THE *IN-VITRO* EFFECT OF HOMOEOPATHICALLY PREPARED *LACHESIS MUTAS* 6XH, 9XH AND 12XH 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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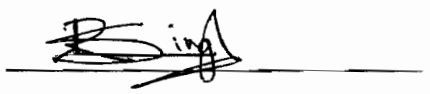
DECLARATION

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



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ABSTRACT

Blood coagulation is part of an important host defence mechanism termed haemostasis. Haemostasis is the cessation of blood loss from a damaged blood vessel. Haemostasis requires the combined activity of vascular, platelet and plasma factors to limit the accumulation of platelets and fibrin in the area of injury.

Snake venoms are complex mixtures of enzymes, polypeptides, glycoproteins and metal ions. Among these components are haemorrhagins that render the vasculature leaky and thus causing both local and systemic bleeding.

The homoeopathic remedy, *Lachesis mutas* is prepared from the venom of the snake *Lachesis mutas*. The action of *Lachesis mutas* is directly on the blood, destroying its vitality and inducing a variety of disorders characterised by disintegration of tissues.

The *in-vitro* effects of homoeopathically prepared *Lachesis mutas* 6XH, 9XH and 12 XH, on the coagulation of blood were assessed. Human plasma was incubated with different potencies of *Lachesis mutas*. Using the Automated Coagulation Laboratory (ACL) machine, prothrombin time (PT) and activated partial thromboplastin time (APTT) tests were carried out on plasma samples. The PT and APTT tests were a measure of the coagulation pathway.

The homoeopathic attenuations of *Lachesis mutas* did not have an *in-vitro* effect on the coagulation of blood. Although this study did not yield substantial results, further *in-vitro* as well as *in-vivo* studies of a similar nature could elucidate the potential therapeutic use of homoeopathic remedies.

To my parents, my sisters and my brother,
for their unsurpassed and unconditional love and blessings.

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CHAPTER ONE
INTRODUCTION

1.1 Statement of the problem

The process of blood coagulation refers to the arrest of bleeding from an injured blood vessel. Disorders of blood vessels (that is, haemostatic abnormalities) may be inherited or acquired, localised or generalised, and results in abnormal bleeding or thrombosis (Beers *et al*, 1992:1195).

Snake venoms have great potential for medical use due to the wide variety of compounds which they contain and the specific action of each compound (Ernst *et al*, 1999:34). The venom of snakes is a complex mixture containing many different biologically active proteins and peptides. A number of these proteins interact with components of the haemostatic system (Markland, 1998:1749). These haemostatically active components are distributed widely in the venom of many different snake species.

Lachesis mutas is a homoeopathic preparation manufactured from the venom of the South American snake, *Lachesis mutas* (Vermeulen, 2001:572). It is the most prominent representative of the snake family in the materia medica (Shemmer, 2000:28). Toxicological studies and experimentation reveal that one of its major branches of activity is on the blood (Jouanny, 1994:215). Like many snake venoms, it decomposes blood and affects circulation (Phatak, 1999:411).

1.2 Aim and Objective of the study

The aim and objective of this study was to treat human plasma with *Lachesis mutas* in decimal potencies (6XH; 9XH and 12XH) and to assess the *in-vitro* effects that these homoeopathic preparations may have on the coagulation of blood.

CHAPTER TWO
LITERATURE REVIEW

2.1 Homoeopathy

2.1.1 The law of similars

The word homoeopathy is derived from the Greek words *homois*, meaning like or similar and *pathos*, meaning suffering (Boyd, 1989:1). Homoepathy is a system of medical practice that originated with the work of Dr. Samuel Hahnemann (Endler and Schulte, 1998:9). Homoeopathic medicine operates on the law of similars which states "like may be cured by like" (Sankaran, 1997:1). This law is also expressed as *"similia similibus curentur"*. This means that a medicine capable of producing certain effects when taken by a healthy human being is capable of curing any illness displaying similar effects. The similia principle, formulated by Hahnemann in the late 18th century, forms the fundamental basis of homoeopathy (Roberts, 1997:57).

When toxic substances are administered to a group of healthy persons, certain signs and symptoms of toxicity are produced. The recording of these signs and symptoms builds up a symptom-complex which is unique to each homoeopathic drug (Boyd, 1989:2). Thus homoeopathic remedies produce similar symptoms as those the sick person complains of and in doing so, the remedy sharply provokes the body into throwing off these symptoms (Boyd, 1989:2-3).

2.1.2 The law of infinitesimals

The law of similars works in conjunction with the homoeopathic law of infinitesimals. This law states that the lower a dose of the curative agent, the more effective it is. A low dose is achieved by diluting the curative substance a number

of times until only a low concentration of the curative substance remains in a large amount of the diluent (Davidson, 2001:18).

2.1.3 Homoeopathic remedies

2.1.3.1 The source of homoeopathic remedies

Homoeopathic remedies can be prepared from any substance which either has a toxic effect on the body or which exerts a chemical change on the body (Boyd, 1989:53). The remedies are extracted from the three kingdoms of nature, namely the vegetable kingdom, the animal kingdom and the mineral kingdom. If the source of the remedies is physiological with active principles, the remedies are called sarcodes, and if the source of the remedies are pathological secretions or products, these remedies are called nosodes (Eizayaga, 1991:162). Imponderabilia, which includes positive and negative magnetic forces, electricity, x-rays etc, also forms a source of homoeopathic remedies (Gunavante, 2000:36).

2.1.3.2 The homoeopathic potency

Homoeopathic remedies are prepared by a process known as dynamisation or potentisation. The process of potentisation involves serial dilution with succussion. Succussion is the vigorous shaking of the solution (Endler and Schulte, 1998:257). Homoeopathic potencies consist of a substance raised to extremely high rates of vibration stimulating the quality and vibratory rate of the life force that animates the body (Bernard, 1999:61).

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).

2.2 Classification of snakes

The classification of the major snake groups is based on evolutionary divergence and monophyletic relationships. These major snake groups include the Scolecophidians, Anomochilus, Basal Macrostonatans and Colubroidea. Fifteen families of snakes are identified (Figure 2.1).

Venomous seakraits and seasnakes are sometimes collectively separated as the Hydophiidea family. The Viperidae, Elapidae and Hydophiidea constitute the poisonous snakes. The Viperidae family is further divided into four subfamilies; namely the Viperinae (true vipers), Crotalinae (pitvipers), Azemiopinae and Causinae (night adder) (Mattison, 1995:246).

Scolecophidia (blindsnakes)

Anomalepididae

Leptotyphlopidae

Typhlopidae

Alethinophidia

***Anomochilus* (dwarf pipesnakes)**

["basal alethinophidians," Macrostomata]

"basal alethinophidians"

Anilius scytale (Red Pipesnake)

Cylindrophis (Asian pipesnakes)

Uropeltidae (shield-tailed snakes)

Loxocemus bicolor (Neotropical Sunbeam Snake)

Xenopeltis (Asian sunbeam snakes)

Macrostomata

"basal macrostonatans"

Boidae (boas, sand boas, etc.)

Pythonidae (pythons)

Bolyeriidae (Round Island boas)

Tropidophiidae (dwarf boas)

Caenophidia (advanced snakes)

Acrochordidae (Australasian filesnakes)

Colubroidea

- Major snake groups
- Snake families

Viperidae (vipers, pitvipers)

Atractaspididae (stiletto snakes, etc.)

Colubridae (ratsnakes, goo-eaters, etc.)

Elapidae (cobras, seasnakes, etc.)

Figure 2.1 Phylogenetic tree of snake classification (Mattison, 1995:193)

2.3 *Lachesis*

2.3.1 *Lachesis mutas*

Lachesis is the venom of the bushmaster snake, *Lachesis mutas* (Russel, 1983:213). *Lachesis mutas,* a tropical snake living in the jungles of central and South America, grows to a length of 12 feet. *Lachesis mutas* is a member of the Crotalidae (pitviper) family. Being the largest poisonous pit viper in the western hemisphere, its venom is deadly and kills rapidly by inhibiting nervous impulses or slowly by interfering with blood clotting and accelerating the destruction of red blood cells (Davidson, 2001:18). Like all Crotalid venoms, *Lachesis mutas* has a special affinity for the blood and circulation and is often indicated for the treatment of bleeding and clotting phenomena (Sankaran, 1998:17). Only small doses of this venom are required to destroy red blood cells, and larger doses poison the heart (Russel, 1983:213).

