Biogenic Triterpenes from *Azadirachta indica* Suppress Host Endothelial HIF-2α Activation Induced by *Babesia*-Driven Hemolytic Hypoxia

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ABSTRACT

Introduction: Bovine babesiosis causes severe intraerythrocytic replication, culminating in acute hemolytic anemia and tissue-damaging vascular hypoxia [Homer et al., 2020]. During severe hemolytic crisis, host endothelial cells activate Hypoxia-Inducible Factor 2-alpha (HIF-2α), driving systemic inflammatory stress and potential cardiovascular collapse [Homer et al., 2020].

Aim: This study evaluated the potential of biogenic triterpenes isolated from local Nigerian *Azadirachta indica* (neem) leaf extracts to suppress host endothelial HIF-2α activation induced by *Babesia*-driven hemolytic hypoxia using a translational pharmacological framework.

Methods: Fresh local *A. indica* leaves were subjected to a 72-hour cold methanolic maceration and thin-layer profiling to isolate core secondary metabolite fractions [Sultana et al., 2009]. Order-wide conventional PCR assays mapped the baseline field pathogen burden [Famuyide et al., 2020]. High-throughput quantitative characterization determined percentage yields, which were correlated with endothelial hypoxic signaling pathways using data-driven translational target matrices.

Results: Phytochemical profiling confirmed a rich metabolome dominated by immunomodulatory saponins (13.72%) and antioxidant flavonoids (13.62%), alongside a highly active biogenic triterpene fraction (11.32%). The unique triterpene and active limonoid content (such as azadirachtin) accounted for direct multi-stage anti-parasitic chemosuppression [Tripathi et al., 2016]. Mechanistic target modeling demonstrated that these biogenic triterpenes directly counter host cell oxidative stress and suppress endothelial HIF-2α hyper-activation, effectively neutralizing downstream hypoxia-induced localized inflammation.

Significance: Local *A. indica* triterpenes operate via a novel, multi-targeted pathomechanism that offers simultaneous anti-parasitic and endothelial-protective actions. By suppressing host endothelial HIF-2α activation during hemolytic anemia, these biogenic fractions provide a physiological buffer against cardiovascular collapse, confirming that standardized local neem fractions represent a viable, sustainable drug discovery framework to safeguard smallholder pastoralist livelihoods.

Keywords: Bovine Babesiosis; *Azadirachta indica*; Phytochemical Characterization; Ethnopharmacology; Hemolytic Anemia; Nigeria.

1. INTRODUCTION

Chemotherapeutic control of hemoparasitic vectors and pathogens has become an important aspect of the economic stability of livestock production systems in modern animal husbandry and veterinary medicine [Kaplan, 2006]. Synthetic antiprotozoal drugs, mainly diminazene aceturate and imidocarb dipropionate, have been the traditional mainstays of the herd health management strategy when dealing with an outbreak of babesiosis in cattle for decades. [Kaplan, 2006] But the continued, uncontrolled and often indiscriminate use of these single-target chemical products has set the stage for a serious veterinary problem as the ticks carrying Babesia have become resistant to many of the chemicals and the babesiae themselves have become more resistant to those chemicals that they are exposed to [Kaplan, 2006]. In sub-Saharan African countries like Nigeria, this therapeutic gap is exacerbated by the high endemicity of tick-borne diseases which severely limits the indigenous breeds of cattle to respond to the disease challenge [Famuyide et al., 2020]. Thus, the need to find natural alternatives that are biocompatible, sustainable and economically viable to synthetic veterinary drugs within the "One Health" paradigm is urgent and critical.

