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Conclusion should elucidate how the results communicate to the theory presented as the basis of the study and provide a concise explanation of the allegation of the findings.

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Advancing Diagnostic Capabilities through Organ-on-a-Chip Technology

OPEN ACCESS

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In the recent years, the field of lab-on-a-chip (LOC) technology has made substantial progress and has transformed the landscape of diagnostic applications. These miniaturized and integrated microfluidic devices have potentially revolutionized medical diagnostics by providing rapid, sensitive, and cost-effective analysis of various biomarkers and analytes. One of the key advancements in this domain is the integration of cellular constructs within micro-engineered platforms. It has enabled to recapitulate the physiological and pathological conditions of complex tissues and organs.

This 'Organ-on-a-Chip' technology holds immense promise for point-of-care diagnostics. These microfluidic devices offer unprecedented insights into disease mechanisms and therapeutic interventions. From mimicking the blood-brain barrier for drug screening to representing the properties of vital organs like the liver, heart, and lungs, organ-on-a-chip systems can revolutionize diagnostic paradigms.

Not only has this technology enhanced the diagnostic accuracy, it is also revolutionizing multiplexing and high-throughput screening. These micro-engineered constructs provide a versatile platform for drug development and toxicology studies, and enable researchers to evaluate multiple parameters simultaneously, which has accelerated the pace of discovery and innovation. In additions, these systems are potentially streamlining the sample preparation and analysis as well. Owing to their miniaturized nature, the organ-on-a-chip devices allow for the use of smaller sample and reagent volumes, leading to more efficient and cost-effective analyses.

Despite the impressive strides made in organ-on-a-chip technology, there are still challenges need to be addressed. Among these hurdles lie standardization, scalability, and regulatory considerations that must be overcome to fully realize the potential of these micro-engineered platforms. However, continued innovation and collaboration can totally alter the future of diagnostic applications. Organ-on-a-chip technology holds the promise of revolutionizing medical diagnostics, offering rapid, sensitive, and cost-effective analysis of biomarkers and analytes. The potential for organ-on-a-chip technology to transform healthcare delivery and improve patient outcomes is limitless.

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Review Article

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A review on Diversity, Mechanism of Action and Evolutionary Significance of Antimicrobial Peptides

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ABSTRACT

Antimicrobial peptides (AMPs) are small, evolutionarily main peptides that widely exist in rich diversity across nature and play a significant role in the innate immunity of various taxa from invertebrates to vertebrates. They are equally targeted as the newest discovered antibiotics against various prokaryotes, including bacteria, viruses, fungi, and parasites. AMPs show broad-spectrum potential with high efficacy and low toxicity via in vivo studies. Undoubtedly, this also confers their specific mechanism of action (MOA) and unique but distinct structures. Already, many studies have reported that AMPs possess diverse MOA against various pathogenic microbes. AMPs also encourage the cells to enhance wound healing, programmed cell death, angiogenesis, and produce chemokines. However, the associated risk is the evolution of resistance to AMPs could lead to possible danger to inherent immunity. From an evolutionary perspective, they are usually considered nonspecific with redundant functions due to the fact that they are easily duplicated and produce pseudogenes, thus showing less evolution at the primary amino acid level. However, the microbial resistance risk against conventional antibiotics can be minimized by using AMPs efficiently and sustainably. Understanding the nature and evolution of AMPs will be beneficial as well. The current review focused on antimicrobial peptides' diversity, history, MOA, and evolutionary significance.

INTRODUCTION

In recent years, microbes' resistance has increased due to the absence of new antimicrobial agents and the reduced ineffectiveness of antibiotics. Thus, the discovery and approval of novel drugs for therapeutic use are of great importance. Among these new drugs, antimicrobial peptides are one of the excellent members for the discovery of new antimicrobial agents [1]. Antimicrobial peptides are peptide-based effectors of the innate immune system in prokaryotic and eukaryotic organisms. AMPs are categorized into various subgroups according to amino acid substitution [2]. They usually contain 12-50 amino acids. Practically, AMPs are part of microorganisms, humans and other living organisms' innate immune system and have been known for an age. Skin infections and wounds are treated by using these peptides [3]. No matter the origin, almost all peptides share some similar characteristics, such as peptides have a net positive charge, they all have amphiphilic activity (both hydrophobic and hydrophilic), and, in some cases, they are also membrane activators [4]. The positive charge of peptides shows more attraction towards negatively charged microorganisms than the host cell because host cells have comparatively less negative charge than prokaryotic cells. Therefore, the antimicrobial peptides bind with the microbes. The ability of AMPs to accumulate at the target location, i.e., the infection site in microbes, makes them more toxic, and their toxicity is more harmful to microorganisms than the host cells; due to this ability, they

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may be called attractive targeting vectors [5].

The AMPs interfere with the synthesis of cytoplasmic and cell membranes. By inhibiting their synthesis, they can kill the microbe and reduce the growth of bacterial cells. The AMPs also hinder the enzymes production in the microbial cell. Thus, weakening the defense of cells [6]. AMPs offer clear and prominent advantages over conventional antimicrobial agents, as they do not tend to induce multidrug resistance in the host. Additionally, AMPs not only exhibit antimicrobial activity but also assist the host's immune system [7]. Biofilms are the communities of surface associated sessile microorganisms and bound in a self-produced extracellular matrix, thus developing the resistance against antimicrobial agents and giving rise to these chemotherapeutic problems [8]. More particularly, these bacterial colonies are physiologically different from those colonies which are planktonic but belong to the same group. They have embedded in a self-secreted matrix that can increase the antimicrobial resistance by one thousand folds by blocking the penetrance of antimicrobial agents [9]. Usually, AMPs were recommended to tackled biofilms because they have broad-spectrum bactericidal action. AMPs are frequently synergistically used with antimicrobial drugs to inhibit the molecular pathways involved in formation of biofilm [10].

AMPs have demonstrated remarkable effectiveness and efficiency in laboratory settings, particularly in cultural tubes. They exhibit significant efficacy against a wide range of bacteria, including both Gram-negative and Gram-positive strains. Moreover, AMPs have shown efficacy against many drug-resistant bacteria, highlighting their ability to overcome microbial resistance mechanisms [11]. The AMPs possess hydrophilic and hydrophobic parts, i.e., they are amphipathic, and they are a-helical peptides. Therefore, they can easily attach themselves to the cell membrane and the proteins in serum. This property helps them remain intact in circulation [12].

However, some limitations in the use of AMPs exist that may be disastrous. These limitations can finish the game of AMPs if serious steps are not taken. These limitations include high cost of discovering the peptides, the synthesis, and the management with screening. Moreover, peptides are toxic for both host and microbial cells; therefore, this is also a limitation in AMPs. In addition, the activity of AMPs is affected by factors such as salt concentration, pH levels, and exposure to serum. Furthermore, they are also sensitive to proteolysis, which can reduce their effectiveness. Additionally, repeated application of AMPs may lead to reduced sensitivity and potential allergic reactions [13].

HISTORY

The first AMP was discovered in 1939, followed by the discovery of some important antimicrobial peptides in the 1980s, initially in insect hemolymph, mammalian neutrophil granules, and the skin secretions of frogs. These peptides, such as defensins and cathelicidins, are key components of the innate immune system and play a crucial role in defending against microbial pathogens. They are typically small, cationic molecules with amphipathic properties, allowing them to interact with microbial membranes and disrupt their integrity [14]. The discovery of antimicrobial peptides has sparked significant interest in their therapeutic potential, particularly in the face of increasing antibiotic resistance. Research continues to uncover new antimicrobial peptides in various organisms, highlighting their diversity and potential for novel antimicrobial therapies [15]. There are almost thousands of AMPs that have been found naturally in microorganisms, plants and from different sources. In addition, several AMPs are synthesized in the laboratory artificially by mimicking the original sequence or with the help of computer design [16]. Since the start of this field, the AMPs have been promoted. From the discovery to some time, these AMPs failed to seek the attraction of scientists and pharmacists. When antimicrobial drug resistance occurs, these peptides become important and promoted well because antimicrobial drug resistance is the leading health crisis in morbidity and mortality globally [17]. In recent times, the significance of AMPs has grown due to the development of some into powerful antimicrobial agents. Several antimicrobial peptides are currently undergoing trials to assess their effectiveness against a wide array of microorganisms and microbial activities [18].

DIVERSITY OF ANTIMICROBIAL PEPTIDES

Antimicrobial peptides are present in great diversity based on their structures (Figure 1), sequences and mechanism of action.

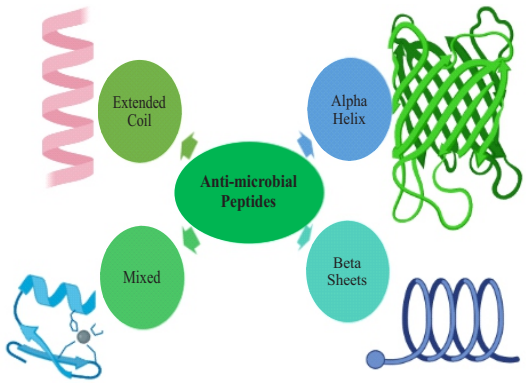

Figure 1: The figure showed types of antimicrobial peptides which are categorized according to their structures. The AMPs are known to have four main types, i.e., extended coil, alpha helix, beta-sheets, and mixed AMPs.

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Diversity Based on the Structure

Based on the structure, the AMPs are categorized into three general subclasses.

1. Antimicrobial Peptides with Alpha-Helical Structure

Antimicrobial peptides present in this subgroup have an alpha-helical structure and these are common in insects and frogs and found in their extracellular matrix.

In Alpha helical peptides, mostly amide groups are present at the C-terminal, enhancing the antimicrobial activity. In addition, the presence of the amide group at the C-terminal increases the electrostatic interaction among the peptide which is charged positively and the bacterial membrane which is negatively charged. This contact helps in stabilization of helical structure at the membrane surface [19].

Few Examples of antimicrobial peptides with α helical cathelicidins [20], LL-37 [21], α helical magainin [22], Aurein [23], pexiganan [18], Mellitin [21], Brevinin [24], Maculatins [25] and Citropin [26].

1. Antimicrobial Peptides with β Sheet Structure

This class comprises Cathelicidin family AMPs [20], such as Protegrins found in pigs, and Bactenecin found in bovine, which contain an arginine-rich disulfide loop. Arginine is crucial in the disulfide loop of Cathelicidin AMPs because it contributes to their antimicrobial activity. Arginine is positively charged, allowing it to interact with negatively charged microbial membranes, disrupting their integrity. This interaction is vital for the peptides' ability to penetrate and destabilize the membranes, leading to microbial cell death. Additionally, the arginine-rich nature of the disulfide loop enhances the peptide's overall cationic charge, which is important for its interaction with microbial membranes and subsequent antimicrobial effects. Defensins are the chief group of β sheet antimicrobial peptides, further divided into three subgroups [27]. Defensins also show antibacterial, antifungal, antiviral, and inflammatory and immune reactions [21, 28]. Tachyplesins and polyphemusins peptides, derived from the hemocytes of horseshoe crabs, are rich in arginine, making up 30% of their sequence [24].

The presence of cysteine residues in almost all β-sheet antimicrobial peptides is of significant importance. Cysteine residues are crucial because they enable the formation of disulfide bonds. These bonds play a critical role in stabilizing the peptide's tertiary structure, enhancing its resistance to degradation by proteases and other enzymes. Additionally, disulfide bonds can contribute to the peptide's antimicrobial activity by facilitating interactions with microbial membranes or other targets. Therefore, cysteine residues are essential for the structural integrity and functional efficacy of β-sheet antimicrobial peptides [29].

2. Antimicrobial Peptides with Extended Coil Structure

The last subclass of antimicrobial peptides has an exceptional extended coil structure. This subclass includes hesitatin, which is found in humans and is known for its antimicrobial properties. Hesitatin contains histidine residues, which are important for its activity. Additionally, this subclass includes members of the Cathelicidin family [20], such as PR-39b, Tritrpticin, Indolicidin, and Crotalicidin, which also exhibit an extended coil structure [30]. Diversity of some antimicrobial peptides is shown in table 1.

Table 1. Diversity of Antimicrobial Peptides

Category	Peptides	Structure	Source	Reference
A Helix	Aurein1-2	Amidated C-Terminus	Frogs	[31]
	Brevinin 1	-	Frogs	[24]
	Mellitin	Amidated C-Terminus	Bees	[21]
	Maculatins	Amidated C-Terminus	Frogs	[32]
	Buforin II	-	Toad	[33]
	Citropin	Amidated C-Terminus	Frogs	[34]
	BMAP-27,28,34	-	Bovine	[20]
	LL-37	Amidated C-Terminus	Humans	[20]
	Cecropin	Amidated C-Terminus	Insect	[20]
	Magainins	-	Frogs	[20]
ß-Sheets	Protegrins	Cysteine Rich	Pigs	[20]
	Bactenecin	Arginine Rich	Bovine	[20]
	α defensins	Disulfide Bonds	Mammals	[27]
	β defensins	Disulfide Bonds	Mammals	[27]
	θ defensins	Disulfide Bonds	Gorilla	[27]
	Tachyplesins	Arginine Rich	Horse Crab	[28]
	Polyphemus	C-Terminus	Horse Crab	[30]
Extended Coil	PR-39	Arginine Rich	Pigs	[20]
	Indolicidin	Tryptophan C-Terminus	Bovine	[20]
	Histatins	Histidine Rich	Humans	[30]
	Tritrpticin	Arginine Rich	Pigs	[20]

HOW ANTIMICROBIAL PEPTIDES WORK/ACT

Antimicrobial peptides act in two different ways. Following are the mechanisms;

Direct Killing: The Membrane-Targeting Mechanism

The antimicrobial peptides with membrane targeting mechanisms have two types of interactions; receptor and non-receptor mediated interactions.

1. Receptor-Mediated Pathway

The pathway involving bacterially produced antimicrobial peptides represents a critical defense mechanism against microbial threats. These peptides, such as nisin, exhibit remarkable activity even at extremely low concentrations in laboratory conditions, typically in the nanomolar range [35]. Nisin, a well-studied antimicrobial peptide, consists of two primary domains, each serving a distinct function. One domain demonstrates a strong affinity for the lipid II molecule, a crucial component involved in bacterial cell wall synthesis. This interaction occurs within the bacterial

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2. Antimicrobial Peptides with Extended Coil Structure

[36]. The second domain known as the proline-developing [illegible] antimicrobial peptides have an domain, becomes embedded within the bacterial membrane. This embedding facilitates the formation of pores in the membrane, which compromises its integrity and leads to microbial cell death. This dual-domain structure and mechanism of action underscore the effectiveness of antimicrobial peptides in combating bacterial infections [37].

2. Non-Receptor-Mediated Pathway

Antimicrobial peptides of vertebrates and invertebrates target the membrane without combining with the receptors [38]. Antimicrobial peptides demonstrate potent activity *in vitro* at micromolar concentrations against various microbes. Their broad-spectrum effectiveness extends to bacteria, fungi, viruses, and some parasites, making them valuable in combating infections. These peptides' ability to act at low concentrations highlights their potential as safe and effective therapeutic agents. Ongoing research aims to enhance their efficacy and develop novel peptide-based treatments, underscoring the importance of antimicrobial peptides in addressing the challenge of antimicrobial resistance.

Wimley and Hristova [39] reported that these antimicrobial peptides play their role by interacting with the membrane's components. For example, Gram-positive and Gram-negative bacteria's outer surface has teichoic and lipopolysaccharide. Surfaces of both contain net negative charge due to which electrostatic attraction with cationic AMPs is possible.

Guilhelmelli *et al.*, [40] reported that AMPs act differently in the bacterial membrane and animals' membrane. The outer leaflet of the lipid bilayer in bacterial membranes is made of lipids that contain head groups, for example, PG and cardiolipin which are negatively charged. They further reported that in animal membranes, zwitterionic phospholipids is present, for example, sphingomyelin, PC, and cholesterol. Guilhelmelli *et al.*, [40] reported that in animal membranes, head groups containing anionic lipids are present in the inner leaflet.

Andersson *et al.*, [41] found that antimicrobial peptides (AMPs) exhibit a stronger electrostatic attraction to the outer leaflet of bacterial membranes compared to animal membranes. AMPs accumulate on the surface through a series of electrostatic and hydrophobic interactions. Once a critical concentration is reached, they begin to self-assemble on the bacterial membrane.

At this stage, various models define the AMPs action. These models are divided into two categories:

- Transmembrane pore which are further divided into two categories: barrel-stave pore and toroidal pore models
- Carpet model (Non-pore models)

Barrel-Stave Pore Model

In the barrel stave model, Kumar *et al.*, [38] reported that the AMPs are oriented parallel to the membrane at the start and then inserted in a perpendicular direction in the lipid bilayer. Wimley [42] reported that it gives rise to lateral peptide-peptide interactions. Ramamoorthy *et al.*, [43] reported another example that is pardaxin. Brogden [44] reported that protegrins also exhibit barrel stave channels.

Toroidal Pore Model

In the toroidal pore model, Wimley [42] reported that the peptides perpendicularly inserted in the lipid bilayer, but no any specific peptide-peptide interactions exist.

However, the peptides cause a local curvature of the lipid bilayer with pores produced in part by peptides and in part by the phospholipid head group. The "toroidal pore" is a dynamic and transient lipid-peptide supramolecule. The distinctive characteristic of this model with the barrel-stave pore model is the net arrangement of the bilayer. In the barrel-stave pore model, the arrangement of the lipids either hydrophilic or hydrophobic is maintained but not maintained in the toroidal pore model, due to which alternative surfaces for the interaction with the lipid's head and tail group arises. As the toroidal pore is transient so, after the disintegration, these peptides move towards the inner cytoplasmic leaflet, so after entering the cytoplasm, they strongly target the components within the cell . The toroidal pore has a discrete size. It exhibits ion selectivity [45].

