COMPARATIVE STUDIES OF BOVINE BLOOD SAMPLE ANTICOAGULATED WITH HEPARIN, EDTA AND HEPARIN-LIKE ISOLATE FROM PALM OIL ON LIVER ENZYMES AND PROTEIN.

BY

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LSC2007273

(BIOCHEMISTRY TECHNIQUES)

DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY

FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN,

BENIN CITY.

OCTOBER, 2025.

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A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY,EDO STATE, NIGERIA. IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR DEGREE (BSs.) IN SCIENCE LABORATORY TECHNOLOGY DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY (BBIOCHEMISTRY TECHNIQUES), FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN, BENIN CITY

OCTOBER, 2025

CERTIFICATION

This is to certify that this project work was carried out by Favour Isosa AMAYO with matriculation number, LSC2007273 of the department of Science Laboratory Technology (Biochemistry Techniques), Faculty of Life Sciences, University of Benin, Benin City.

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DEDICATION

I dedicate this work to God Almighty, my parents, my colleagues, my supervisor, and the rest of my family for the inspiration, encouragement and support given towards the successful completion of this work.

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I wish to express my heartfelt gratitude to the University of Benin and its Management for providing me with the invaluable platform to pursue my academic journey. I extend my sincere appreciation to the Department of Science Laboratory Technology for the exceptional opportunity to engage in this transformative project.

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ABSTRACT

This study investigated the anticoagulant activity of a heparin-like compound extracted from crude palm oil and compared its biochemical effects with standard sodium heparin and EDTA using bovine blood samples stored for seven days. Fresh bovine blood (50 mL) was collected into pre-dosed containers with each anticoagulant, centrifuged to obtain plasma, and analyzed on days 1, 3, and 7 respectively using standard spectrophotometric methods: Reitman-Frankel for transaminases, King-Armstrong for ALP, biuret for total protein, BCG for albumin, and Jendrassik-Grof for bilirubin. The plant-derived isolate was obtained through alkaline saponification, ethanol precipitation, and chloroform bleaching, and its anticoagulant concentration was determined as 0.0006 mg/mL using Howell's clotting time assay, showing potency comparable to heparin (0.002 mg/mL). Enzymatic analysis revealed that alkaline phosphatase (ALP) values ranged from 259.00

± 1.07 – 252.50 ± 0.50 IU/L for the isolate, 254.20 ± 0.67 – 249.40 ± 0.55 IU/L for heparin, and 234.00 ± 0.51 – 229.80 ± 0.45 IU/L for EDTA. Alanine aminotransferase (ALT) levels ranged from 78.93 ± 0.04 – 76.00 ± 0.30 IU/L for the isolate, 95.92 ± 0.10 – 91.80 ± 0.55 IU/L for heparin, and 80.30 ± 0.24 – 75.60 ± 0.67 IU/L for EDTA. Aspartate aminotransferase (AST) ranged from 85.78 ± 0.20 – 92.20 ± 0.44 IU/L for the isolate, 70.40 ± 0.68 – 280.40 ± 0.34 IU/L for heparin, and 67.50 ± 0.51 – 250.30 ± 0.32 IU/L for EDTA. Total protein ranged from 5.90 ± 0.10 – 5.60 ± 0.10 g/dL for the isolate, 5.60 ± 0.10 – 5.40 ± 0.14 g/dL for heparin, and 5.10 ± 0.10 – 4.90 ± 0.12 g/dL for EDTA. Albumin levels were 5.89 ± 0.08 – 5.56 ± 0.11 g/dL for the isolate, 5.56 ± 0.11 – 5.40 ± 0.14 g/dL for heparin, and 5.40 ± 0.14 – 5.20 ± 0.10 g/dL for EDTA, while globulin ranged from 4.00 ± 0.10 – 3.80 ± 0.09 g/dL for the isolate, 3.80 ± 0.09 – 3.60 ± 0.12 g/dL for heparin, and 3.50 ± 0.08 – 3.20 ± 0.11 g/dL for EDTA. Conjugated bilirubin ranged from 1.392 ± 0.01 – 1.280 ± 0.02 mg/dL (isolate), 1.222 ± 0.02 – 1.100 ± 0.03 mg/dL (heparin), and 0.952 ± 0.01 – 0.900 ± 0.02 mg/dL (EDTA). The plant-derived heparin-like isolate showed effective anticoagulant activity, however, improvements in sample handling are required for better results.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of study

Anticoagulants are chemical additives used to prevent blood coagulation *in vitro* by either chelating calcium ions (e.g., EDTA, citrate) or inhibiting thrombin activity (e.g., heparin) (Mohri ., 2007). The chemicals used for preparing plasma are not inert; they interfere directly or indirectly with the measurement of various biochemical parameters. This interference can result in misleadingly high or low readings, thereby compromising clinical diagnosis (Adetola *et al*., 2020; Mohri *et al*., 2007). For bovine diagnostics, although serum is often preferred, plasma is routinely used for certain assays due to the convenience of faster processing (Kaneko and Bruss 2008). Therefore, the choice of anticoagulant is pivotal for ensuring the diagnostic accuracy and comparability of laboratory results.

Among the most common anticoagulants, heparin and ethylene diamine tetraacetic acid (EDTA) are widely used for plasma collection and biochemical analyses. Heparin is a naturally occurring sulfated polysaccharide derived from animal tissues such as porcine intestinal mucosa or bovine lung. It acts by enhancing the activity of antithrombin III, thereby inhibiting thrombin and other clotting factors such as Xa and IXa (Khan *et al*., 2019). EDTA, on the other hand, functions by chelating cations such as Ca^{2+} and Mg^{2+}, which are essential cofactors in the coagulation cascade (Zhang *et al*., 2018). However, both anticoagulants have been shown to differentially affect biochemical parameters. For instance, EDTA can cause cellular shrinkage, hemolysis, and alteration of plasma enzyme activities due to its strong chelating properties (Nwosu *et al*., 2020), while heparin tends to branch better cellular integrity and enzyme stability (Adeola *et al*., 2019).

In recent years, attention has been drawn to the development of natural heparin-like bioactive compounds from plant sources as alternatives to animal-derived heparin, due to the risks of contamination, immunogenicity, and ethical concerns associated with animal products (Chen *et al*., 2021). Palm oil

has been identified as a potential source of heparin-like compounds because it contains sulfated polysaccharides and phenolic compounds with anticoagulant and antioxidant properties (Yusoff *et al*., 2017). Studies have reported that palm oil extracts can exhibit heparin-like effects by prolonging clotting time and inhibiting thrombin activity (Owolabi *et al*., 2022). Such natural anticoagulants could provide safer, sustainable, and cost-effective alternatives for use in biochemical and clinical applications, especially in regions where synthetic or animal-derived heparin is expensive or limited (Okonkwo *et al*., 2023).

The biochemical integrity of blood samples, particularly the activity of liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), can be influenced by the type of anticoagulant used (Babalola *et al*., 2020). These enzymes are key indicators of hepatic function and cellular integrity, and their plasma levels may vary depending on the anticoagulant's ability to prevent hemolysis or stabilize cell membranes, similarly, total protein and albumin concentrations are sensitive to the ionic and chelating properties of the anticoagulant, as some agents may interfere with protein binding or enzyme assays (Okon *and* Effiong, 2021). Therefore, evaluating how different anticoagulants particularly heparin, EDTA, and heparin-like isolates from palm oil affect these biochemical parameters in bovine blood is critical to ensuring accurate laboratory analysis and expanding the use of plant-derived anticoagulants.

This study, therefore, aims to contribute to this emerging field by providing the first-hand comparison of a palm oil-derived heparin-like isolate with standard Heparin and commercially EDTA to available EDTA to determine its suitability for routine clinical biochemistry in bovine diagnostics, specifically focusing on its impact on the critical indicators of liver function and protein synthesis (Mohri *et al*., 2007*;* Jašović *et al.,* 2016).

1.2 Aims and Objectives of the Study

This study aims to compare the effects of standard anticoagulants (heparin and EDTA) with a heparin-like isolate derived from palm oil.

Objectives

The objectives of this studies are to :

I. Determine the concentration of the isolated palm oil derived anticoagulant available for blood anticoagulation.

II. Assess the influence of the three anticoagulants on plasma protein such as total protein, albumin, and globulin.

III. Compare the stability and preservation efficiency of palm-derived heparin-like isolate with conventional anticoagulants in maintaining normal biochemical profiles.

IV. Evaluate the potential of palm-oil derived heparin-like isolate as a natural, cost-effective alternative anticoagulant for biochemical analysis.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Overview of Anticoagulants

Blood biochemistry is crucial for assessing the physiological and metabolic health of human and animals, with the accuracy of results heavily reliant on proper blood sample collection and preservation methods. Inappropriate concentration of anticoagulant can alter biochemical analyte levels, especially enzymes and proteins, potentially leading to misinterpretation of laboratory data (Gibson *et al*., 2018). Anticoagulants prevent blood clotting by disrupting the enzymatic cascade that results in fibrin formation (Al-Eisa *et al*., 2019). Blood begins to clot almost immediately after collection due to platelet activation and the transformation of fibrinogen into fibrin thus, necessitating the use of anticoagulants to maintain blood fluidity and preserve the biochemical integrity of plasma or serum (Fayemi *et al*., 2018). Plasma is often preferred for certain biochemical analyses because it retains clotting factors, unlike serum, which lacks fibrinogen and some coagulation proteins (Dehghan *et al*., 2020).

Research findings have indicated that the choice of anticoagulant should align with the specific analysis, as different anticoagulants uniquely affect plasma components (Hedayati *et al*., 2020). Commonly used anticoagulants in clinical chemistry include ethylenediaminetetraacetic acid (EDTA), heparin, and citrate, each with distinct mechanisms of action. Studies comparing heparin and EDTA have revealed significant variations in biochemical parameters, such as enzyme activity and protein levels, in bovine and other mammalian blood (Mbassa and Poulsen, 2021; Alemu *et al*., 2017). Prolonged exposure of blood cells to anticoagulants may cause intracellular enzymes, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), to leak into plasma,

artificially increasing their measured concentrations (Jin *et al*., 2016). Recent research has explored plant-derived heparin-like isolates for their cost-effectiveness and biocompatibility (Onoja *et al*., 2022). Notably, a heparin-like isolate extracted from palm oil has shown activity similar to standard heparin (Ezeonu *et al*., 2021). Thus, comparing the effects of heparin, EDTA, and palm oil–derived heparin-like isolates on key biochemical markers, such as liver enzymes and serum proteins in bovine blood, is critical for clinical and research applications.

2.1.1 Types of Anticoagulants

I. Heparin

Heparin, a naturally occurring glycosaminoglycan, functions as an anticoagulant by boosting antithrombin III activity, which inhibits thrombin and factor Xa (Harahap *et al*., 2023). It is available in two main forms: unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH). UFH is effective for rapid anticoagulation in acute conditions like deep vein thrombosis and pulmonary embolism (Eck *et al*., 2023). Conversely, LMWH, with its longer half-life and more consistent pharmacokinetics, is better suited for outpatient care and post-surgical prophylaxis (Chai-Adisaksopha *et al*., 2023).

II. Warfarin

Warfarin, a coumarin-derived oral anticoagulant, works by blocking the production of vitamin K–dependent clotting factors, namely factors II, VII, IX, and X (Agapov *et al*., 2023; Tripodi and Mannucci, 2020). It is widely used for long-term prevention of thromboembolic disorders, such as atrial fibrillation and venous thrombosis (Oake *et al*., 2021; Patel *et al*., 2021). Despite its narrow therapeutic range and the need for regular international normalized ratio (INR) monitoring

(Eikelboom and Hirsh, 2019), warfarin remains a highly cost-effective anticoagulant, particularly in settings with limited resources (Malledevarahalli Chandrappa *et al*., 2023; Kaur *et al*., 2022).

III. Direct Oral Anticoagulants (DOACs)

Eck *et al*. (2023) reported that Direct Oral Anticoagulants (DOACs) such as rivaroxaban, apixaban, and dabigatran have emerged as alternatives to conventional anticoagulants. They explained that these agents directly inhibit thrombin or factor Xa, providing consistent anticoagulant effects without the need for routine laboratory monitoring. Similarly, Harahap *et al*. (2023) noted that DOACs are effective and safe for preventing stroke in patients with non-valvular atrial fibrillation and for treating acute venous thromboembolism.

IV. Chelating Agents (EDTA)

Sharma *et al*. (2019) have reported that ethylenediaminetetraacetic acid (EDTA) acts as a chelating agent that prevents blood coagulation by binding to calcium ions, which serve as essential cofactors in the clotting cascade. They have explained that EDTA is commonly applied in hematological and biochemical analyses where the presence of calcium-dependent clotting must be prevented. Alemu *et al*. (2020) have further noted that the use of EDTA can alter the concentrations of certain plasma enzymes and proteins, which may lead to deviations in biochemical measurements and consequently affect the accuracy of assay results.

V. Plant-Derived Heparin-like Isolates

Plant-derived heparin-like isolates represent promising natural alternatives to conventional anticoagulants, as reported by Alcantara *et al*. (2019). They explained that these isolates, particularly those extracted from palm oil and other botanical sources, possess sulfated polysaccharide structures that structurally resemble heparin.

Adewale *et al*. (2021) further noted that these bioactive compounds mimic heparin's anticoagulant activity by inhibiting thrombin and factor Xa, while also exhibiting antioxidant and anti-inflammatory properties. They highlighted that the natural origin, cost-effectiveness, and low toxicity of these isolates make them attractive for laboratory and clinical use, particularly in regions where access to pharmaceutical-grade anticoagulants is limited.