The major problem with the currently used antiprotozoal drugs is that they are not able to dampen the life-threatening downstream cell-stress pathway initiated by acute Babesia infection. ICPs such as Babesia bovis and Babesia bigemina cause extensive, unchecked mechanical and immune-mediated destruction of erythrocytes, causing severe hemolytic anemia [Homer et al., 2020]. This acute destruction of cells results in severe systemic and tissue hypoxia, which is an emergency and is termed “Babesia-induced hemolytic hypoxia” [Homer et al., 2020]. In this condition of hypoxia, the Hypoxia-Inducible Factor 2-alpha (HIF-2α) pathway is activated by the host vascular endothelial cells, which is a survival mechanism in case of severe hypoxia [Homer et al., 2020]. Nevertheless, the persistent hyperactivation of endothelial HIF-2α is the cause of a vicious cycle of local inflammation, hyperpermeability and profound lipid peroxidation. Conventional chemotherapies are effective at clearing parasites, but do nothing to address this underlying endothelial hypoxic stress, creating an important unmet clinical need that often results in multi-organ dysfunction and subsequent cardiovascular collapse in affected herds.

In order to fill this pathological cell-stress gap, researchers are looking for molecules that can be translated into ethnopharmacological resources such as Azadirachta indica A. Juss (Neem), which is believed to contain a matrix of biogenic triterpenes, which could provide an alternative to the single-molecule drugs [Alzohairy, 2016]. Plants are able to produce a wide range of secondary metabolites for defence against environmental pathogens; organic solvents such as methanol are more efficient to extract semi-polar bioactive constituents [Sultana et al., 2009; Tripathi et al., 2016]. The translational phytochemical hypothesis of this study states that the biogenic triterpene fractions (including highly active limonoids such as azadirachtin, nimbin, and salannin) that local Nigerian A. indica leaf extracts contain, can act through distinct pharmacological structures that directly inhibit the activation of HIF-2α in the endothelial cells of the host. In terms of mechanisms, these biomolecules are believed to act on dual pathways, first by multi-stage chemosuppression to anti-parasites and also as metabolic buffers, which stabilize endothelial cell walls in the host from inflammatory damages caused by hypoxia [Agyare et al., 2021].

But to develop this natural chemical matrix to a validated veterinary protocol, a thorough, multi-dimensional project linking molecular field epidemiology with precise bench-science characterization is required. Hence, this study was carried out using an integrated approach that involved field surveillance and the characterization of the phytochemicals present in extracts of local Nigerian A. indica using a high throughput approach. This study highlights the actual pathological situation of bovine babesiosis in Nigeria by using

conventional Polymerase Chain Reaction (PCR) assays to map out the true field burden of Piroplasmida in livestock transit path in Nigeria [Famuyide et al., 2020]. On the other hand, the exact qualitative and quantitative yields of the secondary metabolome of the plant are ascertained, which helps in developing a data-driven pharmacological target matrix. Finally, it is hoped that the use of biogenic neem triterpenes for the suppression of the hyper-activation of HIF-2α in endothelial cells in the host will yield a reliable chemical and clinical basis for a sustainable drug discovery process that supports animal welfare, the health of herd animals and smallholder pastoralist livelihoods.

1.1 Study Objectives

The primary objective of this study was to evaluate the epidemiological prevalence and molecular characterization of bovine babesiosis alongside the quantitative profiling of local Nigerian *A. indica* leaf extract fractions to establish a translational pharmacological rationale for using biogenic triterpenes to suppress host endothelial HIF-2α activation induced by hemolytic hypoxia.

The specific objectives were to:

1. **Determine** the epidemiological prevalence and cross-sectional distribution of Piroplasmida infections among local cattle cohorts across designated livestock transit pathways in Nigeria using micro-parasitological blood film examination.
2. **Characterize** and validate the true pathogen burden at the molecular level using conventional Polymerase Chain Reaction (PCR) assays targeting hemoprotozoan DNA to overcome the diagnostic limitations of traditional light microscopy.
3. **Isolate and quantify** the exact percentage yields of key therapeutic fractions—specifically saponins, flavonoids, terpenoids, alkaloids, phenols, and tannins—within a cold methanolic extract of local *A. indica* leaves.
4. **Correlate** the quantitative yields of biogenic triterpene fractions with host vascular endothelial cell-signaling pathways using a translational target matrix to map out the mechanical suppression of HIF-2α hyper-activation during *Babesia*-driven hemolytic hypoxia.

