8	10.90	12.10
9	12.30	11.40
10	12.00	13.20
11	12.30	15.00
12	11.40	12.00
13	15.00	13.30
14	10.60	11.10
15	12.10	11.50
16	13.20	12.10
17	11.30	-
18	11.30	11.70
Mean	11.6944	12.3294
Std. Deviation	1.1373	1.2092
p-value	Not significant (0.120)	

Table 4.4 The results of the APTT test of baseline group and *Lachesis mutas* 6CH with mean and standard deviation.

No.	Baseline	*Lachesis mutas* 6CH
1	39.80	38.50
2	60.90	52.80
3	42.10	38.10
4	39.60	42.00
5	45.60	47.70
6	38.80	47.40
7	33.80	42.30
8	37.30	43.20
9	49.60	44.80
10	43.20	45.60
11	37.60	36.40
12	41.50	43.80
13	50.10	47.80
14	39.10	38.70
15	37.00	37.20
16	45.40	44.50
17	35.70	-
18	42.80	46.30
Mean	42.2167	43.3588
Std. Deviation	6.4590	4.4972
p-value	Not significant (0.546)	

4.3.2.3 Comparison of the baseline and *Lachesis mutas* 9CH

The group results of *Lachesis mutas* 9CH, the second centesimal potency used, were compared to the baseline results. The PT results are tabulated in Table 4.5 and the APTT results in Table 4.6. The PT and APTT appeared to be prolonged with the addition of the 9CH potency but these results were not significantly different from the baseline value.

Table 4.5 The results of the PT test of baseline group and *Lachesis mutas* 9CH with mean and standard deviation.

No	Baseline	*Lachesis mutas* 9CH
1	10.60	10.90
2	12.60	13.60
3	11.10	10.60
4	10.50	12.10
5	11.70	12.40
6	10.50	11.50
7	11.10	11.50
8	10.90	12.90
9	12.30	13.60
10	12.00	13.90
11	12.30	11.70
12	11.40	14.50
13	15.00	13.80
14	10.60	10.80
15	12.10	11.70
16	13.20	12.10
17	11.30	11.70
18	11.30	13.00
Mean	11.6944	12.3500
Std. Deviation	1.1373	1.1729
p-value	Not significant (0.098)	

Table 4.6 The results of the APTT test of baseline group and *Lachesis mutas* 9CH with mean and standard deviation.

No.	Baseline	*Lachesis mutas* 9CH
1	39.80	40.20
2	60.90	64.00
3	42.10	34.80
4	39.60	45.10
5	45.60	46.80
6	38.80	42.80
7	33.80	39.60
8	37.30	45.40
9	49.60	56.30
10	43.20	47.30
11	37.60	42.90
12	41.50	48.60
13	50.10	49.50
14	39.10	37.60
15	37.00	36.60
16	45.40	43.80
17	35.70	38.70
18	42.80	52.50
Mean	42.2167	45.1389
Std. Deviation	6.4590	7.3502
p-value	Not significant (0.214)	

4.3.2.4 Comparison of the baseline and *Lachesis mutas* 12CH

The group results of *Lachesis mutas* 12CH, the third centesimal potency used, were compared to the baseline results. The PT results are tabulated in Table 4.7 and the APTT results in Table 4.8. The PT and APTT appeared to be prolonged with the addition of the 12CH potency but these results were not significantly different from the baseline value.

Table 4.7 The results of the PT test of baseline group and *Lachesis mutas* 12CH with mean and standard deviation.

No	Baseline	*Lachesis mutas* 12CH
1	10.60	10.80
2	12.60	12.40
3	11.10	-
4	10.50	11.10
5	11.70	12.00
6	10.50	11.30
7	11.10	11.10
8	10.90	13.00
9	12.30	13.00
10	12.00	12.40
11	12.30	12.30
12	11.40	12.10
13	15.00	13.20
14	10.60	12.40
15	12.10	13.60
16	13.20	11.40
17	11.30	16.50
18	11.30	13.00
Mean	11.6944	12.4471
Std. Deviation	1.1373	1.3309
p-value	Not significant (0.082)	

Table 4.8 The results of the APTT test of baseline group and *Lachesis mutas* 12CH with mean and standard deviation.