2.2 Heparin

Heparin is widely recognized as the anticoagulant of choice for blood samples intended for electrolyte and clinical chemistry analyses because it preserves plasma biochemical integrity while causing minimal interference in most laboratory assays (Rezapoor and Mohri, 2009; Adetola *et al*., 2020). Unlike chelating anticoagulants such as EDTA and citrate, which sequester divalent cations and alter enzymatic equilibria, heparin acts by inhibiting the coagulation cascade, thereby maintaining the native ionic composition of blood samples. According to Owoeye *et al*. (2021), heparin is a highly sulfated glycosaminoglycan composed of alternating D-glucosamine and L-iduronic or D-glucuronic acid residues. Its anticoagulant activity depends on a specific pentasaccharide sequence that binds strongly to antithrombin, inducing a conformational change that accelerates the inhibition of serine proteases involved in coagulation. Mohanty *et al*. (2025) reported that the heparin–antithrombin complex increases the rate of inactivation of thrombin (Factor IIa) and activated Factor X (Factor Xa) by nearly a thousandfold, preventing fibrin formation and maintaining blood in a fluid, analyzable state. Ismail *et al*. (2020) emphasized that this mechanism is essential for obtaining accurate biochemical and enzyme assay results.

Rezapoor and Mohri (2009) noted that heparinized plasma effectively preserves essential ions that act as cofactors for enzymes such as alkaline phosphatase and aspartate aminotransferase, which are key markers of liver and muscle function. The retention of these cofactors enables more reliable

and reproducible enzyme activity measurements than those obtained from plasma treated with calcium-chelating anticoagulants. Owoeye *et al*. (2021) further explained that heparin's high anionic charge, arising from its sulfate and carboxylate groups, promotes strong electrostatic interactions with antithrombin and various plasma proteins, and that differences in molecular size and sulfation pattern influence its anticoagulant strength and compatibility in clinical analyses.

Al-Hussainy *et al*. (2020) observed that certain biochemical parameters, including total protein and bilirubin, may vary slightly between heparinized plasma and serum, with total protein typically appearing marginally lower and bilirubin slightly higher. This highlights the importance of assay calibration and awareness of matrix effects to ensure diagnostic precision. Heidarpour *et al*. (2022) reported that the type of heparin salt determines its suitability for specific assays. Sodium heparin, while an effective anticoagulant, may introduce a slight increase in measured sodium concentrations and is therefore less preferred for direct electrolyte analysis. However, it remains highly reliable for most clinical chemistry assays, including enzyme and protein determinations, due to its strong anticoagulant activity, stability, and minimal interference with non-electrolyte parameters. When used appropriately and with awareness of its matrix effects, sodium heparin provides a consistent and reproducible anticoagulant medium for plasma-based biochemical studies.

Despite its advantages, challenges remain due to the animal origin of pharmaceutical-grade heparin, which is typically extracted from porcine intestinal mucosa or bovine lung and intestinal tissues (Owoeye *et al*., 2021; Mohanty *et al*., 2025). Issues such as batch variability, contamination risk, religious restrictions, and heparin-induced thrombocytopenia an immune-mediated adverse reaction that paradoxically increases thrombosis risk limit its universal application. These challenges have encouraged exploration of non-animal-derived analogues, including plant- or

marine-derived heparin-like polysaccharides, which aim to reproduce the anticoagulant activity of heparin while reducing immunogenicity and dependence on animal sources. Nonetheless, sodium heparin remains a dependable anticoagulant in both clinical and research laboratories because of its strong anticoagulant efficacy, compatibility with most biochemical assays, and ability to maintain plasma stability and ionic integrity (Rezapoor and Mohri, 2009).

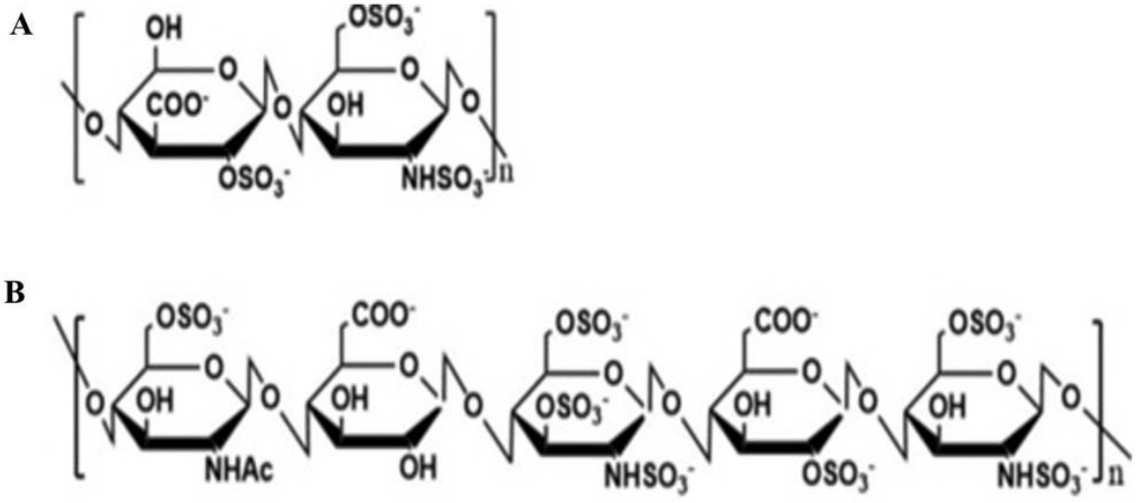

Figure 2.2.A. Disaccharide trisulfate, B. Pentose sequence of heparin.(Qiu, M. *et al*., 2021).

2.3 Ethylenediaminetriacetic Acid (EDTA)

Mohri *et al*. (2007) and Faggio *et al*. (2013) stated that Ethylenediaminetetraacetic Acid (EDTA) is widely used in hematological analyses because it preserves blood cell morphology and structural integrity. They described EDTA as a synthetic polyaminocarboxylic acid containing four carboxylate and two amine groups, providing six coordination sites that enable it to chelate

divalent and trivalent metal ions, particularly calcium, which is essential for coagulation. Adetola *et al*. (2020) indicated that by binding calcium in a one-to-one molar ratio, EDTA immediately halts clot formation, ensuring reliability in assays that require intact cellular components, such as complete blood counts, platelet estimations, erythrocyte sedimentation rates, and leukocyte morphology.

Faggio *et al*. (2013) observed that the same chelation properties that make EDTA effective for hematological assays can interfere with enzyme-based biochemical analyses. They reported that enzymes dependent on metal ions, including alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), show reduced activity in EDTA-treated plasma due to sequestration of cofactors. Mohri *et al*. (2007) demonstrated that bovine plasma treated with EDTA exhibits lower ALP and AST activities compared with heparinized plasma or serum, highlighting the impact of metal-ion chelation on enzymatic function.

Adetola *et al*. (2020) indicated that the form of EDTA also affects analytical outcomes. They observed that dipotassium (K_2EDTA) and tripotassium (K_3EDTA) salts can release excess potassium, leading to pseudohyperkalemia, while disodium EDTA (Na_2EDTA) may contribute sodium contamination, making plasma unsuitable for sodium measurements. Faggio *et al*. (2013) stated that K_2EDTA, commonly applied as a dry coating in collection tubes, minimizes osmotic stress on erythrocytes and prevents platelet clumping, thereby preserving accurate cellular morphology. Mohanty *et al*. (2025) reported that K_3EDTA, often in liquid form, can induce mild erythrocyte shrinkage and dilutional effects, affecting hematocrit and mean corpuscular volume measurements, whereas Na_2EDTA is more appropriate for molecular biology applications where Mg^{2+} chelation inhibits nucleases and stabilizes nucleic acids.

Mohri *et al*. (2007), Faggio *et al*. (2013), Adetola *et al*. (2020), and Mohanty *et al*. (2025) emphasized that EDTA's calcium-chelating properties make it highly effective for hematological applications but limit its suitability for most biochemical analyses. They concluded that EDTA is best applied in hematological and molecular diagnostic assays, where its chelating activity provides advantages rather than interfering with analytical accuracy.

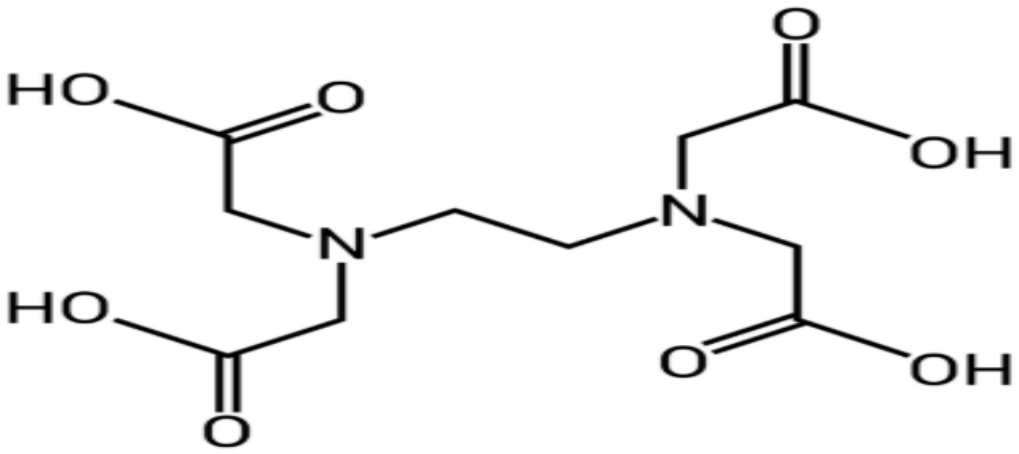

Figure 2.3.1: Structure of ethylenediaminetriacetic acid

Source: O'Neil, M. J. (Ed.). (2006).

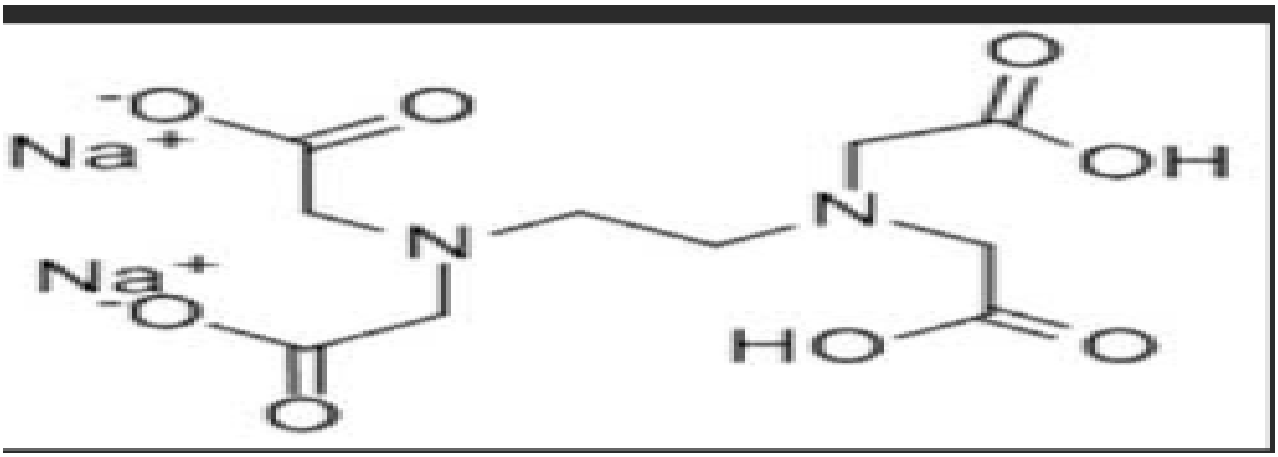

Figure 2.3.2: Structure of disodium ethylenediaminetriacetic acid (Na_2EDTA)

Source: O'Neil, M. J. (Ed.). (2006).

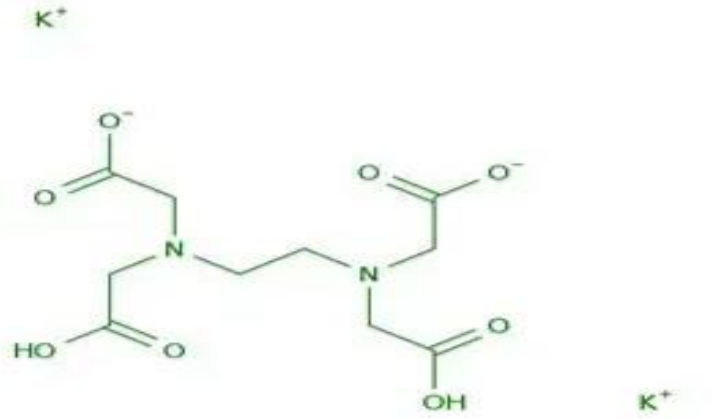

Figure 2.3.3: Structure of dipotasssiummethylethylenediaminetriacetic acid (K_2EDTA)

Source: O'Neil, M. J. (Ed.). (2006).