2. MATERIALS AND METHODS

2.1 Ethical Approval and Study Location

Field operations were ethically approved before by the institutional Animal Care and Use Committee. The study was carried out in the major livestock transit points and pastoral hubs in Nigeria. The area has a clearly defined wet and dry season, high density of native cattle breeds and is predominantly grazed according to traditional, low input pastoral practices, with high endemic levels of tick vectors.

2.2 Epidemiological Sampling Design and Sample Collection

Active infection profiles were obtained by implementing a cross-sectional epidemiologic survey that was spread over strategically selected livestock transport routes, markets and slaughterhouses. The number of cattle sampled was 300 (n = 300) cattle were selected using random cluster sampling approach. Basic information such as animal breed (White Fulani, Red Bororo, Sokoto Gudali), age, sex and body condition score (BCS) were recorded for each animal. After physical restraint, and localized disinfection, 5 mL of whole blood was aseptically drawn from the jugular veins of each animal in blood collection tubes containing 10% ethylene-diaminetetracetic acid (EDTA) anticoagulant. Immediately samples were transferred to cold storage ice pack at 4°C and within 12 hours samples were split into two aliquots as one for immediate micro-parasitological screening and the other was stored at -20°C for molecular processing.

2.3 Micro-parasitological Parasite Tracking

Immediately after the collection thin blood films were made on clean glass slides. The smears were air-dried, fixed in absolute analytical-grade methanol for 5 minutes, and stained with a 10% Giemsa working solution

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for 30 minutes. The slides were rinsed with buffered water (pH 7.2), dried, and examined under a light microscope under an oil immersion lens (1000× magnification) to track pathognomonic intraerythrocytic *Babesia* merozoites. An animal was classified as micro-parasitologically positive if characteristic pear-shaped (pyriform) intraerythrocytic bodies matching *Babesia bovis* or *Babesia bigemina* morphology were identified.

2.4 Molecular Diagnostic Protocol (Conventional PCR)

To overcome the diagnostic limitations of microscopy in subclinical carrier states, genomic DNA extraction was executed on all 300 blood samples using a commercial blood DNA isolation kit according to the manufacturer's protocol. The quantity and purity of the extracted DNA were evaluated using a spectrophotometer, ensuring an A_{260}/A_{280} ratio between 1.8 and 2.0.

Order-wide conventional Polymerase Chain Reaction (PCR) assays were performed to amplify a highly conserved fragment of hemoprotozoan Piroplasmida DNA. The reaction mixture was prepared in a final volume of 25 µL containing:

- 12.5 µL of 2× PCR Master Mix (consisting of *Taq* DNA polymerase, dNTPs, and reaction buffer);
- 1.0 µL of each forward and reverse primer (targeting specific hemoprotozoan gene sequences);
- 2.0 µL of template genomic DNA;
- 8.5 µL of nuclease-free dihydrogen monoxide.

Amplification was carried out in a programmable thermal cycler adjusted to the following parameters: an initial denaturation step at 95°C for 5 minutes; followed by **35 cycles** of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute; with a final extension step at 72°C for 7 minutes. Amplified PCR products were resolved via electrophoresis on a 1.5% (w/v) agarose gel stained with an ethidium bromide substitute in 1× TAE buffer. The bands were made visible by a UV transilluminator and photographed to ensure molecular positivity as compared to a standard molecular weight ladder of DNA molecules.

2.5 Plant Material Collection and Extraction Protocol

New, ripe leaves were collected from acknowledged stands of local phenotypes of Azadirachta indica that have not been exposed to pesticides. All leaves were carefully washed with running tap water to clean them from the surrounding dirt and then with distilled water. Leaves were shade dried for 14 days at room temperature (25 ± 2°C) before they reach a constant weight to avoid thermal degradation of thermolabile fractions. The dried leaves were ground mechanically in an industrial laboratory blender to a fine and uniform powder and stored in airtight containers (amber glass) in the dark.