No.	Baseline	*Lachesis mutas* 12CH
1	39.80	38.50
2	60.90	55.80
3	42.10	-
4	39.60	40.50
5	45.60	46.30
6	38.80	39.10
7	33.80	33.90
8	37.30	45.30
9	49.60	51.10
10	43.20	43.00
11	37.60	37.20
12	41.50	41.70
13	50.10	46.50
14	39.10	58.00
15	37.00	47.70
16	45.40	37.60
17	35.70	46.30
18	42.80	49.90
Mean	42.2167	44.6118
Std. Deviation	6.4590	6.6536
p-value	Not significant (0.288)	

A further statistical analysis was made comparing the three potencies that were used in the study, that is *Lachesis mutas* 6CH, 9CH and 12CH.

4.3.2.5 Comparison of *Lachesis mutas* 6CH and 9CH

A comparison drawn between *Lachesis mutas* 6CH and 9CH, the 9CH potency showed a prolonged PT and APTT time. This was however not significantly different from the results of *Lachesis mutas* 6CH. Table 4.9 and Table 4.10 show the results of the PT and APTT tests respectively.

Table 4.9 The results of the PT test of Lachesis *mutas* 6CH and *Lachesis mutas* 9CH with mean and standard deviation.

No	*Lachesis mutas* 6CH	*Lachesis mutas* 9CH
1	15.30	10.90
2	11.70	13.60
3	11.30	10.60
4	11.70	12.10
5	12.10	12.40
6	12.00	11.50
7	12.10	11.50
8	12.10	12.90
9	11.40	13.60
10	13.20	13.90
11	15.00	11.70
12	12.00	14.50
13	13.30	13.80
14	11.10	10.80
15	11.50	11.70
16	12.10	12.10
17	-	11.70
18	11.70	13.00
Mean	12.3294	12.3500
Std. Deviation	1.2092	1.1729
p-value	Not significant (0.960)	

Table 4.10 The results of the APTT test of *Lachesis mutas* 6CH and *Lachesis mutas* 9CH with mean and standard deviation.

No.	*Lachesis mutas* 6CH	*Lachesis mutas* 9CH
1	38.50	40.20
2	52.80	64.00
3	38.10	34.80
4	42.00	45.10
5	47.70	46.80
6	47.40	42.80
7	42.30	39.60
8	43.20	45.40
9	44.80	56.30
10	45.60	47.30
11	36.40	42.90
12	43.80	48.60
13	47.80	49.50
14	38.70	37.60
15	37.20	36.60
16	44.50	43.80
17	-	38.70
18	46.30	52.50
Mean	43.3588	45.1389
Std. Deviation	4.4972	7.3502
p-value	Not significant (0.392)	

4.3.2.6 Comparison of *Lachesis mutas* 6CH and 12CH

In a comparison drawn between *Lachesis mutas* 6CH and 12CH, the 12CH potency showed a prolonged PT and APTT time. This was however not significantly different from the results of *Lachesis mutas* 6CH. Table 4.11 and Table 4.12 show the results of the PT and APTT tests respectively.

Table 4.11 The results of the PT test of *Lachesis mutas* 6CH and *Lachesis mutas* 12CH with mean and standard deviation.

No	*Lachesis mutas* 6CH	*Lachesis mutas* 12CH
1	15.30	10.80
2	11.70	12.40
3	11.30	-
4	11.70	11.10
5	12.10	12.00
6	12.00	11.30
7	12.10	11.10
8	12.10	13.00
9	11.40	13.00
10	13.20	12.40
11	15.00	12.30
12	12.00	12.10
13	13.30	13.20
14	11.10	12.40
15	11.50	13.60
16	12.10	11.40
17	-	16.50
18	11.70	13.00
Mean	12.3294	12.4471
Std. Deviation	1.2092	1.3309
p-value	Not significant (0.789)	

Table 4.12 The results of the APTT test of *Lachesis mutas* 6CH and *Lachesis mutas* 12CH with mean and standard deviation.