2.3.2 Toxicological action

Snake venoms can be divided into two basic types; namely haemotoxic (attacking tissues and blood) or neurotoxic (attacking the nervous system). Some snake venoms may also exhibit cytotoxic effects where the tissues are affected (van de Merwe, 1990:743).

2.3.2.1 Neurotoxic group

The neurotoxic group of venom causes the following signs and symptoms:

- Neuromuscular blockade with flaccid paralysis of the skeletal muscles (the smooth muscles and the central nervous system are usually not seriously affected)
- Weakness of the eye muscles
- Halting or slurred speech
- Impaired swallowing

- Shallow and laboured breathing with a diminished peak flow due to muscles of respiration being affected
- Total paralysis of the respiratory muscles
- Death, unless resuscitation is instituted immediately.

Symptoms and signs of neurotoxic envenomation usually start within fifteen to thirty minutes and increase gradually. Although these patients are completely paralysed, they may be conscious to their surroundings (van de Merwe, 1992:747).

2.3.2.2 Cytotoxic group

The following signs and symptoms are common in the cytotoxic group of snake venom:

- Severe local damage to the tissues and vascular endothelium
- Local swelling which occurs within about 10 to 30 minutes, increasing gradually
- Blisters, blood blisters, ecchymoses and deeper haemorrhages
- Swollen and tender lymph nodes
- Systemic symptoms including, nausea, vomiting and dizziness
- Hypovolaemic shock in serious cases of envenomation.

The late complications of cytotoxic envenomation include: necrosis, ulcers, impaired function and gangrene. Although death may occur, most patients are less seriously affected and recover well (van de Merwe, 1992:747).

2.3.2.3 Haematoxic group

Most pit viper venoms are of the haemotoxic type (Russel, 1983:211). *Lachesis mutas* is the largest of the pit viper family. The venom of *Lachesis mutas*, comprising of a vast range of potent enzymes, yields an approximate 280-450mg

of the dry weight of the venom. The lethal dose fifty (LD 50) is calculated at 5,93mg/kg. Since the blood is primarily attacked, the main affinities are within the blood. The venom causes red cell disintegration and lowered coagubility after an initial tendency to thrombosis (Gibson, 1996:89). Since the venom of these snakes consists mainly of elements which break down blood and affect the tissues, the region of the bite is painful and the following symptoms are characteristic of haematoxic envenomation:

- Intense burning pain at the site of the bite
- Swelling of the affected area, which becomes livid and soon spreads to distend the whole limb
- Ulceration, abscesses and gangrene may develop around the bite with watery blood oozing from fang punctures
- Muscle fasiculations
- Vomiting
- Confusion and seizures
- Bleeding disorders
- The patient has cold and clammy sweat and pronounced shock (Fitzsimons, 1984:215).

These signs and symptoms are slight at first and may only become evident after several hours, developing gradually over a few days (van de Merwe, 1992:750).

Following a short period of hypercoagubility, the blood quickly becomes hypocoaguble (Jouanny, 1984:215). Thus in addition to these symptoms the patient develops haematological manifestations including bruises, haemorrhages under the skin and elsewhere. Epistaxis, haemolysis, haematuria, haematamesis and other haemorrhages may occur (van de Merwe, 1992:750). Significant hypofibrinogenemia and thrombocytopenia lasting up to two weeks may be common following envenomation. Advanced stages of poisoning results in involuntary passing of faeces and urine, difficulty breathing, dilated pupils with

photophobia and finally collapse with a loss of consciousness. Extreme exhaustion leads to death (Boyer *et al.*, 1999:159).

2.3.3 The clinical picture of *Lachesis mutas*

In homoeopathic terms, Dr. Constantine Hering proved the venom of *Lachesis mutas* as a remedy in 1828 when he handled the deadly snake himself and recorded the symptoms after regaining consciousness (Mathur, 1999:544). Like all snake venoms, *Lachesis mutas* acts directly upon the blood, destroying its vitality. This remedy induces a great variety of disorders characterised by disintegration of tissues (Allen, 1994:626). The homoeopathic picture of *Lachesis mutas* shows it to be suited to septic illness and decomposition of blood as well as thrombocytopaenic (haemorrhagic) purpura or a haemorrhagic diathesis (Reckeweg, 1991:244).

According to Boericke (1998:387), *Lachesis mutas* decomposes the blood rendering it more fluid; thus having a marked haemorrhagic tendency. Purpura, septic states, diphtheria and other low forms of disease occurs when the system is thoroughly poisoned. The haemorrhages produced by the remedy are thin and contains dark particles like charred straw (Murphy, 1995:951). This may be from the uterus, bowels, nose, stomach, and lungs or at the base of an ulcer.

It is also of use in haemorrhages occurring in typhoid fever (Lilienthal, 1996:481). These haemorrhages are usually dark and decomposed (Farrington, 1995:35). This remedy is often indicated in purpura haemorrhagica, where the whole body is swollen and extremely sore. This renders the patient intolerant to clothing. There is a tendency to ecchymoses and bed sores (Allen, 1994:636).

The circulatory complaints are marked and include the following symptoms:

- Swollen and engorged veins that give a bluish tinge to the skin
- Varicose veins
- Nose bleeds
- Bluish wounds which are slow to heal
- Weak irregular pulse and palpitations are also marked (Hering, 1995:605).

2.4 Haemostasis

Haemostasis or the coagulation of blood may be defined as a process which maintains the flow of blood in a fluid state and is confined to the circulatory system (Pallister, 1998:447). The haemostatic mechanism has several important functions. The first is to maintain blood in a fluid state whilst it remains circulating within the vascular system. The second is to arrest bleeding at the site of injury or blood loss by the formation of a haemostatic plug. Thirdly, it must ensure the eventual removal of the plug when healing is complete. A deficiency or exaggeration of any one may lead to either thrombosis or haemorrhage (Dacie and Lewis, 1996: 297).

Thus the normal haemostatic system exists as a balance between haemorrhage and thrombosis. This balance is maintained through the interaction of blood vessels, platelets, the coagulation and anticoagulation factors, and the fibrinolytic factors.

2.4.1 The mechanism of blood coagulation

The cascade theory of blood coagulation, introduced by Biggs and MacFarlane in the early part of 1964, is composed of an extrinsic and intrinsic pathway (Rice, 1996:11). According to this coagulation mechanism, the extrinsic pathway begins with interaction of the phospholipid tissue factors with factor VII to activate factor X. Furthermore, factor X activation also occurs in the intrinsic pathway, which is initiated by contact activation of factor XII (Hageman factor) when it comes in contact with the negatively charged surfaces underlying the endothelium (Rice, 1996:11).

Although this concept of "intrinsic" and "extrinsic" pathways served as a basis of explaining the mechanism of blood coagulation for many years, recent studies have shown that there is no separation of the two pathways (Rice, 1996:12). The two pathways are highly interconnected and the central controlling key in the entire haemostatic mechanism is factor VII and tissue factor.

The mechanism of blood coagulation begins with damage to the vessel wall. At the site of vessel injury, factor VII binds to tissue factor (Rice, 1996:12). Tissue factor is a non-enzymatic lipoprotein occurring on the surface of cells that are not normally in contact with plasma (Colwell and Tollefsen, 1998:5). Fibroblasts and macrophages are examples of cells that are not normally in contact with plasma. The initiation of blood coagulation is seen in Figure 2.2 (Colwell and Tollefsen, 1998:6).

Following the binding to tissue factor, factor VII is immediately activated from its zymogen to its enzymatically active form. The resultant can be called a "two-unit" enzyme, where the activated factor VII is the catalytic component and the tissue factor constitutes the regulatory or rate controlling part.

The tissue factor or the factor VIIa complex then binds and activates both factor X and factor IX. The activation of factor X results in a small component of factor X being split off and washed away in plasma. In the presence of factor V, Ca^{++} and the platelet membrane, the activated factor X converts prothrombin (factor II) to thrombin (factor IIa). The thrombin generated converts fibrinogen to fibrin (Rice, 1996:13). The factor VII or tissue factor complex also activates factor IX. In the presence of factor VIII, Ca^{++}, and the platelet membrane, the activated factor IX activates more factor X which in turn generates more thrombin. Some of this thrombin activates factor XI which accelerates the activation of factor IX. The result is an increased process of large amounts of thrombin formation and the conversion of fibrinogen to a fibrin clot.