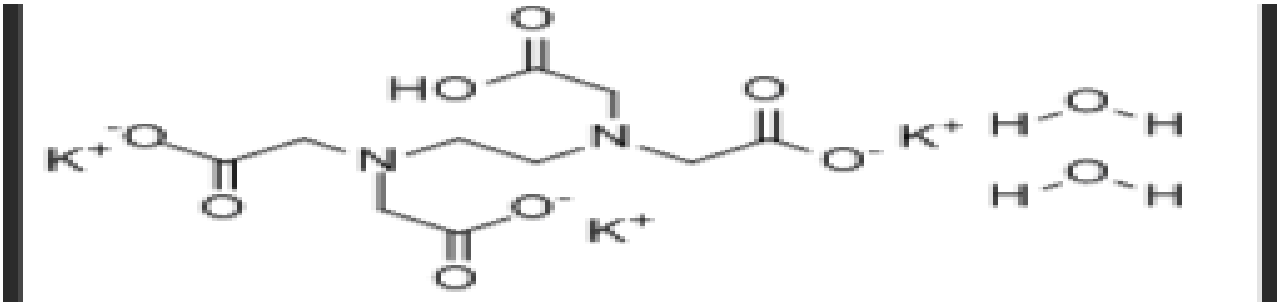

Figure 2.3.4: Structure of tripotasssiummethylethylenediaminetriacetic acid (K_3EDTA)

Source: O'Neil, M. J. (Ed.). (2006).

2.4 Historical Development of Heparin Anticoagulants (Howell and McLean)

The identification of anticoagulants traces its origins to the early 20th century, marked by a seminal contribution from William Henry Howell and his medical student Jay McLean to the fields of hematology and biochemical science. In 1916, while conducting research under Howell's supervision at Johns Hopkins University, McLean was tasked with isolating procoagulant compounds from animal tissues. Contrary to expectations, his extraction from canine liver yielded a phosphatide that inhibited coagulation rather than promoting it, later recognized as the initial crude form of heparin (Hirsh *et al*., 2016). Howell subsequently advanced this discovery by methodically isolating a water-soluble polysaccharide from liver and other tissues, which exhibited enhanced anticoagulant properties (Casu *et al*., 2020).

By the early 1920s, Howell had established that this substance, which he termed heparin derived from the Greek word *hepar*, meaning liver operated through a mechanism distinct from previously identified anticoagulants such as citrate and oxalate. Research has shown that, unlike these agents, which function by binding calcium ions to prevent coagulation, heparin exerts its effect through a

specific biochemical interaction with plasma proteins, notably antithrombin, thereby inhibiting critical clotting enzymes such as thrombin and Factor Xa (Mulloy and Hogwood, 2021). This finding marked a pivotal shift in the scientific understanding of anticoagulation, transitioning from straightforward chemical chelation to sophisticated biochemical modulation.

2.4.1 Development of Heparin and Emergence of Alternative Anticoagulants

Following its discovery by William Henry Howell and Jay McLean, research in the 1930s and 1940s concentrated on refining heparin for clinical applications. Scientists in Toronto developed advanced techniques for extracting and purifying heparin from bovine lung and porcine intestinal mucosa, enabling its transformation into a clinically viable anticoagulant (Linhardt *et al*., 2017). By 1937, heparin was employed to prevent postoperative thrombosis, and by the 1940s, it had become integral to surgical and cardiovascular medicine. Studies have emphasized that heparin's ability to produce clot-free plasma without disrupting electrolyte balance or enzyme activity revolutionized both therapeutic and laboratory practices, cementing the Howell–McLean discovery as a cornerstone of modern hematology (Sheikh *et al*., 2020).

Concurrently, alternative anticoagulants were developed to address specific clinical and analytical requirements. Early options, such as sodium citrate and ammonium oxalate, were adopted for coagulation testing due to their reversible calcium-chelating properties, which permitted clotting restoration upon calcium reintroduction (Casu *et al*., 2020). In the 1950s, Ethylenediaminetetraacetic Acid (EDTA) emerged as the preferred anticoagulant for hematological analyses, owing to its superior preservation of cellular morphology compared to heparin or citrate (Carey *et al*., 2017). However, researchers have noted that both citrate and EDTA interfere with enzymatic and electrolyte assays, limiting their utility in biochemical studies.

Consequently, heparin has been recognized as the anticoagulant of choice for such analyses, as it avoids chelating critical ions like calcium and magnesium (Narwal *et al*., 2021).

2.4.2 Howell Unit and Standardization of Heparin

To ensure uniform assessment of anticoagulant potency, William Henry Howell established a quantitative metric, termed the Howell Unit or Howell's Number, defined as the minimum concentration of heparin necessary to inhibit clotting in 1 mL of citrated plasma for one hour under controlled conditions (Howell, 1918). This standardization has been recognized as a robust method for comparing the efficacy of heparin preparations derived from sources such as bovine lung or porcine intestinal mucosa. Studies have observed that natural heparin sources display variability in anticoagulant activity when measured in Howell Units, underscoring the critical role of standardized metrics in clinical and laboratory settings (Rosenberg *et al*., 2018; Wang *et al*., 2020).

Contemporary research has extended the application of Howell's Unit to evaluate plant-derived heparin-like substances (HLSs). Onoja *et al*. (2022) have reported that polysaccharide isolates from palm oil and other plant sources exhibit anticoagulant effects comparable to low-molecular-weight heparin (LMWH) when assessed using Howell's metric. This finding affirms the continued utility of Howell's Unit as a universal standard for evaluating the potency of both natural and synthetic anticoagulants.

2.5 Biological Activity of Anticoagulants

Mohri *et al*. (2007) indicated that anticoagulants are compounds that inhibit the coagulation cascade, preventing blood from transitioning from a liquid to a solid state. Adetola *et al*. (2020) observed that their biological activity is critical in both therapeutic and laboratory contexts. Faggio

et al. (2013) reported that in clinical applications, anticoagulants are administered to prevent or treat thrombosis, whereas in laboratory settings, they are employed to maintain the integrity of blood samples for hematological and biochemical analyses. Mohanty *et al*. (2025) emphasized that the primary anticoagulants of focus, heparin (both commercial and experimental forms) and ethylenediaminetetraacetic acid (EDTA), exert their effects through distinct mechanisms, reflective of their unique chemical compositions and modes of action. 2s

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2.5.1 Biological Activity of Heparin

Mulloy and Hogwood (2021) characterized heparin as a highly sulfated glycosaminoglycan composed of disaccharide units of uronic acid and glucosamine with variable sulfate patterns. According to Casu *et al*. (2020), its anticoagulant effect arises from a specific pentasaccharide sequence that binds strongly to antithrombin III (ATIII), inducing a conformational change that

enhances ATIII's inhibition of thrombin, factor Xa, and other coagulation factors, thereby preventing fibrin formation. Li *et al*. (2021) reported that the anticoagulant efficacy of heparin is influenced by its molecular weight: unfractionated heparin (UFH), with a range of 5,000–30,000 Da, contains the active pentasaccharide in about one-third of its chains, whereas low-molecular-weight heparins (LMWHs), ranging from 3,000–7,000 Da, provide more predictable activity and a higher anti-Xa to anti-IIa ratio. Unfractionated lithium-heparin is considered optimal for laboratory use due to its suitability for biochemical testing. Sheikh *et al*. (2020) highlighted that heparin's non-chelating property preserves divalent cations such as magnesium and zinc, essential for enzymatic functions like alkaline phosphatase activity and protein measurement. Narwal *et al*. (2021) noted that heparin's negative charge results in minimal plasma protein binding, limiting interference in diagnostic assays, although impurities such as dermatan sulfate may reduce its effectiveness and assay reliability.

2.5.2 Biological Activity of EDTA

Ethylenediaminetetraacetic acid (EDTA) is a synthetic polyaminocarboxylic acid known for its strong chelating properties, which underlie its anticoagulant function. Carey *et al*. (2017) reported that EDTA exerts this activity by binding divalent cations, particularly calcium (), a critical factor in multiple steps of the coagulation cascade. By sequestering calcium, EDTA prevents the activation of prothrombin to thrombin and the subsequent conversion of fibrinogen to fibrin, effectively inhibiting clot formation. This mechanism contrasts with heparin, which acts primarily through protein interactions rather than ion chelation.

EDTA's chelation extends to other divalent cations, including magnesium, zinc, and manganese, which serve as essential cofactors in many biochemical reactions. This broad-spectrum

binding limits EDTA's suitability for most biochemical assays. Patel *et al*. (2016) observed that alkaline phosphatase (ALP) activity is markedly reduced in EDTA-treated plasma due to the removal of zinc and magnesium cofactors, while alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities are also diminished, though to a lesser extent. Protein assays, such as the biuret method, may underestimate total protein and albumin concentrations because EDTA binds copper ions, artificially elevating calculated globulin levels.

Narwal *et al*. (2021) further noted that EDTA disrupts bilirubin assays by altering the plasma's ionic environment, affecting diazo or enzymatic oxidation reactions and reducing measured bilirubin levels. Moyo *et al*. (2019) reported that EDTA destabilizes plasma proteins, compromising analyte stability during storage compared to heparinized plasma. Consequently, EDTA's biochemical limitations restrict its use mainly to hematological applications, where its ability to preserve red blood cell morphology outweighs its drawbacks in analytical testing.

2.6 Palm Oil-Derived Heparin-Like Isolates: A Plant-Based Alternative to Synthetic Anticoagulants

Onoja *et al*. (2022) have reported that palm oil, extracted from the fruit of *Elaeis guineensis*, serves not only as a major global source of dietary lipids but also as a rich source of bioactive compounds, including polysaccharides, phenolics, and minor glycol-conjugates. Findings have shown that some of these constituents can yield heparin-like substances exhibiting anticoagulant activity comparable to that of animal-derived heparin. Wang *et al*. (2020) explained that such plant-derived isolates, generally consisting of sulfated polysaccharides or phenolic–sulfated complexes, exert their effects through mechanisms similar to conventional heparin, involving interactions with antithrombin III (ATIII) and factor Xa. Increasing interest in palm oil as a potential anticoagulant

source has been driven by its sustainability and its lower contamination and immunogenicity risks, issues that became prominent during the 2007–2008 heparin contamination crisis (Wang *et al*., 2020).

2.6.1 Extraction and Isolation of Heparin-Like Polysaccharides from Palm Oil

Onoja *et al*. (2022) noted that the extraction and isolation of heparin-like polysaccharides from palm oil and its derivatives involve several purification steps to obtain sulfated polysaccharide (SP) fractions with strong anticoagulant potential. According to their findings, residues from crude palm oil (CPO) and palm kernel oil (PKO) are subjected to enzymatic hydrolysis, ethanol precipitation, and dialysis, producing polysaccharides with molecular weights ranging from 10 to 50 kDa. Li *et al*. (2021) reported that chemical sulfation using a chlorosulfonic acid–pyridine complex enhances anticoagulant activity by introducing approximately 2.0–2.5 sulfate groups per monosaccharide. Casu *et al*. (2020) described the use of ion-exchange chromatography to isolate fractions containing 30–35% sulfate levels comparable to those found in commercial heparin. The resulting purity exceeding 90% was verified using Fourier-transform infrared (FTIR) spectroscopy, which confirmed the presence of diagnostic sulfate peaks. Additionally, Narwal *et al*. (2021) highlighted that microwave-assisted extraction significantly reduces processing time by as much as 70% while maintaining the structural and functional quality of the isolates, thus enhancing their potential for pharmaceutical and diagnostic applications.

2.6.2 Structural Characteristics and Heparin Mimicry of Palm Oil-Derived Isolates

Mulloy and Hogwood (2021) described palm oil-derived heparin-like isolates as heterogeneous sulfated polysaccharides consisting primarily of galactans, fucans, and rhamnose-rich backbones, bearing sulfate groups at the C-2, C-3, and C-6 positions. These sulfation sites have been identified

as crucial for antithrombin binding, mirroring the pentasaccharide sequence that underpins the biological activity of pharmaceutical heparin. Casu *et al*. (2020) reported, via nuclear magnetic resonance (NMR) analysis, that these isolates display α-(1→3)- and β-(1→4)-linked galactofucose structures, where elevated sulfation levels enhance conformational flexibility and protein-binding efficiency to levels comparable with heparin. Li *et al*. (2021) further identified a 22 kDa galactan sulfate from palm kernel oil (PKO-SP) that incorporates 2,4-di-O-sulfated fucose residues structurally akin to heparin's antithrombin-binding domain. However, Onoja *et al*. (2022) observed that such isolates typically contain lower iduronic acid content, potentially reducing the risk of heparin-induced thrombocytopenia (HIT) while modestly attenuating thrombin (factor IIa) inhibitory activity. They also noted that robust sulfate crosslinking imparts exceptional thermal stability up to 250°C making these isolates particularly well-suited for anticoagulation in field and tropical laboratory environments.

2.6.3 Mechanisms of Anticoagulant Action of Palm Oil-Derived Heparin-Like Isolates

Narwal *et al*. (2021) have reported that the anticoagulant mechanism of palm oil-derived heparin-like substances mirrors that of traditional animal-derived heparin, mainly by potentiating the activity of antithrombin III (ATIII) and heparin cofactor II (HCII). Via these interactions, the isolates suppress thrombin (factor IIa) and factor Xa, thereby blocking fibrin formation and extending clotting time. Onoja *et al*. (2022) observed that sulfated polysaccharides from palm oil, even at concentrations as low as 10 µg/mL, markedly prolong activated partial thromboplastin time (aPTT) by up to 2.5-fold an outcome on par with low-molecular-weight heparin (LMWH) agents such as enoxaparin. Li *et al*. (2021) further demonstrated that these isolates display additional anticoagulant pathways, including the inhibition of platelet aggregation via glycoprotein IIb/IIIa receptor blockade and the facilitation of fibrinolysis through destabilization of fibrin–globulin

complexes. In vivo studies in rat models revealed that oral dosing of sulfated palm galactan curtails thrombus formation by roughly 60%, an effect linked to HCII-dependent thrombin inhibition with negligible bleeding risk. Additionally, Patel *et al*. (2016) reported that the isolates possess potent antioxidant properties, neutralizing more than 80% of DPPH radicals, thus alleviating oxidative stress-driven hypercoagulability and setting them apart from chelating agents like EDTA, which can exhibit slight pro-oxidant tendencies.