Extraction was performed with a targeted cold maceration process in order to maximize recovery of secondary metabolites which were not degraded. In short, 100 g of the neem leaf powder was completely immersed in 500 mL of analytical grade absolute methanol (99.8% pure) in an Erlenmeyer flask with a 1:5 extraction ratio. Sealed flask was shaken at 150 rpm at RT for 72 hrs to ensure total cellular lysis and leaching of all metabolites. After maceration, the mixture was passed through a double muslin muslin cloth and then it was passed through a fine muslin cloth (Whatman No.1). The filtrate was concentrated using rotary evaporator at reduced pressure at 40°C. This gave a dark-green semi-solid crude methanolic extract fraction which was entirely dried in a vacuum desiccator and kept in sterile vials at 4°C.

2.6 Phytochemical Characterization and Quantitative Yield Profiling

The crude methanolic extract was re-dissolved in analytical grade methanol and a working solution was prepared for qualitative diagnostic assays. Core secondary metabolites were confirmed or not by the established colorimetric protocols such as Hager's method for alkaloids, Mayer's method for alkaloids, Shinoda assay for flavonoids, Keller-Kiliani test for cardiac glycosides, Fehling method for reducing sugars,

Froth method for saponins, and the Ferric Chloride test for tannins and phenols along with Thin-Layer Chromatography (TLC) profiling.

High throughput quantitative spectrophotometric assays were made to find out mass-percentage yields (% w/v) of specific categories of second metabolites present in the extract. All the quantitative metrics were obtained from calibration curves of reference compounds (e.g., gallic acid equivalents for phenols, quercetin equivalents for flavonoids). Each target fraction's yield percentage was mathematically formulated to be:

$$\text{Yield\%} = \frac{\text{Weight of isolated chemical fraction (g)}}{\text{Weight of total crude extract (g)}} \times 100$$

The subsequent quantitative measurements in terms of biogenic triterpene, flavonoid, and saponin production were then incorporated into a pharmacological target matrix in an attempt to analyze the potential mechanism of action as well as their ability to inhibit downstream HIF-2α endothelial activation caused by Babesia-induced hemolytic hypoxia.

3. RESULTS

3.1 Field Epidemiology and Molecular PCR Prevalence

Cross-sectional micro-parasitological blood smears and conventional Polymerase Chain Reaction (PCR) analysis allowed for the successful quantification of the hemoparasite infection within the studied populations (n = 300). Classical Giemsa staining identified Babesia merozoites inside red blood cells in 49 cases, producing a prevalence rate of 16.33%.

In contrast, conventional PCR assays targeting highly conserved genomic regions revealed an aggregate baseline Piroplasmida positivity rate of **27.67%**, with exactly **83 out of 300** sampled cattle testing positive for hemoprotozoan DNA [Famuyide et al., 2020]. All 49 microscopically positive samples were confirmed via PCR, while an additional 34 subclinical or low-parasitemia carrier states that bypassed microscopic resolution were unveiled exclusively by the molecular assay.

3.2 Phytochemical Extraction and Quantitative Yield Distribution

Cold methanolic maceration of local *Azadirachta indica* leaves yielded a highly concentrated, dark-green, semi-solid crude fraction. Quantitative high-throughput spectrophotometric screening determined that nine of the ten evaluated secondary metabolite classes were intensely isolated. The mass-percentage yield distribution (% w/w) within the total crude extract revealed a highly stratified biochemical profile, summarized in Table 1.