Lee *et al.*, [29] reported that AMPs such as magainin 2 and lacticin Q exhibit this model activity.

Both toroidal pore and barrel which are pore forming models cause membrane depolarization and lead to cell death.

Carpet Model

Lee *et al.*, [29] reported the model in which antimicrobial peptides act without the formation of specific pores. Wimley and Hristova [39] reported that antimicrobial peptides oriented parallel to the lipid bilayer. They cover the surface of the membrane which looks like a "carpet" when they reached threshold concentration. This is disapproving of interactions on the surface of the membrane. As a result, membrane integrity is lost. The same happened in the detergent model, in which the membrane disintegrates at last by forming micelles. The peptide doesn't have to put into the hydrophobic core for the formation of trans-membrane channels. The membrane-bound peptide monomers' relations to one another are not shown in the carpet model.

Direct Killing: Mechanisms of Action without Targeting Membrane

The non-membrane targeting antimicrobial peptides

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classified into two groups

- Bacterial cell wall target
- Intracellular targets

1. Bacterial Cell Wall Target

Malanovic and Lohner [46] reported that, like antibiotics (which were used conventionally), AMPs obstruct the synthesis of cell wall. These antibiotics attach to particular proteins which involved in synthesis of cell wall's components. On the other hand, AMPs show interaction with a variety of precursor molecules used to synthesize the cell wall. Highly conserved lipid II is one of the molecules which is a major target. Münch and Sahl [47] reported that AMPs like defensins bind with negatively charged pyrophosphate sugar precursor of the lipid II molecule.

Münch and Sahl [47] reported that AMPs, for example, defensin 3, put heads together with the bactericidal activity by the selective binding with lipid II molecule.https://www.ncbi.nlm.nih.gov/core/lw/2.0/html/tileshop_pmc/tileshop_pmc_inline.html?title=Click on image to zoom&p=PMC3&id=5871973_biomolecules-08-00004-g006.jpg

2. Intracellular Targets

It was believed that AMPs have no intracellular targets. Currently it is recognized that many AMPs have intracellular targets because these AMPs lead to bacterial death without causing permeabilization of the membrane at their minimum concentration.

In this mechanism, the AMPs show interaction with the membrane of cytoplasm and then they store intracellularly, where they disrupt cellular processes like inhibition of nucleic acid or protein synthesis and block the protein as well as enzymatic activity.

3. Immune Modulation Mechanism of Action

Hilchie *et al.*, [48] Reported that antimicrobial peptides are involved in the direct killing of microbes and activating the immune cells. These cells, as a result, increase microbial killing and control inflammation.

Antimicrobial peptides are produced from certain immune cells like neutrophils and macrophages, so they are considered the first molecules interacting with the attacking microbes [41]. The examples of these antimicrobial peptides are LL-37 and β defensins that induce activation of immune cells by chemoattraction like mast cells, microglia, and monocyte. In addition, the activation of another group of immune cells (leukocytes) is also reported.

EVOLUTIONARY SIGNIFICANCE OF AMPs

AMPs are evolutionarily conserved components of innate immunity of invertebrates against pathogens. Various AMPs in invertebrates showed significant diversity in their amino acid structure, sequence, and biological activity. AMP genes have evolved rapidly, probably due to a co-evolutionary arms race among host and pathogens and allowing organisms to survive in different microbial environments. Even though AMPs have been used extensively for most of the time, they have retained their antimicrobial activity during evolution. Therefore, the sequence diversity of AMPs probably indicates organisms' ability to adapt to live in various microbial-infested environments[49].

The amino acid composition of natural AMPs plays a crucial role in their structure, function, and evolution. In higher organisms, the preference of arginines in AMPs is supposed to have performed a key part in the evolution of adaptive immune systems and provided a regulatory and integrative role to natural AMPs in host immune responses. Likewise, it appears that different natural AMP structures are directly influenced by the composition of amino acid [50].

Survival of host can significantly be affected during infection due to few evolutionary variations in composition of AMPs amino acid. In *D. melanogaster*, alleles of Diptericin A have pathogen-specific action against *Providencia rettgeri* and not show against other bacteria, including *P. rettgeri* cousins. To specifically change resistance to *P. rettgeri,* Diptericin A just to have a single polymorphic amino acid change. These findings indicate previously unrecognized AMP activity specificity [51]. Loss of gene, duplication of exon and gene and exon shuffling have all extensively occurred in AMPs. In insects, AMPs reveal the existence or lack of a gene family in general as well as lineage-specificity in copy counts within a gene family. For example, the Drosomycin family of AMPs is present in certain *Drosophila,* and coleoptericin belong to order Coleoptera [52]. Evolution of the pathogens might be restricted due to the release of multiple AMPs simultaneously during an immune response. In insects, AMPs as immune proteins evolved faster than non-immune proteins. In crustaceans, Shrimps produce AMPs in response to an infection. Shrimps have evolved and use a variety of AMPs to prevent being exposed to various harmful microbes[49].

AMPs have not lost their ability to kill the microbes totally, despite their long history of co-evolution. Microbes also have not learned to evade the lethal hit of AMPs. Therefore, AMPs can provide a significant advancement and form the foundation for a new group of antibiotics[53].

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CONCLUSIONS

It is challenging to treat biofilm-linked persistent and chronic infections with traditional antibiotics. AMPs are novel therapeutic agents that are used to treat biofilm-associated diseases. It is not easy for microbes to develop resistance against AMPs compared to conventional antibiotics. AMPs have a variety of structures and kill microbes in various ways, including interaction with biological membranes and activity at specific extracellular and intracellular targets. However, function of AMPs to control different infections is still hampered by various problems, including poor pecularity, high toxicity to animal cells, deficiency of a rational design guidelines and high expenses of production.

Nevertheless, AMPs are attractive candidates for translational application due to their potency and diversity, and many are already in clinical trials. Additionally, the research could explain both sides of a co-evolutionary arms race among host and pathogen by recognizing the alteration in microbial genes that can cause resistance to AMPs. However, to use AMPs effectively and sustainably, it will be essential to understand their evolution and natural biology to reduce the danger of collateral harm and avoid the resistance crisis that traditional antibiotics are now facing.

Authors Contribution

Conceptualization: NM

Writing-review and editing: NM, WA, AU, IL, ZN

All authors have read and agreed to the published version of the manuscript.

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The authors declare no conflict of interest.

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Assessing the Impact of Xenobiotic (Bisphenol A) on Blood Physiology and Biochemical Alterations Using *Labeo rohita* Fish as a Model Organism

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ABSTRACT

Bisphenol A (BPA) is an emerging pollutant that is extensively used in the manufacturing of various industrial products and is associated with adverse effects on both human and wildlife health. **Objective:** Present study aimed to evaluate the effects of bisphenol A on hematobiochemical biomarkers in freshwater *Labeo rohita*. **Methods:** For the purpose of this investigation, healthy fish were divided into four groups (A-D). Group A was treated as a BPA-free control group, while Group B, Group C, and Group D were exposed to various doses of BPA such as 400, 800, and 1600 µg/L, respectively for 21 days. **Results:** BPA-exposed fish showed different physical and behavioral abnormalities in dose-dependent ways. Results indicated significant increase in the concentrations of various hematobiochemical parameters, such as WBCs, MCHC, RDW, RDW-SD, platelets, neutrophils, triglycerides, cholesterol, ALT, AST, blood glucose, urea, T_3, TSH and creatinine, while HGB, RBCs, HCT, MCV, MCH, PDW, lymphocytes, HDL, LDL, VLDL, total protein, globulin, albumin and T_4 concentrations were decreased. **Conclusions:** The current study concluded that bisphenol A causes deleterious effects by disrupting physiological and hematobiochemical parameters alteration in exposed fish.

INTRODUCTION

The global focus on monitoring the impacts of pollutants, including agricultural and industrial wastes, has grown substantially in recent years [1]. Several contaminants from various industries, including textile mills, pharmaceuticals, paper industry, chemical manufacturing, and plastic industry, add many pollutants, such as pesticides, flame retardants, plasticizers, and heavy metals, to water bodies. A number of these chemicals are endocrine disrupters, such as bisphenols [2]. The release of these chemicals into aquatic ecosystems poses a risk to aquatic organisms, especially fish. Thus, fish are particularly susceptible to these contaminants because they are often exposed to numerous waterborne pollutants throughout their lives, especially during important stages of development [3, 4]. Human activities are increasing the level of these toxic substances in our rivers and lakes, posing a threat to aquatic life [5]. Bisphenol A (BPA) is an organic, colorless, synthetic chemical that has a global application. BPA, an organic compound with two phenol functional groups, is a key precursor in many plastics, including epoxy resins and polycarbonate polymers [6]. BPA is widely utilized in many sectors, including car lenses, compact discs, construction materials, water pipes, electrical components, dyes, protective coatings, paints, plastic bottles and food containers [7]. With a global production of 3 billion kg of BPA per year, around 100 tons of the chemical are released into the environment annually [8]. Government agencies in the United States and Europe have classified bisphenol A as an endocrine disruptor and a "moderately toxic" chemical [9]. Due to its detrimental effects on fish and other aquatic animals, it has recently seen extensive use in aquatic toxicity research. Humans

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and ecosystems, especially aquatic environments, are continually exposed to BPA via discharges from the petrochemical sector, municipal sewage and landfill effluent. The paper recycling and packaging industries release wastewater containing high quantities of BPA, which can also contaminate aquatic ecosystems [10]. Fish are among aquatic animals that are vulnerable to high levels of BPA exposure because surface waters absorb it. BPA at elevated concentrations can harm aquatic life over time, hence the highest quantity recorded at a place is the optimum exposure limit [11]. BPA concentrations in water ranged from 1 to 1000 µg/L [12]. The presence of BPA in aquatic systems leads to significant health issues for aquatic animals, and its negative impact on aquatic ecology has raised considerable alarm [13]. Previous studies reported that BPA causes alteration in different hematobiochemical parameters such as hemoglobin, white blood cells, MPV, hematocrit, RBCs and serum proteins in yellow perch, Korean rockfish and albino rats [14-16]. Fish, as members of the food chain, are sensitive to even small quantities of xenobiotics like Bisphenol A (BPA) and bioaccumulative harmful chemicals. So, they are used as indicators to detect pollutants in water bodies [17]. The carp *Labeo rohita* was used in the present experimental trial. Rohu *(Labeo rohita),* a member of the *Cyprinidae* family, is an economically significant and cultivable fish found in freshwater lakes and rivers in Asia, especially in Pakistan, India, and Bangladesh [18]. Serum biochemical indices and hematological alterations are important biomarkers for detecting physiological changes and assessing toxicity. With these alterations, the fish health status and the toxicological indicators for organisms may be better understood [19].

Therefore, we conducted the current study to examine the effects of graded concentrations of BPA on *Labeo rohita* using hematological and biochemical biomarkers.

METHODS

Bisphenol A was obtained from MACLIN, China. We dissolved an adequate amount of BPA in ethanol to prepare the stock solution for use in the experiments following the protocol described by Kwak *et al* [20]. Rohu (*Labeo rohita*) with an average weight of 32.40 ± 4.53 g and length of 18.40 ± 0.28 cm were purchased from Balloki fish farm, Pakistan and transported to fish lab of the University of Okara. No mortality was found during transportation. Fish were acclimatized in a glass aquarium having dimensions of 40" W × 30" H × 35" L with 100 liters of water for one week and fed once a day. 90% of the water was changed after one day during the whole experiment. After acclimatization, four groups were made (A-D). Group A was treated as a BPA-free control group, while groups B, C, and D were exposed to various concentrations of BPA such as 400, 800, and 1600 µg/L, respectively for 21 days. Temperature, pH, hardness of water and dissolved oxygen were maintained. Bisphenol A concentrations were decided based on earlier research [21, 22]. At day 21, fish (n = 12 for each group) were taken from the control and treated groups. Blood was collected through a BD syringe from the abdominal vein and placed in EDTA and gel vials for hematology and serology. This examination was carried out in a laboratory using specific experimental techniques. Fish were anesthetized by using clove oil. All hematological parameters were analyzed by a hematological analyzer. The number of RBCs and WBCs were counted using a hemocytometer. The amount of hemoglobin was measured using a UV spectrophotometer set to 540 nm and a cyanmethemoglobin diagnostic reagent kit. The measurement of HCT was performed using the microhematocrit technique. Erythrocyte indices, viz., MCHC, MCH, and MCV, were calculated from RBC, HCT, and HGB values following the method described by Ramesh *et al* [23]. The lipid profile (cholesterol, LDL, HDL, VLDL, triglyceride), liver enzymes (ALT and AST), and some other biochemical parameters (total proteins, globulin, albumin, urea creatinine, T3, T4, TSH and blood glucose) were analyzed using a chemistry analyzer using the method of Ghaffar *et al* [24]. For the assessment of protein and glucose levels, blood samples were centrifuged at 9392 × g, for 20 minutes at 4 °C to separate the blood plasma. Using a diagnostic reagent kit, blood glucose level was determined as described by Abraham and Gerarge [25]. Blood proteins were assessed by using the method of Kumar *et al* using bovine serum albumin as standard [26]. ALT and AST levels were determined by using a special kit (Spectrum AST – kit, Egypt) [27]. Creatinine and urea were estimated using kits supplied by Biomerieux (France). Using standard kits, serum cholesterol, HDL, and triglyceride levels were measured following the method of Hassan *et al* [28]. VLDL and LDL levels were calculated according to the standard formula described by Zaahkouk, *et al* [29]. T_3, T_4 and TSH values were assessed by following the method of Hadie *et al.*, using standard kits [30]. Statistical analysis was done by applying one-way ANOVA on GraphPad Prism (V 9.5.1) software at $p<0.05$ level of significance. GraphPad Prism (Version 9.5.1) was also used for graphical representations.

RESULTS

In chemical-free group A, no physical and behavioral abnormalities and mortality were observed. Bisphenol-treated low to high-dose groups showed different physical and behavioral responses, from mild to severe. Physical and behavioral responses include loss of equilibrium, faintness, black spots on the whole body surface (changed skin color), operculum movement, fins tremor, gulping of air, mucosa secretion from mouth and gills, eyes bulging, jerking and laying on one side during uneven swimming.

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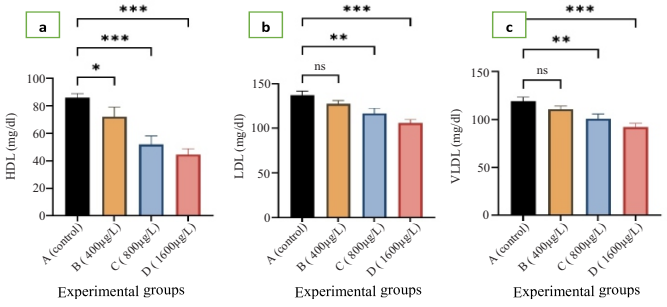
Group C (800 g/L) and D (1600 g/L) BPA-exposed fish showed more severe physical and behavioral signs. In the chemical-free control group, all hematological parameters, including HGB, WBCs, HCT, RBCs, RDW-SD, PCT, neutrophils, lymphocytes, monocytes, MCHC, MCV, RDW, platelets, MCH, MPV, PDW, and eosinocytes were observed as normal. As compared with the BPA-free group, the values of MCHC, WBCs, RDW, RDW-SD, platelets and neutrophils were significantly increased with increasing dose concentration, while HGB, RBCs, HCT, MCV, MCH, PDW and lymphocytes were significantly decreased as compared with chemical-free group A. Other parameters (MPV, PCT, monocytes, and eosinocytes) were not significantly affected by BPA exposure, as shown in table 1.

Table 1: Showing the Hematological Profile Of *Labeo Rohita* Exposed To Various Doses Of Bpa.

Variables	A (Control)	B (400µg/L)	C (800µg/L)	D (1600µg/L)
HGB (g/dl)	5.63 ± 0.35	4.96 ± 0.40	3.16 ± 0.50*	2.36 ± 0.35*
WBC (x10^3/µL)	15.27 ± 2.21	20.83 ± 2.82*	25.27 ± 4.20*	31.80 ± 2.75*
RBC (x10^6/ µL)	2.03 ± 0.14	1.66 ± 0.11	1.29 ± 0.16*	0.71 ± 0.13*
HCT (%)	14.87 ± 0.35	12.13 ± 0.75*	9.85 ± 1.03*	8.61 ± 0.52*
MCV (FL)	142.02 ± 3.48	129.6 ± 4.38	102.9 ± 7.46*	95.57 ± 8.27*
MCH (pg)	47.07 ± 2.12	42.03 ± 2.63	35.30 ± 2.60*	23.53 ± 3.05*
MCHC (g/dl)	30.87 ± 2.13	34.70 ± 1.51	38.10 ± 2.68*	43.67 ± 2.15*
RDW (%)	23.13 ± 3.01	28.03 ± 1.94	36.10 ± 2.45*	42.87 ± 2.85*
RDW-SD (%)	31.90 ± 1.99	36.80 ± 3.03*	45.33 ± 3.11*	49.93 ± 2.30*
PLT (x10^3/µL)	24.35 ± 3.03	30.63 ± 2.84*	39.60 ± 2.09*	45.23 ± 3.27*
MPV (FL)	5.46 ± 0.40	5.03 ± 0.30	4.56 ± 0.25	4.20 ± 0.45
PDW (%)	15.30 ± 0.36	14.40 ± 0.45	12.93 ± 0.41*	12.17 ± 0.30*
PCT (%)	0.33 ± 0.03	0.19 ± 0.05	0.08 ± 0.02	0.06 ± 0.02
Neutrophils (%)	70.70 ± 3.05	87.07 ± 3.64	111.10 ± 4.54*	164.10 ± 4.59*
Lymphocytes (%)	27.00 ± 3.60	23.97 ± 1.15	21.57 ± 1.80*	16.15 ± 1.94*
Monocytes (%)	1.97 ± 0.65	1.70 ± 0.08	1.26 ± 0.08	0.77 ± 0.14
Eosinocytes (%)	1.90 ± 0.07	1.83 ± 0.06	1.39 ± 0.11	1.20 ± 0.08

The values are shown as mean ± SD. Asterisk (*) bearing values show significant differences ($p< 0.05$) as compared to the BPA-free group A (control). The statistical values of biochemical parameters including cholesterol, triglycerides, VLDL, HDL and LDL were presented in figure 1. A significant increase was observed in triglycerides, and cholesterol while a decrease in HDL, LDL and VLDL levels (Figure 1).