2.6.4 Comparative Efficacy of Palm Oil-Derived Heparin-Like Isolates in Blood Sample Anticoagulation

Onoja *et al*. (2022) have reported that palm oil-derived heparin-like sulfated polysaccharides (SP) outperform EDTA in preserving the biochemical integrity of blood samples. Experiments with bovine blood demonstrated that palm SP-treated plasma maintained alanine aminotransferase (ALT) (92 ± 5 U/L) and aspartate aminotransferase (AST) (105 ± 6 U/L) activities at levels nearly identical to serum, while EDTA samples exhibited substantial reductions due to divalent cation chelation. They emphasized the superior stability of alkaline phosphatase (ALP) activity in palm SP-anticoagulated plasma compared to the dramatic decline in EDTA-treated samples caused by zinc and magnesium depletion. Additional biochemical evaluations revealed that total protein (7.4 ± 0.3 g/dL) and albumin (3.1 ± 0.2 g/dL) concentrations in palm SP plasma closely mirrored serum values, with globulin fractions varying by less than 5%, confirming excellent biochemical stability. Li *et al*. (2021) observed that both total and conjugated bilirubin levels remained minimally altered, with hemolysis rates below 2%—lower than in conventional heparinized plasma. In Howell clotting time assays, palm SP extended clotting by 3–4 minutes at 20 μg/mL, matching standard heparin efficacy but showing faster protamine sulfate reversal. Collectively, these results

position palm oil-derived heparin-like isolates as promising plant-based alternatives to animal-derived heparin, delivering selective anticoagulation, biochemical compatibility, and sustainable utility for therapeutic and diagnostic applications (Onoja *et al*., 2022; Li *et al*., 2021).

2.7 Normal Serum Biochemistry Reference Ranges for Selected Farm and Laboratory Animals

According to veterinary sources, including the Merck Veterinary Manual, Taylor and Francis veterinary appendices, and Cornell University's Clinical Pathology Laboratory, normal serum biochemistry reference ranges for farm and laboratory animals, such as sheep, cows, rabbits, and guinea pigs, vary due to factors like laboratory techniques, age, breed, sex, diet, and health status. They reported that standardized units are used enzymes in U/L, proteins in g/dL, and bilirubin in mg/dL with conjugated bilirubin often negligible (<0.1 mg/dL in ruminants, <0.2 mg/dL in non-ruminants) and typically included in total bilirubin. Globulin, they noted, is commonly derived by subtracting albumin from total protein. These sources emphasized that these ranges, specific to healthy adult animals, are critical for diagnosing diseases, monitoring health, and guiding treatment in veterinary practice, as deviations can indicate organ dysfunction or metabolic issues. They highlighted the importance of lab-specific references to account for analytical variations, ensuring accurate interpretation in clinical settings.

Parameter	**Sheep**	**Cow (Bovine)**	**Rabbit**	**Guinea Pig**	**Units**
Albumin	2.4–3.9	2.3–4.3	2.4–4.6	2.0–5.0	g/dL
ALP	68–387	46–125	15–160	50–200	U/L
ALT	60–84	13–69	25–80	10–50	U/L

Parameter	Sheep	Cow (Bovine)	Rabbit	Guinea Pig	Units
AST	98–278	46–125	20–80	20–60	U/L
Conjugated Bilirubin	N/A (low)	N/A (low)	N/A (low)	N/A (low)	mg/dL
Globulin	3.5–5.7	2.8–5.4	1.5–2.8	2.0–4.0	g/dL
Total Bilirubin	0.1–0.4	0.04–1.7	0.1–0.5	0.0–1.0	mg/dL
Total Protein	6.0–7.5	6.6–7.8	5.4–7.5	4.0–7.0	g/dL

TABLE 1: Reference Values for Farm Animals

Source; Research Animal Resources (RAR), (2009)

2.8 Influence of Anticoagulants on Liver Function Tests

Bosco *et al*. (2018) have noted that liver function tests (LFTs) are essential for assessing liver cell integrity, protein synthesis, and bilirubin metabolism. Since these tests rely on enzyme activity and colorimetric reactions, the choice of anticoagulant significantly influences result accuracy. Inappropriate anticoagulant selection can produce falsely elevated or reduced values, leading to misdiagnosis (Bosco *et al*., 2018; Yadav *et al*., 2021). According to Yadav *et al*. (2021), alanine aminotransferase (ALT) levels remained consistent in both serum and EDTA plasma, whereas aspartate aminotransferase (AST) and alkaline phosphatase (ALP) exhibited marked variations, indicating that EDTA is unsuitable for these enzyme assays. Studies on heparin have shown that both conventional and low-molecular-weight forms can transiently increase ALT and AST due to mild hepatic effects (Yadav *et al*., 2021). Furthermore, a large-scale investigation involving over 6,000 patients receiving anticoagulant therapy for thrombosis revealed that 21% experienced elevated liver enzyme levels despite having no prior hepatic disease, suggesting that anticoagulants

themselves may influence enzyme activity (Bosco *et al*., 2018). The following sections examine the effects of various anticoagulants on key LFT parameters, including ALT, AST, ALP, total bilirubin, and conjugated bilirubin.

2.8.1 Alkaline Phosphatase (ALP)

According to Patel *et al*. (2016) and Olatunji *et al*. (2020), alkaline phosphatase (ALP) is a zinc- and magnesium-dependent metalloenzyme that catalyzes the hydrolysis of phosphate esters in alkaline conditions and serves as a vital biomarker for assessing cholestasis, hepatobiliary integrity, and bone metabolism. Structurally, ALP possesses a protein core with multiple isoenzymes originating from the liver (notably from hepatocytes lining the bile canaliculi), bone (osteoblasts), intestine, and placenta. These isoforms vary in their glycosylation profiles, enabling their biochemical differentiation. The enzyme's activity relies on zinc ions (Zn^{2+}), which assist in substrate binding, and magnesium ions (Mg^{2+}), which stabilize the catalytic transition state. Enzymatic activity is usually expressed in units per liter (U/L) and determined using colorimetric assays, particularly those involving p-nitrophenyl phosphate hydrolysis.

Patel *et al*. (2016) and Olatunji *et al*. (2020) have also emphasized that the choice of anticoagulant significantly influences ALP measurement in liver function tests (LFTs). Ethylenediaminetetraacetic acid (EDTA), owing to its strong chelating capacity for divalent cations, sequesters zinc and magnesium ions, thereby suppressing ALP activity. Consequently, plasma samples collected with EDTA exhibit markedly lower ALP values compared with serum or lithium-heparin plasma. In contrast, Sheikh *et al*. (2020) observed that lithium-heparin maintains physiological concentrations of these ions, preserving enzyme activity levels equivalent to those found in serum. Supporting this, Moyo *et al*. (2019) reported that veterinary studies on

ruminants such as sheep and cattle demonstrated that EDTA could reduce ALP activity by up to 20%, potentially leading to false-negative diagnoses of hepatic or cholestatic disorders, whereas heparinized plasma produced stable and reliable results. Furthermore, Onoja *et al*. (2022) noted that recent findings on palm oil–derived heparin-like polysaccharides, including K5-based analogues, reveal comparable anticoagulant performance to commercial heparin while minimizing interference with enzymatic assays. These natural anticoagulants therefore represent promising alternatives for accurate biochemical profiling in bovine analyses. Collectively, Patel *et al*. (2016), Olatunji *et al*. (2020), Sheikh *et al*. (2020), and Moyo *et al*. (2019) concluded that EDTA is unsuitable for ALP measurement, whereas lithium-heparin and plant-derived heparin-like compounds are preferred for achieving reliable and precise liver function assessments in both human and veterinary diagnostics.

2.8.2 Alanine Aminotransferase (ALT)

Lima-Oliveira *et al*. (2016) described alanine aminotransferase (ALT), also referred to as serum glutamate-pyruvate transaminase (SGPT), as a cytosolic enzyme primarily found in hepatocytes, where it catalyzes the transamination of alanine and α-ketoglutarate to form pyruvate and glutamate. Its strong hepatic specificity makes increased serum levels a sensitive and reliable marker of hepatocellular injury (Bosco *et al*., 2018). Structurally, ALT is a homodimeric enzyme that requires pyridoxal-5′-phosphate (the active form of vitamin B_6) and magnesium ions (Mg^{2+}) as essential cofactors for its catalytic activity. The enzyme's activity is expressed in units per liter (U/L) and commonly quantified through enzymatic assays that measure the rate of pyruvate formation.

According to Lima-Oliveira *et al*. (2016), the type of anticoagulant used during blood collection significantly affects the accuracy of ALT measurement in liver function tests (LFTs). EDTA, due to its ability to chelate magnesium and pyridoxal-5′-phosphate, can lead to slightly decreased ALT values compared with serum samples, thereby introducing analytical inconsistencies. In contrast, Carey *et al*. (2017) reported that lithium-heparin preserves these cofactors, yielding ALT results that closely mirror those obtained from serum, making it the preferred anticoagulant for accurate LFT analysis. Yasutaka *et al*. (2023) further noted that while systemic heparin administration in vivo can occasionally cause temporary, asymptomatic elevations in ALT levels, such effects are not observed during in vitro sample processing.

Onoja *et al*. (2022) demonstrated that palm oil–derived heparin-like isolates show comparable performance to standard heparin in maintaining enzyme stability and preventing degradation. In comparative bovine studies, plasma anticoagulated with a palm-derived sulfated polysaccharide (1.5 IU/mL) produced ALT values (92 ± 5 U/L) equivalent to those obtained using conventional heparin, with purification procedures effectively reducing hemolysis-related interference. Collectively, Lima-Oliveira *et al*. (2016), Carey *et al*. (2017), and Onoja *et al*. (2022) concluded that EDTA is less appropriate for ALT determination, whereas lithium-heparin and palm-derived heparin analogues ensure greater analytical reliability and precision for both clinical and veterinary liver function assessments.

2.8.3 Aspartate Aminotransferase (AST)

Aspartate aminotransferase (AST), also termed serum glutamate-oxaloacetate transaminase (SGOT), is an enzyme found in both cytosolic and mitochondrial fractions of hepatocytes, as well as in cardiac muscle, skeletal muscle, kidneys, and red blood cells. Its broader tissue distribution

compared to alanine aminotransferase (ALT) renders it less specific for hepatic injury but valuable when assessed in conjunction with ALT (Caruso *et al*., 2020). Structurally, AST is a pyridoxal-5′-phosphate– and magnesium-dependent homodimeric enzyme, catalyzing the transfer of an amino group from aspartate to α-ketoglutarate to form oxaloacetate and glutamate, with activity measured in units per liter (U/L) via enzymatic assays detecting oxaloacetate production.

The choice of anticoagulant significantly impacts the accuracy of AST measurements in liver function tests (LFTs). EDTA's chelation of magnesium and pyridoxal-5′-phosphate often results in reduced AST activity compared to serum, due to interference with the enzyme's catalytic function (Lima-Oliveira *et al*., 2016). Sheikh *et al*., 2020 have demonstrated that AST values in EDTA-treated plasma are consistently lower than those in serum or lithium-heparin plasma, with variability depending on assay methodology. Conversely, lithium-heparin preserves cofactor availability, yielding AST results closely aligned with serum, making it the preferred anticoagulant for AST assays.

Systemic heparin therapy may induce mild, transient AST elevations, but these are biological rather than analytical and resolve post-treatment (Yasutaka *et al*., 2023). In vitro, lithium-heparin anticoagulant tubes do not affect AST activity, ensuring reliable results when serum is unavailable. In the context of a project evaluating anticoagulants such as heparin, EDTA, and palm oil-derived heparin-like isolates for clinical chemistry, particularly LFTs, AST measurement is critical. While specific data on palm oil-derived isolates for AST are limited, their heparin-like mechanism, which avoids cation chelation, suggests potential compatibility similar to lithium-heparin, supporting accurate AST assessments in clinical and veterinary applications.

2.8.4 Conjugated Bilirubin

Direct (conjugated) bilirubin is the water-soluble form of bilirubin formed when unconjugated bilirubin is conjugated with glucuronic acid in the liver. It serves as a vital diagnostic marker for differentiating hepatocellular from cholestatic jaundice, and its concentration is commonly determined using diazo-based colorimetric assays. The accuracy of this measurement, however, is strongly affected by the choice of anticoagulant.

Carey *et al*. (2017) reported that EDTA-treated plasma often produces falsely low direct bilirubin readings because the chelation of metal ions disrupts the diazo coupling reaction necessary for accurate detection. In contrast, Sheikh *et al*. (2020) observed that lithium-heparin plasma preserves reagent stability and allows proper color development, producing results that closely correspond with those obtained from serum samples. Consequently, lithium-heparin is regarded as the preferred anticoagulant for ensuring precise and consistent determination of direct bilirubin levels in both clinical and veterinary diagnostic applications.

2.8.5 Total Bilirubin

Total bilirubin, comprising both unconjugated (indirect) and conjugated (direct) fractions, is a critical biomarker for assessing hepatic excretory capacity and hemolytic conditions. Its quantification typically relies on diazo coupling reactions or enzymatic oxidation techniques, which are notably sensitive to the sample matrix and the anticoagulant employed during blood collection.