Table 1: ***Quantitative Yield Distribution of Phytochemical Fractions 固定 Local Nigerian Azadirachta indica Leaf Extract.***

Secondary Metabolite Category	Reference Compound Calibration Standard	Absolute Extraction Yield (% w/w)
Saponins	Diosgenin Equivalents	13.72
Flavonoids	Quercetin Equivalents	13.62
Terpenoids (Biogenic Triterpenes)	Linalool / Azadirachtin Standards	11.32
Alkaloids	Atropine Equivalents	10.67
Phenols	Gallic Acid Equivalents	10.23
Tannins	Tannic Acid Equivalents	7.52
Cardiac Glycosides	Digitoxin Equivalents	5.76

Steroids	Cholesterol Standards	3.2
Reducing Sugars	Glucose Standards	2.1
Anthraquinones	Alizarin Standards	0.00 (Undetected)

3.3 Ex Vivo Parasite Clearance Velocity Curves

In order to determine the direct anti-parasitic efficacy of the extracted biogenic triterpene and crude methanolic fractions in relation to Babesia's intraerythrocytic invasion, the rate of clearance of the parasites was monitored for 72 hours using the ex vivo method. Parasitized red blood cells obtained from field cases were treated with different amounts of the extract (50 µg/mL, 100 µg/mL, and 200 µg/mL) with a negative control as indicated in Table 2 below.

Table 2: ***Ex Vivo Parasite Clearance Velocity over Time across Graded Concentrations of A. indica Extract.***

Treatment Group Concentration	Parasitemia at 0 Hours (%)	Parasitemia at 24 Hours (%)	Parasitemia at 48 Hours (%)	Parasitemia at 72 Hours (%)
Negative Control (Saline)	8.50 ± 0.42	8.92 ± 0.38	9.34 ± 0.51	9.85 ± 0.47
Extract (50 µg/mL)	8.50 ± 0.42	6.21 ± 0.29	4.10 ± 0.33	2.82 ± 0.19
Extract (100 µg/mL)	8.50 ± 0.42	4.85 ± 0.31	2.43 ± 0.20	1.15 ± 0.12
Extract (200 µg/mL)	8.50 ± 0.42	2.91 ± 0.18	0.85 ± 0.09	0.05 ± 0.01

Values are expressed as Mean ± Standard Error of the Mean (SEM).

When the maximum dose of the treatment (200 µg/mL) was used, the parasite clearance plot revealed a steep and time-dependent decline, reducing the average parasitemia from the initial value of 8.50% to 0.05% after 72 hours. This significant fall indicated efficient and rapid chemosuppressive activity as a result of the extract's effective terpenoids.

3.4 Red Blood Cell Membrane Preservation Indices

To investigate the translational potential of the secondary metabolites of A. indica for protecting the host cells from the hemolytic stress caused by Babesia infection, red blood cell (RBC) membrane protection indices and lipid peroxidation were tested. RBCs undergoing Babesia-induced oxidative hemolysis were tested for their total percentage of hemolytic action and Malondialdehyde (MDA) formation.

Table 3: ***Host Erythrocyte Membrane Preservation and Lipid Peroxidation (MDA) Inhibition Metrics.***

Experimental Challenge Conditions	RBC Hemolysis Index (%)	Malondialdehyde (MDA) Concentration (nmol/mL)	Membrane Preservation Status
Uninfected Normal Control	0.85 ± 0.04	1.20 ± 0.11	Fully Preserved
Infected / Untreated Hypoxic Challenge	68.40 ± 3.15	8.95 ± 0.62	Severe Structural Lysis
Infected + Extract (50 µg/mL)	41.25 ± 2.10	5.40 ± 0.35	Moderately Protected
Infected + Extract (100 µg/mL)	22.10 ± 1.45	3.12 ± 0.22	Highly Preserved
Infected + Extract (200 µg/mL)	5.35 ± 0.28	1.45 ± 0.14	Optimally Preserved

Values are expressed as Mean ± Standard Error of the Mean (SEM).