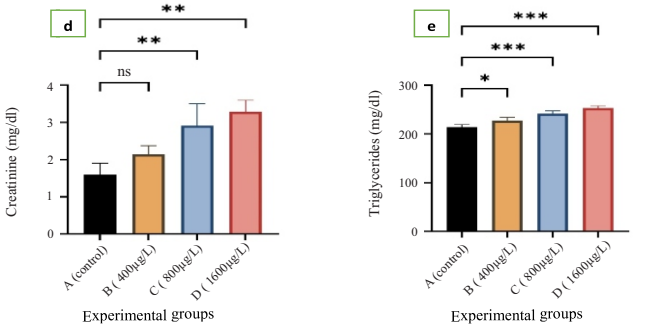


Figure 1: Change in (a) HDL, (b) LDL, (c) VLDL, (d) creatinine and (e) triglycerides in BPA-exposed groups as compared with control group. The data are shown as mean ± SD. Asterisk was shown different significant levels ($p\leq0.05$).

Results of ALT, AST, total protein, albumin, globulin and blood glucose are presented in figure 2. Results showed significant elevation in ALT, AST and blood glucose levels while a decline in total protein, globulin and albumin levels. In figure 2, the change in blood glucose level, ALT, AST, albumin, globulin, and serum total proteins in BPA-exposed groups compared to the control group is presented as mean ± SD, with asterisks indicating significant differences ($p\leq0.05$).

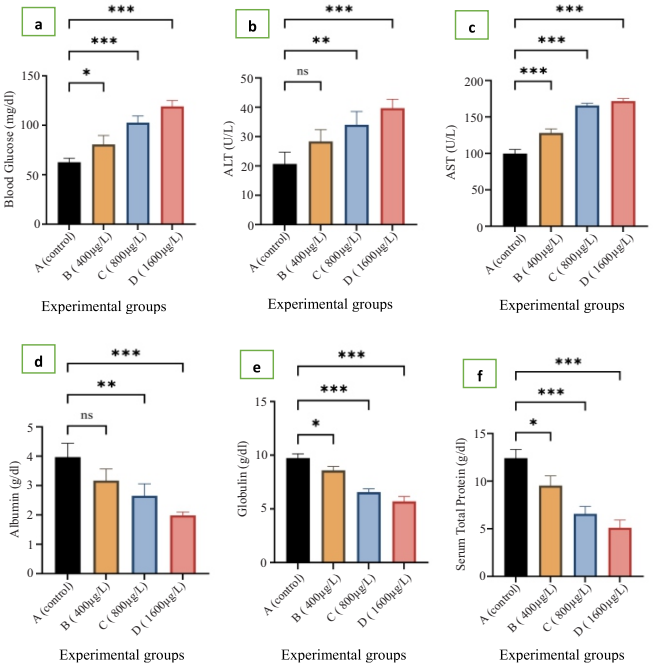


Figure 2: Change in (a) blood glucose level, (b) ALT, (c) AST, (d) albumin, (e) globulin and (f) serum total proteins in BPA-exposed groups as compared with control group. The data are shown as mean ± SD. Asterisk was shown different significant levels ($p\leq0.05$)

T_3, T_4, TSH, urea, creatinine and blood urea nitrogen were presented in figure 3. The result of the one-way ANOVA statistic showed a significant ($p\leq0.05$) increase in, urea, T_3, TSH, blood urea nitrogen, and creatinine levels in treated groups as compared with the chemical-free group A

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(control). A dose-dependent decrease in T4 level was observed

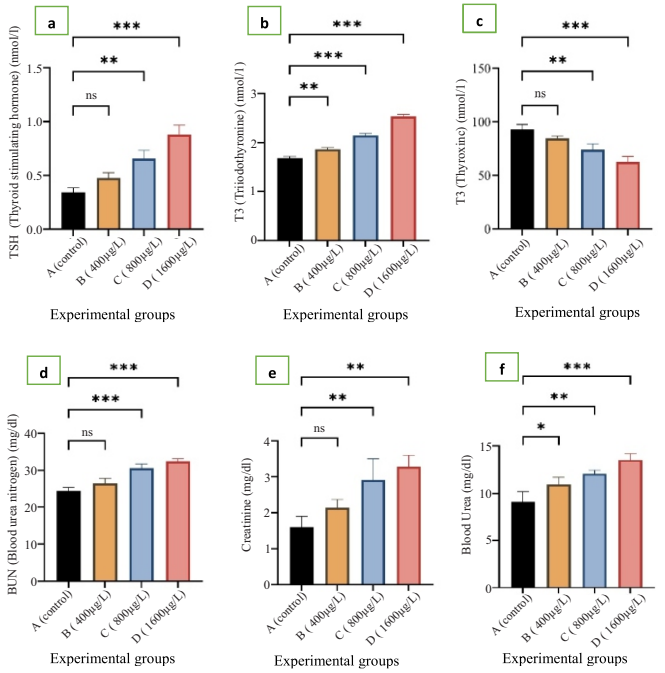

Figure 3: Change in (a) TSH level, (b) T_3, (c) T_4, (d) blood urea nitrogen, (e) creatinine and (f) urea in BPA-exposed groups as compared with control group.The data are shown as mean ± SD. Asterisk was shown different significant levels ($p \leq 0.05$).

DISCUSSION

In toxicological studies, exposing organisms to specific dosages at different acute or sublethal concentrations helps better understand the hazardous levels of chemicals, including bisphenol A [31]. The extent to which environmental contaminants harm aquatic life is crucial [32]. Pollutants may harm fish fauna via physiological, biochemical and histological alterations [33]. Over the last few decades, there has been a worldwide rise in efforts to monitor and record the impacts of environmental toxins such as herbicides, pesticides, and industrial effluents [24]. Many pollutants from different kinds of sources readily and instantly enter water bodies. Therefore, aquatic organisms are more vulnerable to damage than terrestrial animals [34]. Many of these synthetic substances, such as bisphenol A, are endocrine disruptors that damage fish tissues [35]. Therefore, to minimize the public health concerns associated with bisphenol A, it is important to conduct ongoing monitoring and evaluation of its toxicological impacts at low levels. The current study aimed to evaluate the sublethal toxicity of bisphenol A in *L. rohita* concerning clinical, behavioral, and hematobiochemical alterations in exposed fish. In present study, results showed that fish exposed to low levels of BPA developed a variety of physical and behavioral symptoms such as loss of balance, faintness, black spots on the whole-body surface (changed skin color), operculum movement, trembling of fins, gulping of air, increased production of mucus from mouth and gills, eyes bulging, and jerking of body and laying on one side during irregular swimming. Previous research revealed that the same findings were seen in *Cirrhinus mrigala* [36], *Ctenopharyngodon* [37], bighead carp [21], zebrafish [38], and *Channa punctatus* [39]. The same observations were also reported by Cervantes *et al.*, and Namratha *et al.*, in vertebrates [40, 41]. Blood serves as a pathophysiological indicator of the health status of an organism [42]. Therefore, hematobiochemical profiles are vital indicators of health, that are frequently employed to understand and diagnose the harmful effects of external environmental stressors and harmful chemicals on fish morphology and physiology [43-45]. Some hematological parameters, like hemoglobin level, hematocrit, white blood cell count, mean corpuscular hemoglobin concentration, mean corpuscular volume and red blood cell count, can be used to find out which organs in fish are most affected by metals, pesticides, and endocrine disruptors [46, 47]. Hematological analysis revealed that in the BPA-treated groups, the concentrations of platelets, neutrophils, White Blood Cells (WBCs), MCHC, RDW, RDW-SD, and RDW significantly increased with increasing dose concentration, compared to the BPA-free control group. Similar findings were reported by Afzal *et al.*, and Asenuga *et al* [48, 49]. Andujar et al., and Senthil *et al.*,reported that bisphenol [33, 50]. A causes reductions in hemoglobin, lymphocytes, PCV, RBCs, and monocytes . Higher levels of WBCs, leucocytes, neutrophils, cholesterol, triglycerides, urea, creatinine, blood glucose, ALT, and AST were observed by exposure to BPA [15, 48, 51]. Various kinds of stressors in animals can increase white blood cells, and MCHC due to immune system activation and inflammation [15]. A rise in WBC level is associated with direct activation of immunological responses and tissue injury exposed to BPA. The elevation in the count of WBCs in treated fish is indicative of a state of toxemia that indicates impairment of the defense system [52]. Increases in WBC concentration may lead to higher numbers of neutrophils. HCT, MCH, RBC, HGB, lymphocytes, MCV and PDW levels were reduced significantly in BPA-treated fish as compared to unexposed fish, leading to anemia in *L. rohita*. A decrease in hematopoiesis and an increase in erythrocyte breakdown in hemopoietic organs like the liver and kidneys can lead to anemia. Similar results are reported by Andujar *et al.*, Abid *et al.*, Hassan *et al.*,and Yaghoobi *et al.*, [33, 51, 53]. One possible explanation for the reduced HGB concentration might be the detrimental impact of BPA on HGB formation. BPA may inhibit HGB synthesis by interfering with the actions of enzymes necessary for HGB formation. Erythrocytes are crucial in assessing the health status of fish in the presence of toxic substances. Changes in the red blood cells of fish are exceptionally reliable indicators of the accumulation of hazardous substances in various

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organs of the fish. RBCs may exhibit responses to certain environmental stressors [54]. Furthermore, decreased levels of HCT, RBC, and HGB suggest the initiation of a defensive reaction in response to exposure to bisphenol A [55]. A low level of red blood cells can lead to oxidative damage in the body, resulting in membrane impairment and eventual cell death [56, 57]. The excessive accumulation of bisphenol A, which results in internal destruction, hemorrhage, and decreased erythrocyte formation, maybe the cause of the observed change in hematological parameters [58]. The decrease in MCV was caused by changes in RBC volume associated with exosmosis and a rise in the concentration of electrolytes inside RBCs following exposure to BPA [59]. Since RBC and HGB produce MCHC and MCH, changes in RGB and HGB concentrations also affect the levels of MCHC and MCH [60]. Highly significant reductions in MCV and MCH levels indicate hypochromic microcytic anemia [61]. This study demonstrated that BPA exposure had no significant effects on mean platelet volume, procalcitonin, monocytes, or eosinocytes. Therefore, work carried out by Asenuga *et al.,* supported present study [49]. Assessing biochemical indicators, such as lipid profile, glucose, and protein levels, is commonly employed to monitor the health of fish in aquatic environments and to understand the physiological responses shown by aquatic species under stress [58]. Aquatic pollutants have the potential to alter the function of enzymes in fish serum, which can serve as an indicator of fish health [62]. Biochemical examination revealed that the current study work showed a substantial drop in HDL, LDL, and VLDL levels and a rise in triglycerides and cholesterol. Similar findings were reported by Ozaydin *et al.*,and Pinafo *et al.,* [63, 64]. A rise in blood total lipids and cholesterol may cause catecholamines, which enhance lipolysis and fatty acid production. Due to an increase in total blood cholesterol, the liver bile duct may become blocked, reducing its secretion to the duodenum and causing cholestasis. ALT, AST, and blood glucose levels were increased by increasing the BPA dose. The present results are supported by Abid and Hassan [51]. The higher levels of ALT (alanine aminotransferase) and AST (aspartate aminotransferase) may be due to the oxidative stress caused by BPA exposure [65]. BPA impacts glucose metabolism via oxidative damage, inflammation, insulin resistance, and β cell malfunction [66]. A significant rise in urea, creatinine, BUN, T_3 and TSH levels while a significant decrease in albumin, total protein, globulin, and T_4 levels, were observed in the BPA-treated groups as compared with the BPA-free group. Similar results have been reported on other fish and rats [67-70]. High levels of urea and creatinine may be due to renal tubule damage, as shown in histological alterations, which indicated that bisphenol A affects muscle and purine metabolism [71]. Damage to the glomerulus, a decrease in glucose metabolism, and an increase in muscle tissue catabolism may all contribute to an elevated blood creatinine concentration [72]. BPA can disrupt the production of thyroid hormones by causing changes in blood protein transporters or by increasing the breakdown of thyroid hormones [70]. Administration of BPA may result in hypothyroidism through the processes of thyroid dyshormonogenesis and dysgenesis [73]. Abdel *et al.,* and Qiu *et al.,* reported that BPA causes a decrease in globulin, total proteins, and albumin in *Cyprinus carpio* and *Oreochromis niloticus* [68, 74]. The liver and kidneys have significant functions in protein metabolism. The liver and kidneys have the role of synthesizing, breaking down, and excreting blood proteins [75]. Therefore, the reduction of total protein, globulin, and albumin in the BPA-treated fish may be a result of liver and kidney damage, as shown by histological changes. Albumins are a group of globular proteins that play an important role in antioxidation, immune function and homeostasis [76, 77]. A reduction in albumin production is linked with inflammation [78].

CONCLUSIONS

Results of the current investigation concluded that bisphenol A causes harmful effects on the hematological and biochemical parameters of *Labeo rohita*. Exposure of *Labeo rohita* to BPA at 800 µg/L and 1600 µg/L induces alterations in hemoglobin, RBC, hematocrit, MCV, MCHC, lipid profile, kidney and liver functioning. Moreover, BPA altered thyroid functioning by altering T_3, T_4 and TSH levels in a dose-dependent manner. According to the findings, BPA is undoubtedly toxic to aquatic life. To lessen their harmful impacts, there is an urgent need to find other eco-friendly chemicals with higher degradation abilities and decrease BPA use.

Authors Contribution

Conceptualization: KS
Methodology: SA
Formal analysis: HA
Writing, review and editing: SA, HA, KS

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Development and Validation of Loop-Mediated Isothermal Amplification (Lamp) Field Assay for the Detection of *Brucella abortus*

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ABSTRACT

Currently, a number of techniques are available for detection of *Brucella abortus (B. abortus)* but these techniques are costly and specialized equipment are needed. Therefore, the development of a rapid, accurate, sensitive, and cost effective technique for identification of *Brucella* species is of high priority. **Objective:** The current research study was designed to detect *Brucella* species more rapidly. The current study area was conducted in district Lodhran, Punjab, Pakistan. **Methods:** A total 100 blood samples (50 cattle and 50 buffaloes) were collected. Serum samples were screened against *B. abortus* antibodies using Rose Bengal plate test (RBPT). The specific gene was designed by using NCBI website and whole genome of *Brucella* species. The primers were designed from Gene accession number 20404. Following primers were designed F3, B3, FIP, BIP, LF, LB, B4, and B5. The LAMP technique for BSCP31 gene was developed by using many concentrations of components and conditions. **Results:** The development and validation of LAMP assay for detection of *B. abortus* from bovine blood in the present study proved helpful in early detection of said pathogen in animal and humans. **Conclusions:** This study will be helpful in prevention and control of animal and human brucellosis in Pakistan.

INTRODUCTION

Brucellosis is a zoonotic ancient disease, which was reported in Egypt. The causative agent of Brucellosis is Brucella species. The Brucella species are Gram-negative cocco-bacilli bacteria have worldwide distribution [1-3]. David Bruce in 1887 isolated Brucella melitensis in British soldier's spleen that died due to Malta fever which is common in the location of Malta station. After Malta fever discovery, 20 years later it was considered as a vector-borne disease, accidently Themistocles Zammit revealed that Malta fever is a zoonotic disease in 1905, when he isolated *B. melitensis* in milk of goat. It was the time when considered that the goats were not the source of disease because the goats were not ill from inoculation of Brucella cultures. In epidemiology discovered new thing that healthy goats were the carriers of brucellosis [4, 5]. Brucellosis outcomes are very important in economic point of view because of reproductive impairment in cattle cause stillbirth, abortion, infertility and neonatal mortality which can affect country's economics [6]. Recently, there are ten species but each specie has characteristics according to their host, *B. melitenesis* (sheep and goats), *B. pinnipedialis* (pinnipeds), *B. neotomae*, *B. microti* (rodents-Microtusarvalis), *B. ceti* (cetacean), *B. canis* (dogs), *B. inopinata* (Breast infections in females), *B. abortus* (cattle), *B. ovis* (rams) and *B. suis* (pigs) [7, 8]. Brucellosis causes febrile disease having the broad spectrum indications

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which were fatal in many cases in humans [9, 2]. Humans are usually infected through the consumption of contaminated food of infected animals, especially unpasteurized milk [10]. Brucellosis species infection has been reported in more than 170 countries every year and about 1/5- 1/6 of world population, the expected loss due to brucellosis in many billions dollars every year [11]. Specific diagnosis normally requires confirmation by isolating the causative agent from clinical samples. Methods of direct Brucella-DNA detection and validation may minimize these drawbacks. Previous researches have cleared the benefits of direct PCR method for Brucella detection [12-14]. The main problem of serological method such as (RBPT) Rose Bengal Plate Test and (STAT) Standard Tube Agglutination Test which are not specific for antibodies test and can affect other Gram-negative microorganisms for example *Yersinia enterocolitica.* At initial stages of infection, antibodies growing period such tests are not of great value in detecting brucellosis. Now a days PCR technique is used for detecting the disease, such commonly used techniques are sensitive and easy to use but require special equipment and skills for detecting the brucellosis, and also require special post protocol for clarification. Anyhow, these above mentioned tests are time consuming, require skilled approach and are not straight forward, whereas, DNA analyses based studies are widely used for diagnosing brucellosis. As these techniques are costly and specialized equipment are needed, so they are not appropriate. Whereas, the development of rapid, accurate, sensitive, cost effective and identification of Brucellosis species is of high priority. All the bacteriological techniques used in past require many expertise and a lot of time to isolate the organism because the growth period of organism requires 2-3 days. Other drawbacks of these techniques are low sensitivity when we load the low amount of the sample pathogen and because brucellosis can spread from animals to human rapidly, so class 3 bio safety cabinets are required to handle its causative agent [15]. LAMP technique is a novel gene amplification which requires 4-6 primers. The target identifies specific regions at constant temperature (60-65ºC) in less than an hour without any special reagent [16]. Accurate development and validation technique for brucellosis is required for achieving accurate and reliable results. LAMP technique is one of such techniques, having all facilities and qualities for diagnosing of brucellosis. LAMP technique is more specific than PCR and more valuable tool for field level assay [17]. The anti- *B. abortus* antibody was reported because of the presence of brucella in semen [18].