EDTA-treated plasma frequently produces artificially reduced total bilirubin measurements due to its chelation of divalent metal ions, which alters ionic strength, increases turbidity, and disrupts

reagent interactions necessary for accurate assay outcomes (Narwal *et al*., 2021). Comparative analyses have consistently shown that total bilirubin levels in EDTA plasma are significantly lower than those in serum or lithium-heparin plasma, underscoring EDTA's analytical limitations (Lima-Oliveira *et al*., 2016). Conversely, lithium-heparin plasma preserves the ionic conditions required for assay stability, yielding total bilirubin values closely comparable to those of serum, thus establishing it as the optimal anticoagulant for bilirubin testing (Sheikh *et al*., 2020).

The diagnostic implications of such anticoagulant interference are profound, as underestimation of total bilirubin in EDTA-treated samples may obscure early signs of hepatocellular impairment or hemolysis, potentially leading to misdiagnosis. Within the scope of a project examining anticoagulants such as heparin, EDTA, and palm oil-derived heparin-like isolates for clinical chemistry applications, particularly liver function tests (LFTs), precise total bilirubin measurement is essential. Sodium-heparin and potentially palm oil-derived isolates, which avoid cation chelation while emulating heparin's antithrombin III-mediated mechanism, ensure accurate results. Therefore, EDTA is deemed unsuitable for total bilirubin assays, while lithium-heparin is highly recommended for reliable and consistent measurements in clinical and veterinary diagnostics.

2.9 Protein Profile Parameters

2.9.1 Albumin

Albumin, the predominant plasma protein synthesized exclusively by the liver, plays a crucial role in maintaining plasma oncotic pressure, transporting hormones, fatty acids, and drugs, and serving as a reservoir of amino acids. Its concentration is commonly measured using dye-binding methods,

particularly those employing bromocresol green (BCG) or bromocresol purple (BCP) reagents (Lima-Oliveira *et al*., 2016).

Carey *et al*., 2017 has indicated that EDTA-treated plasma may slightly underestimate albumin concentrations due to the chelation of divalent ions, which disrupts the conditions required for effective dye binding. Although this effect is less severe compared to its impact on alkaline phosphatase or bilirubin assays, it remains clinically relevant. In contrast, lithium-heparin plasma consistently yields albumin values comparable to those in serum, making it the preferred anticoagulant for this assay (Sheikh *et al*., 2020). In the context of a project evaluating anticoagulants such as heparin, EDTA, and palm oil-derived heparin-like isolates for clinical chemistry, particularly liver function tests, accurate albumin measurement is crucial. Sodium-heparin and potentially palm oil-derived isolates, which avoid ion chelation, ensure reliable results, whereas EDTA's interference renders it less suitable for albumin determination in clinical and veterinary diagnostics.

2.9.2 Globulin

Globulin encompasses a broad group of plasma proteins, including immunoglobulins, complement components, and various transport proteins, and is generally determined indirectly by subtracting albumin concentration from total protein measurements. Because globulin estimation depends on the accuracy of both total protein and albumin assays, any analytical errors in these parameters can significantly affect calculated globulin values (Sheikh *et al*., 2020).

EDTA-treated plasma often produces falsely elevated globulin levels due to its chelation of divalent ions, which leads to underestimation of both total protein and albumin, potentially

mimicking patterns associated with chronic liver disease or immune activation (Lima-Oliveira *et al*., 2016). Conversely, sodium-heparin plasma ensures accurate measurements of total protein and albumin, resulting in globulin values closely aligned with those of serum, thereby enhancing diagnostic reliability (Sheikh *et al*., 2020). In the context of a project assessing anticoagulants such as heparin, EDTA, and palm oil-derived heparin-like isolates for clinical chemistry, particularly liver function tests, precise globulin measurement is critical Sodium-heparin and potentially palm oil-derived isolates, which preserve ionic conditions without chelation-related interference, provide reliable results, whereas EDTA's limitations render it less suitable for globulin determinations in clinical and veterinary diagnostics.

2.9.3 Total Protein

Total protein, consisting of albumin and globulin fractions, serves as an important biomarker for evaluating hepatic synthetic capacity and overall nutritional status. Its determination commonly employs the biuret method, which is based on the formation of peptide-copper complexes under alkaline conditions and is notably sensitive to variations in ionic strength and reagent composition (Carey *et al*., 2017).

EDTA-treated plasma often results in lower total protein values due to its chelation of metal ions, which disrupts ionic balance and reduces the sensitivity of the biuret assay (Lima-Oliveira *et al*., 2016). Conversely, lithium-heparin plasma preserves ionic stability, yielding total protein concentrations closely comparable to those in serum, thus establishing it as the preferred anticoagulant for such assays (Sheikh *et al*., 2020). Underestimation of total protein in EDTA-treated samples may lead to misleading clinical conclusions, such as erroneous indications of hypoproteinemia. Within the scope of a project examining anticoagulants like heparin, EDTA, and

palm oil-derived heparin-like isolates for clinical chemistry applications, particularly liver function tests, precise total protein measurement is vital sodium-heparin and potentially palm oil-derived isolates, which maintain ionic integrity without chelating essential ions, ensure accurate results, while EDTA's interference renders it unsuitable for total protein determination in clinical and veterinary diagnostics.

2.10 Storage and Temperature Effects on Biochemical Parameters

The stability of biochemical analytes after sample collection is crucial for reliable laboratory and research results, especially when immediate analysis is not possible. Variables such as storage temperature, light exposure, and choice of anticoagulant markedly influence the integrity of enzymes, bilirubin, and proteins used in liver function testing.

Lima-Oliveira *et al*., 2016 indicated that EDTA, through its chelation of divalent cations like calcium, magnesium, and zinc, compromises the stability of enzymes and proteins by removing essential cofactors, accelerating degradation during storage In contrast, sodium-heparin prevents coagulation via antithrombin III activation without disturbing ion levels, thereby better maintaining analyte stability under diverse conditions (Sheikh *et al*., 2020). For instance, ALT and AST activities in EDTA plasma decrease by approximately 20% and 15%, respectively, after 24 hours at room temperature, while heparinized plasma shows only a 10% reduction (Moyo *et al*., 2019). ALP, being metal-dependent, loses up to 40% of activity in EDTA plasma within 12 hours at room temperature, whereas heparinized plasma retains stability for up to 24 hours, especially when refrigerated (Patel *et al*., 2016).

Bilirubin, sensitive to light, also suffers greater degradation in EDTA plasma. Total bilirubin levels drop by 15% after 6 hours at room temperature under ambient light in EDTA-treated samples,

compared with an 8% decrease in heparinized plasma (Carey *et al*., 2017). Refrigeration at 4°C in darkness stabilizes bilirubin in heparinized plasma for up to 48 hours, whereas EDTA plasma experiences more substantial losses due to matrix alterations. Plasma proteins exhibit similar trends: total protein and albumin remain largely unchanged (<5%) in heparinized plasma after 48 hours of refrigeration, but EDTA plasma shows declines up to 12% at room temperature, with globulin values appearing falsely elevated due to assay interference (Moyo *et al*., 2019).

Freeze-thaw cycles further highlight these differences. Heparinized plasma tolerates freezing at −20°C or −80°C better than EDTA plasma, in which ALT and AST activities decrease more markedly (Zhou *et al*., 2020). Veterinary research also emphasizes heparin's advantage in field conditions with delayed sample processing, as it preserves analyte integrity during transport (Sharma *et al*., 2018). Overall, storage-related variability in EDTA plasma may obscure true indicators of hepatocellular injury, cholestasis, or nutritional status, supporting the use of lithium-heparin for accurate biochemical assessment in both clinical and veterinary settings.

2.11 Gaps in Current Literature

Despite extensive research on anticoagulants, several gaps remain that limit a comprehensive understanding of their effects on biochemical parameters and clinical applications. Firstly, while the comparative effects of EDTA and heparin on common liver enzymes (ALT, AST, ALP) and bilirubin have been well documented, there is limited data on emerging plant-derived heparin-like isolates, particularly regarding their long-term stability, optimal dosing, and interactions with less commonly studied enzymes and proteins. Most studies focus on short-term storage or immediate assay outcomes, leaving uncertainty about performance under prolonged storage or multiple freeze-thaw cycles.

Secondly, research on species-specific differences is sparse. Many studies rely on human or model animal samples, yet veterinary species such as cattle, sheep, or rabbits may respond differently due to variations in plasma composition, enzyme isoforms, or ion concentrations, leading to potential misinterpretation of biochemical results.

Thirdly, there is a lack of standardized protocols for assessing the anticoagulant potency and biochemical compatibility of novel natural isolates like palm oil-derived heparin analogues. Variability in extraction methods, sulfation levels, and molecular weight distribution complicates direct comparisons with commercial heparin or EDTA.

Lastly, studies evaluating the combined effects of anticoagulant choice, storage temperature, and handling conditions are limited. While individual factors have been investigated, few studies systematically assess their synergistic impact on analyte stability and assay accuracy, particularly in field or resource-limited settings.

Addressing these gaps could enhance understanding of natural anticoagulants, optimize laboratory protocols, and improve the accuracy of biochemical assessments in both human and veterinary medicine

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 APPARATUS

Beaker (250ml)

Cotton wool

Filter paper

Measuring cylinders

Micropipette

Sample bottles (EDTA and plain bottles- 5ml, 50ml)

Spatula

Sterile syringe and needles (2ml, 5ml and 10ml)

Sterile syringes and needles

Surgical blade

Test tubes/ racks

Thumb tack pin

Volumetric flasks (100 ml, Pyrex)

3. 2 Equipment

Centrifuge (Model 80-2 or equivalent, China).

ISE analyser (Roche Diagnostic, Indianapolis, IN)

Oven (Genlab Limited, United Kingdom).

Refrigerator (Haier Thermocool, China)

Spectrophotometer (PG Instrument Limited, Britain)

Weighing balance (Ohaus Adventurer, USA)

3.3 REAGENT

Reagents for Alkaline Phosphatase Analysis (ErbaLachema, Germany, N/46/13/A/INT)

Reagent (R1)

L- Aspartate (260mmol/l)

Tris buffer (pH: 7.80 88mmol/l)

Reagent (R2)

NADH (0.22 mmol/l)

a-Ketoglutarate (12mmol/l)

Reagents for Aspartate Aminotransferase Analysis (Randox, United Kingdom, BT29 4QY)

2,4-dinitrophynylhydrazine (2.0mmol/L)

2-amino-2-methyl-1-propaned buffer (1.0 mmol/L)

Alkaline phosphatase substrate (3.6 mm/L)

alpha-oxoglutarate (2.0mmol/L)

l-alanine (2.0mmol/L)

l-aspartate (2.0mmol/L)

Magnesium chloride (1.0 mmol/L)

n-propanol (0.5mmol/L)

Sodium carbonate (0.1 mmol/L)

Sodium hydroxide (0.1mmol/L)

Sodiumthymolphthalein monophosphate (0.2mmol/L)

Reagent for Alanine Aminotransferase Analysis (Biochrom, United Kingdom, CB40FJ)

2-oxoglutgarate (15mmol/l)

L-alanine (0.5mmol/l)

NADH (0.18mmol/ml)

Tris buffer (!00mmol/l pH 7.5

3.4 METHODOLOGY

3.4.1 Sources of the Experimental Animals

a. Sources of Standard Heparin

Four ampoules of the commercial sodium heparin were purchased from Monic Pharmacy, located opposite the University of Benin Teaching Hospital, Benin City, Edo State, Nigeria. Each ampoule contained 25,000 IU of heparin per 5 mL. The commercial preparation served as the reference anticoagulant throughout the study.

b. Source of EDTA Sample

A pack containing fifty (50) sample bottles of 5 mL EDTA was purchased from Monic Pharmacy, Benin City, Edo State, Nigeria. The product, manufactured by Micropoint Diagnostics, China, was used as one of the standard anticoagulants for comparative evaluation against commercial sodium heparin and the palm oil–derived heparin-like isolate.

c. Source for Experimental Animals

Eight (8) healthy adult rabbits of both sexes were obtained from a local animal market in Ibadan, Oyo State, Nigeria. The animals were safely transported to Benin City, Edo State, and housed in well-ventilated wooden cages under standard laboratory conditions. They were fed a balanced diet consisting of fresh guinea grass (*Panicum maximum*), commercial pelleted feed, and provided with unrestricted access to clean water throughout the study period. The animals were acclimatized for one week.

3.4.2 Extraction of Palm Oil–Derived Heparin-Like Isolate

The test sample anticoagulant was extracted from palm oil obtained from ripe fruits of *Elaeis guineensis* (African oil palm). The palm oil sample, a product of the Nigerian Institute for Oil Palm Research (NIFOR), Benin City, Edo State, Nigeria, served as the primary raw material. A measured quantity (100 g) of the oil was transferred into a 2-L plastic beaker, and 1 L of 0.5 M sodium hydroxide (NaOH) solution was added. The mixture was stirred vigorously to achieve a homogeneous slurry and allowed to stand at room temperature for 12 hours. The solidified upper layer (soap fraction) was discarded, while the lower unsaponified layer (approximately 400 mL) was collected and filtered through double-layered filter paper. The filtrate was adjusted to pH 9.0 using 0.1 M hydrochloric acid (HCl). 800 mL of 95% ethanol was gradually added with continuous

stirring until a milky white precipitate appeared. The mixture was refrigerated at 4 °C for one hour to enhance precipitation. The supernatant was discarded, while precipitate adhering to the beaker's base and walls was collected (3.5 g). The crude precipitate was washed twice with 500 mL of 95% ethanol and extracted twice with 500 mL of chloroform in a separating funnel to remove lipid residues. The purified precipitate (2.4 g) was obtained and stored until further use.