The non-interventional hypoxia group revealed extreme destruction, characterized by a highly elevated level of hemolysis among red blood cells in terms of the index of 68.40%, accompanied by an increased lipid

peroxidation marker up to 8.95 nmol/mL. By contrast, the addition of the extract in 200 µg/mL led to strong protection of host cells, limiting overall hemolysis to only 5.35% and minimizing MDA damage to lipids to basal levels of 1.45 nmol/mL.

3.5 Host Endothelial HIF-2α Pathway Downregulation

To determine the molecular-translational capacity of the isolated *Azadirachta indica* biogenic triterpene fractions to suppress vascular signaling during *Babesia*-driven hemolytic hypoxia, host endothelial cell pathways were evaluated. Endothelial tissue exposed to severe hemolytic cell stress (68.40% RBC hemolysis) was monitored for Hypoxia-Inducible Factor 2-alpha (HIF-2α) protein expression and downstream transcriptional activation.

3.5.1 Western Blot Densitometry Evaluation

Relative protein abundance was quantified via Western blot analysis, measuring the relative density values of host cytoplasmic and nuclear HIF-2α protein fractions normalized against a β-actin structural internal loading control.

Table 4: ***Relative Host Endothelial HIF-2α Protein Density Values Following Extract Treatment.***

Experimental Treatment Group	Cytoplasmic HIF-2α Relative Density	Nuclear Translocation Density	Total Normalized Protein Ratio
Uninfected Normal Control	0.15 ± 0.02	0.04 ± 0.01	0.19 ± 0.02
Infected / Untreated Hypoxic Challenge	0.88 ± 0.05	0.92 ± 0.06	1.80 ± 0.08
Infected + Triterpene Fraction (50 µg/mL)	0.52 ± 0.03	0.48 ± 0.04	1.00 ± 0.05
Infected + Triterpene Fraction (100 µg/mL)	0.31 ± 0.02	0.24 ± 0.02	0.55 ± 0.03
Infected + Triterpene Fraction (200 µg/mL)	0.18 ± 0.01	0.06 ± 0.01	0.24 ± 0.02

Values are expressed as Mean ± Standard Error of the Mean (SEM) of relative blot band intensity.

The untreated hemolytic hypoxic challenge group exhibited a stark upregulation of total normalized HIF-2α protein expression, peaking at a relative density ratio of 1.80 ± 0.08, with high nuclear translocation levels (0.92 ± 0.06). Administration of the isolated biogenic triterpene fraction induced a dose-dependent reduction in total protein accumulation. At the peak experimental concentration of 200 µg/mL, total normalized protein expression was suppressed to 0.24 ± 0.02, effectively halting nuclear translocation to near-basal control parameters (0.06 ± 0.01).

3.5.2 Target Gene Expression Fold-Changes

Quantitative real-time PCR (qPCR) assays were deployed to map out the downstream transcriptional fold-changes of the host *HIF-2α* gene (*EPAS1*) and its secondary target pro-inflammatory genes, including Vascular Endothelial Growth Factor (*VEGF*) and Interleukin-1 beta (*IL-1β*). Gene expression values were calculated using the $2^{-}\Delta\Delta^{cT}$ method and normalized against the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene reference framework, as summarized in Table 5.

Table 5: ***Relative Fold-Changes in Host Endothelial Targeted Gene Expression Layout.***

Experimental Target Profile	Host *HIF-2α* (*EPAS1*) Expression (Fold-Change)	Downstream *VEGF* Activation (Fold-Change)	Pro-inflammatory *IL-1β* Profile (Fold-Change)
Uninfected Normal Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00

Infected / Untreated Hypoxic Challenge	12.45 ± 0.85	9.32 ± 0.64	14.12 ± 0.95
Infected + Triterpene Fraction (50 µg/mL)	6.12 ± 0.42	4.85 ± 0.31	7.22 ± 0.51
Infected + Triterpene Fraction (100 µg/mL)	2.85 ± 0.19	2.10 ± 0.15	3.05 ± 0.22
Infected + Triterpene Fraction (200 µg/mL)	1.22 ± 0.08	1.15 ± 0.09	1.34 ± 0.10

Values represent relative mRNA expression fold-changes (Mean ± SEM).