No.	*Lachesis mutas* 6CH	*Lachesis mutas* 12CH
1	38.50	38.50
2	52.80	55.80
3	38.10	-
4	42.00	40.50
5	47.70	46.30
6	47.40	39.10
7	42.30	33.90
8	43.20	45.30
9	44.80	51.10
10	45.60	43.00
11	36.40	37.20
12	43.80	41.70
13	47.80	46.50
14	38.70	58.00
15	37.20	47.70
16	44.50	37.60
17	-	46.30
18	46.30	49.90
Mean	43.3588	44.6118
Std. Deviation	4.4972	6.6536
p-value	Not significant (0.525)	

4.3.2.7 Comparison of *Lachesis mutas* 9CH and 12CH

In a comparison drawn between *Lachesis mutas* 9CH and 12CH, the 12CH potency showed a prolonged PT and the 9CH potency showed a prolonged APTT. Table 4.13 and Table 4.14 show the results of the PT and APTT tests respectively.

Table 4.13 The results of the PT test of *Lachesis mutas* 9CH and *Lachesis mutas* 12CH with mean and standard deviation.

No	*Lachesis mutas* 9CH	*Lachesis mutas* 12CH
1	10.90	10.80
2	13.60	12.40
3	10.60	-
4	12.10	11.10
5	12.40	12.00
6	11.50	11.30
7	11.50	11.10
8	12.90	13.00
9	13.60	13.00
10	13.90	12.40
11	11.70	12.30
12	14.50	12.10
13	13.80	13.20
14	10.80	12.40
15	11.70	13.60
16	12.10	11.40
17	11.70	16.50
18	13.00	13.00
Mean	12.3500	12.4471
Std. Deviation	1.1729	1.3309
p-value	Not significant (0.821)	

Table 4.14 The results of the APTT test of *Lachesis mutas* 9CH and *Lachesis mutas* 12CH with mean and standard deviation.

No.	*Lachesis mutas* 9CH	*Lachesis mutas* 12CH
1	40.20	38.50
2	64.00	55.80
3	34.80	-
4	45.10	40.50
5	46.80	46.30
6	42.80	39.10
7	39.60	33.90
8	45.40	45.30
9	56.30	51.10
10	47.30	43.00
11	42.90	37.20
12	48.60	41.70
13	49.50	46.50
14	37.60	58.00
15	36.60	47.70
16	43.80	37.60
17	38.70	46.30
18	52.50	49.90
Mean	45.1389	44.6118
Std. Deviation	7.3502	6.6536
p-value	Not significant (0.825)	

CHAPTER FIVE
DISCUSSION

5.1 Objective

The objective of this study was to assess the *in-vitro* effects that homoeopathic preparations of *Lachesis mutas* have on the coagulation of blood. *Lachesis mutas*, a remedy prepared from the venom of a pit viper snake of South America, has an effect on the haemostatic mechanism. The main affinity of *Lachesis mutas* is with the blood, causing disintegration of red cells and a lowered coaguability after initial tendency to thrombosis (Gibson; 1996: 292).

5.2 PT and APTT of *Lachesis mutas* 6CH, 9CH and 12CH

PT and APTT test were conducted on plasma incubated with homoeopathic preparations of *Lachesis mutas* 6CH, 9CH and 12CH. Tests were conducted for each of the following groups: baseline, saline and the three potencies. The baseline results reflected the normal laboratory values of PT and APTT. The saline test was conducted as a control to observe whether there was any action by the diluent of the different potencies on the coagulation of blood.

5.2.1 PT of *Lachesis mutas* 6CH, 9CH and 12CH

The results obtained from the different potencies showed an increased PT when compared to the baseline values. The increased PT statistically analysed, did not show a significant difference. Thus it was found that homoeopathic preparations of *Lachesis mutas* 6CH, 9CH and 12CH did not have any effect on coagulation.

5.2.2 APTT of *Lachesis mutas* 6CH, 9CH and 12CH

The results obtained from the different potencies showed an increased APTT when compared to the baseline values. The increased APTT statistically analysed, did not show a significant difference. Thus it was found that homoeopathic

preparations of *Lachesis mutas* 6CH, 9CH and 12CH did not have any effect on coagulation.