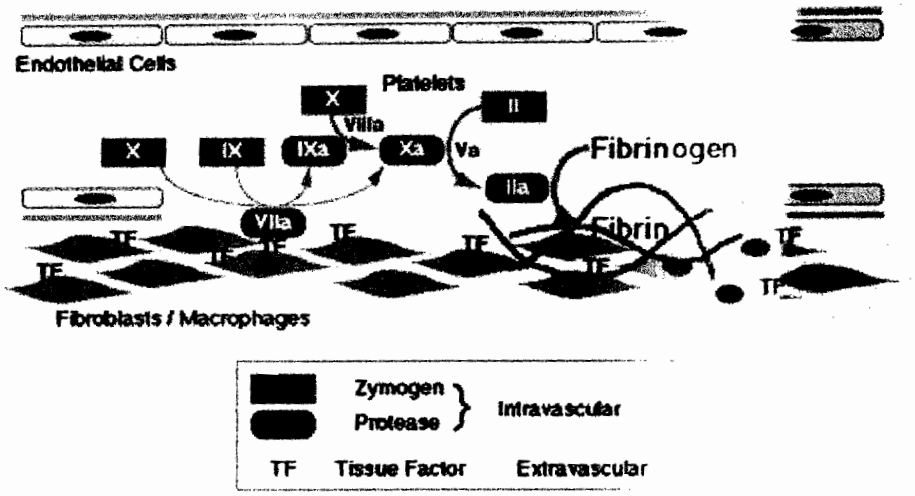

Figure 2.2 Initiation of blood coagulation (Colwell and Tollefsen, 1998:6)

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 with a molecular weight of 7200 (Beck, 1985:517). After the removal of a single peptide, a carboxylase resident in the endoplasmic reticulum or Golgi binds to the peptide region of the prothrombin, converting ~10-12 glutamate (Gla) residues to γ-carboxyglutamate (Gla) in the adjacent "Gla domain" (Colwell and Tollefsen, 1998:10). Figure 2.3 shows the γ-carboxylation of prothrombin (Colwell and Tollefsen, 1998:11). Rapid activation occurs when prothrombin and factor Xa contain Gla residues. The Gla residues bind calcium, which alters the conformation of the Gla domains of these factors. This alteration allows for interaction with a membrane surface provided by platelets *in-vivo*.

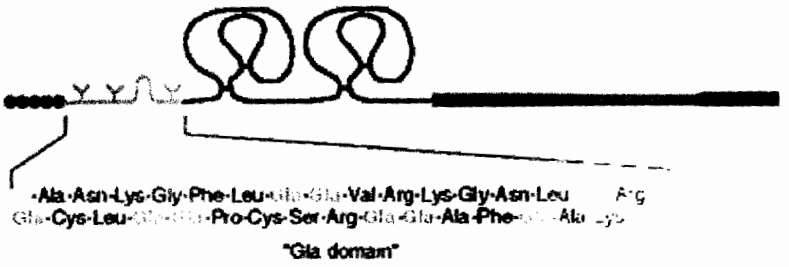

Figure 2.3 γ-Carboxylation of prothrombin (factor II) (Colwell **and Tollefsen,** 1998:11)

2.4.2.2 Conversion of prothrombin to thrombin

A complex of substances called prothrombin activators is formed **in response to** rupture of the vessel or damage to the blood itself **(Guyton, 1991:391).** Prothrombin activator is a complex of an enzyme, factor Xa **and two co-factors;** factor Va and factor X. The activation of prothrombin by factor **X, in the presence** of factor Va, phospholipid and calcium, results in the cleavage **of two peptide** bonds. In the first step the prodomain is cleaved away **leaving prethrombin 2,** while in the second step the prethrombin 2 is cleaved, generating **active thrombin** (Markland, 1998:1753). Prothrombin activation complex **is shown schematically** in Figure 2.4 (Colwell and Tollefsen, 1998:13).

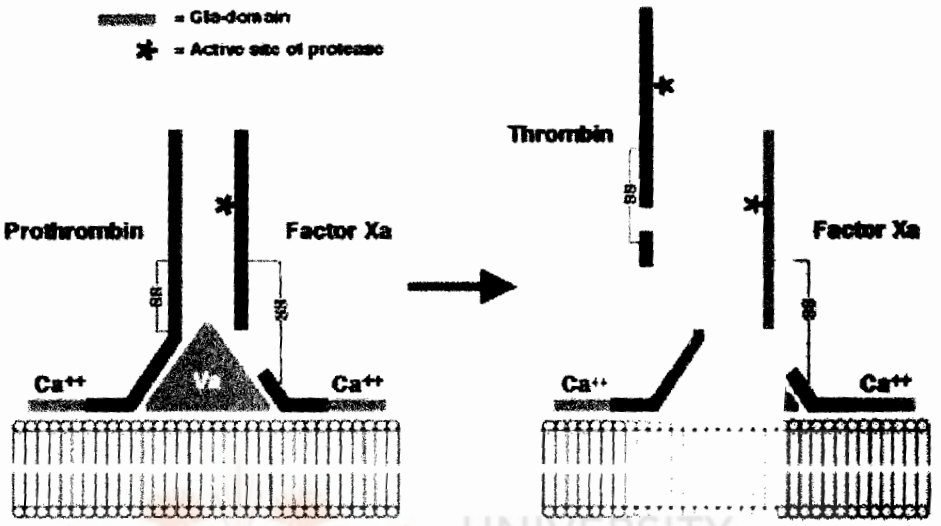

Figure 2.4 Prothrombin activation complex (Colwell and Tollefsen, 1998:13)

2.4.3 Snake venoms containing prothrombin activators

Based on the structural properties and the mechanism of prothrombin activation, the venom prothrombin activators can be divided into four groups. Group I acts by converting prothrombin directly into meizothrombin. This meizothrombin or prethrombin is then autocatalytically converted to thrombin. Group II is able to cleave both bonds in prothrombin leading to the activation of the two-chain thrombin. Activators of Group II are inactive against prothrombin in the absence of cofactors. However, their activity is strongly stimulated by phospholipids and factor Va in the presence of calcium (Markland; 1998:1753). Group III members only require phospholipid and calcium for the activation of prothrombin. Group

III members have a cofactor which plays the role of factor V, thus not requiring factor V. Group IV activators are unable to generate thrombin but rather convert prothrombin into modified forms which are enzymatically active.

Ecarin, the prothrombin activator from the saw scaled viper (***Echis carinatus***) venom; trigramin, the disintegrin from the green tree viper (***Trimeresurus gramineus)*** venom and jararhagin, the haemorrhagic protein from the venom of jararaca (*Bothrops jararaca*) are venom prothrombin activators belonging to Group I. Venom enzymes of Group II include the tiger snake (***Notechis scutatus scutatus***) venom. Taipan venom (*Oxyuramus scutellatus scutellatus*) belongs to Group III activators (Markland; 1998:1754).

2.4.4 Fibrinogen and the conversion of fibrinogen to fibrin

2.4.4.1 Fibrinogen

Fibrinogen is a plasma protein composed of three pairs of polypeptide chains termed Aα, Bß, and γ chains. These six chains are held together by means of disulphide bridges, which maintain the N-terminals of all the chains in a rigid, symmetrical configuration at one end of the molecule. This region of the fibrinogen molecule is referred to as the N-terminal disulphide knot or N-DSK region (Figure 2.5) (Beck, 1995:416). The N-terminal disulphide knot forms part of a larger nodular structure termed the E domain that is made up of intertwined polypeptide chains. The six chains emerge from this area of the molecule to form two lateral bundles of three each. Each bundle contains a single Aα, Bß, and γ polypeptide chain (Beck, 1995:417).

The C-terminal region of each of these bundles intertwines to form two separate nodular structures termed the D domains. The C-terminal regions of the Aα and γ chains are in particularly exposed position with the D domain. The last 12 residues of the C-terminal segment of the γ chain constitute the major domain by which fibrinogen binds to platelet membranes. The final phases of platelet aggregation require the interaction of divalent fibrinogen with exposed

glycoprotein IIb/IIIa receptors of separate platelets in order to bridge the distance between these cellular elements (Beck, 1995: 417). Figure 2.5 shows the structure of (A) fibrinogen and (B) the fibrin clot.