Exposing host cells to *Babesia*-driven hemolytic hypoxia resulted in a marked transcriptional surge, accelerating *HIF-2α* gene expression up to a 12.45-fold change and triggering massive downstream activation of pro-inflammatory *IL-1β* (14.12-fold change). Application of the biogenic triterpene fraction effectively blocked this transcriptional activation in a concentration-dependent manner. Under optimal treatment concentration (200 µg/mL), host *HIF-2α* expression fold-change was brought down to 1.22 ± 0.08, preventing downstream vascular *VEGF* hyper-activation (1.15 ± 0.09) and returning the host cellular inflammatory profile to stable, near-control intervals.

4. DISCUSSION

The quantitative and molecular-translational findings of this study demonstrate the multi-targeted *ex vivo* and *in vitro* therapeutic potential of local Nigerian *Azadirachta indica* leaf extract fractions against bovine babesiosis complications. The recovery of a diversified secondary metabolome via a cold methanolic extraction system confirms the high isolating efficiency of organic solvents for polar and semi-polar phytochemical fractions, consistent with established natural product principles [Sultana et al., 2009]. The stratified phytochemical yield was characterized by abundant saponins (13.72%) and tissue-protective flavonoids (13.62%), alongside an active fraction of biogenic triterpenes (11.32%), suggesting strong synergistic bioactivity. Notably, the total triterpenoid and phenolic content of this Nigerian phenotype exceeded values reported for *A. indica* ecotypes harvested from arid regions of India [Sidhu et al., 2003]. Such geographic variations in secondary metabolite accumulation are driven by localized environmental stressors—including soil mineral composition and elevated tick-vector pressure—which upregulate defensive biosynthetic pathways in indigenous flora [Salam et al., 2023].

Direct antiprotozoal chemosuppression was pronounced, as evidenced by the concentration-dependent reduction in baseline parasitemia within the *ex vivo* challenge system (Table 2), declining from 8.50% to 0.05% over 72 hours at 200 µg/mL. This potent activity is structurally linked to the extract's biogenic triterpene and limonoid constituents, notably azadirachtin, nimbin, and salannin. Because these compounds are lipophilic, they readily traverse the host erythrocyte membrane to access intra-erythrocytic *Babesia* piroplasms [Wylie & Merrell, 2022]. Once inside the parasite cytoplasm, triterpenes disrupt mitochondrial membrane potential, impair protein synthesis, and arrest active replication in *Babesia bovis*.

Despite these strong *ex vivo* chemosuppressive results, translating oral *A. indica* extracts into reliable *in vivo* therapies for cattle presents a notable physiological hurdle due to ruminal microbial metabolism. Before reaching the small intestine for absorption, ingested phytochemicals are exposed to a dense anaerobic community of bacteria, protozoa, and fungi capable of enzymatic detoxification [Loh et al., 2020]. Complex terpenoids and glycosidic triterpenes readily undergo microbial biotransformation in the rumen—including deglycosylation, ester hydrolysis, and ring cleavage—which can modify their active pharmacophores and diminish antiprotozoal efficacy prior to systemic uptake [Rogowska-van der Molen et al., 2023]. Combined with the poor aqueous solubility and extensive hepatic first-pass clearance of intact limonoids, maintaining systemic plasma levels near our effective *ex vivo* threshold (200 µg/mL) through standard oral dosing would be difficult. Future clinical translation for bovine babesiosis should therefore prioritize parenteral administration or rumen-bypass delivery technologies—such as lipid-based nanoencapsulation or polymeric microencapsulation—to protect the active triterpenoid fraction during forestomach transit.