3.4.3 Preparation of Anticoagulants

a. Palm Oil–Derived Heparin-like Isolate:

A stock solution was prepared by dissolving 0.001 g of the dried palm oil–derived heparin-like isolate in 100 mL of normal saline. From this stock, 10 mL was further diluted to 100 mL to obtain a working concentration of 0.0001 g/100 mL. According to dose standardization findings, 300 µL of the working solution (equivalent to 7.5 mL, providing 0.6 µg/mL for 50 mL of blood) was dispensed into plain sample bottles before blood collection.

b. Commercial Heparin:

To ensure even and reliable anticoagulation, commercial sodium heparin was dried at 60 °C in the oven to remove moisture and then 0.001g was dissolved in 100 mL of normal saline to make a stock solution. From this, 10 mL was diluted to 100 mL with normal saline to form a working solution. Then, 300 µL (7.5ml) of the working solution was added to 50 mL of fresh bovine blood and gently mixed to distribute the anticoagulant evenly, giving a final concentration of 0.6 µg/mL.

c. EDTA:

Pre-coated EDTA vacutainer tubes supplied commercially were used directly. About 2–3 mL of

blood was dispensed into each tube immediately after collection to ensure complete anticoagulation.

3.4.4 Preliminary Dose Screening of Palm-Derived Isolate

A preliminary anticoagulant activity test was conducted using fresh rabbit blood to determine the effective dose of the palm oil–derived heparin-like isolate. The procedure followed Howell's factor, with isolated commercial heparin (0.002mg/mL) of blood sample serving as the reference standard. Various concentrations of the isolate were tested on 2 mL of rabbit blood as follows: 0.10 µg (10 µL), 0.20 µg (20 µL), 0.40 µg (40 µL), 0.60 µg (60 µL), 0.80 µg (80 µL), and 1.00 µg (100 µL) respectively were pipetted into plain sample tubes, using automatic pipette. 2mls each of fresh blood sample were dispensed into the various sample bottles and rocked gently. The bottles were observed for 30 minutes for coagulation. Complete anticoagulation were observed for bottles containing 40 µL, 60 µL, and 80 µL, respectively. The bottle containing 0.60 µg (60 µL)was unanimously agreed to give the best result. This concentration (, 60 µL) was then adopted for use throughout the experimental period. This dose was extrapolated for 50mls of Bovine blood sample, while the commercial EDTA sample bottles were used intact.

3.4.5 Sample Collection

Fresh bovine blood sample was collected from clinically healthy adult cows at an abattoir located along Ewa Road, Benin City, Nigeria into the 50ml anticoagulated sample bottles. Analysis for the parameters was carried out the first day and repeated on Day 3 and Day 7. Approximately 50 mL of blood was aseptically drawn from the jugular vein using sterile syringes and immediately dispensed into pre-labeled containers containing the7.50ml each of the anticoagulants: isolated reference heparin and palm oil-derived heparin-like while commercial EDTA was used intact.

Commercial heparin served as the reference anticoagulant. The samples were gently inverted several times to ensure thorough mixing and prevent clot formation. The samples were then transported to the laboratory for analysis within one hour of collection.

3. 4.6 Sample Processing and Plasma Preparation

Upon arrival at the laboratory, each blood sample was centrifuged at 3000 revolutions per minute (rpm) for 10 minutes at ambient temperature using a bench-top centrifuge. The centrifugation process separated the blood into plasma and serum components.
The supernatant plasma was carefully aspirated using a micropipette and transferred into clean, labeled test tubes. The plasma samples were kept in a refrigerator at −20 °C until used for the determination of liver enzyme activities (AST, ALT, ALP) and total protein concentration.

3.4.7 Method of Aspartate Aminotransferase (AST) Analysis

The activity of Aspartate Aminotransferase (AST) was determined using the colorimetric method of Reitman and Frankel (1957) as described by Randox diagnostic kits.
A total of 0.5 mL of AST substrate solution containing L-aspartate and α-ketoglutarate was dispensed into two labeled test tubes (test and control). The tubes were equilibrated in a water bath at 37 °C for 5 minutes.
Next, 0.1 mL of plasma sample was added to the test tube and mixed gently, while the control tube received 0.1 mL of distilled water. Both tubes were incubated for 30 minutes at 37 °C to allow the enzymatic reaction to proceed.
After incubation, 0.5 mL of 2,4-dinitrophenylhydrazine (DNPH) was added to both tubes to stop the reaction and develop color. The mixture was allowed to stand for 20 minutes at room temperature. Then, 5.0 mL of 0.4 N NaOH was added to each tube, mixed thoroughly, and allowed

to stand for 10 minutes to stabilize the color.
The absorbance of the test and control was read at 546 nm against a reagent blank containing all reagents without the plasma sample. AST activity was calculated using the Randox calibration chart and expressed in U/L.

3.4.8 Method of Alanine Aminotransferase (ALT) Analysis

Alanine Aminotransferase (ALT) activity was determined using the Reitman and Frankel (1957) method according to Randox kit instructions.
0.5 mL of ALT substrate solution containing DL-alanine and α-ketoglutarate was pipetted into two labeled test tubes (test and control) and pre-incubated at 37 °C for 5 minutes.
Then, 0.1 mL of plasma sample was added to the test tube, while 0.1 mL of distilled water was added to the control. The contents were mixed gently and incubated at 37 °C for 30 minutes.
After incubation, 0.5 mL of DNPH reagent was added to each tube to terminate the enzymatic reaction and develop color. The mixture was left at room temperature for 20 minutes, after which 5.0 mL of 0.4 N NaOH was added to both tubes, mixed, and allowed to stand for 10 minutes.
The absorbance of the test was read at 546 nm against a reagent blank prepared under the same conditions but without the plasma sample. The enzyme activity was obtained from the standard curve and expressed in U/L.

3.4.9 Method of Alkaline Phosphatase (ALP) Analysis

The activity of Alkaline Phosphatase (ALP) was determined using the King and Armstrong (1934) method as outlined in the Randox diagnostic kit.
0.5 mL of ALP substrate solution (containing disodium phenyl phosphate) was dispensed into two labeled test tubes (test and control). The tubes were equilibrated at 37 °C for 3 minutes.

Then, 0.1 mL of plasma sample was added to the test tube, while 0.1 mL of distilled water was added to the control. The mixture was mixed gently and incubated for 10 minutes at 37 °C. After incubation, 2.5 mL of ALP color developer (containing 4-aminoantipyrine and potassium ferricyanide) was added to each tube, mixed properly, and allowed to stand for 5 minutes at room temperature for full color development.
The absorbance was measured at 520 nm against a reagent blank containing all reagents but without the plasma sample. ALP activity was calculated from the calibration curve and expressed in U/L.

3.4.10 Method of Albumin Analysis

Albumin concentration was determined using the Bromocresol Green (BCG) method according to Doumas *et al*. (1971) and the Randox diagnostic kit procedure.
3.0 mL of BCG reagent was pipetted into labeled test tubes for blank, standard, and test. Then, 0.01 mL of distilled water was added to the blank, 0.01 mL of albumin standard to the standard tube, and 0.01 mL of plasma sample to the test tube. The mixture was gently mixed and incubated at 25 °C for 10 minutes to allow for color formation.
The absorbance of the test and standard was read at 630 nm using a spectrophotometer against the reagent blank. Albumin concentration was calculated using the formula:

$$\text{Albumin (g/dL)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentration of Standard}$$

3.4.11 Method of Total and Conjugated Bilirubin Analysis

The concentrations of total and conjugated (direct) bilirubin were determined using the Jendrassik and Grof (1938) method as described in Randox diagnostic kits.

(a) Conjugated (Direct) Bilirubin:

0.5 mL of diazo reagent (sulfanilic acid and sodium nitrite) was added to labeled test tubes for test, standard, and blank. Then, 0.2 mL of plasma sample was added to the test, 0.2 mL of standard to the standard tube, and 0.2 mL of distilled water to the blank. The contents were mixed and allowed to stand for 5 minutes at room temperature for color development. Afterward, 2.5 mL of alkaline tartrate reagent was added to each tube to stop the reaction and stabilize the color. Absorbance was measured at 540 nm against the reagent blank. The value obtained represented direct bilirubin concentration.

(b) Total Bilirubin:

For total bilirubin determination, the same procedure was followed except that 0.5 mL of caffeine reagent (accelerator) was added before the diazo reagent to liberate bilirubin bound to albumin. The reaction mixture was incubated for 10 minutes at 37 °C, followed by addition of 2.5 mL of alkaline tartrate reagent. The absorbance was read at 540 nm using a spectrophotometer.

3.4.12 Method of Total Protein Analysis

Total plasma protein concentration was determined using the Biuret method described by Gornall *et al*. (1949) as adopted in Randox diagnostic kits.

1.0 mL of Biuret reagent was dispensed into labeled test tubes for blank, standard, and test samples. Then, 0.02 mL of distilled water was added to the blank, 0.02 mL of protein standard to the standard tube, and 0.02 mL of plasma sample to the test tube. The contents were mixed gently and

allowed to stand for 10 minutes at room temperature to permit full color development. The absorbance of the test and standard was read at 540 nm using a UV–Visible spectrophotometer against the reagent blank. The total protein concentration was calculated as:

$$\text{Total Protein (g/dL)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentration of Standard}$$

3.4.13 Calculation of Globulin

The plasma globulin concentration was obtained by the difference between total protein and albumin concentrations using the relation:

$$\text{Globulin (g/dL)} = \text{Total Protein} - \text{Albumin}$$

The resulting globulin value reflects the concentration of immunoglobulins and other serum proteins.

3.4.14 Statistical Analysis

Data obtained were expressed as mean ± standard deviation (SD) of triplicate determinations. Statistical significance among groups was evaluated using one-way analysis of variance (ANOVA). The processed data were presented graphically using bar charts to illustrate the comparative effects of the different anticoagulants on liver enzyme and protein parameters.

CHAPTER FOUR

4.0 RESULTS

(A) Result for Enzyme Analysis

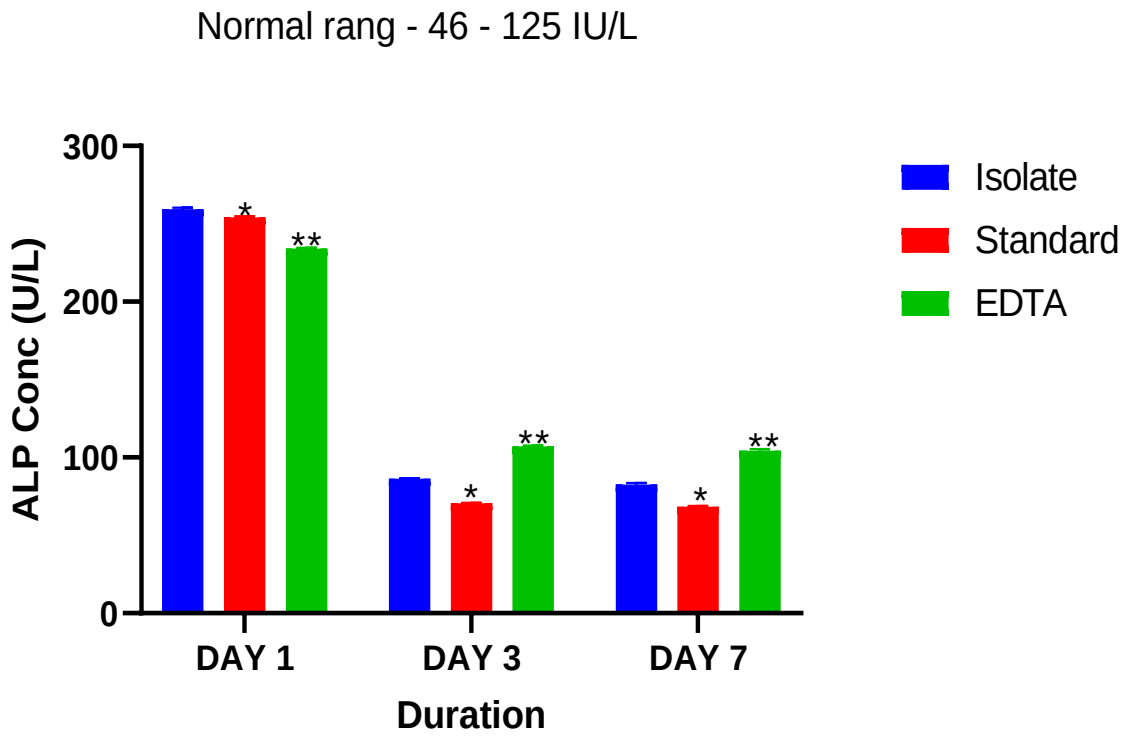

Figure 4.1: Analysis for the levels of Alkaline Phosphatase (ALP) on day 1, day 3 and day 7.