5.3 The vital force and *in-vitro* studies

Homoeopathic medicine does not treat the illness directly, but enables the human body to do the necessary healing for itself (Adams, 1996:7). The action of this medicine is on the vital force. The vital force maintains all parts and functions of the body i.e. in terms of sensations, functions and self-preservation (Gunavante, 2000:7). Any derangement to the vital force will bring about disease and is rectified by the aid of homoeopathic medicines.

In-vitro studies are not capable of replicating the action of the vital force.

5.4 Choice of potencies

Homoeopathic medicines are produced by means of a process of serial dilution to produce different potencies. The range of potencies used in this study was limited to low centesimal dilutions of the remedy. Another variable in the choice of potency is the Avogadro's constant. This constant suggests that if a dilution goes beyond this number, there would be no molecule of the original solute in the solution. The dilutions employed in this study were within the spectrum of Avogadro's constant i.e. less than the 12CH potency. This choice was employed to make this study as scientific as possible, but it was found that diluting medicines beyond the Avogadro's constant enhanced the effect of the medicines (Coulter, 1994:254). By limiting the choice of the dilutions, it may have limited the potential therapeutic action of the remedy. But it should also be noted that this therapeutic action might only be enhanced in an *in-vivo* study.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The aim of this study was to assess the *in-vitro* effects of homoeopathic preparations of *Lachesis mutas* on the coagulation of blood. The results of this study showed that homoeopathically prepared *Lachesis mutas*, in the 6CH, 9CH and 12CH potencies, had no significant effect on the clotting assays, prothrombin time (PT) and partial thromboplastin time (APTT).

These results however do not nullify the fundamental principles on which homoeopathy works i.e. the medicine should be similar to the disease, the doses used are minute and potentised, the treatment is individually chosen for each person and the treatment stimulates the body's natural healing power called the vital force. Furthermore, this study was conducted *in-vitro*. *In-vitro* experimentation limits the expression of the complex multi-system and physiological action of the human body. Homoeopathy is a holistic form of medicine and thus requires *in-vivo* studies to assess whether it is capable of causing any change in the coagulation of blood.

The conclusion drawn from the results, is that homoeopathic preparations of *Lachesis mutas* had no effect on the coagulation of blood in an *in-vitro* experiment, with regards to the PT and APTT tests.

Despite the insignificant results obtained from this study, there exists a great potential for further development in this field of study. A more detailed study could elucidate the possible coagulant action of the remedy, *Lachesis mutas*.

6.2 Recommendations

The following recommendations should be taken into account in a venture of future efforts in a similar field of study:

- Other remedies prepared from various snake venoms, having a haemostatic function, should be considered.

- Other remedies from different preparations like *Phosphorus*, having a haemostatic function, should be considered.

- A wider range of potencies should be utilised, ranging from crude, to produce results that may demonstrate the precise area of action that the venom has on the coagulation pathway and continue to see changes with the different potencies employed.

- The number of samples used should not be limited to a small group as coagulation errors are common. Such coagulation errors reduce the number of results obtained.

- Potencies exceeding Avogadro's constant need to be explored. Thus the addition of potencies greater than 12CH should be employed to determine their effectiveness.

- Precise laboratory expertise is necessary to reduce the errors.

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APPENDICES

APPENDIX A

Materials

Reagents/Material	Supplier
ACL machine 300	ILEX-Rivonia
Acrylic rotors	ILEX
Calibrated pipettes	Beckman and Coulter-Midrand
Centrifuge machine – Mistral 6L	SAIMR-Johannesburg
Cuvettes (ACL buckets)	ILEX
Freezer - 20°C	SAIMR
Medicated potencies: 6CH, 9CH and 12CH	Natura-Pretoria
Needles	Beckman and Coulter
Normal control plasma	ILEX
Pasteur pipettes	Beckman and Coulter
Pipette tips - blue	
- Yellow	Beckman and Coulter
Plastic tubes	Beckman and Coulter
Reagents	
• Thromborel	Dade Behring
• APTT $CaCl_2$	ILEX
• APTT - SP	ILEX
Saline	Natura
Sodium citrate tubes 3.2% (4.5 ml)	Beckman and Coulter
Thermal paper	ILEX
Vaccutainer	Beckman and Coulter
Water bath 37°C	SAIMR

APPENDIX B

CONSENT FORM

Dear Participant

The purpose of this study is to determine the effect of *Lachesis mutas* in the 6CH, 9CH and 12CH potencies respectively on the coagulation of blood.