The current research study was designed to detect *Brucella* species more rapidly. The current study area was conducted in district Lodhran, Punjab, Pakistan.

METHODS

Sample Collection and Serology

The current study was conducted in District Lodhran, Punjab, Pakistan. A total of 100 blood samples (50 cattle and 50 buffaloes) were collected. Serum samples were separated after centrifugation at 3000 rpm for 5 minutes. These serum samples were screened for B. abortus antibodies using Rose Bengal plate test RBPT [19].

Primer Designing

The specific gene was taken by using NCBI website and whole genome of Brucella species. The primers were designed from Gene accession number 20404. Following primers were designed F3, B3, FIP, BIP, LF, LB, B4, and B5 as shown in figure 1.

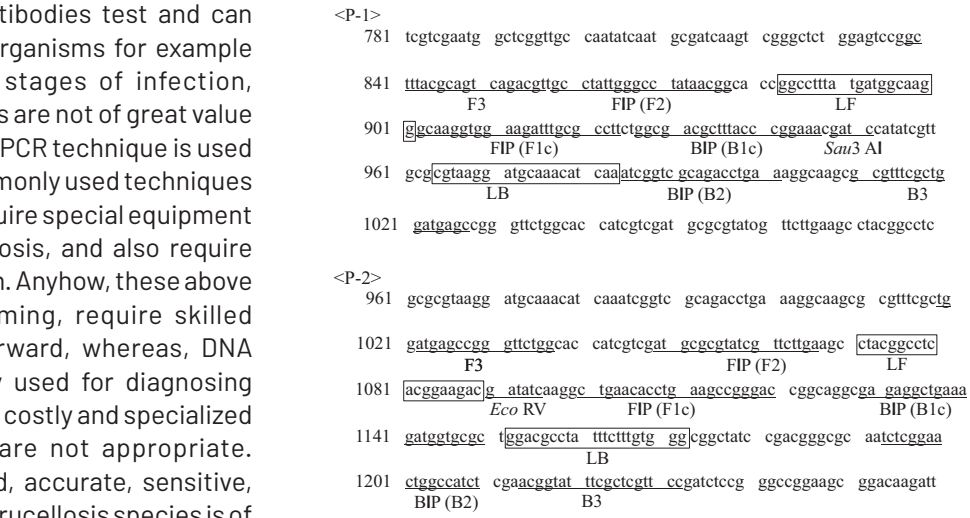

Figure 1: Primer Design for LAMP to Detect *Brucella* DNA. Nucleotide Sequence of BCSP31 Gene (GenBank accession no M20404), Used to Design LAMP Primers. Underlining Indicates the Positions of Targeting Sequences

Bacterial DNA Extraction

Extraction of DNA was done using the PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instruction.

DNA Extraction from Whole Blood

Extraction of DNA was performed by organic DNA extraction method involving PCI [20]. The extraction of all 50 samples was performed by the following protocol.

Loop Mediated Isothermal Amplification Assay

The LAMP technique for BSCP31 gene was developed by using various concentrations of components and conditions. The constant temperature 60-68ºC was provided.

LAMP Products Detection

The amplified outcomes of LAMP were observed through naked eye. The color of product changed from violet to sky blue in positive result with no change in negative result. The result was observed by agarose gel documentation apparatus.

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LAMP Assay

The loop-mediated isothermal amplification assay was processed in 25µl of reaction mixture consisting 40 $pmol^{-1}$ of forward inner primer(1µl) and 40 $pmol^{-1}$ of BIP (1µl), 5 pmol l^{-1} of F3(0.5µl) and 5 pmol l^{-1} of B3(0.5µl), 20 $pmol^{-1}$ of LF(1µl) and 20 $pmol^{-1}$ of LB(1µl), betain (2.5µl), 20 mmol l^{-1} Tris-HCl, 10 mmol l^{-1} KCl, 10 mmol l^{-1} (NH4)2SO4, 8 mmol l^{-1} MgSO4 (1.5µl), 0.1% Tween 20,1.4 mmol l^{-1} each deoxynucleoside triphosphates, 8 units of Bst DNA polymerase (2µl) isothermal lamp buffer (2.5µl) (New EnglandBiolabs, Berverly, MA, USA), 2µl of template DNA and 8.5µl distilled water. The reaction mixture was incubated at 63ºC for 35 min. Heated to 95ºC for 2 minutes to end the reaction. The loop-mediated isothermal amplification amplicon was identified as the dimension of fluorescence. An aggregate of 2µl was analyzed by electrophoresis in 2% agarose gel [16].

RESULTS

A total of 100 samples (50 samples from buffalo and 50 from cows) were collected from district Lodhran. Sixty samples out of 100 were found seropositive for brucellosis based on RBPT.

Intensification of BCSP31 by Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification reaction for LAMP assay was modified and ended at 95ºC for 2 minutes. We attained the same results from this modified protocol. By using primers, P-1(Table 1),

Table 1. Sequences Set of Primers (Primer-1) Used for LAMP

Primer	Sequence
F3	5'-GCTTTACGCAGTCAGACGT-3'
B3	5'-GCTCATCCAGCGAAACGC-3'
FIP	5'-AGGCGCAAATCTTCCACCTTGCGCCTATTGGGCCTATAACGG-3'
BIP	5'-GGCGACGCTTTACCCGGAAATTCAGGTCTGCGACCGAT-3'
LF	5'-CCTTGCCATCATAAAGGCC-3'
LB	5'-CGTAAGGATGCAAACATCAA-3'

FIP, forward inner primer; F3, forward outer primer; BIP, backward inner primer; B3, backward outer primer; LF, forward loop primer; LB, backward loop primer.

The loop-mediated isothermal amplification assay successfully amplified the target sequence of BCSP31 of *B. abortus* DNA at 63ºC for 35 minutes. After intensification, items were likewise specifically seen by the bare eye with a fluorescent location reagent. The pattern was affirmed by gel electrophoresis (Figure 2).

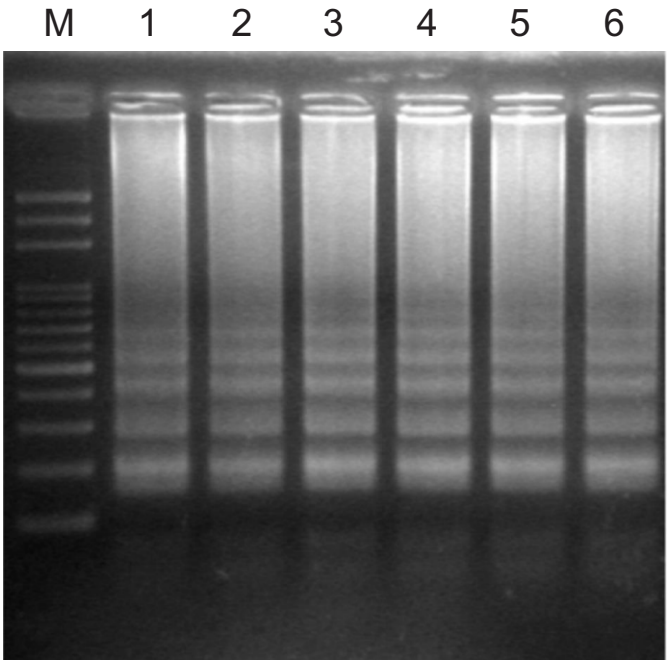

Figure 2: Result of LAMP PCR on Gel Electrophoresis. Lane M = DNA Ladder of 100-bp; Lane 1-6 = DNA samples.

Specificity of the Loop-Mediated Isothermal Amplification examine with different Sorts of Bacterial Species

To assess the specificity of the loop-mediated isothermal amplification primers, *Brucella* species and other non *Brucella* species were used. Huge and explicit enhancement of deoxyribonucleic acid was observed after 35 minutes of all *Brucella* species used, consisting reference, antibody, clinical segregates, while other non-*Brucella* species demonstrated no intensification (Table 2)

Table 2. Bacterial Strains Used in This Study and Results OF LAMP Amplification

Sources No.					
***Brucella* Species**			**Non *Brucella* Species**		
Species	**Source**	**LAMP Result**	**Species**	**Source**	**LAMP Result**
Brucella abortus	Microbiology Laboratory, UVAS, Ravi campus, Pattoki	Positive	*Escherichia coli*	Microbiology Laboratory, UVAS, Ravi campus, Pattoki	Negative
Brucella melitensis	Microbiology Laboratory, UVAS, Ravi campus, Pattoki	Positive	*Staphylococcus aureus*	Microbiology Laboratory, UVAS, Ravi campus, Pattoki	Negative

The figure 3 illustrated the specificity of the loop-mediated isothermal amplification (LAMP) assay when tested on various species.

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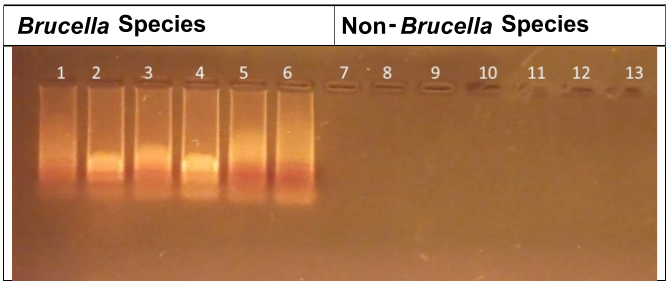

Figure 3: Specificity of the Loop-Mediated Isothermal Amplification Assay with Different Types of Species. Lane 1-6 are Bacterial Species and 7-13 are non-brucella Species Per Products Were Identified on the Base of Specific Primers Used for Identification.

DISCUSSION

This study is a principal report of use of loop-mediated isothermal amplification for recognition of *Brucella* species. Brucellosis has great importance in human health, in both veterinary medicine and public health because it is a zoonotic disease [21]. Different *Brucella* species influence steers, deer, elk, sheep, goats, pigs, hounds and a few different living organisms, including marine warm blooded animals [21, 22]. In people, the infection shows exceedingly assorted side effects, for example, fever that forms into a chronic sickness influencing different parts of body. Epidemic breakout of brucellosis in lab labors have additionally been reported [23-25]. As medication treatment is drawn out and powerful anti-infection agents are restricted, a dependable and early finding of brucellosis is of real significance for starting satisfactory treatment. However advantageous serological diagnostic techniques, for example, Rose Bengal method for identification of *Brucella-explicit* antibodies were accessible. The helpfulness was restricted by a great commonness of *Brucella-explicit* antibodies in disease territories of brucellosis, low amount in the intense stage, and cross-responses with different gram-negative microscopic organisms, for example, Y. *enterocolitica* O:9 [26, 27]. Likewise, developing an assay for the identification of *Brucella* bacteria involves creating a test that can accurately and reliably detect the presence of this pathogen [28, 29]. Biochemical identification is an economical approach, even due to its slow developmental rates (of *Brucella spp). Brucella spp* has a potential natural causative agent of brucellosis [30, 10]. By and large, a quick, explicit, straightforward and safe identification framework for *Brucella spp.* should be set up. The loop-mediated isothermal amplification measure is beneficial as a result of its basic task, quick response and simple detection [16]. A straightforward and economical mechanical assembly, for example, a water bath that gives a steady of 63ºC is adequate for test, not at all like polymerase chain reaction, reaction is specifically seen with exposed vision refuting requirement for agarose gel electrophoretic examination. In addition, loop-mediated isothermal amplification test can be processed nearby equipment for example, a PCR machine is not required. By utilizing our loop-mediated isothermal amplification test, 10 fg of *Brucella* DNA is effectively processed inside 35 minutes, and assessed to relate to 2.8 DNA duplicates per response [31]. The specificity of Brucella loop-mediated isothermal amplification was relatively equivalent to that of real time PCR recently detailed by [32]. Consequently, the specificity of the loop-mediated isothermal amplification measure was better than that of real time PCR. At the point when melt cover examination was incorporated, continuous PCR took around 50 min, while the Brucella loop-mediated isothermal amplification can be done for 35 minutes. We likewise assessed the loop-mediated isothermal amplification through which *Brucella* was identified. In the defected spleen, loop-mediated isothermal amplification distinguished as few as 8.2x102 CFU of *B. abortus.* These outcomes propose that the loop-mediated isothermal amplification measure would be valuable for quick finding of brucellosis at beginning of disease and also for diagnosis of microorganisms. *Brucella* loop-mediated isothermal amplification strategy created in this examination is a quick, fast and very explicit technique that can be substituted for polymerase chain reaction or constant polymerase chain reaction tests. It is a valuable technique for determination and reconnaissance of brucellosis. Dairy cattle entire blood tests were handled by regular techniques for DNA extraction and henceforth, PCR inhibitors may exist, yet the LAMP test is a solid examine as the compound Bst polymerase is not hindered by inhibitors, which is an additional credit to the technique of LAMP [33]. Isolation and distinguishing proof of the species was endeavored in the underlying stages of the examination without productive outcomes, as entire blood is not a good for the isolation of *Brucella*. Traditional PCR is ordinarily utilized as molecular device for recognition of brucellosis and henceforth efforts were made to contrast LAMP and ordinary PCR, as opposed to the more refined real time PCR. The clinical affectability, specificity and sensitivity of the indicative test were altogether observed to be right around 100% and thus the LAMP test can be used as a helpful demonstrative apparatus for screening of brucellosis in field conditions, with more extensive appropriateness around the world. The LAMP measure demonstrated promising outcomes when utilized for epidemiological screening of brucellosis in cows and this investigation additionally uncovered the pervasiveness of cow-like brucellosis in various parts of Pakistan. In the LAMP assay blend is lyophilized, it might be utilized as a field test to distinguish *Brucella spp.* outwardly inside 30 min, which would examine the sub-atomic the study of disease transmission of this essential zoonotic pathogen.

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CONCLUSIONS

This is principal report of use of loop-mediated isothermal amplification for recognition of *Brucella* species. Our study will be helpful in prevention and control of animal and human brucellosis in Pakistan.

Authors Contribution

Conceptualization: MJ
Methodology: MJ, SA, AH[1], MAR, AA
Formal analysis: YA
Writing, review and editing: MJ, AH[2]

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Molecular Docking-Aided Identification of Natural Bioactive Molecules as Potential Cancer Cell Proliferation Inhibitors

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ABSTRACT

Cancer is the second leading cause of death worldwide. Uncontrolled proliferation of cells is a hallmark of cancer development and progression. Ki-67 (a marker of proliferation Kiel-67) and Proliferating Cell Nuclear Antigen (PCNA) are two major proliferations, diagnostic and prognostic biomarkers as these are over expressed in cancerous cells. Pharmacological inhibition of Ki-67 and PCNA could effectively inhibit the growth of cancer cells. **Objective:** To identify Sesquiterpene Lactones (SLs) as potential inhibitors of Ki-67 and PCNA to reduce cancer burden. **Methods:** The inhibitory potential of SLs, namely sulfocostunolide A, sulfocostunolide B, ilicol, eucalyptone, and ascleposide E, were investigated using Molecular Docking (MD) analysis. MD analysis and visualization of ligand-protein complexes were performed using softwares such as MGL tools, BIOVIA Discovery Studio visualizer and LigPlot plus. Additionally, drug likeness and pharmacokinetic properties of SLs were assessed via pkCSM and ADMET analysis. **Results:** Results showed that eucalyptone with binding energy of -8.1 kcal/mol with Ki-67 while sulfocostunolide B with -6.4 kcal/mol binding energy with PCNA are the most potent proliferative inhibitors of Ki-67 and PCNA. ADMET properties, MD studies and toxicity prediction shows that current investigated ligands bind effectively with Ki-67 and PCNA without showing any toxicity. **Conclusions:** Current study concludes that eucalyptone with Ki-67 and sulfocostunolide B with PCNA made stable complexes and can be considered as novel inhibitors. In addition to that, these suggested ligands have also shown effective drug likeness and ADMET profile. Further, *in-vitro* and *in-vivo* studies are required to validate these findings.

INTRODUCTION

Cancer is a group of diseases consisting of a combination of genetic, epigenetic, signaling, and metabolic anomalies which critically disrupt the regular homeostasis of cell survival, growth and death [1]. Cancer is the second leading cause of death. The cancer diagnosis is difficult due to the wide range of symptoms that appear during different stages of cancer [2]. MRI scan, CT scan, ultrasound, biopsy, and X-rays are being used to detect abnormalities and presence of tumor within the body [3]. Besides these diagnostic tests, two other diagnostic biomarkers, Ki-67 (a marker of proliferation Kiel-67) and Proliferating Cell Nuclear Antigen (PCNA) are effective diagnostic tools as both these proteins are over-expressed in cancerous cells. Uncontrolled proliferation of cells is the one of the major hallmark of developing cancer, and expression of those genes that are involved in proliferation are up-regulated in cancerous cells [4]. Ki-67 and PCNA are regular biomarkers of proliferation that are usually used to measure the growth fraction of the population of a cell. Both proteins have proliferation markers characteristics as well as predictive and prognostic importance [5]. Molecular docking (MD) is structure-based in-silico method that is commonly used in drug discovery. In-silico docking enables to identify the novel bioactive compounds of high therapeutic interests and predicts interactions between ligand and receptor at the molecular level [6]. Currently, this computational technology is widely used for initial stages of drug design. For researchers, it is convenient to use the compound database to synthesize and complete pharmacological tests. It greatly reduces cost, time wastage and improves

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the efficacy of research in drug development [7]. For decades plants are a widespread natural product reservoir and have been used for the treatment of numerous ailments including cancer [8]. Sesquiterpene lactones (SLs) are the utmost dominant group among all the secondary metabolites that are present in plants. SLs displayed various biological activities such as anti-oxidant, anti-tumor, anti-microbial, and hepatoprotective activities that are reported in a various studies [9]. In current study, MD analysis between Ki-67, PCNA and five compounds of SLs has been carried out for evaluating their anticancer potential.