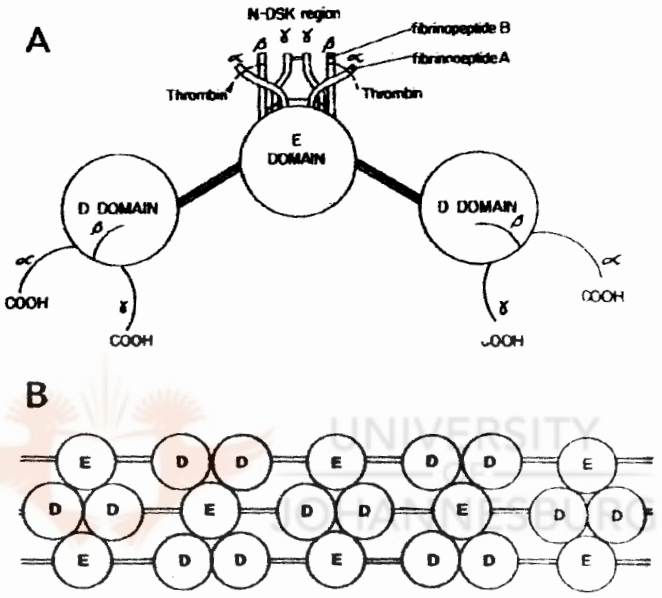

Figure 2.5 Structure of (A) fibrinogen and (B) the fibrin clot **(Beck, 1995:416)**

2.4.4.2 Conversion of fibrin to fibrinogen

Thrombin is a protein enzyme possessing proteolytic capabilities. Thrombin acts on fibrinogen, removing four low molecular weight peptides from each fibrinogen molecule, resulting in the formation of a molecule of fibrin monomer (Beck, 1995:415). The fibrin monomers are automatically capable of polymerising with other fibrin monomer molecules to form long fibrin polymers. Fibrin polymerisation is seen in Figure 2.6 (Colwell and Tollefsen, 1998:14). This polymerisation takes place within seconds. In the beginning stages the clot is weak and can easily be broken apart. This occurs as a result of fibrin monomer

molecules being held together by weak noncovalent hydrogen bonding and the threads are not cross-linked with each other. A substance called the fibrin stabilising factor strengthens the fibrin reticulum. Before it can have an effect on the fibrin, the fibrin stabilising factor must be activated itself. Its activation is by the same thrombin that causes fibrin formation. Once activated the fibrin stabilising factor acts as an enzyme to form covalent bonds between the fibrin monomer molecules. The activated substance also forms multiple cross linkages between adjacent fibrin threads adding to the three dimensional strength of the fibrin meshwork (Beck, 1995:416).

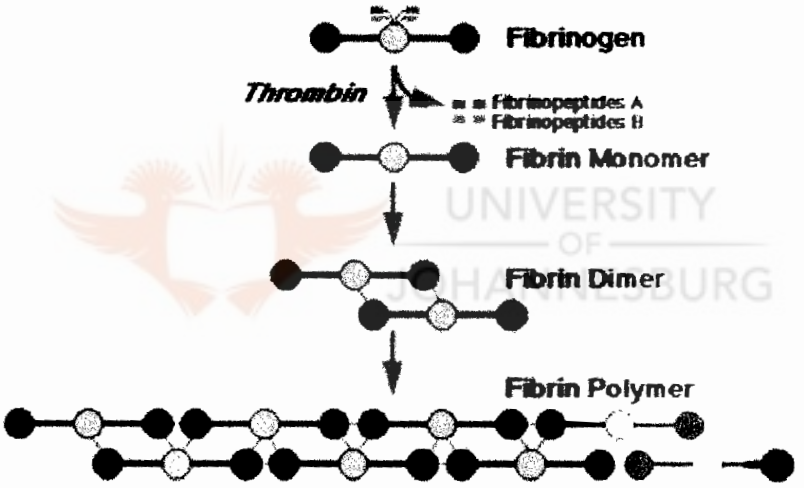

Figure 2.6 Fibrin polymerization (Colwell and Tollefsen, 1998:14)

2.4.5 Snake venoms able to clot fibrinogen

Enzymes that are able to clot fibrinogen are found mainly in venoms of snakes from the true vipers and pit vipers. These snake venom fibrinogen-clotting enzymes are divided into three groups depending on their rate of release of fibrinopeptides A and B from fibrinogen (Markland, 1998:1754). The first group (venombin A group) preferentially releases fibrinopeptide A. The second group

(venombin AB group) releases both fibrinopeptide A and B and the third group (venombin B group) releases fibrinopetide B preferentially.

2.4.5.1 Venombin A group

Three proteins having similar properties represent this class of enzymes. These include 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 to act as defibrinogenating agents to remove fibrinogen from blood. In the test tube they act as procoagulants and form a fibrin clot (Markland, 1998:1755). 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, resulting in the release of fibrinopeptide A and the conversion of fibrinogen to a fibrin clot (Markland, 1998:1755).

Ancrod was found to remove a protein, which augments clot formation from the blood, thus improving blood flow. Blood supply is interrupted to parts of the brain in the course of a stroke and Ancrod may be able to reduce serious neurological damage and reduce mortality rate (Cook, 2000:86).

2.4.5.2 Venombin AB group

This is represented by the enzyme from Gaboon viper (*Bitis gabonica*) venom. The cleavage of the Arg16-Gly17 bond nearest to the amino terminus of the Aα-chain of the fibrinogen, releasing fibrinopeptide A, is triggered by the coagulant action of gabonase. The cleavage of the Arg15-Gly16 bond nearest to the amino terminus of the Bβ-chain causes a slow release of fibrinopeptide B (Markland, 1998:1758).

2.4.5.3 Venombin B group

Venombin B group is represented by enzymes from the southern copperhead (*Agkistrodon contortrix contortrix*) venom. This enzyme is called venzyme and

causes more rapid release of fibrinopeptide B then fibrinopeptide A. Venzyme only clots fibrinogen after prolonged incubation (Markland, 1998.1758).

2.4.6 Activation of thrombin

Thrombin or factor IIa is a protein enzyme with proteolytic capabilities (Dacie and Lewis, 1995:298). It is the final protease produced in the clotting cascade. Activated factor X activates prothrombin (factor II) to thrombin (factor IIa). Thrombin in turn converts fibrinogen to fibrin. The activation of thrombin occurs on the surface of activated platelets and requires the formation of a prothrombinase complex.

Thrombin is capable of several actions. It may be able to activate factor XI. Thrombin can also convert factors V and VIII into forms that are more potent than the precursor circulating factors, thus promoting its own production (Babior and Stossel, 1994:192). Its main action, however, is the conversion of fibrinogen to form fibrin.

In addition to its role in activation of fibrin clot formation, thrombin plays an important regulatory role in coagulation. Thrombin combines with thrombomodulin (present on endothelial cell surfaces) to form a complex that converts protein C to protein Ca (Babior and Stossel, 1994: 198). The cofactor protein S and protein Ca degrade factors Va and VIIIa. This limits the activity of factor Va and factor VIIIa in the coagulation cascade.

2.4.7 Thrombin-like snake venom enzymes

As mentioned above, thrombin has many actions. Thrombin activates factor V and Factor VIII into more potent forms than the precursor circulating factors. Thrombin also causes activation of factor XIII and protein C. However; thrombin's main function is the conversion of fibrinogen to form fibrin.

Enzymes that activate or inactivate zymogens, cofactors or inhibitors of the prothrombin activation pathway, and proteinases that exert one or several of the multiple functions of thrombin have been detected in snake venoms (Meier and Stocker, 1996:67). These thrombin-like snake venoms have an action upon factor V, factor VIII, factor XIII, protein C and fibrinogen.

2.4.7.1 Action on factor V

Russel's viper venom is a potent activator of factor X (RVV-X). The venom also contains a serine protease, which is able to convert factor V into factor Va, by cleaving a single peptide bond (Meier and Stocker, 1996:70).

2.4.7.2 Action on factor VIII

Thrombocytin from *Bothrops atrox* venom causes an increase in factor VIII clotting activity.

2.4.7.3 Action on factor XIII

When factor XIII is treated with thrombin or thrombocytin, it causes proteolytic cleavage of the α-chain and release of a peptide. The activated factor XIII (factor XIIIa) causes fibrin cross-linkage. Both ancrod and batroxobin cause a partial activation of factor XIII (Meier and Stocker, 1996:71).