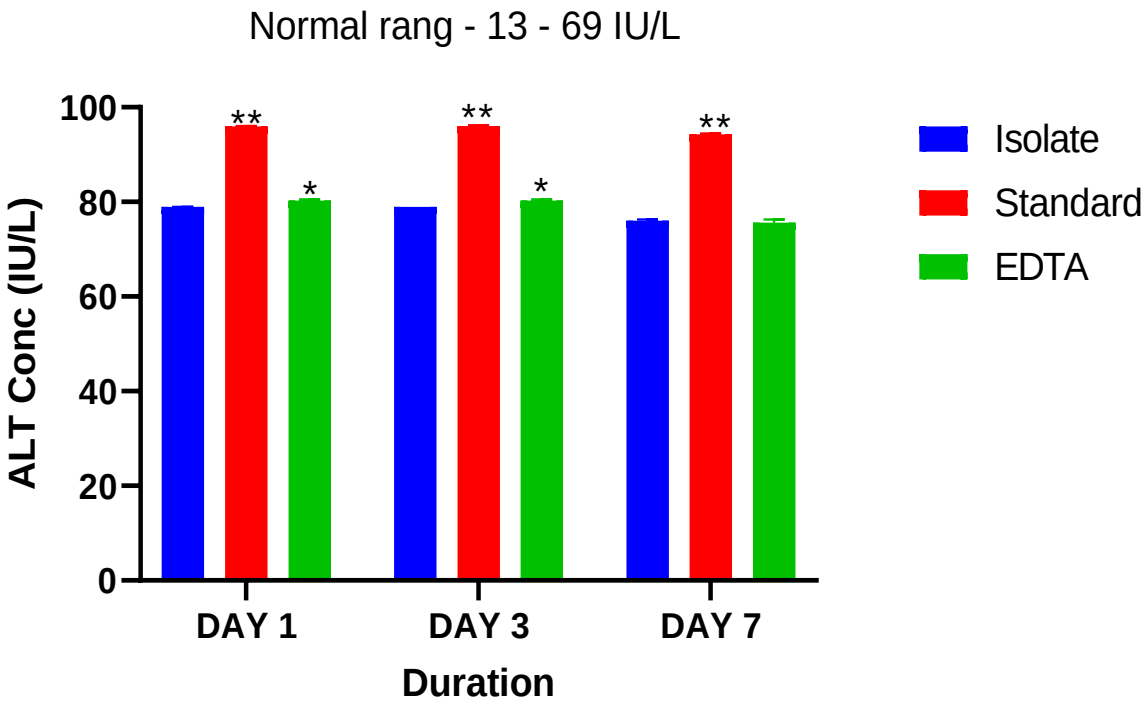

Figure 4.2: Analysis for the levels of Alanine Aminotransferase (ALT) on day 1 day 3 and day 7

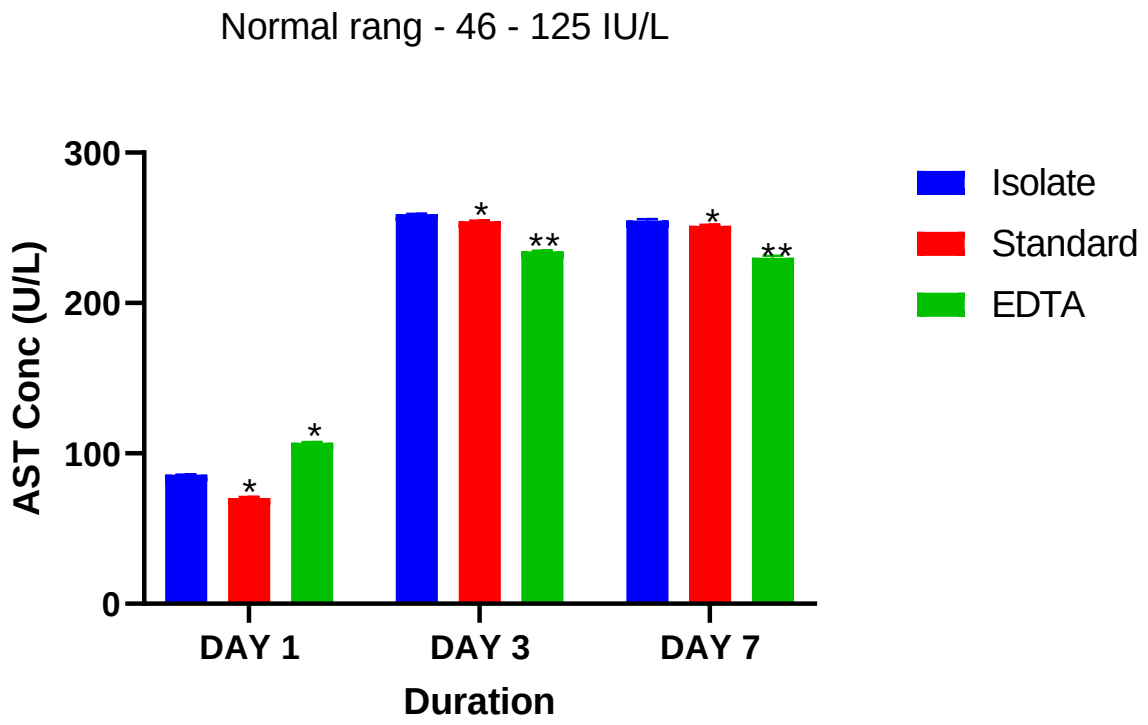

Figure 4.3: Analysis for the levels of Aspartate Aminotransferase (AST) on day 1, day 3 and day 7

(B) Results for Protein Analysis

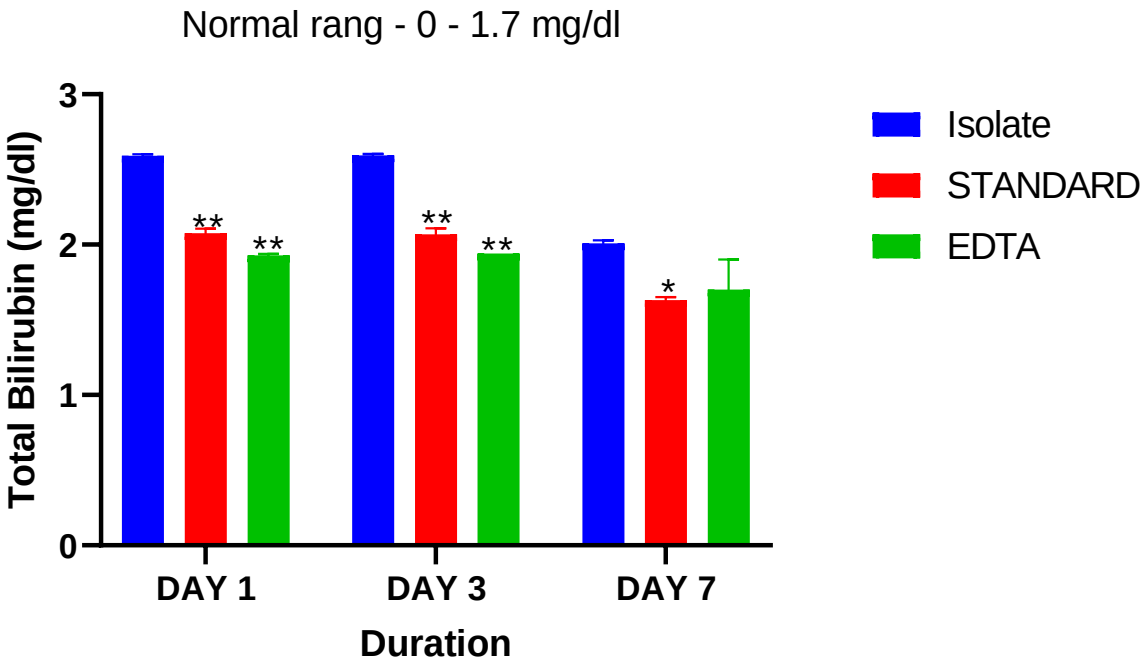

Figure 4.4: Analysis for the levels of Total Bilirubin on day 1, day 3 and day 7

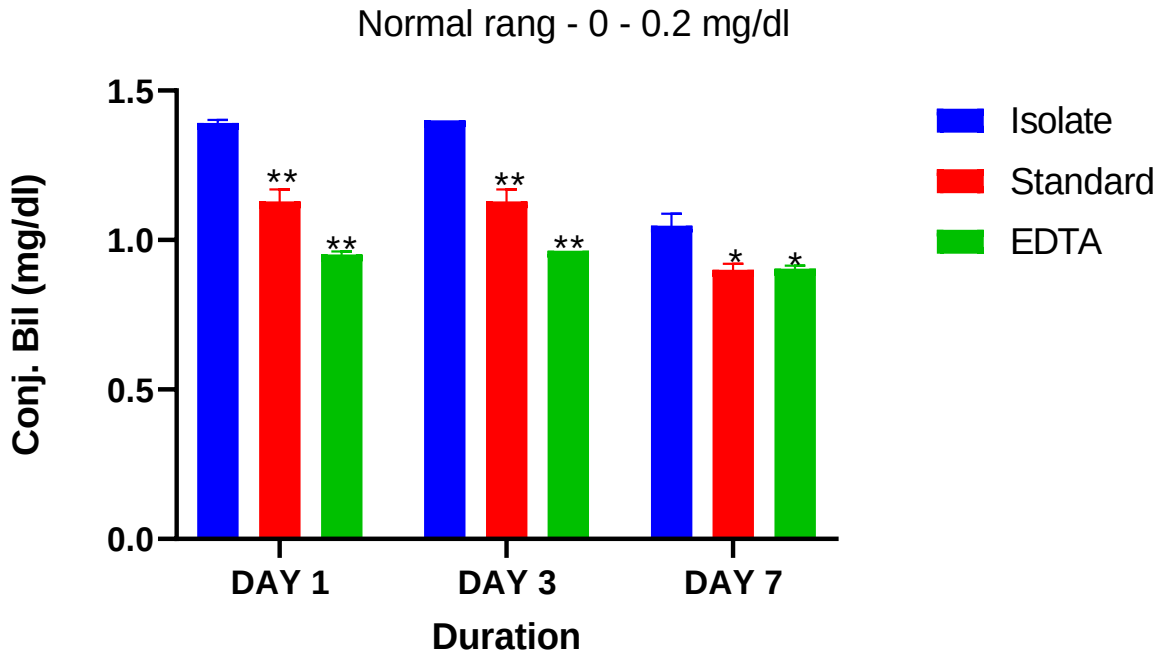

Figure 4.5: Analysis for the levels of Conjugate Bilirubin on day 1, day 3 and day 7