METHODS

Molecular docking was done by retrieval of ligands and proteins from databases, optimization of their conformations, binding of ligands with proteins and analysis of the interactions occurring between them [10, 11]. Crystal structure of Ki-67 (PDB ID: 5J28) and PCNA (PDB ID: 1VYM) were retrieved by using RSCB protein data bank and was prepared in PDBQT format by Autodock Vina. Five ligands namely sulfocostunolide A, sulfocostunolide B, eucalyptone, ilicol and ascleposide E were recovered in SDS-3D format by using PubChem Database and were also prepared PDBQT formats by AutoDock Vina. Afterwards, AutoDock vina 1.5.7 was used to dock these SLs with Ki-67 and PCNA. In this case, size of designed grid box was 40x40x40Å in X, Y, Z dimension, along with 0.375 spacing. With each ligand, both proteins generated nine diverse poses out of which only one pose is taken as probable binding mode due to its highest binding energy as given in the previous studies [12]. After analysis of MD, Biovia discovery studio visualizer was used for visualization of different interactions occurring between ligand and protein. Ligplot+ 4.5.3 was used for visualization of hydrophobic and hydrogen interactions. In addition to that, pharmacokinetic properties of ligands were analyzed by using pkCSM and SwissADME to assess their toxicity and drug likeness. For this purpose, SMILES of ligands were taken from PubChem and analyzed using pkCSM [13, 14]. pkCSM is a computational tool and used to predict ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties of a candidate drug. These are the major pharmacokinetic properties for drug likeness of a molecule by giving canonical SMILES as an input. These properties can reduce the late stage failure or withdrawal of drug in drug discovery process to save money and time and ensuring the stability and safety of designed drug in future. Drug likeness scores by these physicochemical properties can be considered as first step of success for any drug candidate molecule [13].

RESULTS

Current study revealed that selected SLs strongly bind with proliferation marker proteins Ki-67 and PCNA. All the ligands showed good binding energy score as well as efficient binding interactions with respective proteins. Among all, eucalyptone with binding energy of -8.1 kcal/mol with Ki-67 while sulfocostunolide B with -6.4 kcal/mol binding energy with PCNA seemed to be the most potent proliferative proteins inhibitors (Table 1).

Table 1: Binding Affinities and Inhibition Constants of SLs with ki-67 and PCNA.

Ligands	Ki-67		PCNA	
	Binding Affinity	Inhibition Constant	Binding Affinity	Inhibition Constant
	Kcal/mol	µM	Kcal/mol	µM
Sulfocostunolide A	-7.4	3.57	-6.3	23.04
Sulfocostunolide B	-7.8	1.81	-6.4	19.4
Eucalyptone	-8.1	1.09	-6.1	32.3
Ilicol	-7.3	4.23	-5.6	75.4
Ascleposide E	-7.9	1.53	-6.1	32.3

Among all SLs, eucalyptone showed more hydrogen bonds with Ki-67. It binds with Ki-67 by forming seven hydrogen bonds with ARG221, ARG221, GLN249, ASP220, GLN249, ASP220 and ASP220 residues with bond distances of 2.66Å, 2.88Å, 2.17Å, 2.43Å, 2.61Å, 2.39Å and 2.75Å, respectively. Eucalyptone also formed an electrostatic bond with ASP220 (3.83Å) as shown in figure 1.

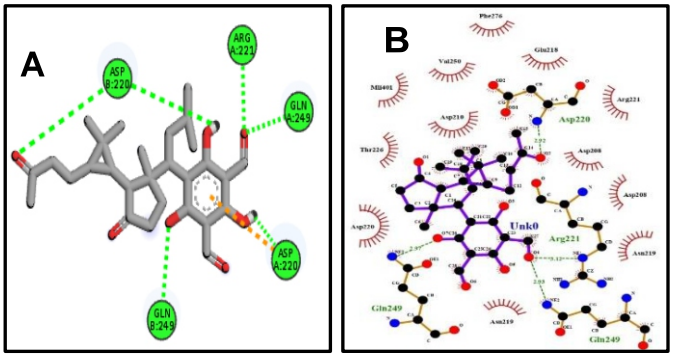

Figure 1: Two-dimensional view of docked eucalyptone with Ki-67 protein (a) visualization by discovery studio showed six hydrogen bonds and one hydrophobic interaction in eucalyptone-Ki67 complex. (b) Ligplot results verified the same interaction of eucalyptone with Ki-67.

Sulfocostunolide B strongly bounded by four hydrogen bonds with ARG149, ARG149, THR216 and ALA145 of PCNA via bond distances 2.51Å, 2.19Å, 2.48Å and 2.42Å respectively (Figure 2).

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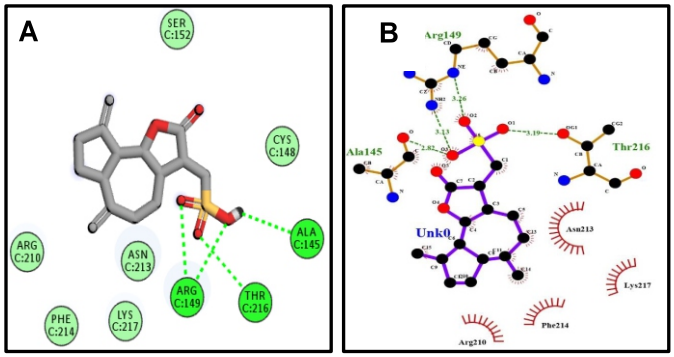

Figure 2: Two-dimensional view of docked sulfocostunolide-B with PCNA protein (a) visualization by discovery studio showed four hydrogen bonds between sulfocostunolide-B and PCNA complex (b) Ligplot results verified the same interaction of sulfocostunolide-B with PCNA.

For assessment of drug likeness of the selected ligands, Lipinski rule of 5 was employed as elaborated in table 2. This rule provides a guideline to predict the bioavailability of the drug candidate. If the drug candidate follows all the criteria of this rule then it is safe for oral administration and effective to use. For this purpose, it should have a molecular weight below 500 Dalton, a log P value not exceeding 5, no more than 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors and polar surface area should be less than 140 Å2[15]. All our ligands have shown no violation than standard values as given in table 2.

Table 2: Lipinski Rule of Five Analysis of Selected Ligands

Ligands	Molecular Weight < 500 (g/mol)	H-Bond Acceptor < 10	H-Bond Donors < 5	log P < 5	Polar Surface Area (Å2)
Sulfocostunolide A	312.387	4	1	1.964	89.05
Sulfocostunolide B	312.387	4	1	1.964	89.05
Eucalyptone	486.605	7	3	5.184	128.97
Ilicol	238.371	2	2	2.892	40.46
Ascleposide E	388.457	8	4	-0.254	125.68

Properties of potential ligands were evaluated by ADMET analysis. ADMET analysis was carried out using pkCSM to ensure ADMET properties of potential drug candidates as given in Table 3. The ADMET profiling of eucalyptone and sulfocostunolide B was determined as they showed greater binding energy with Ki-67 and PCNA respectively. For absorption, Water solubility of sulfocostunolide B and eucalyptone were -2.185 and -3.977 (log S) mol/L with 95.978 and 91.219 intestinal absorption respectively. Log value of volume distribution of sulfocostunolide B and eucalyptone were -0.503 and 0.068 (L/KD) respectively. Substrate and inhibitors of CYP2D6 and CYP3A4 protein of both ligands were found to be absent expect CYP3A4 substrate of eucalyptone for metabolism. Total clearance of both candidate drugs were 0.009 and 0.24 Log/ml/min/kg for both drugs respectively with no OCT2 substrates. Both ligands showed no AMES and hepatotoxicity.

Table 3: Drug Likeness Prediction Using pk CSM Online Database Server for the Selected Ligands.

ADMET	Variables	Sulfocost-unolide B	Eucalyptone
Absorption	Water Solubility (LogS) ml/L	-2.185	-3.977
	Intestinal Absorption	95.978	91.219
	P-Glycoprotein I/II Inhibitors	No	Yes
Distribution	Log VDs (L/Kg)	-0.503	0.068
Metabolism	CYP2D6 Substrate	No	No
	CYP3A4 Substrate	No	Yes
	CYP2D6 Inhibitor	No	No
	CYP3A4 Inhibitor	No	No
Excretion	Total Clearance (Log ml/ min/ kg)	0.009	0.24
	Renal OCT2 Substrtae	No	No
Toxicity	AMES	No	No
	Max. Tolerable Dose (log mg/kg/day)	0.856	-0.203
	Hepatotoxicity	No	No

DISCUSSION

SLs display higher varieties in structures and exhibit many biological activities. SLs have been proved to exert anti-cancer efficiency and tumor cell cytotoxicity and are currently in clinical trials [16, 17]. In present study, docking results revealed that protein-ligand complexes have shown good binding affinities and hydrogen bonding. Current study revealed that desired ligands strongly bind with Ki-67 through different hydrogen, hydrophobic and electrostatic interactions, and inhibited Ki-67 efficiently in the same way as reported in previous studies as follows. In an in-vitro study, it has been shown that a natural compound arglabin reduces Ki-67 positive cells by inhibiting mTOR/Akt/PI3K pathway group. It increases SCC-4 cells growth, apoptosis and induces arrest. Arglabin induces apoptosis by chromosomal condensation, SCC-4 cell fragmentation, and bleb formation. These findings were further confirmed by in vivo studies and Ki-67 was down-regulated after treatment with arglabin indicating that the growth of OSCC cells was inhibited by arglabin which is SLs [18]. Recently reported in-vitro findings showed similar binding of other SLs such as costunolide Trilobolide-6-O-Isobutyrate (TBB) with Ki-67. Results revealed the inhibition of proliferation and HCC cell colony formation by TBB. Similarly, TBB inhibited the STAT3 signaling pathway which in turn influences and inhibited the expression and transcription of P21, Ki-67 and PCNA genes [19]. In another in-vivo study, Micheliolide (SLs) effected growth of tumor cells (AGS and N87) of gastric cancer analyzed through an MTT assay. Results revealed that Ki-67 and PCNA expression in AGS and N87 was reduced significantly after treatment with Micheliolide [20]. These results are in accordance to our selected SLs as they inhibited the proliferation markers by MD analysis as well. Favorable bioavailability and drug-likeness of a drug is generally evaluated by Lipinski's rule of

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5. All our selected ligands followed this rule as explained in methodology section. It confirms their bioavailability and oral administration. Eucalyptone showed deviation (logP=5.1849) from the threshold value (logP< 5.00). However, it is important to note that the Lipinski rule of 5 is a guideline rather than an absolute rule and deviations from these parameters can still result in successful drug candidates [21]. Absorbance of drug candidate was examined by analyzing various parameters such as water solubility (log S) mol/L, P-glycoprotein I/II inhibitor and intestinal absorption. Water solubility (log S) in mol/L measures drug's availability in aqueous solution. Higher value signifies higher absorbance and both drug candidates fall within its range of -4 to -2 mol/L [22]. P-glycoprotein I/II is an efflux membrane transporter which is responsible for hindering the absorption and bioavailability of chemotherapeutic drugs. P-glycoprotein I/II inhibitors have ability to enhance the consumption of potential drug many folds leading to adverse drug-drug interactions [23]. As shown in table 3, eucalyptone being an inhibitor compromise higher drug absorbance at the risk of unfavorable pharmacokinetic interactions. Both compounds exhibit intestinal absorption value greater than 30% indicating their significant absorption in intestine [24]. LogVDss (volume of distribution at steady state) ensures the steady concentration of drug in blood plasma and its ideal value must be in the range of -0.15 to 0.45. Both drug candidate's falls within this range and depict optimal values [25, 26]. Cytochrome P450 (CYP450) plays significant role in metabolism of drugs. Its two isoforms, CYP3A4 and CYP2D6, oxidize and modify chemical structures of drugs allowing their biotransformation. Sulfocostunolide B does not show any interaction with CYP450 isoforms. However, eucalyptone, being CYP3A4 substrate can facilitate elimination and clearance of drug from body yet it can also impose drug-drug interaction due to increased enzyme activity [26-28]. Both of the ligands are not renal OCT substrate which ensures reduced renal clearance leading to increased therapeutic effect. Total clearance represent sum of all clearance mechanism and both the ligands seemed to be eliminated from the body [29]. In addition to that, it is evident from table 3 that both the drug candidates are neither hepatotoxic nor AMES toxic. AMES toxicity assay analyze drug's ability to induce genetic mutations [30]. Both ligands possess fewer values for maximum tolerable dose. Lower values of maximum tolerable dose signifies maximum limit of drug's administration in the body so that it might impose adverse effects [29].

CONCLUSIONS

In the current MD-aided study, we analyzed some SLs as potential inhibitors of cell proliferation markers of Ki-67 and PCNA. All these compounds showed effective binding energies and molecular interactions. However, eucalyptone and sulfocostunolide B can be considered to have more potential as a cancer cell proliferation inhibitors. In addition to that, both the suggested compounds have shown effective ADMET profile, drug likeness and bioavailability. To further validate their inhibitory effect, *in-vitro* and *in-vivo* investigations are required to have much deeper insights in their inhibition potential.

Authors Contribution

Conceptualization: MK
Methodology: IH, SG, EZ
Formal analysis: IH, AZ, EZ
Writing, review and editing: IH, MK, AZ, SG

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Evaluating the Composition of Biodiesel Synthesized from Black Soldier Fly (*Hermetia illucens*) Larvae

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ABSTRACT

Biodiesel is considered a viable alternative to conventional diesel, particularly for the ground transportation industry. While different plant seeds oils have been the dominant feedstocks for biodiesel synthesis to date. However, they are often expensive due to their limited supply and low reproductive rate. **Objective:** To present a sustainable approach by using the black soldier fly (*Hermetia illucens*) larvae as an alternative feedstock. **Methods:** The larvae were fed with waste chicken rice, fish, soft vegetables and fruits. These wastes provide food and shelter for disease-causing insect larvae and contributes to land pollution. Using petroleum ether as a solvent, 12.2 g of crude grease was extracted from ~ 500 larvae, resulting in about 11.8 g of biodiesel through a two-step acid-base catalyzed transesterification process. **Results:** The resultant biodiesel was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS), revealing a Fatty Acid Methyl Ester (FAME) profile predominantly comprising dodecanoic acid, 9-Hexadecenoic acid, 9- octadecenoic acid and 11- octadecenoic acid etc, emphasizing its potential as a high-quality alternative to conventional diesel fuel. **Conclusions:** This study contributed to develop biodiesel as an eco-friendly renewable energy technology by using BSFL.

INTRODUCTION

In recent decades, the global ground transportation sector has seen a significant rise in the use of non-renewable fossil fuels, propelled by rapid population growth. These fuels are finite, deplete quickly, and come with environmental risks, raising concerns about their sustainability [1, 2]. Therefore, the search for new renewable energy resources is becoming increasingly critical [3, 4]. Biodiesel is emerging as a compelling alternative to petroleum-based diesel for heavy vehicle fueling especially for trains, trucks, buses and tractors [5]. Many researchers encourage the utilization of biodiesel than conventional diesel fuel as it is less explosive, forms low carbon deposits in engine on combustion and hence causes less wear and tear of engine [6]. Biodiesel is not only renewable but also non-toxic, biodegradable, highly combustible and economically competitive [7]. It contains Fatty Acid Methyl Esters (FAME) produced through a transesterification reaction involving lipids, alcohol, and a catalyst [8]. For the synthesis of Black Soldier Fly (BSF) based biodiesel through transesterification reaction, the larval grease is allowed to react with alcoholic solvents (petroleum ether, n-hexane, ethanol) in the presence of acid (sulphuric acid), base (sodium hydroxide) or enzyme (lipase) catalyst [9, 10]. Currently, the production of biodiesel primarily relies on vegetable oils like palm, soybean, sunflower oil, and others [11]. Their limited supply and ethical concerns surrounding the use of such plant-based feedstocks have led to the exploration of alternative

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sources. Moreover, the cost of biodiesel using these feedstocks is significantly higher (1.5 times) than that of traditional petroleum diesel. This expense hampers its large-scale adoption, creating a need for more cost-effective raw materials [12]. Black Soldier Fly (BSF) (Hermetia illucens) is widely distributed non-pest insect, present most commonly in temperate and tropical areas [13]. Black Soldier Fly Larvae (BSFL) have recently gained scientific attention as a potential and more sustainable biodiesel feedstock [14]. They offer advantages such as a rapid reproductive rate, short life cycle, ease of cultivation, high lipid contents and ultimately a higher biodiesel yield [15]. Meanwhile, food waste can be a potential source of the development of many pathogenic insects as well as land pollution. BSFL can feed on a variety of these waste food materials and incorporate them into their bodies as lipids through metabolic processes [16, 17]. The residual material left after lipid extraction can also serve as animal feed [18]. This study was specifically aimed at producing biodiesel from BSFL, fed on food waste, using acid-based catalyzed transesterification. We assessed the composition of the resulting biodiesel to evaluate its suitability as a potential fuel for transportation.