2.4.7.4 Action on protein C

The combination of thrombin with thrombomodulin present on the endothelial cell surface forms a complex converting protein C to protein Ca. In the absence of thrombomodulin, calcium inhibits the thrombin-mediated activation of protein C. Incubation of zymogen with the factor X-activating proteinase (RVV-X), from Russel's viper venom, causes slow protein C activation. Fast acting protein C activators are found in the venom of *Agkistrodon contortrix contortrix* (Meier and Stock, 1996:73).

2.4.7.5 Action on fibrinogen

'Thrombin-like' snake venom refer to those enzymes that exert prominent thrombin action (Markland, 1998:1754). Depending on their rate of fibrinopeptide release, these thrombin-like snake venoms are divided into three groups, namely, Venombin A group (releasing mainly FPA), venombin AB group (releasing both FPA and FPB) and venombin B group (releasing mainly FPB) (Markland, 1998:1754).

2.4.8 The fibrinolytic system

The major function of the fibrinolytic system is the degradation and dissolution of formed fibrin within the circulation. Fibrinolysis is required to degrade small quantities of fibrin, which are continually being deposited within the circulation. This is the body's first line of defence against thrombosis. There is a balance between the coagulation and fibrinolytic pathways *in-vivo* (Pallister, 1998: 473). The enzyme responsible for fibrin degradation is called plasmin. Plasmin is produced in the area of the clot by means of plasminogen activation. Two activators convert plasminogen to plasmin. The first activator is tissue plasminogen activator, which initiates clot lysis by activating plasminogen bound to the fibrin in the clot. The second activator, urokinase, keeps hollow organs free from clots. Stimulated endothelial cells and mononuclear phagocytes secrete both activators, while urokinase is secreted by epithelial cells as well (Babior and Stossel, 1994:199).

Plasmin digests the fibrin molecule in an orderly manner. Figure 2.7 shows the process of fibrin degradation (Babior and Stossel, 1994:200). Firstly, plasmin releases the outer parts of the α-chains, which produces the X-fragment. This fragment splits on one side of the disulfide knot to form a D (small) and a Y (large) fragment. A similar cleavage on the other side of the disulfide knot releases a second D fragment which leaves behind a small E fragment. (Babior and Stossel, 1994:200). When plasmin digests a clot, it cuts the covalently linked fibrin polymers in the same places, that is, at the outer portions of the α-chains

and next to the disulfide knots. The fragments of polymers cut away by plasmin are released from the clot. The resultant fragments are called fibrin-degradation products (FDPs). Raised levels of FDPs are potent inhibitors of clotting (Babior and Stossel, 1994:201). Plasmin is also capable of degrading other proteins of the clotting cascade, such as factors V and VIII, but the most important is fibrinogen.

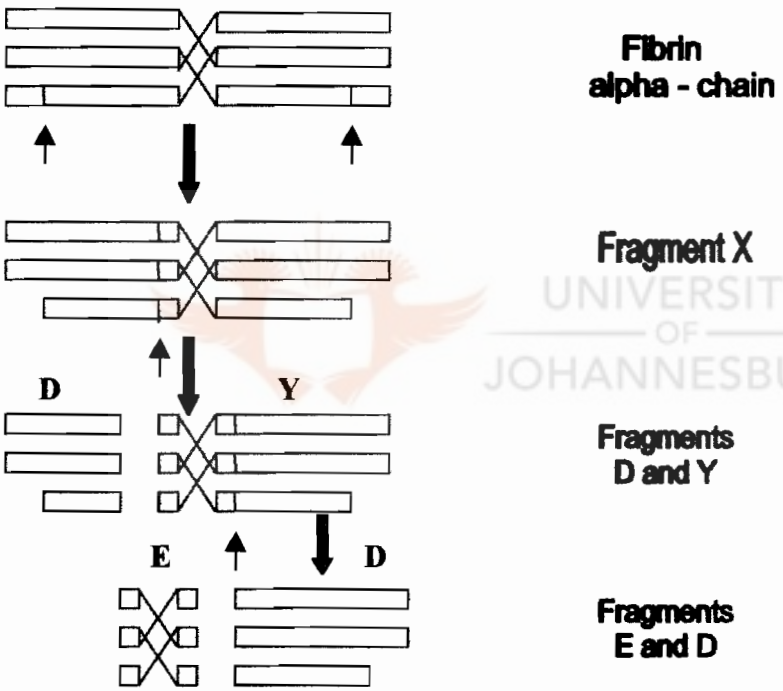

Figure 2.7 The process of fibrin degradation (Babior and Stossel, 1994:200)

2.4.9 Snake venoms acting on fibrinolysis

Venom fibrinolytic enzymes may either be α- or β- chain fibrin(ogen)ases. These venom enzymes differ from plasmin. Plasmin is a serine proteinase that is readily inactivated by plasma serine proteinase inhibitor (SERPINS). Plasmin also cleaves peptide bonds at the carboxy-terminal side of lysine residues in the α- β- and γ-chains of fibrin(ogen). These sites are different then those cleaved by fibrin(ogen)olytic enzymes (Markland, 1998:1761). Majority of these fibrin(ogen)olytic enzymes are metalloproteinases with specificity directed toward the Aα-chain and with lower activity toward the Bβ-chain. Most metalloproteinases are fibrinolytic. Serine proteinases with fibrinolytic activity cleaves mainly the Bβ-chain with lower activity toward the Aα-chain. Most serine proteinases are both fibrinolytic and fibrinogenolytic (Markland, 1998:1762).

Purified fibrin(ogen)ases from snake venoms devoid of haemorrhagic activity include, atroxase (venom from the western diamondback rattle snake) and fibrolase (venom from the southern copperhead snake) which degrades the α-chain of fibrinogen and fibrin more rapidly than the β-chain. The fibrin(ogen)olytic enzyme from the northern copperhead (*Agkistrodon contortrix mokasen*) venom degrades only the Aα-chains of fibrinogen. Two fibrin(ogen)olytic enzymes from Mexican West Coast rattlesnake (*C.b.basiliscus*) venom degrade the Aα- and Bβ-chains of fibrinogen. None of these chains show activity with the γ-chain of fibrin or fibrinogen (Markland, 1998:1763).

CHAPTER THREE
MATERIALS AND METHODS

3.1) Materials

See Appendix A

3.2) Description of the Automated Coagulation Laboratory (ACL) machine

The ACL is a fully automatic microcomputer with a controlled microcentrifugal analyser. The ACL system incorporates a video display unit (VDU) which, continually displays the status of the instrument and gives instructions, to the user on how to proceed. By means of a membrane keyboard, instructions are added to the ACL system. When a sampling cycle is initiated, the samples and the reagents are sequentially pipetted into a twenty place acrylic rotor. Via a centrifugal force, the sample and the reagents are mixed and the readings are made while the rotor is spinning.

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. Chromogenic analysis is performed in the chromogenic channel. The radiation from a halogen light source is directed to the cuvette of the rotor via a quartz optic fibre system. The results are displayed on the VDU and printed by the thermal printer.

3.3 Principles of the laboratory tests for the coagulation pathways

There are a number of laboratory tests available to evaluate the coagulation pathways. These tests include the prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and assays for fibrinogen concentration. Of the four laboratory tests that are mentioned for evaluating the coagulation pathways, the activated partial thromboplastin time (APTT) and the prothrombin time (PT) tests were used during the laboratory study of this research project.

3.3.1 Activated Partial Thromboplastin Time (APTT)

3.3.1.1 Principle

The APTT is a screening test used to measure the intrinsic pathway of coagulation. Only if the factor involved in the intrinsic pathway (factors XII. XI. IX and VII) and the common pathway (factors I, II, V, X and XIII) are present in normal concentration, will the formation of fibrin occur at a normal rate. Optimal activation was achieved by the addition of a platelet phospholipid substitute which eliminated the tests sensitivity to platelet number and function, as well as the addition of activators such as kaolin, celite and ellagic acid which eliminated the variability of the activation by glass contact.

3.3.2 Prothrombin Time (PT)

3.3.2.1 Principle

The PT is the time required to form a fibrin clot when plasma is added to a thromboplastin-calcium mixture. The test is a measure of the extrinsic and common pathway of coagulation involving factors II, V, VII, X as well as fibrinogen. Tissue thromboplastin activates factor VII, which then proceeds through a cascade, eventually generating thrombin. The resultant thrombin converts fibrinogen to fibrin. The rate of fibrin formation thus depends on the

level of the factors II, V, VII and X and fibrinogen, thus measuring the overall activity of these factors.