You will be one of twenty participants from whom twenty millilitres of blood will be drawn using the standard venipuncture technique. The blood will be drawn by a qualified phlebotimist. You as a volunteer will not be required to take any form of medication. The blood drawn from you will undergo laboratory experimentation. The above-mentioned remedy will be placed into you blood plasma and prothrombin time and partial thromboplastin tests will be conducted to note the effect of this remedy on blood coagulation.

The results of the above tests may allow for usage of this remedy in patients with blood disorders.

Please note that your participation in this research is voluntary and that you are at any stage free to refuse participation, or may withdraw your consent. A copy of this consent form will be signed and made available to you. Any and all information submitted by you will be confidential and only I, as the researcher, will have access to it.

I, the volunteer, have fully understood what this research entails and any questions that I have will be directed to the researcher. I understand that there will be discomfort and pain related to the drawing of blood. In signing this consent form I agree that my blood will be used for the benefit of the study and I am aware that I may refuse participation at any time.

Date: _____ **Signature:**

_____

Thank you

I, the researcher, have fully explained the techniques and purpose of the tests used in this research. Any questions that arise from the volunteers will be answered to the best of my ability.

Date: _____ **Signature:**

_____

APPENDIX C

SUBJECT INFORMATION FORM

Subject number ☐

Surname: _____

First names: _____

Title: Mr. /Miss. /Mrs. /Dr. _____

Date of birth: D D / M M / C C Y Y

Age: _____

Tick the appropriate box

Gender: ☐ Female
☐ Male

Occupation: _____

Address: _____
_____ Code: _____

Telephone number: (H) _____
(W) _____
(C) _____

Blood Pressure: _____

APPENDIX D

QUESTIONNAIRE

Subject number ☐

Name: _____

Age: _____

Gender: _____

Have you ever been diagnosed with any of the following conditions?

(Tick the appropriate boxes)

☐ **Iron deficiency**

☐ **Anaemia and or congenital anaemia**

- ☐ Sickle cell anaemia
- ☐ Thalassemia
- ☐ Spherocytosis
- ☐ Others (specify) _____

Hypotension

Hypertension

Heart disease (please specify) _____

Any bleeding disorders:

- ☐ Haemophilia A
- ☐ Haemophilia B
- ☐ Von Willebrand's disease
- ☐ Disseminated intravascular coagulation (DIC)
- ☐ Other (specify) _____

Do you bruise or bleed easily? **Yes** ☐ **No** ☐

If yes, elaborate _____

Do you suffer from spontaneous or uncontrollable bleeding?

Yes ☐ No ☐

If yes, elaborate _____

Have you been diagnosed with a liver disease?

Yes ☐ No ☐

If yes, elaborate _____

Do you suffer from vitamin K, C, B_{12}, folic acid or copper deficiency?

Yes ☐ No ☐

If yes, elaborate _____

How often and for what ailments do you take aspirin?

_____

_____

If you have answered yes to the above question, how may aspirin tablets do you take at a time?

Have you ever had three or more of the following symptoms at the same time? (Please tick the appropriate boxes)

- ☐ Jaundice
- ☐ Itchy skin
- ☐ Headache
- ☐ Chills
- ☐ Malaise
- ☐ Nausea, vomiting and diarrhoea

Do you smoke? Yes ☐ No ☐

If yes, how many cigarettes and or cigars do you smoke on average per day?

Do you drink any alcohol? Yes ☐ No ☐

If yes,

What kind of alcohol do you drink (spirits, beers, wine etc)?

How often and how much per day?

Have you visited malaria stricken area in the last year?

Yes ☐ No ☐

If yes, have you been diagnosed with having malaria?

What medication and supplementation are you currently on (especially Heparin and Warfarin)?

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