METHODS

BSFL Rearing

The study involved the sustenance of BSFL colony over two generations from November 2023 to February 2024 in the General Zoology Lab at the University of Okara and was originated by the courtesy of Dr. Hafiz Kamran Yousaf from Thal university Bhakkar, Punjab, Pakistan. A feed comprising waste chicken rice, fish, partially rotten vegetables and fruits was offered to provide all essential nutrients for their optimal development. Cultivation conditions were maintained at a temperature of 27 ± 2°C and a humidity range of 60-75%. For this experiment, fully grown, fifth-instar larvae were selected as older larvae yield higher lipid content.

Crude Lipid Extraction from BSFL

In March 2024, approximately 500 BSFL were first cleaned with water and then inactivated by boiling at 80°C for five min. The inactivated larvae were subsequently oven-dried at 70°C for four hours and stored at 4°C. These prepared larvae were then ground into a fine powder using a micro-mill grinder. The resulting powder was immersed in 100 ml of petroleum ether, and allowed to dissolve for 48 hours. To purify the crude lipid extracted, 1 ml of 0.5% sulphuric acid was added to remove impurities like phospholipids, pectin and other solid matter. The purified crude lipid was then evaporated using a rotary evaporator to eliminate any remaining solvent, followed by drying the sample overnight at room temperature. A centrifuge was used at 1000 rpm for two min to separate any residual undesired molecules from the larval lipid. Finally, the acidity level of the prepared sample was measured using a pH meter to ensure that the crude lipid was not overly acidic.

Production of Biodiesel from Crude Larval Lipids

For the synthesis of biodiesel from free fatty acids present in the grease obtained from BSFL, a two-step acid-base catalyzed transesterification was employed to normalize the acidity of the extracted grease [19]. The reaction took place in a sealed system, equipped with a thermometer, reflux condenser, 100 ml reactor vessel, a sample outlet for periodic testing and an electromagnetic stirrer for thorough mixing as mentioned by Jain *et al.*, in 2011.

Acid-Catalyzed Transesterification

A mixture of methanol and grease in an 8:1 molar ratio, along with 1 ml of 0.5% sulphuric acid as a catalyst, was maintained at a temperature of 75°C for 60 min. This was done in a rotatory evaporator operating at a rotational speed of 75 rpm. The outcome of this reaction was a mixture comprising unreacted biodiesel crude grease and some residual solvent. To separate the resultant layers, the mixture was centrifuged at 400 rpm for 10 min. The upper layer, which was the desired product, was then dried in an oven set at 60°C for 20 min. This procedure was specifically designed to reduce the acidity of the crude larval grease.

Alkaline-Catalyzed Transesterification

After acid-transesterification, the resulting mixture was kept in a new reactor for carrying out alkaline-catalyzed transesterification. During this process, the temperature was kept at 65°C for 30 min and methanol: lipid (6:1) was mixed with the catalyst 1 ml of 0.8% sodium hydroxide by using a magnetic stirrer at 500 rpm.

Separation and Purification of Synthesized Biodiesel

After the acid-base transesterification process, the mixture contained two distinct layers: the upper layer comprised biodiesel, and the lower layer contained impurities. These layers were isolated using a centrifuge operating at 400 rpm for 10 min. The biodiesel was then distilled at 80°C to remove any remaining traces of methanol.

Analysis of Biodiesel Composition

The composition of the biodiesel, specifically the FAME profile, was analyzed using gas chromatography-Mass Spectroscopy (GC-MS). In the case of gas chromatography, nitrogen gas was used as the carrier at a flow rate of 29 ml/min. While a capillary column with a flame ionization detector was used during mass spectroscopy. Before analysis, the biodiesel sample was diluted with dichloromethane. The detector and injector temperatures were set at 250°C and 220°C, respectively. The column temperature was initially held at 140°C for 5 min and then raised to 240°C for an additional 15 min to analyze FAME profile as mentioned by Pauline, Sivaramakrishnan, Pugazhendhi, Anbarasan and Achary [19].

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RESULTS

Crude Lipid Extraction

Approximately 500 larvae were used as the larval biomass for petroleum ether extraction, yielding 12.2 g of grease. Additionally, Gas Chromatography-Mass Spectrometry (GC-MS) analysis revealed the composition of the extracted grease as 40.8% unsaturated fatty acids and 57.2% saturated fatty acids. The biodiesel yield was calculated to be 96.7% as outlined in table 1.

Table 1: The Quantities of Larval, Grease and Biodiesel Mass and Biodiesel Yield

Number of BSF	BSFL biomass in Powdered Form (g)	Crude BSFL Grease Biomass (g)	Biodiesel Biomass (g)	Biodiesel Yield (%)
~500	32.5	12.2	11.8	96.7%

Biodiesel Production

Following grease extraction, the mixture was allowed to settle for three hours, resulting in the formation of two separate layers. The upper layer, less dense and consisting mainly of fatty acids and triglycerides, was isolated for further processing. This layer was subjected to a two-step acid-base transesterification process. Initially, 0.5% sulphuric acid (1 ml) was used as a catalyst for acid-catalyzed transesterification. This was followed by alkaline-catalyzed transesterification using 0.8% sodium hydroxide (1 ml). After these steps, the resulting biodiesel weighed 11.8 g formed again the upper less dense layer settling below the impurities. Residual solvents were removed using a rotary evaporator at 75°C and 80 rpm for 20 min.

Chemical Composition

The chemical composition of the produced biodiesel was analyzed using GC-MS, identifying 15 FAME. Among these, the most abundant were 9-octadecenoic acid (22.6%), dodecanoic acid (22.3%), 9-hexadecenoic acid (15%), 11-octadecenoic acid (9%), and myristic acid (5.5%). These results, presented in table 2 , suggest that the composition may vary based on the diet provided to the BSFL Retention time is the time period that molecules stay in the column of gas chromatography

Table 2: Composition of biodiesel derived from BSFL through acid-base catalyzed transesterification, (values have been written as mean ± standard deviation)

S. No.	Biodiesel Composition (FAME)	Number of Carbon Atoms	Concentration (%) ± SD	Retention Time (min)
1	Nonanoic Acid	9	1.0 ± 0.02	4.4
2	Dodecanoic Acid	10	22.3 ± 0.8	6.3
3	Undecanoic Acid	11	2.6 ± 0.1	10.2
4	Myristic Acid	14	5.5 ± 0.5	7.8
5	Pentadecanoic Acid	15	1.0 ± 1.0	8.7
6	Hexadecanoic Acid	16	8.0 ± 0.1	18.3
7	9-Hexadecenoic Acid	16	15.0 ± 0.3	9.1
8	7-Hexadecenoic Acid	16	5.2 ± 0.0	4.2
9	Heptadecanoic Acid	17	12.7 ± 0.3	7.9
10	Linoleic Acid	18	2.3 ± 0.5	14.8
11	11- Octadecenoic Acid	18	9.0 ± 1.0	5.6
12	9- Octadecenoic Acid	18	22.6 ± 1.2	8.2
13	Noadecanic Acid	19	1.4 ± 0.8	16.9
14	Nonadecanoic Acid	19	0.9 ± 1.1	11.2
15	Docosanic Acid	22	0.5 ± 1.2	19.9

DISCUSSION

Petroleum diesel, currently the predominant fossil fuel for ground transportation, will no longer be available in the near future [21]. It was now imperative to shift from non-renewable diesel fuel to renewable biodiesel to avoid any future energy crisis [22]. The present study was conducted to investigate the potential of BSFL as a feedstock for biodiesel synthesis. Grease was extracted from these oleaginous larvae using petroleum ether solvent through the chemical extraction method, which was then transformed into fatty acid methyl esters containing biodiesel through two-step transesterification. In terms of biodiesel production, the study was successfully carried out by the acid-base transesterification process, which yielded 11.8 ± 0.4 g of biodiesel from 12.2 ± 3.5 g crude grease, with 96.7 ± 0.76% biodiesel yield. A similar result was reported by in which 23.6 ± 0.5 g of biodiesel was extracted from 25.4 ± 3.5 g larval grease with a 93 ± 0.78% yield of biodiesel [23]. The current study also revealed that the chemical composition of biodiesel further reinforces its viability as a renewable energy source. The significant presence of dodecanoic acid, 9-Hexadecenoic acid, 9-octadecenoic acid, 11- octadecenoic acid, and among other FAME indicate a rich and balanced fatty acid profile, and these results are in line with previous studies [24]. Furthermore, the feeding substrate has an impact on the nutritional profile of the larvae, which may have an impact on the quantity and quality of biodiesel [25]. The life cycle of BSFL was heavily dependent on optimal temperature of 25 to 35°C, any slight change in maintenance of this temperature would negative affect their growth [26]. The constraints pertaining to process scalability, feedstock's impact on biodiesel quality, and the improvement of lipid extraction techniques require more investigation. Further research was required to produce healthier BSFL and ultimately quality biodiesel. In this regard, BSF can be genetically modified to easily ingest or digest the food to accumulate more fat reserves during their larval stage, produce more eggs, and extend the life of their adults through emerging CRISPR Cas9 technology. Future studies would explore how controlled feeding experiments could establish the optimal diet for maximizing biodiesel yield and quality. Additionally, the stability of the produced biodiesel should be investigated further, particularly

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focusing on its long-term storage and how the higher acidic value could be managed effectively. This could include exploring additives or refining processes that could enhance its stability. Furthermore, an economic analysis could be conducted to evaluate the cost-effectiveness of the entire biodiesel production cycle, from larval rearing to grease extraction to transesterification. This could help in formulating business models and policy guidelines for promoting BSFL-based biodiesel as a sustainable alternative. By pursuing these future research directions, a more comprehensive, practical, and economically viable framework for biodiesel production from BSFL can be developed. Moreover, additional studies are needed to investigate how to make BSF grow fast with maximum biomass, the scalability of this process, and to further fine-tune the biodiesel properties to meet varying energy requirements.

CONCLUSIONS

The current study highlighted that the BSFL, a non-pest oleaginous insect larva, carries the capability to consume waste materials, has a higher reproductive rate and a short life cycle, can potentially be used as a biodiesel feedstock. Through experimentation, it was demonstrated that the 12.2 g grease extracted from ~500 BSFL can be effectively converted into 11.8 g of biodiesel that contains the desirable profile of fatty acid methyl esters. Moreover, it elaborated the importance of BSFL-based biodiesel as a biodegradable, harmless, cheap and sustainable energy source that produces the least number of pollutants upon combustion. Hence, it can potentially meet future fuel demands in the transportation sector and can replace the use of fossil fuels. Overall, the study sets the stage for more in-depth investigations that could pave the way for more sustainable energy solutions.

Authors Contribution

Conceptualization: MSS, HKY
Methodology: MSS, FK
Formal analysis: FK, HKY
Writing, review and editing: FK, SH

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Original Article

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First Evidence of Haplotypes of *Babesia bigemina* from District Sialkot Pakistan and their Relation to Other Countries

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ABSTRACT

Babesiosis is a tick borne disease of animals and causes significant losses to livestock sector and in turn to the economy of Pakistan. **Objective:** To analyze the molecular and phylogenetic analysis of *Babesia* species for inter and intra specific genetic variations in district Sialkot, Pakistan. **Methods:** Total 150 (Cow=120, Buffalo=20 and Sheep=10) blood samples were collected. Microscopic examination of blood smears stained by field stain method was performed. DNA was extracted by phenol chloroform extraction method and 18S rRNA gene was targeted for PCR amplification. MEGAX software was used to perform phylogenetic analysis. Haplotype analysis was performed with Popart software by using median joining network method. **Results:** Out of 150 blood samples, 50 (33%) were positive for *Babesia bigemina (B. bigemina).* Out of these, 36 showed 446bp bands specific for 18S rRNA gene of *B. bigemina.* In Phylogenetic tree one sequence from cow had 98% similarity with sequence from Uganda while all other sequences from cow had 98% similarity with sequence from China. Isolate from buffalo showed 98% similarity with sequence of buffalo previously reported from Pakistan and sequence of sheep appeared as more related to buffalo samples. Haplotype analysis from current study revealed four haplotypes, two from cow, one each from buffalo and sheep. **Conclusions:** This study was helpful for molecular and phylogenetic analysis of *Babesia* isolates from district Sialkot, Pakistan. Haplotyping has revealed the genetic variants among different and same host species.

INTRODUCTION

Babesiosis is one of the most significant Tick-Borne Diseases (TBDs) that affects Bovids in Pakistan [1]. It is a deadly disease which is caused by an intra-erythrocytic protozoan parasite belonging to genus *Babesia.* The important vectors for transmission of this disease is *Boophilus* genus of ticks which is extensively available in tropical and subtropical countries [2]. *Babesiosis* is also known as the red-water disease which is caused by different species of genus *Babesia.* Both subclinical and acute infections are caused by *Babesia* spp. [3]. The parasite multiplies in red blood cells that results in the demolition of a huge number of red blood cells. The most important characteristic feature of this disease is coffee colored urine. Affected animals experience the noticeable increase in body temperature (40-41°C), struggle for breathing, loss of appetite, termination of rumination, jaundice of various degrees from paleness in case of mild infection to severe yellow coloration of mucous membranes of vagina and conjunctiva in more progressive cases, abnormal thinness of body (emaciation), progressive hemolytic anemia, increased heart rate, increased respiratory rate, haemoglobinuria, weakness in body, unwillingness to move and eye problems. The high fever in infection causes abortion to pregnant females in some cases [4]. It also reduces the fertility in males specially bulls [5]. Traditionally, the microscopic examination is used for identification of parasites in stained blood smears. The sensitivity of this method is low and it cannot differentiate morphologically indistinguishable organisms [6]. Polymerase Chain

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Reaction (PCR) is a nucleic acid based assay that is far more sensitive than conventional methods for the identification of parasites including *B. bigemina* [7, 8]. Polymerase Chain Reaction (PCR) is sensitive to the extent that, it can detect parasite even if there is only one parasitic cell present in the sample. The PCR based methods have been proved as almost 100 times more specific and sensitive than microscopic examination. This method is highly specific and consumes less time which makes it more satisfactory for diagnostic purposes [8-10].

The current study was for molecular characterization of *Babesia* species in District Sialkot, Pakistan and to investigate about the evolutionary relationship of isolated species with reported *Babesia* spp. throughout the world. This type of study has not been previously done from District Sialkot, Pakistan. Hence, this study on molecular analysis of *B. bigemina* from Bovids may be contemplated as first report of this kind in District Sialkot, Pakistan.

METHODS

Sample Collection

Blood samples for detection of *Babesia spp.* were collected from January 2019 - June 2020 from district disease diagnostic laboratory, Sialkot. Total 150 blood samples were collected from different host animals in sterilized vacutainers containing EDTA. Out of 150 blood samples 120 were collected from cows, 20 from buffalos and 10 samples were collected from sheep.

Microscopic Examination

The microscopic examination of *Babesia spp.* was performed at District disease diagnostic laboratory, Sialkot. Blood samples were analyzed for identification and morphological differentiation of piroplasms of *Babesia spp.* by field stain method by following the standard protocol. Finally, the stained slide was observed under oil immersion lens of light microscope.

DNA Extraction

The genomic DNA extraction was performed by Phenol: Chloroform: Isoamyl alcohol (PCI) method as previously described [11]. The extracted DNA was stored at -20ºC till further utilization. DNA extraction was confirmed by running the samples on 1% agarose gel.

PCR Amplification

A pair of primers specific for 18S rRNA gene of *B. bigemina* i.e., for forward reaction **F**-5 CCCAATCCTGACACAGGGAG3' and for reverse reaction **R**-5 GCAAATGCTTTCGCAGTGGT3' was used to amplify the amplicon of 446bp [12]. PCR amplification was performed by using GeneDireX, Inc. amaR OnePCR kit. A 50µl PCR mixture was prepared by adding 30µl PCR mix from kit, 3µl of each forward and reverse primers, 10µl of distilled water and 4µl of template DNA. Thermal cycler (BIO-RAD T100™) was set for 35 cycles under conditions of thermal cycler for PCR amplifications as given in table 1.

Table 1: Thermal Cycler Conditions for PCR Amplification

Steps	Temperature (°C)	Time	Cycles
Initial Denaturation	95°C	5 min	-
Denaturation	94°C	5 sec	35 Cycles
Annealing	57°C	1 min	
Extension	72°C	1 min	
Final Extension	72°C	10 min	
Infinite Hold	4°C	∞	

PCR products were run on 1% agarose gel. PCR products in gel were visualized in gel documentation system for the desired amplicon size specific for species under examination.

Sequencing and Bioinformatics Analysis

PCR products were sent for sequencing to 1st BASE, Singapore and Malaysia. Purification of PCR products was also performed. PCR products were sequenced in forward direction. Nucleotide BLAST was used for the analysis of 18S rRNA gene sequences for similarity and accuracy level. Nucleotide sequence from different animal hosts showing genetic diversity were submitted to Genbank and accession numbers was received. MEGA X software was used for construction of phylogenetic tree by neighbor joining method with Kimura 2 parameter model and bootstrap support from 1000 replicates.

Haplotype Analysis

A haplotype network to show the relationship of different haplotypes derived from 18S rRNA gene sequence of B. *bigemina* generated from samples of present study and those reported from different selected countries was done by using Popart software 4.1. Haplotypes were generated by median joining network method which is a character based method of haplotype analysis.

RESULTS

Microscopic Examination

Microscopic identification of this species was accomplished according to the morphological characteristics reported by [13] which indicated the presence of *B. bigemina* species in samples of current study as shown in figure 1.

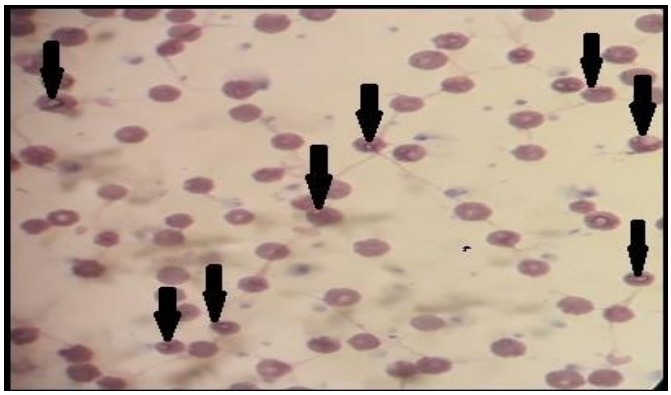

Figure 1: Field Stained Blood Smear Showing the *B. Bigemina* (Black arrows) Under 100X Oil Immersion Lens

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Out of 150 blood samples 50 blood samples were positive by microscopic examination indicating the 33% rate of prevalence and frequency in District Sialkot as shown in table 2.