3.4 Homoeopathically prepared *Lachesis mutas*

3.4.1 Potencies used

For the purpose of this study, *Lachesis mutas* was prepared in decimal potencies. The decimal potencies used were the 6XH, 9XH, and 12XH. These potencies were obtained from a manufacturer of homoeopathic remedies, Natura, Pretoria and were suspended in normal saline.

3.4.2 Preparation of the Potencies

The attenuations of the remedy *Lachesis mutas* are prepared from the venom of the snake *Lachesis mutas*. The venom collected is dried into crystals at low temperatures and the secretion then constitutes the stock. Following the preparation of the stock, the decimal potencies are prepared as follows. The decimal dilution method is prepared using the process of potentisation, where the substance is succussed and diluted, increasing the therapeutic effect while simultaneously nullifying the toxic effect. This process involves adding one drop of the stock to nine drops of the diluent. The solution resulting from the add-mixture of the two liquids is subject to vigorous shaking with an impact known as succussion. The succussion is carried out a hundred times. This yields the first decimal potency. The number followed by the letters 'XH' (Decimal Hahnemannian) designates the potencies. The process is repeated for each successive potency.

3.4.3 Avogadro's number

The law of chemistry suggests a limit of how many serial dilutions can be made without losing the original substance altogether. This limit is known as

Avogadro's number. Avogadro's number is the number of molecules in one mole of any substance is $6.02554X10^{23}$. It more or less corresponds to the homoeopathic potency of 24XH. Depending on what material is used, once it has been diluted beyond 24XH, the Avogadro's number has been exceeded and no molecules of the medicine are theoretically left in solution. Potencies as the 12XH are frequently used and at this level there are still molecules present in solution.

3.5 Methodology

3.5.1 Subjects

The study was conducted using blood samples obtained from twenty subjects. These subjects, qualifying for the study, were recruited between the age group of eighteen and fifty-five years. Participants were selected by means of random stratified sampling and were part of an interethnic group. An information and consent form (Appendix C and Appendix B), explaining the purpose of the study and the drawing of blood, was completed by each subject. The subjects were made aware that they were not required to take any form of medication and that their participation in this research was strictly on a voluntary basis. All information submitted by the subjects was confidential and only accessible to researcher.

3.5.2 Selection criteria

- The study eliminated subjects who had been diagnosed with any bleeding disorder.
- Subjects using heparin, warfarin and asprin were also excluded from the study.
- Subjects diagnosed with having malaria in the last year were excluded from the study.

- Participants having iron deficiency, anaemia, hypotension, hypertension, heart disease and jaundice would have been taken into consideration if the results were significant.
- Alcohol consumption and smoking were also taken into account.

3.5.3 Sample Collection

For the purpose of sample collection, blood samples were obtained from five subjects per day over a period of four days. This constituted the total amount of twenty samples required for the study. Twenty millilitres of blood was drawn per subject. The samples were collected by a phlebotomist at the WITS Medical School Day Care Ward. Venous blood was drawn with minimum stasis into 4.5 ml 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.

3.5.4 Sample Preparation

During drawing of blood samples precautions were taken to avoid haemolysis as damaged red blood cells affect coagulation. Therefore, the samples collected were immediately centrifuged, at 3000g for 10 minutes at 4°C. The resultant supernatant (plasma) was allocated into 1 ml volumes per plastic tube. A total number of ten labelled tubes were used per subject sample. 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. The plasma samples were then divided into aliquots of 400 µl into five cuvettes:

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

Calibrated pipettes were used for both allocating the plasma into the cuvettes as well as for adding 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 machine. Numbers 1-8 were used on the loading disk, 1 being the plasma pool (normal control plasma), 2 the baseline sample, 3 the saline sample, 4 the 6 XH potency and 5 and 6 the 9XH and 12XH potencies respectively. The ACL operative conditions requires a pool of normal plasma (calibration plasma). The calibration plasma consisted of lyophilised normal human plasma which had the same characteristics of normal human fresh plasma. The main purpose of this calibration plasma 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, single factors and chromogenic cycles. Secondly, during analysis a position of 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/ normal control plasma (supplied by ILEX) was refilled as often as every run on the ACL machine.

Following the loading of samples on the disk, the disk was placed into the ACL machine. Prior to commencement, reference emulsion needed to be checked. The ACL system measured direct haemostasis and calculated parameters of plasma samples. For this study the coagulimetric test PT-FIB/APTT was used. The screen was checked to set the PT-FIB/APTT test. The reagents were then filled into their respective cups. In the first cup thromborel was added, in the second cup APTT-Sp was added and APTT-$CaCl_2$ was added to the third cup. Once the thermal paper was checked, the machine was programmed to start. At this stage, 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 results were statistically analysed using the independent 2-sample T-test on the SPSS statistic program.

CHAPTER FOUR

RESULTS

4.1 Introduction

The haematoxic class of snake venoms contains a variety of procoagulant and anticoagulant proteins. These snake venoms also contain enzymes that directly activate factor X, enzymes that directly activate prothrombin and enzymes that directly cleave fibrinogen (Gandolfi *et al*, 1997:295). Toxicological studies and experimentation reveals that homoeopathically prepared *Lachesis mutas* should exhibit red blood cell disintegration and lowered coagubility after an initial tendency to thrombosis (Gibson, 1996:89). Additionally, *in-vitro* studies of the venom of *Lachesis mutas,* has shown it to have proteolytic and indirect haemolytic activities (Otero, 1998:1226).

4.2 Group analysis

The results obtained after each subsequent run on the ACL machine, were statistically analysed using the independent 2-sample T-test on the SPSS statistic program. The statistics 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 and groups 3, 4, and 5 represented the 6XH, 9XH, and 12XH potencies respectively. The saline served as a control for the experiment, to detect these results comparable to the results of the attenuations of the remedy, *Lachesis mutas*.

The potencies of the remedy employed in the study were also prepared in saline. Alcohol, being the normal vehicle of homoeopathically prepared liquid potencies, was avoided to nullify the possible effects that the alcohol may have had on the coagulation of blood.

4.3 Statistical analysis

The analysis of the statistic report took into account the mean, standard deviation and 2-tailed significance values of both the prothrombin time (PT or PI) and partial thromboplastin time (PTT). The mean and standard deviation of the PT and PTT tests of each group were drawn in comparison to the baseline results. Depending on the reagents used and the pre-incubation period, the normal values for the PT and PTT tests differ (Dacie and Lewis, 1995:308). Each laboratory should thus establish its own normal range. The baseline offered the normal values for these coagulation tests.

A further comparison was drawn between the three potencies that were utilised in this study.

4.3.1 Descriptive results

4.3.1.1 Comparison between the baseline and saline

The group results of the saline were compared to the baseline results. The mean value of the saline appears to be prolonged in both the PT and PTT tests but with no significant difference. Table 4.1 shows the PT results and Table 4.2 shows the PTT results of the baseline and saline groups. The PI and PTT values of group 2 (that is, the saline group) are thus within normal range.

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

No	Baseline	Saline
1	12.40	19.30
2	10.60	11.50
3	16.30	17.30
4	13.00	16.00
5	10.20	11.40
6	15.60	16.30
7	11.80	12.60
8	10.80	11.30
9	10.80	11.10
10	12.30	11.70
11	13.90	14.40
12	16.50	12.60
13	14.70	-
14	11.50	11.80
Mean	12.8857	13.6385
Std. Deviation	2.1704	2.7309
P-value	Not significant	

Table 4.2 The results of the PTT test of baseline and saline groups showing the comparison between the mean and standard deviation.

No.	Baseline	Saline
1	39.90	29.50
2	36.00	41.70
3	43.60	47.70
4	43.60	50.70
5	35.50	41.70
6	40.20	42.60
7	44.40	50.70
8	40.50	43.30
9	33.10	33.40
10	43.50	42.40
11	54.90	50.70
12	48.80	42.60
13	34.30	-
14	39.80	39.60
Mean	41.2929	42.8154
Std. Deviation	5.8852	6.3877
P-value	Not significant	

4.3.1.2 Comparison between the baseline and *Lachesis mutas* 6XH

The group results of the first decimal potency used, *Lachesis mutas* 6XH, were compared to the baseline results. The comparison indicated a prolonged PT (seen in Table 4.3) and PTT (seen in Table 4.4). The calculated 2-tailed significance of the 6XH potency showed that these prolonged PT and PTT values were not significantly different from the baseline values.