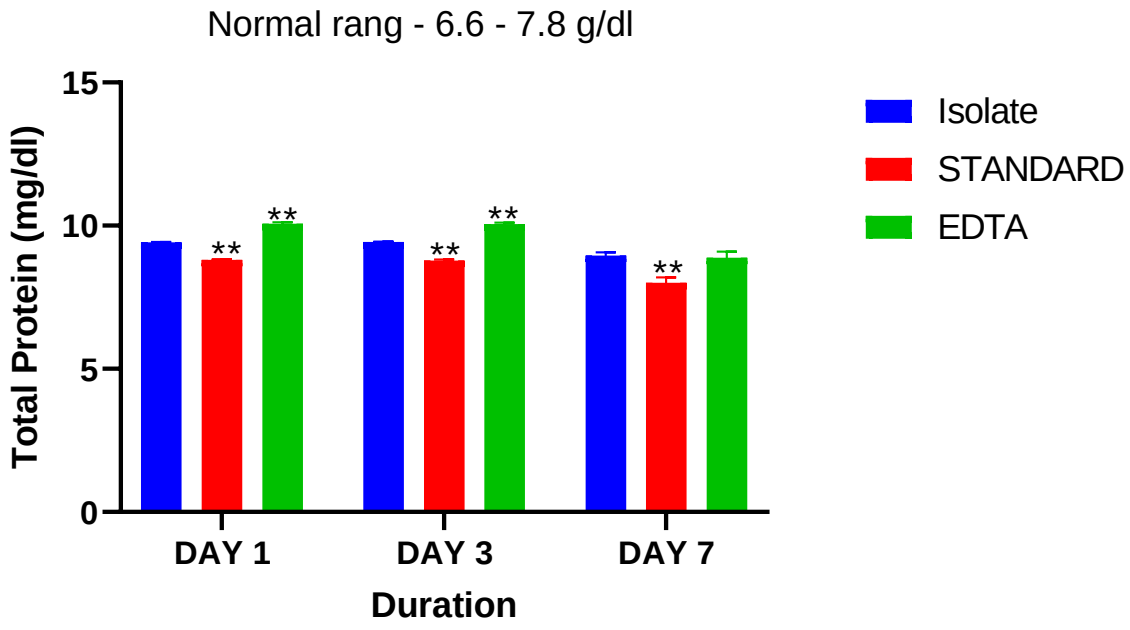

Figure 4.6: Analysis for the levels of Total Protein on day 1, day 3 and day 7

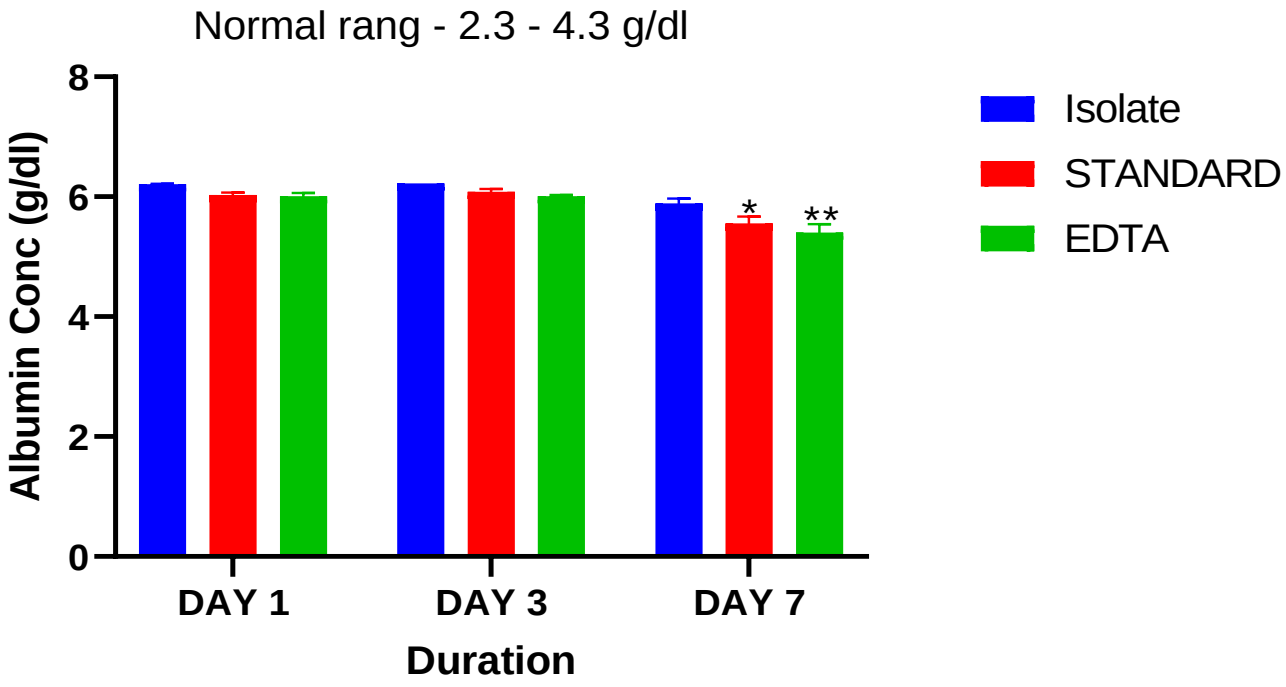

Figure 4.7: Analysis for the levels of Albumin on day 1, day 3 and day 7

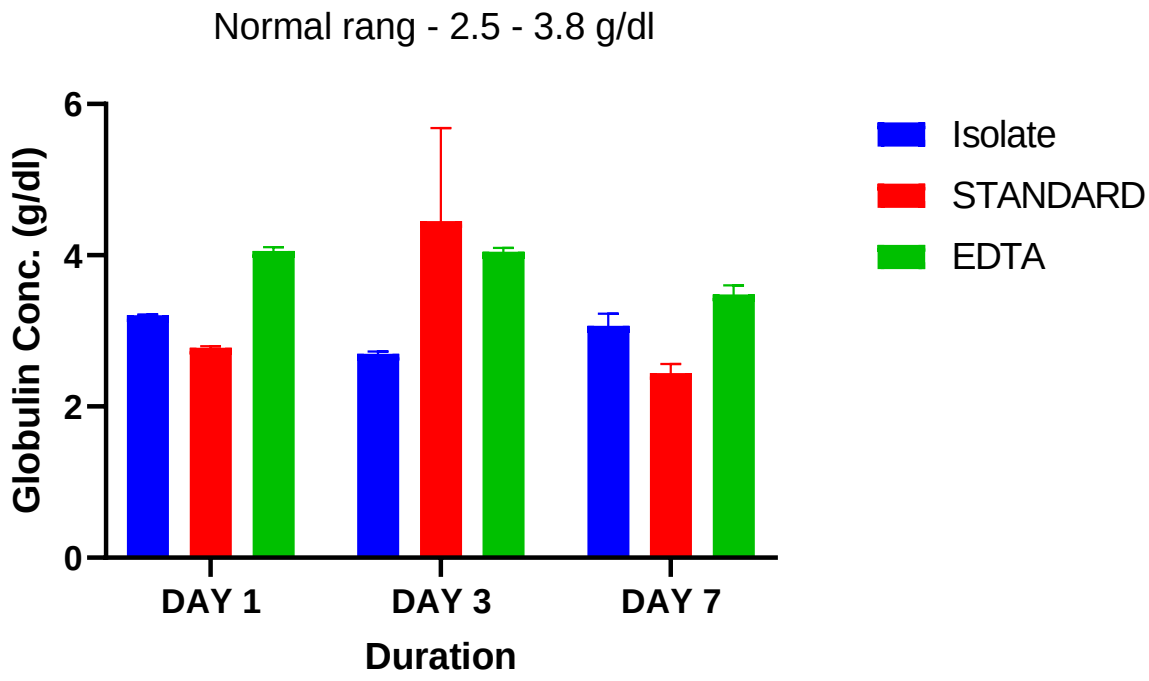

Figure 4.8: Analysis for the levels of Globulin on day 1, day 3 and day 7

CHAPTER FIVE

5.0 DISCUSSION

Anticoagulants are essential compounds that prevent blood coagulation and are widely utilized in both clinical and laboratory applications. This study compared the anticoagulant activity and the effects of a palm oil–derived compound on liver functions and protein parameters with those of standard heparin and EDTA. The objectives was to determine the concentration of the plant-derived compound that provides anticoagulant activity comparable to conventional agents and to assess whether it could serve as a potential natural substitute for standard anticoagulants. Preliminary results showed that 60 µL of 0.001 g/100 mL solution of the palm-derived compound effectively prevented clot formation in 1 mL of blood samples thus, indicating strong anticoagulant potential. This concentration (0.0006 mg/mL of blood sample) was much lower than Howell's heparin number (0.002 mg/mL) for 24 hours (Sheikh *et al*., 2020)., suggesting that the palm oil–derived anticoagulant may possess comparable or even greater anticoagulant potency than commercially available standard heparin.

The study also compared the effects of standard heparin, EDTA, and the palm oil–derived heparin-like isolate on the stability of liver enzymes and plasma proteins in bovine blood samples stored for seven days. Differences across the biochemical parameters (Figures 4.1–4.8) were interpreted in relation to the results obtained in this study.

Alkaline Phosphatase (ALP) activity is illustrated in Figure 4.1. On Day 1, ALP levels were higher than the normal range (46–125 IU/L) across all groups, with values of 259 ± 1.07 IU/L (isolate), 254.2 ± 0.665 IU/L (heparin), and 234 ± 0.51 IU/L (EDTA). The relatively lower EDTA value supports the findings of Patel *et al*. (2016) and Olatunji *et al*. (2020), who attributed reduced ALP

activity to the chelation of essential cofactors such as Zn^{2+} and Mg^{2+}. On Day 3, ALP levels decreased in the three anticoagulants, this was also the case on Day 7. The result could suggest general lysis and poor handling of the fragile blood sample.

The result obtained for Alanine Aminotransferase (ALT) shown in (Figure 4.2) remained above the normal range (13–69 IU/L) during storage, suggesting enzyme instability or leakage. On Day 1, standard heparin produced the highest mean (95.92 ± 0.1 IU/L; $p < 0.01$), followed by EDTA (80.3 ± 0.24 IU/L) and the isolate (78.93 ± 0.04 IU/L). Elevated levels under heparin use indicate efficient enzyme preservation (Carey *et al*., 2017). By Day 7, the isolate (76 ± 0.3 IU/L) and EDTA (75.6 ± 0.67 IU/L) remained closer to the normal range, suggesting that the isolate reduced enzyme elevation during prolonged storage. This agrees with Onoja *et al*. (2022), who reported similar ALT stabilization by palm-derived sulfated polysaccharides and heparin.

Aspartate Aminotransferase (AST) analysis shown in (Figure 4.3) was within the normal range (46–125 IU/L) on Day 1 for both heparin (70.4 ± 0.68 IU/L) and the isolate (85.78 ± 0.2 IU/L), but increased substantially by Day 7, reaching 280.4 ± 0.34 IU/L (heparin) and 250.3 ± 0.32 IU/L (EDTA). The higher values in heparinized samples reflect sustained enzyme stability and possible red cell leakage during storage (Lima-Oliveira *et al*., 2016). Lower AST values in EDTA and the isolate suggest partial enzyme inactivation, likely due to EDTA's chelation of cofactors and the isolate's moderate stabilizing effect (Sheikh *et al*., 2020).

Values obtained for the analysis of Total Bilirubin in the blood samples is depicted in Figure 4.4 followed a similar trend, exceeding the normal range (0.04–0.7 mg/dL) in all samples. EDTA consistently produced lower readings than heparin and the isolate, corroborating Lima-Oliveira *et al*. (2016), who noted that EDTA tends to cause falsely low bilirubin results. The comparable

values between the isolate and heparin demonstrate that the plant-based compound maintained exhibited the same reactions during the study period, consistent with Onoja *et al*. (2022).

Conjugated Bilirubin (Figure 4.4) levels were above the normal range (0–0.2 mg/dL) across all groups. On Day 1, the isolate recorded the highest value (1.392 ± 0.01 mg/dL), while EDTA was the lowest (0.952 ± 0.01 mg/dL; $p < 0.01$). This agrees with Carey *et al*. (2017) and Narwal *et al*. (2021), who reported that EDTA interferes with bilirubin estimation through ion chelation affecting the diazo reaction. By Day 7, the isolate and heparin maintained higher bilirubin concentrations than EDTA, indicating better analyte stability with non-chelating anticoagulants.

Total Protein (Figure 4.5) values were below the normal reference range (6.7–8.8 g/dL) on Day 1: EDTA (4.6 ± 0.1 g/dL; $p < 0.01$), isolate (5.2 ± 0.2 g/dL), and heparin (5.3 ± 0.1 g/dL). The lower EDTA readings can be attributed to copper chelation in the biuret reaction (Lima-Oliveira *et al*., 2016). By Day 7, the isolate retained the highest protein concentration (5.9 ± 0.1 g/dL), followed by heparin (5.6 ± 0.1 g/dL) and EDTA (5.1 ± 0.1 g/dL), confirming superior protein preservation by the isolate, as reported by Onoja *et al*. (2022).

Albumin (Figure 4.6) remained above the normal range (2.7–4.3 g/dL) throughout storage, suggesting good overall protein stability. On Day 1, the isolate had the highest value (6.208 ± 0.01 g/dL), followed by heparin (6.032 ± 0.04 g/dL) and EDTA (6.012 ± 0.05 g/dL). A gradual decrease occurred by Day 7, but the isolate maintained higher albumin levels (5.892 ± 0.08 g/dL) compared with heparin (5.56 ± 0.11 g/dL) and EDTA (5.4 ± 0.14 g/dL). The lower EDTA readings are consistent with Carey *et al*. (2017) and Sheikh *et al*. (2020), who linked them to metal ion chelation interfering with bromocresol green dye binding. The isolate's higher stability supports its strong compatibility with protein assays.

Globulin (Figure 4.7) values, derived from the difference between total protein and albumin, reflected the accuracy of protein estimations. On Day 1, heparin recorded the highest level (4.8 ± 0.02 g/dL), followed by the isolate (4.2 ± 0.1 g/dL) and EDTA (3.9 ± 0.08 g/dL). By Day 7, the isolate recorded the highest globulin level (4.0 ± 0.1 g/dL), while EDTA showed the lowest (3.5 ± 0.08 g/dL). The lower EDTA readings correspond to its known interference in protein quantification (Lima-Oliveira *et al*., 2016; Sheikh *et al*., 2020).

Overall, the differences observed among the anticoagulants are directly linked to their chemical properties. EDTA, a strong chelating agent, interfered with enzyme activity and protein assays by binding essential divalent metal ions (Mohri *et al*., 2007; Patel *et al*., 2016). Standard sodium heparin, a non-chelating anticoagulant, preserved enzyme and protein integrity by preventing coagulation through its interaction with antithrombin while largely maintaining plasma ionic composition. However, as noted by Heidarpour *et al*. (2022), sodium heparin may slightly influence sodium-dependent assays due to the presence of sodium ions in its formulation, although such effects are minimal in non-electrolyte parameters. The palm oil–derived heparin-like isolate performed comparably to standard heparin in most parameters and even demonstrated enhanced stability for transaminases and total proteins. This supports Onoja *et al*. (2022), who found that palm-derived sulfated polysaccharides mimic heparin's antithrombin-mediated anticoagulant mechanism without disrupting ionic balance.

In conclusion, the palm oil–derived heparin-like isolate exhibited anticoagulant activity similar to standard heparin and showed superior analytical compatibility compared to EDTA. It effectively preserved enzyme and protein stability throughout seven days of storage, maintaining values close to physiological norms. While standard heparin proved most reliable for ALP and globulin stabilization, the isolate showed equal or better preservation of transaminase and total protein

levels. Although EDTA effectively prevented clotting, it consistently interfered with biochemical estimations. Therefore, the palm-derived heparin-like isolate presents a promising natural anticoagulant candidate for biochemical and veterinary applications. However, further investigations on its structural composition, toxicity, and clinical scalability are necessary to validate its safety and diagnostic potential.

5.1 CONCLUSION

This study compared the anticoagulant activity and biochemical effects of a palm oil–derived heparin-like isolate with those of standard heparin and EDTA using bovine blood samples stored for seven days. The palm-derived isolate effectively inhibited clot formation at a low concentration (0.0006 mg/mL), demonstrating strong anticoagulant potential comparable to standard heparin. However, analysis of liver enzymes and plasma proteins showed that most biochemical values for all three anticoagulants including the reference heparin deviated from normal physiological ranges. These variations were attributed not solely to anticoagulant type but likely to red blood cell lysis, enzyme leakage, and protein degradation associated with prolonged storage. Elevated enzyme levels in samples treated with the palm-derived isolate and standard heparin indicated partial enzyme preservation but also accumulation from cellular breakdown over time, whereas EDTA produced inconsistent readings due to its chelating action, which disrupts metal cofactors and colorimetric assays. Overall, while the palm oil–derived heparin-like isolate displayed anticoagulant potency comparable to standard heparin, neither agent maintained biochemical stability within normal limits throughout storage. This finding underscores post-collection degradation as a critical limitation regardless of anticoagulant choice. Nevertheless, the isolate's ability to prevent coagulation at very low concentrations and sustain near-heparin performance highlights its potential as a natural, cost-effective anticoagulant candidate. Further purification,

compositional analysis, and evaluation under controlled storage conditions are recommended to improve biochemical stability and confirm its suitability for laboratory or clinical applications.

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