Table 2: Result of Screening of Blood Smears by Microscopic Examination

Total Number of Blood Samples Collected	Number of Positive Samples N (%)	Positive Samples From Different Host Animals	
		Host	N (%)
150	50 (33%)	Cow	45 (90%)
		Buffalo	3 (6%)
		Sheep	2 (4%)

PCR Amplification

DNA was successfully extracted by Phenol chloroform extraction method. PCR was applied on all 50 samples that were confirmed as positive by microscopic examination. Out of 50 samples 36 produced the characteristic band of 446bp in 1% agarose gel. No amplification was seen in case of negative control as shown in figure 2.

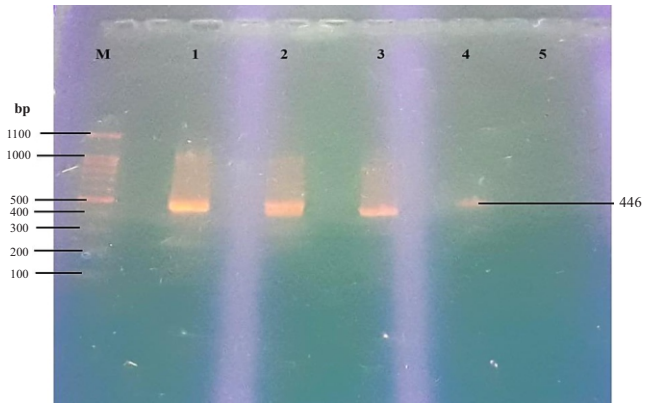

Figure 2: PCR amplification of 18S rRNA gene of *B. bigemina*. Lane M=DNA marker (100bp), Lanes 1, 2 (Cow), 3 (Buffalo), 4 (Sheep) showing positive samples and Lane 5=negative control

Sequencing Analysis

Nucleotide BLAST results confirmed that sequences of our samples were of *B. bigemina*. All the 18S gene sequences generated were aligned by using CLC viewer software to observe the nucleotide polymorphism. Multiple sequence alignment showed both single and multiple nucleotide polymorphism at various positions along the whole length of sequences. Inter and Intra-specific variations were also noted in sequences as shown in figure 3.

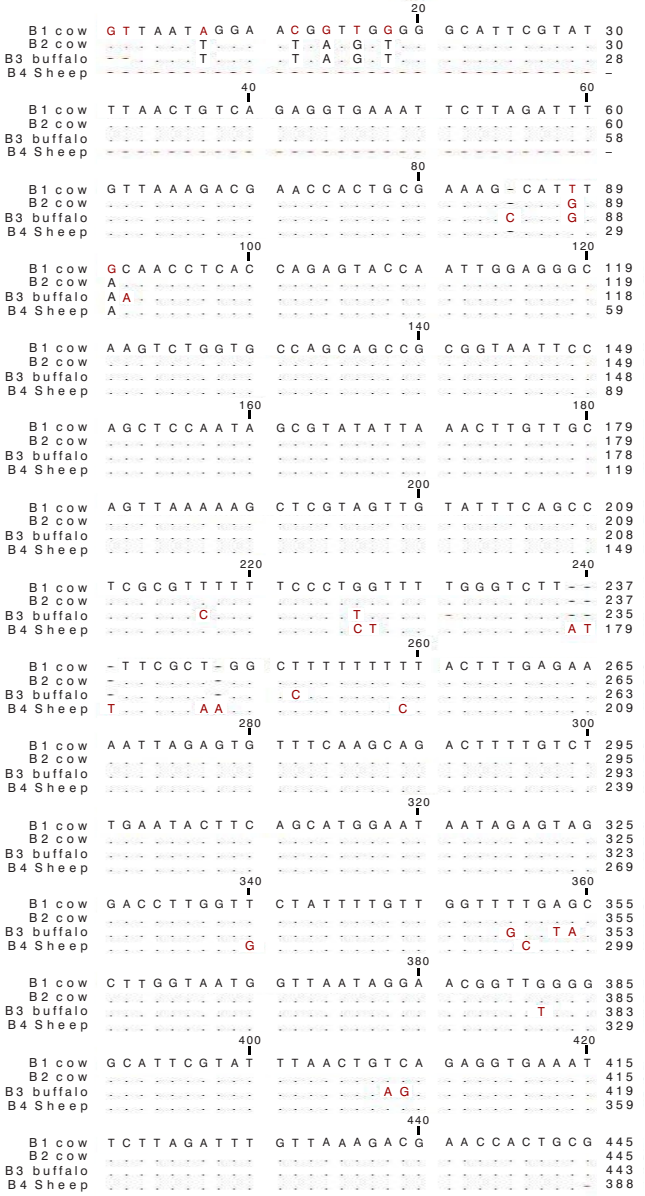

Figure 3: Multiple Sequence Alignment of 18S rRNA gene of Selected Samples of *B. bigemina* Showing Genetic Variability are Designated as B1(Cow), B2(Cow), B3(Buffalo) and B4(Sheep)

Phylogenetic Analysis

Phylogenetic analysis of 18S rRNA gene sequence of *B. bigemina* revealed that all the sequences of cow (B1-MZ817010) showed similarity with sequences from China (KY805824) except one B2-MZ817011 showed 98% with Uganda (KU206297). The sequence B3-MZ817012 from Buffalo showed 98% identity with sequence of 18S rRNA gene sequence reported from Pakistan (KY765562). While the sample from sheep i.e. B4-MZ817013 did not shared any clade with reported sequences as shown in figure 4.

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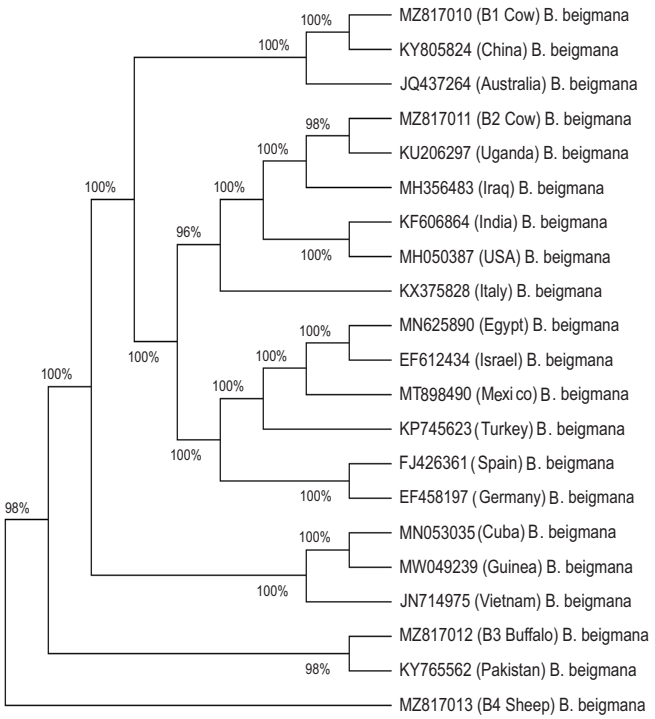

Figure 4: Phylogenetic Tree Constructed by Neighbor Joining Method from 18S rRNA Gene Sequence of *b. Bigemina*

Haplotype Analysis

Four haplotypes were recognized in current study. In haplotype network, Hap 2 haplotype was dominant as indicated by the size of circle. Hap 2 and Hap 1 represented the sequences from cow, Hap 3 from buffalo while Hap 4 from Sheep (figure 5A) [14].

Haplotype Networking of 18S rRNA Sequences from Current Along With Sequences from Other Countries

Haplotype network generated between current study sample and other selected sample from 24 countries represented total 16 haplotypes. Hap 1 haplotype of our study from cow and China shared the same circle indicating same evolutionary lineage. Hap 2 from cow made network with the haplotype reported from eight other countries while Hap 3 from buffalo form network with another Haplotype reported from Pakistan from same host and Hap 4 from sheep showed large number of mutation before making network with haplotype from Cuba and West Africa (figure 5B). Nucleotide diversity and neutrality test statistics is given in table 3, where Tjima's D represented the negative value which indicates population growth [14].

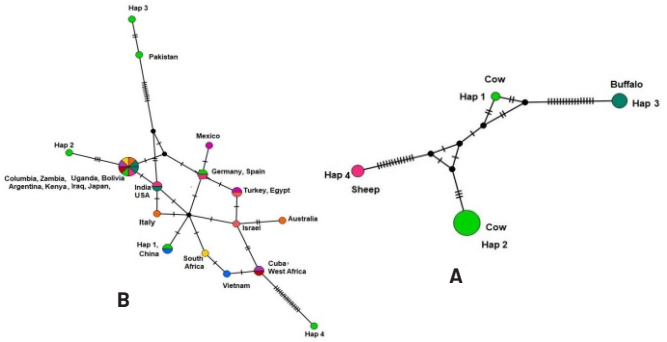

Figure 5: Haplotype Network of 18S rRNA: a) Sequences Generated from Samples of Current Study. b) Haplotype Network of Sequences from Current Study and Those Reported from other Countries

Size of circles is proportional to haplotype frequency and no. of dashes on lines exhibit the number of mutations between nodes representing different sequences. Nucleotide diversity and neutrality test statistics of *B. bigemina* is given in table 3, where the positive value of Tajima's D shows reduction in population size.

Table 3: Nucleotide Diversity and Neutrality Test Statistics of *B. bigemina*

Variables	Analysis of Sequences from Current Study	Current Study with Reported Sequences
Nucleotide Diversity (N)	0.0160036	0.154468
No. of Segregation Sites	14	36
No. of Parsimony-Informative Sites	0	16
Tajima's D	0.691885	-1.47792

DISCUSSION

Babesiosis is well known tick borne disease that causes enormous losses to livestock sector in Pakistan. In present study microscopic examination indicated 33% rate of prevalence in District Sialkot, Pakistan. Our results are in agreement with the study reported (34%) from Southern Punjab [15]. Other workers also reported prevalence of *Babesia* from some areas of Pakistan. Prevalence of *Babesia* in Kasur was reported 33.33% in 2008 [1]. From Khyber Pakhtunkhawa 27.5% prevalence was reported by PCR analysis and 9.83% by microscopic examination [16]. Another report from Khyber Pakhtunkhawa showed overall prevalence of 20.66% [17]. A report from Qadirabad revealed 18% positive by microscopic examination while 29% were positive by PCR [18]. While from Malakamd Agency lower prevalence was reported i.e., 12.49 % [19]. These variations in rate of prevalence can be attributed to the differences in climatic zones and environmental conditions of areas under study and rate of exposure to vector ticks and breeds of animals. The animals which are mostly at risk are exotic and their different breeds than local animal breeds [20]. For molecular and phylogenetic analysis 18S rRNA gene of *B. bigemina* was amplified as it is

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a part of functional core of ribosomes and it is also exposed to same selection pressure in every living organism [21]. Various studies have reported 18S rRNA gene as useful marker for molecular detection of *Babesiosis* [12, 22-26]. Sequence analysis showed genetic variations between samples from different hosts and even intra-specific variations in case of cow. The bioinformatics analysis revealed that samples of our study were also distinct from sequences reported from other countries and Pakistan, although, samples from cow showed close relationship with China, Uganda and Iraq, from buffalo to other samples of buffalo from Pakistan and sheep samples were not closely related genetically to other samples. The *B. bigemina* species with similar sequences having slight or more variations correlates to increase geographical distribution of this parasite species where it has potential disease causing effect in Mammals [27]. Migration of hosts between different areas and colonization of new areas has potential effect on dynamics of parasite [28]. Seasonal migrations and trade has an impact on diversity, epidemiology and load of parasites [29]. Long distance migration of host animals increases the risk of spread of pathogenic organisms and facilitate their transmission. When organisms invade new areas different traits and environmental conditions affect their survival. The mutation and adaptive radiations play their role which makes organisms able to survive by adaptation and also causes these species to diverge from their ancestral species [30]. In present study four haplotypes were recognized from 18S rRNA gene sequences from District Sialkot, Pakistan while overall 16 haplotypes were recognized from analysis of 18S rRNA gene sequences from selected countries of world and current study. Presence of haplotypes in 18S rRNA gene sequence of *B. bigemina* can be attributed to the lack of bottleneck effect and unequal rate of crossing over [31]. In addition to the prevalence of emergent strains of *B. bigemina* in District Sialkot, Pakistan, existence of haplotypes can also be attributed to the pathogenicity of Babesia species and resistance of B. *bigemina* against immune response of host [32, 33].

CONCLUSIONS

It can be concluded that this study provides insight into molecular and phylogenetic analysis of *B. bigemina* to assess the relationship between isolated strains and previously reported strains. In District Sialkot, *B. bigemina* was detected as dominant species responsible for *babesiosis* which was confirmed by PCR and sequencing of 18S rRNA gene of *B. bigemina.* This study was helpful in tracking *babesiosis* in district Sialkot, Pakistan. Haplotyping has revealed the presence of genetic variants among different and same host species.

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Author's Contribution

Conceptualization: AWQ
Methodology: DM
Formal analysis: AWQ
Writing, review and editing: AWQ, DM

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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Physiological Effects of Alloxan on Serum Glucose Levels and Liver Function Test in Male Rabbit

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ABSTRACT

Diabetes is a metabolic disorder characterized by elevated blood glucose levels that can lead to various complications. Exploring the physiological alterations in rabbits can provide valuable insights for the development of therapeutic interventions. This research delves into the impact of diabetes on the physiological and biochemical parameters of male rabbits. **Objectives:** To compare the physiological parameters like body temperature, heart rate, respiration rate, and oxygen saturation) and body weight and biochemical parameters, including blood glucose levels, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, lactate dehydrogenase (LDH), and total protein levels in diabetic and non-diabetic rabbits. **Methods:** An experiment on 30 male rabbits divided into diabetic and control groups measured physiological parameters like body temperature, heart rate, respiration rate, and oxygen saturation. Body weight and blood glucose levels were tracked, and blood samples were taken for ALT, AST, creatinine, LDH, cholesterol, triglycerides and total protein levels. Statistical analysis was conducted to compare the physiological and biochemical parameters between the diabetic and control groups. **Results:** The results showed that induced diabetes in male rabbits affects their physiological and biochemical parameters significantly. Diabetic rabbits had lower body temperature, heart rate, respiration rate, and oxygen saturation compared to the control group. They also had higher body weight and blood glucose levels. Biochemical analysis showed increased ALT, AST, and creatinine levels, and decreased LDH and total protein levels in diabetic rabbits. **Conclusions:** These results demonstrate the extensive impact of diabetes on rabbit physiology and biochemistry, offering insights for future diabetes research.

INTRODUCTION

Diabetes is a metabolic disorder characterized by persistent high levels of glucose in the blood due to abnormalities in insulin regulation. It poses a significant global health challenge, leading to mortality, morbidity, and substantial economic consequences. Type I diabetes arises from an insufficient production of insulin, while type II diabetes stems from the body's inability to effectively utilize insulin; both types require treatment for over 40% of affected individuals [1]. It is unsurprising that a substantial amount of research is currently underway, aimed at investigating the etiology, diagnosis, and management of this debilitating condition [2]. Animal testing for diabetes uses toxic chemicals like streptozotocin [3, 4] transgenic rodents used for studying diabetes are becoming increasingly popular. A significant critique of utilizing rodents stems from their abbreviated lifespan, which may preclude the manifestation of clinical complications commonly observed in humans who have suffered from diabetes for a prolonged period. In some cases, felines, canines, porcine species and nonhuman primates are employed as alternatives [5]. Rabbits present a viable and often neglected option for conducting chronic diabetes experiments due to their manageable nature. Rabbits are less phylogenetically advanced than cats, dogs, pigs, and primates. Rabbits live 5-8 years, longer than rodents. As research subjects, rabbits possess advantageous attributes such as their manageable size, extended lifespan, amiable dispositions, ease of handling, and cost-effectiveness [6]. Our extensive exploration of PubMed unveiled that within the past decade, a multitude of

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studies, exceeding 1000 in number, have harnessed rabbits as a viable model for diabetes [7]. Nonetheless, the majority of these investigations involved rabbits with diabetes for a limited duration, typically ranging from weeks to a mere two months [8]. Studies of such brevity have severely restricted the ability to delve into diabetic research, given the multitude of complications linked to diabetes that require a prolonged period to manifest [5]. Elevated blood glucose is a feature of all types of diabetes because of a relative or total lack of the hormone insulin, which is secreted by the islets of Langerhans in the pancreas. Insulin lowers blood sugar and regulates metabolism [9]. Substances influence the production and release of hormone. Nutrient secretagogues raise ATP levels, while non-nutrient secretagogues stimulate brain pathways. Certain sugars do not require insulin to enter cells. Pancreatic secretion is influenced by glucose levels. Insulin binds with a receptor in the cell membrane to activate tyrosine kinase activity [10]. Guides metabolism and muscle transfer, using muscle glycogen for energy and stimulating lipid/glycogen production while inhibiting lipolysis gluconeogenesis. Insulin and growth hormone/IGF-I work together to prevent hypoglycemia, with growth hormone being produced when the extracellular component activates AT. Counter-regulatory hormones such as Glucocorticoids and catecholamines regulate metabolic processes by modulating the activity of enzymes through phosphorylation/dephosphorylation mechanisms, which are influenced by the balance between insulin and glucagon levels [11]. We have established a robust rabbit model for type 1 diabetes research. Alloxan disrupts pancreatic cells, resulting in hyperglycemia. This model proves to be an effective tool in exploring new treatments for diabetes.