Table 4.3 The results of the PT test of baseline group and *Lachesis mutas* 6XH showing the comparison between the mean and standard deviation.

No	Baseline	*Lachesis mutas* 6XH
1	12.40	21.30
2	10.60	27.80
3	16.30	15.60
4	13.00	15.30
5	10.20	11.70
6	15.60	9.75
7	11.80	12.90
8	10.80	11.50
9	10.80	12.40
10	12.30	-
11	13.90	11.10
12	16.50	18.10
13	14.70	12.60
14	11.50	18.00
Mean	12.8857	15.2346
Std. Deviation	2.1704	5.0392
P-value	Not significant	

Table 4.4 The results of the PTT test of baseline group and *Lachesis mutas* 6XH showing the comparison between the mean and standard deviation.

No.	Baseline	*Lachesis mutas* 6XH
1	39.90	36.90
2	36.00	52.30
3	43.60	46.90
4	43.60	56.30
5	35.50	33.80
6	40.20	38.10
7	44.40	52.80
8	40.50	42.80
9	33.10	34.30
10	43.50	-
11	54.90	40.80
12	48.80	57.30
13	34.30	34.90
14	39.80	55.80
Mean	41.2929	45.5385
Std. Deviation	5.8852	8.5947
P-value	Not significant	

4.3.2.3 Comparison between the baseline and *Lachesis mutas* 9XH

The group results of *Lachesis mutas* 9XH, the second decimal potency used, were compared to the baseline results. The PT and PTT appeared to be prolonged with the addition of the 9XH potency but these results were not significantly different from the baseline value. The PT results are tabulated in Table 4.5 and the PTT results in Table 4.6. *Lachesis mutas* 9XH did not effect the clotting time of plasma, both in the presence of an optimal concentration of tissue extract, namely the PT time and after the activation of contact factors, in the absence of the tissue extract, namely the PTT time.

Table 4.5 The results of the PT test of baseline group and *Lachesis mutas* 9XH showing the comparison between the mean and standard deviation.

No	Baseline	*Lachesis mutas* 9XH
1	12.40	11.70
2	10.60	18.40
3	16.30	15.30
4	13.00	14.80
5	10.20	12.10
6	15.60	10.30
7	11.80	12.00
8	10.80	11.50
9	10.80	12.40
10	12.30	-
11	13.90	11.40
12	16.50	13.30
13	14.70	12.40
14	11.50	11.70
Mean	12.8857	12.8692
Std. Deviation	2.1704	2.1519
P-value	Not significant	

Table 4.6 The results of the PTT test of baseline group and *Lachesis mutas* 9XH showing the comparison between the mean and standard deviation.

No.	Baseline	*Lachesis mutas* 9XH
1	39.90	39.00
2	36.00	45.60
3	43.60	45.40
4	43.60	52.90
5	35.50	32.40
6	40.20	40.20
7	44.40	45.90
8	40.50	42.10
9	33.10	35.40
10	43.50	-
11	54.90	44.80
12	48.80	44.40
13	34.30	40.80
14	39.80	39.90
Mean	41.2929	42.2154
Std. Deviation	5.8852	5.2070
Sig.	Not significant	

4.3.2.4 Comparison between the baseline and *Lachesis mutas* 12XH

The group results of *Lachesis mutas* 12XH, the third potency used, exhibited similar results as the 9XH potency when compared to the baseline values. The resultant prolonged PT (shown in Table 4.7) and PTT (shown in Table 4.8) time of *Lachesis mutas* 12XH was not significantly different from the baseline value. This thus depicted that *Lachesis mutas* 12XH had no effect on the PT and PTT time.

Table 4.7 The results of the PT test of baseline group and *Lachesis mutas* 12XH showing the comparison between the mean and standard deviation.

No	Baseline	*Lachesis mutas* 12XH
1	12.40	-
2	10.60	10.30
3	16.30	12.00
4	13.00	15.80
5	10.20	10.80
6	15.60	10.80
7	11.80	12.10
8	10.80	11.70
9	10.80	11.50
10	12.30	11.40
11	13.90	12.10
12	16.50	13.20
13	14.70	11.50
14	11.50	15.10
Mean	12.8857	12.1769
Std. Deviation	2.1704	1.6285
P-value	Not significant	

Table 4.8 The results of the PTT test of baseline group and *Lachesis mutas* 12XH showing the comparison between the mean and standard deviation.

No.	Baseline	*Lachesis mutas* 12XH
1	39.90	-
2	36.00	36.80
3	43.60	46.00
4	43.60	57.30
5	35.50	37.30
6	40.20	38.80
7	44.40	45.30
8	40.50	43.50
9	33.10	34.80
10	43.50	42.30
11	54.90	47.10
12	48.80	45.40
13	34.30	36.40
14	39.80	48.80
Mean	41.2929	43.0615
Std. Deviation	5.8852	6.2919
P-value	Not significant	

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

4.3.2.5 Comparison between *Lachesis mutas* 6XH and 9XH

In a comparison drawn between *Lachesis mutas* 6XH and 9XH, the 6XH potency showed a prolonged PT and PTT time. This was however, calculated not to be significantly different from the results of *Lachesis mutas* 9XH. Table 4.9 and Table 4.10 show the results of the PT and PTT tests respectively.

Table 4.9 The results of the PT test of *Lachesis mutas* 6XH and *Lachesis mutas* 9XH showing the comparison between the mean and standard deviation.

No	*Lachesis mutas* 6XH	*Lachesis mutas* 9XH
1	21.30	11.70
2	27.80	18.40
3	15.60	15.30
4	15.30	14.80
5	11.70	12.10
6	9.75	10.30
7	12.90	12.00
8	11.50	11.50
9	12.40	12.40
10	-	-
11	11.10	11.40
12	18.10	13.30
13	12.60	12.40
14	18.00	11.70
Mean	15.2346	12.8692
Std. Deviation	5.0392	2.1519
P-value	Not significant	

Table 4.10 The results of the PTT test of *Lachesis mutas* 6XH and *Lachesis mutas* 9XH showing the comparison between the mean and standard deviation.

No.	*Lachesis mutas* 6XH	*Lachesis mutas* 9XH
1	36.90	39.00
2	52.30	45.60
3	46.90	45.40
4	56.30	52.90
5	33.80	32.40
6	38.10	40.20
7	52.80	45.90
8	42.80	42.10
9	34.30	35.40
10	-	-
11	40.80	44.80
12	57.30	44.40
13	34.90	40.80
14	55.80	39.90
Mean	45.5385	42.2154
Std. Deviation	8.5947	5.2070
P-value	Not significant	

4.3.2.6 Comparison between *Lachesis mutas* 6XH and 12XH

A greater PT and PTT time was also seen in *Lachesis mutas* 6XH when this group was compared to the 12XH potency. The prolonged PT (seen in Table 4.11) and PTT (seen in Table 4.12) of the 6XH potency was however, not significantly different from the 12XH potency.

Table 4.11 The results of the PT test of *Lachesis mutas* 6XH and *Lachesis mutas* 12XH showing the comparison between the mean and standard deviation.

No	*Lachesis mutas* 6XH	*Lachesis mutas* 12XH
1	21.30	-
2	27.80	10.30
3	15.60	12.00
4	15.30	15.80
5	11.70	10.80
6	9.75	10.80
7	12.90	12.10
8	11.50	11.70
9	12.40	11.50
10	-	11.40
11	11.10	12.10
12	18.10	13.20
13	12.60	11.50
14	18.00	15.10
Mean	15.2346	12.1769
Std. Deviation	5.0392	1.6285
P-value	Not significant	

Table 4.12 The results of the PTT test of *Lachesis mutas* 6XH and *Lachesis mutas* 12XH showing the comparison between the mean and standard deviation.

No.	*Lachesis mutas* 6XH	*Lachesis mutas* 12XH
1	36.90	-
2	52.30	36.80
3	46.90	46.00
4	56.30	57.30
5	33.80	37.30
6	38.10	38.80
7	52.80	45.30
8	42.80	43.50
9	34.30	34.80
10	-	42.30
11	40.80	47.10
12	57.30	45.40
13	34.90	36.40
14	55.80	48.80
Mean	45.5385	43.0615
Std. Deviation	8.5947	6.2919
P-value	Not significant	

4.3.2.7 Comparison between *Lachesis mutas* 9XH and 12XH

The comparative PT and PTT results of *Lachesis mutas* 9XH and 12XH are seen in Table 4.13 and Table 4.14 respectively. The 9XH potency showed a greater PT and PTT time when compared to the 12XH potency. This was however, not significantly different from the 12XH potency.