This study aimed to investigate the effects of this disease on rabbits, and this paper expounds upon management strategies, as well as the physiological, biochemical, and hepatic glucose test results of the rabbits.

METHODS

A total of 30 young male rabbits of 8–10 weeks old with an average weight of 1.85 ±0.13 kg was divided into two groups: 15 with diabetes and 15 healthy. They were housed individually in cages with controlled temperature and humidity, and provided with a specific diet and water. Environmental enrichment was also provided. The animals were parasite-free and acclimatized for at least seven days before experimentation began. Rabbits were systematically weighed on a weekly basis over the six-week duration of the study, with their weights being meticulously documented. Prior to the administration of alloxan, the rabbits were gently anesthetized with ketamine hydrochloride at a dosage of 30 mg/kg and xylazine at 3 mg/kg (administered intramuscularly). To prevent dryness, a carefully applied ointment of artificial tears (Butler Animal Health Supply, Dublin, OH) was administered to the surface of each eye. The rabbit cohorts were induced into a diabetic state through the administration of a solitary intravenous dose of 150 mg/kg of alloxan monohydrate (Sigma, St. Louis, MO) dissolved in 0.9% NaCl [12]. The rabbit's body temperature was meticulously maintained using a warm water circulating blanket (Gaymar T Pump, Gaymar Industries Inc., Orchard Park, NY). Parameters such as heart rate, respiratory rate, body temperature, and SpO2 were closely monitored while the animals were under anesthesia and during their recovery. Alloxan monohydrate was dissolved in saline and administered to rabbits intravenously. To prevent hypoglycemia, glucose was administered at intervals and oral glucose solution provided. Rabbits with low blood glucose levels received a second dose of alloxan to maintain levels above 300 mg/dl. Rabbits injected with alloxan to induce diabetes had blood glucose monitored regularly. Insulin given based on glucose levels. Samples collected weekly for one months of experiment. Insulin dosage for rabbits determined by blood glucose curve after trial dose. Final dosage set if peak BGL over 350 mg/dl and trough BGL 50 mg/dl or higher. Illness symptoms prompt new curve. Regular monitoring with blood glucose meter. Morning BGL over 350 mg/dl, Novolin-R insulin administered SC daily. Blood specimens were obtained for examination. The blood was drawn from the central auricular artery using a 25-gauge needle. Plasma was acquired through the process of centrifugation of the blood at 2500 g for 20 minutes at a temperature of 4°C, and was subsequently stored at -20°C. The aforementioned analyzer was employed to assess levels of LDH, AST, ALT, total protein, cholesterol, TG, BUN, and creatinine. The examination was performed using the Roche Cobas Mira Plus chemistry analyzer and associated reagents. The results were presented as the mean value accompanied by its corresponding standard error (SEM). The Student's t-test was used to compare the two groups using statistical software from GraphPad Prism Software, Inc. based in San Diego, California. A significance threshold of $P < 0.05$ was deemed statistically significant.

RESULTS

Body weight of Group A (induced) and Group B (Control) rabbits were observed before and after 6 weeks of induction. Significant difference ($P<0.05$) was observed in body weight of rabbits among A and B groups (Figure 1).

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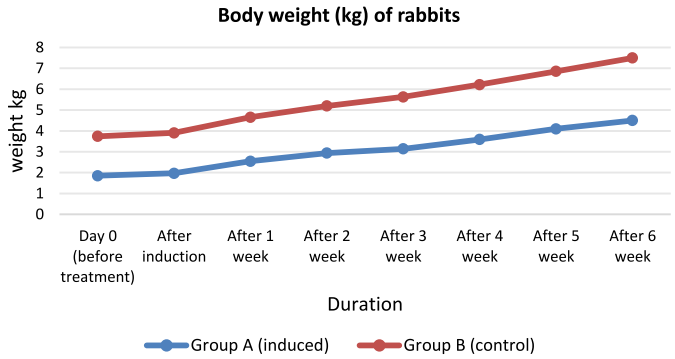

Figure 1: Body weight (kg) of Rabbits from Day 0 to 6-week Duration in Group A(Induced)and Group B(Control).

Different physiological parameters such as body temperature, heart rate, respiration rate and oxygen saturation of Group A (induced) and Group B (Control) rabbits were observed. There was a decline in body temperature, heart rate, respiration rate and oxygen saturation of induced rabbits as compared to non-induced rabbits. Significant difference (P<0.05) was observed in body temperature, heart rate, respiration rate and oxygen saturation of rabbits among A and B groups(Figure 2).

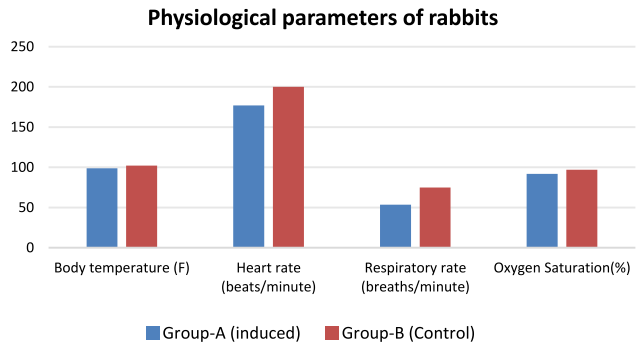

Figure 2: Physiological Parameters of Rabbits

Blood glucose level of Group A (induced) and Group B (Control) rabbits were observed before and after 24 hours of treatment. Significant difference (P<0.05) was observed in blood glucose level of rabbits among A and B groups (Table 1).

Table 1: Hourly Blood Glucose Level of Rabbits

Host	Group A (induced) Mean ± SEM	Group B (control) Mean ± SEM
Day 0 (before treatment)	141.4 ± 6.44[a]	144.3 ± 5.82[a]
After 2 hours of Alloxan Injection	350.20 ± 18.99[a]	142.4 ± 4.33[b]
After 4 hours of Alloxan Injection	280.64 ± 15.46[a]	141.3 ± 4.22[b]
After 8 hours of Alloxan Injection	211.55 ± 11.36[a]	145.1 ± 3.85[b]
After 12 hours of Alloxan Injection	200.84 ± 8.33[a]	138.2 ± 2.33[b]
After 24 hours of Alloxan Injection	196.35 ± 5.33[a]	140.3 ± 5.66[b]

Alphabets (a, b) among the mean indicates significant (P<0.05) difference between the groups.

Blood glucose level of Group A (induced) and Group B (Control)rabbits were observed before and after 4 weeks of induction. Significant difference (P<0.05) was observed in blood glucose level of rabbits among A and B groups (Table 2).

Table 2: Weekly Blood Glucose Level of rabbits

Duration	Group A (induced) Mean ± SEM	Group B (control) Mean ± SEM
Week 1	205.44 ± 15.4[a]	150.5 ± 3.58[b]
Week 2	200.66 ± 11.52[a]	147.4 ± 2.33[b]
Week 3	198.97 ± 6.39[a]	145.3 ± 2.52[b]
Week 4	199.5 ± 5.22[a]	142.1 ± 2.11[b]
Week 5	201 ± 4.2[a]	140 ± 2.22[b]
Week 6	205 ± 3.2	141 ± 3.2[b]

Alphabets (a, b) among the mean indicates significant (P<0.05) difference between the groups.

Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) of Group A (induced) and Group B (Control) rabbits were determined before and after injecting Alloxan. ALT and AST values of induced rabbits were increased as compared to control group rabbits. Significant difference (P<0.05) was observed in ALT and AST of rabbits among A and B groups(Table 3).

Table 3: Liver Function Test of Rabbits

Duration	Group A (induced) Mean ± SEM	Group B (control) Mean ± SEM
ALT (U/L) Before	21 ± 1.55[a]	20.6 ± 1.43[a]
ALT (U/L) After Alloxan Injection	37.8 ± 2.66[a]	20.6 ± 1.50[b]
AST (U/L) Before	97 ± 1.59[a]	97 ± 1.66[a]
AST (U/L) After Alloxan Injection	195 ± 2.55[a]	97 ± 1.64[b]

Alphabets (a, b) among the mean indicates significant (p<0.05) difference between the groups.

Plasma lactate dehydrogenase (LDH), creatinine and total protein levels of Group A (induced) and Group B (Control) rabbits were determined before and after injecting Alloxan. The results showed reduction in LDH and total protein levels of induced rabbits as compared to non-induced rabbits. Significant difference (P<0.05) was observed in LDH and total protein of rabbits among A and B groups. Creatinine of induced rabbits was increased as compared to control group rabbits. Significant difference (P<0.05) was observed in creatinine of rabbits among A and B groups (Table 4).

Table 4: Serum Biochemical Parameters in Diabetic and Control Group Rabbits

Duration	Group A (induced) Mean ± SEM	Group B (control) Mean ± SEM
LDH (mg/dl) before	69.44 ± 1.14[a]	69.55 ± 1.23[a]
LDH (mg/dl) after Alloxan Inj.	123 ± 2.33[b]	70.11 ± 4.66[a]
Creatinine (mg/dl) value before	1.264 ± 0.13[a]	1.257 ± 0.12[a]
Creatinine (mg/dl) after Alloxan Inj.	2.476 ± 0.88[a]	1.261 ± 0.09[b]
Total protein(mg/dl) before treatment	5.50 ± 1.44[a]	5.54 ± 1.22[a]
Total protein (mg/dl) after Alloxan Inj.	7.80 ± 1.53[a]	5.40 ± 1.64[b]

The blood urea, triglyceride and cholesterol levels of

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rabbits in the Group A (induced) and Group B (Control) were assessed both before and after the administration of Alloxan. Th results showed a decrease in urea, triglyceride and cholesterol levels in the induced rabbits as compared to the non-induced rabbits. A statistically significant difference ($P<0.05$) was noted in the urea, triglyceride and cholesterol levels between groups A and B (Figure 3).

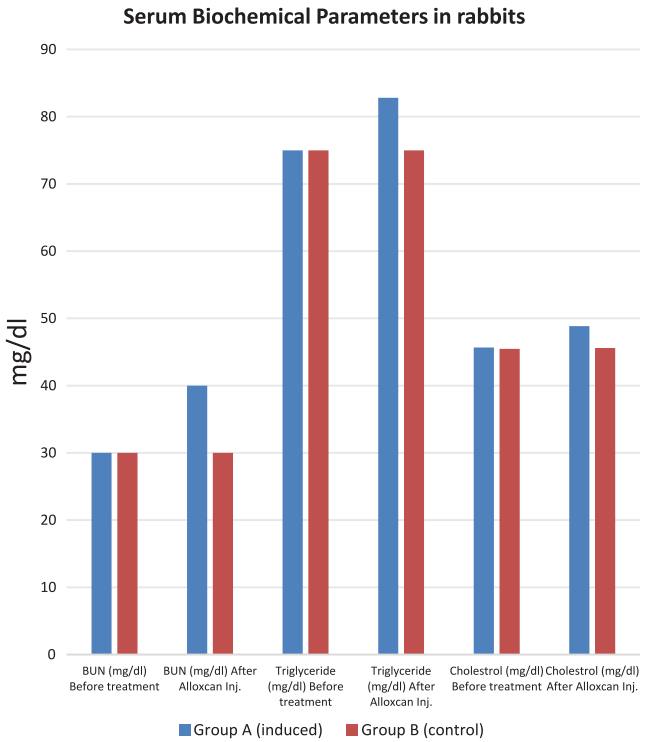

Figure 3: Comparison of Blood Urea, Triglyceride and Cholesterol Levels in Induced and Control Group Rabbits

DISCUSSION

In recent years, there has been a surge in research delving into the impacts of diabetes on rabbits during relatively short timeframes. However, the utility of these investigations is inherently constrained by the use of short-term diabetic rabbit models, given that human diabetes is characterized by its chronic nature and the gradual onset of diabetic complications. A perusal of the PubMed database yielded a limited number of studies in which diabetic rabbits were maintained for 3-6 months for the purpose of pharmacological or growth factor research [13], with only a singular study documenting the year-long survival of three alloxan-induced diabetic rabbits for the evaluation of their aortic intima-media [14]. Arif *et al.* [15], studied the effects of alloxan on kidney injury in toxin-induced diabetic rabbits. The treated animals showed unconsciousness, hypothermia, high blood urea, and low blood glucose levels. Hyperglycemia and pancreatic islet necrosis were observed, leading to the rapid induction of diabetes in animals like rats. This research indicates similarities between animal and human diabetes symptoms [16, 17]. The onset of diabetes was correlated with a significant elevation in plasma glucose concentrations. The high blood sugar levels observed in Tables 1 and 2 are linked to a notable decrease in insulin levels in fully developed diabetes mellitus as well as a decrease in glucose uptake by muscle and fat cells [18]. The higher glucose levels in the diabetic group supplemented with starch clearly stem from dietary factors. Research has indicated that total carbohydrate intake is a reliable predictor of postprandial glucose levels [19, 20]. The glucose concentration exhibited a notable increase in diabetic rabbits (as illustrated in table 1 & 2). The glucose concentration exhibited a notable increase in diabetic rabbits (as illustrated in table 3 & 4). Flockhart & Larsen [21] illustrated variations in blood sugar levels and insulin sensitivity among the elderly, showing elevated levels, while Bando *et al.* [22] found no seasonal fluctuations in glucose levels. The heightened glucose concentration observed in diabetic rabbits. Furthermore, a decline in physical activity during the winter months has been documented in various studies [23]. This situation necessitates a rapid release of insulin from deteriorating beta cells, ultimately resulting in a hypoglycemic state around six hours after injection, particularly in animals that have been fasting. This increased hypoglycemia is highlighted [24]. After the administration of Alloxan into an animal's body [4] explains that blood glucose levels follow a predictable pattern, increasing within the first two hours primarily due to the breakdown of liver glycogen [25]. The response to toxic and diabetogenic doses varies greatly among different species and even within the same species when it comes to the use of Alloxan [26]. Therefore, the safe diabetogenic dosage is relatively restricted in each animal, as even a slight overdose can be harmful, eventually leading to death primarily due to renal tubular cell necrosis resulting from high doses administered. In this study, a less toxic/diabetogenic dose of 100mg/kg was used, but it did not always result in persistent diabetes in the tested rabbits, as sixty percent required a secondary dose to maintain chronic hyperglycemia, although all rabbits survived until the end of the experiment. Additionally, recovery from this condition could occur either through the multiplication of surviving beta cells after the initial injection or through the production of new beta cells generated from the exocrine pancreas' duct epithelium [27]. The slight increase in serum total protein levels in diabetic rabbits (Tables 4) is generally in line with previous studies [28, 29] that have shown elevated serum total protein levels in diabetic rats. Conversely, other researchers [28, 30] have reported a notable decrease in total protein levels in alloxan-induced diabetic rats. However, our findings indicate that diabetic rabbit groups exhibited lower levels of albumin. Hypoalbuminemia is a

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prevalent issue in diabetic animals and is often associated with diabetic nephropathy [30]. Microalbuminuria is frequently used as a diagnostic marker for early-stage diabetic nephropathy in humans [31]. The decline in serum total protein levels in diabetics has been linked to the inhibition of oxidative phosphorylation, leading to reduced protein synthesis, increased catabolic processes, and diminished protein absorption [31]. The decrease in serum total protein levels in both diabetic and non-diabetic groups (Table 4 & Fig. 3). Previous research has documented a decrease in serum total protein levels in rabbits exposed to heat stress [32, 33]. In the diabetic rabbit group, elevated serum urea levels were sustained (Figure 3). This discovery is in line with studies that have documented increased urea levels in alloxan-induced diabetic rabbits [34, 35] and diabetic individuals [36, 37]. The surge in urea nitrogen in diabetes can be attributed to heightened catabolism of both hepatic and plasma proteins that coincide with gluconeogenesis [38]. It has been proposed that the increase in urea synthesis in streptozotocin-induced diabetes in rats is a result of enzyme induction by glucagon [39]. The higher serum urea level in starch-supplemented diabetic rabbits in the current investigation is congruent with other studies [40] that have reported heightened urea levels in rabbits fed a high carbohydrate-low fat diet. The escalation of urea observed during winter in the present study may be linked to increased food consumption by rabbits and/or hemoconcentration. Elevated serum urea levels were also observed in New Zealand White rabbits [41]. The data suggests that diabetic rabbits exhibited decreased levels of serum creatinine (Table 4). This discovery aligns with previous studies [28, 29] that demonstrated a significant reduction in creatinine levels in alloxan-induced diabetic rats. Analysis of the impact of a high carbohydrate-low fat diet in rabbits showed a marked decrease in serum creatinine levels [41]. However, elevated levels of creatinine may be attributed to conditions related to extensive muscle breakdown, as observed in poorly managed diabetes mellitus [42]. The heightened creatinine levels in the test groups (Table 3) could be linked to increased tissue breakdown associated with elevated levels of glucocorticoid hormones. The current response of creatinine in rabbits corroborates earlier research [43]. Furthermore, diabetic rabbits exhibited elevated serum cholesterol levels (Figure 3). The escalation in cholesterol levels, linked to insulin deficiency, is attributed to heightened plasma concentrations of VLDL and LDL. Similarly, previous studies have documented a rise in cholesterol levels in experimentally induced diabetes in alloxan-diabetic rats [44].

CONCLUSIONS

Based on the findings delineated in this research, it is deduced that the induction of diabetes in rabbits through alloxan administration results in noteworthy modifications in blood biochemical, physiological parameters, and liver function evaluations. Biochemical analysis showed increased ALT, AST, and creatinine levels, and decreased LDH and total protein levels in diabetic rabbits. Diabetic rabbits had lower body temperature, heart rate, respiration rate, and oxygen saturation compared to the control group. They also had higher body weight and blood glucose levels. Additional investigations are necessary to elucidate the concomitant variations in electrolyte levels and acid-base equilibrium.

Author's Contribution

Conceptualization: IAP, ABK
Methodology: IAP
Formal analysis: RSB
Writing, review and editing: JS

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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