Table 4.13 The results of the PT test of *Lachesis mutas* 9XH and *Lachesis mutas* 12XH showing the comparison between the mean and standard deviation.

No	*Lachesis mutas* 9XH	*Lachesis mutas* 12XH
1	11.70	-
2	18.40	10.30
3	15.30	12.00
4	14.80	15.80
5	12.10	10.80
6	10.30	10.80
7	12.00	12.10
8	11.50	11.70
9	12.40	11.50
10	-	11.40
11	11.40	12.10
12	13.30	13.20
13	12.40	11.50
14	11.70	15.10
Mean	12.8692	12.1769
Std. Deviation	2.1519	1.6285
P-value	Not significant	

Table 4.14 The results of the PTT test of *Lachesis mutas* 9XH and *Lachesis mutas* 12XH showing the comparison between the mean and standard deviation.

No.	*Lachesis mutas* 9XH	*Lachesis mutas* 12XH
1	39.00	-
2	45.60	36.80
3	45.40	46.00
4	52.90	57.30
5	32.40	37.30
6	40.20	38.80
7	45.90	45.30
8	42.10	43.50
9	35.40	34.80
10	-	42.30
11	44.80	47.10
12	44.40	45.40
13	40.80	36.40
14	39.90	48.80
Mean	42.2154	43.0615
Std. Deviation	5.2070	6.2919
P-value	Not significant	

Thus, it can be seen that none of the potencies employed in this study exhibited a prolonged PT nor PTT time that was significant when compared to each other as well as to the baseline values.

CHAPTER FIVE
DISCUSSION

5.1 Objectives

The objective of this study was to assess the effect of homoeopathically prepared *Lachesis mutas* on the *in-vitro* coagulation of blood. *Lachesis mutas* is a homoeopathic remedy prepared from the venom of the snake, *Lachesis mutas.* It belongs to the haematoxic group of snakes (Russel, 1983:211) and primarily attacks the blood, thus the main affinities are within the blood (Gibson, 1996:89). *Lachesis mutas* causes red blood cell disintegration and following a short period of hypercoagubility, the blood quickly becomes hypocoaguble (Jouanny, 1994:215).

5.2 Procedure

Human plasma was incubated with different potencies of *Lachesis mutas* at room temperature for a period of one hour. Using the ACL machine, prothrombin time (PT or PI) and partial thromboplastin time (PTT) tests were run on each plasma sample. The PT and PTT tests were a measure of the coagulation pathway.

5.3 PT and PTT of *Lachesis mutas* 6XH, 9XH and 12XH

The homoeopathic preparations of *Lachesis mutas* employed in this study were in the decimal dilution form, that is, 6XH, 9XH and 12XH. The results obtained showed an apparent prolonged PT and PTT time of these potencies when compared to the baseline results. The baseline results offered the normal laboratory values of the PT and PTT tests. However, statistical analysis revealed that the resultant prolonged PT and PTT time were not significantly different to that of the baseline value.

Based on the outcome of this study, it was found that homoeopathic preparations of *Lachesis mutas* 6XH, 9XH and 12XH did not show any coagulant action.

5.4 Haemolytic activity of *Lachesis mutas*

In-vitro studies of the venom of *Lachesis mutas* showed the venom to have proteolytic and indirect haemolytic activities (Otero, 2000:1226). According to Salveira *et al* (1989:863-871), the venom contains serine proteases similar to components isolated from the *Bothrops* species. The enzyme cleaves only fibrinopeptide A from fibrinogen, it does not activate factor XIII and is devoid of kallikrein activity. Therefore, the above-mentioned studies show that the venom of *Lachesis mutas* does have haemolytic activity.

5.5 The vital force in homoeopathy

Homoeopathy is a true science of healing based on fixed laws and principles. The principles of homoeopathy that merit attention in this study, includes the role of the vital force to achieve cure and individualisation of the patient. The vital force, life force or dynamis is that force which maintains all parts and functions of the body in admirable harmonious, vital operation as regards to sensations, functions and even self preservation (Gunavante, 2000:7). This life force is disturbed by a disease state and the aim of homoeopathic medicine is to restore the deranged life force to its full power. The individualisation of the patient is based on the principle that, since individual susceptibility varies, it is necessary to study each patient as an individual (Gunavante, 2000:12). This involves taking the mental, emotional and physical aspects of the patient into consideration. Thus a short coming of this study may be attributed to the methodology employed. *In-vitro* studies are not capable of considering all the variables of the human body.

5.6 Choice of potencies

Virtually no evidence exists regarding the correct choice of potencies to be used when administering a homoeopathic remedy (Kleijnen, 1998:128). The range of potencies used in this study was limited to low decimal dilutions of the remedy. This study made use of only 6XH, 9XH and 12XH potencies. The limit to how many serial dilutions that can be made without loosing the original substance altogether is know as Avogadro's number (Kayne, 1997: 49). Thus, in a medicine diluted beyond this limit (that is, 24XH level) it is improbable that a single molecule of the original medicinal substance remains present. However, it was found that diluting medicines beyond the "Avogadro Limit" enhanced the effect of the medicines (Coulter, 1994:254). Scientific basis of potency research and elucidation of the mechanism of action of homoeopathic dilutions beyond Avogadro's number needs to be explored. The low dilutions employed in this study may have limited the potential therapeutic action of the remedy.

Despite the insignificant results obtained from this study and the obvious short comings, 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*.

CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study aimed to assess the *in-vitro* effects of homoeopathically prepared *Lachesis mutas* on the coagulation of blood. The potencies of *Lachesis mutas* employed in this study, showed no effect on the tests used to measure the coagulation pathway, namely the prothrombin time (PT) and partial thromboplastin time (PTT). It can therefore be concluded that homoeopathic preparations of *Lachesis mutas* had no effect on the coagulation of blood in the specified *in-vitro* studies.

Although this study did not enumerate the phenomenon of the microdose (that is, the law of infinitesmals) and the similar principle (the law of similars) of homoeopathy through an *in-vitro* experiment, research on homoeopathy should not be termed as mandatory without the conduction of extensive *in-vitro* as well as *in-vivo* studies. Homoeopathy is a holistic approach toward the sick individual and treats the patient's disturbances on the physical, emotional and mental levels. The aim of homoeopathic medicine is to restore the equilibrium of the sick individual on all three levels. *In-vitro* studies do not take such physiological factors into account. Thus, *in-vivo* studies, where all the variables of the human body are considered, needs to be explored.

Lachesis mutas is one of an array of homoeopathic drugs prepared from snake venom. Many remedies fall into the snake venom group and are used to treat various haemostatic abnormalities. Current and future efforts of research in this field of study should test the potentiality of other such remedies.

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 taken under study. This could be beneficial to elucidate the therapeutic action of such homoeopathic drugs on the coagulation of blood.

- Potencies exceeding Avogadro's number need to be explored. Thus the addition of potencies greater than 24XH should be employed to determine their effectiveness.

- A wider range of potencies should be utilised to produce results that are comparable for each significant potency.

- 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.

- Precise laboratory expertise is necessary to reduce the errors in coagulation experienced.

- *In-vitro* studies, taking into account all the variables of the human body, should be conducted as an improvement to the methodology employed in this study.

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APPENDICES

Appendix A

Materials

Reagents/Material	Supplier
ACL machine 300	ILEX
Acrylic rotors	ILEX
Calibrated pipettes	Beckman and Coulter
Centrifuge machine – Mistral 6L	SAIMR
Cuvettes (ACL buckets)	ILEX
Freezer - 20°C	SAIMR
Medicated potencies :	
6XH, 9XH and 12XH	Natura
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 6XH, 9XH and 12XH potency 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 a 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 will allow the further 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 from 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. /Mrs. /Dr. _____

Date of birth: _____

Age: _____

Tick the appropriate boxes

Gender: ☐ Female
☐ Male

Race: ☐ White
☐ Asian / Indian
☐ African
☐ Coloured

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?

(Thick 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, B12, 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 a 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)?