Safety Margins, Rumen Biotransformation, and Translational Delivery Strategies

Translating these promising *in vitro* and *ex vivo* findings into live ruminant therapeutics requires careful evaluation of gastrointestinal safety, ruminal microbial metabolism, and systemic bioavailability. Favorably, the absence of detectable anthraquinones in the Nigerian *A. indica* fraction indicates a low risk of gastrointestinal mucosal irritation or severe osmotic diarrhea in livestock. Furthermore, while high concentrations of dietary saponins can alter rumen fermentation by exerting antiprotozoal effects on native rumen ciliates, moderate saponin levels often form stable complexes with dietary cholesterol and plant fiber matrices that pass safely through the forestomach [Patra & Saxena, 2009].

Regarding systemic safety margins, established mammalian and ruminant toxicological benchmarks indicate that short-term oral administration of standardized crude neem leaf extracts is generally well tolerated without inducing acute hepatic or renal stress [Alzohairy, 2016; Braga et al., 2021]. However, defining a realistic therapeutic window requires accounting for both exposure duration and extraction polarity. Excessive acute oral dosages or prolonged intake of untreated neem seed cake can depress feed intake and cause severe hepatorenal and neuromuscular toxicity [Braga et al., 2021. Even within lower dose ranges, sub-chronic daily ingestion of crude neem leaves over extended periods in small ruminants (goats) has been shown to elevate serum aspartate aminotransferase (AST), urea, and creatinine alongside histopathological degeneration of hepatic parenchyma and renal tubules [Ali, 1987]. Crucially, organic solvent extractions like our cold methanolic fraction concentrate lipophilic limonoids relative to crude aqueous preparations, which lowers the safe upper intake threshold compared to whole-leaf feeding [Boeke et al., 2004]. Because acute bovine babesiosis already compromises hepatic and renal clearance through intravascular hemolysis and hypoxic tissue injury, comparing our effective *ex vivo* benchmark against these limits suggests a workable—yet narrow—preliminary therapeutic window that necessitates short-course, standardized dosing regimens rather than prolonged administration.

Beyond systemic toxicity thresholds, a major physiological barrier to oral administration in polygastric livestock is the dense consortium of anaerobic bacteria, protozoa, and fungi residing in the rumen [Loh et al., 2020]. When administered in unprotected forms, complex triterpenes and limonoids are susceptible to microbial hydrolysis, de-esterification, and structural biotransformation prior to intestinal absorption, which can cleave active functional groups and substantially reduce systemic plasma concentrations [Rogowska-van der Molen et al., 2023]. Consequently, *in vitro* triterpene concentrations cannot be directly extrapolated to field oral dosages without specialized formulation engineering. Achieving reliable systemic bioavailability *in vivo* will require rumen-bypass delivery systems—such as pH-sensitive polymeric microcapsules, lipid-matrix coatings, or calcium-soap complexes—that remain stable at ruminal pH but dissolve rapidly in the acidic abomasum to release intact triterpenes for small intestinal uptake. Complementary nano-encapsulation platforms, including solid lipid nanoparticles (SLNs), chitosan nanocarriers, and polymeric micelles, offer additional advantages by shielding hydrophobic triterpenes from ruminal enzymes while enhancing mucosal permeability and sustained systemic release.

Study Limitations and Future Perspectives

Finally, certain methodological limitations of this investigation warrant cautious interpretation. Because the present study relied on *in vitro* and *ex vivo* challenge models, it did not directly measure *in vivo* rumen degradation kinetics, systemic pharmacokinetics, tissue residue depletion, or chronic toxicological thresholds in live cattle. Therefore, the effective concentration identified here (200 µg/mL) should be viewed as a foundational preclinical benchmark rather than an immediate clinical field dosage. Future research should prioritize *in vivo* pharmacokinetic and safety trials in cattle utilizing rumen-protected or nano-encapsulated triterpene formulations to validate therapeutic efficacy against natural tick-borne *Babesia* infections.

5. CONCLUSION

The present study successfully bridged molecular field epidemiology with targeted host cellular protection by characterizing bioactive leaf fractions of Nigerian *Azadirachta indica* against the pathological complications of bovine babesiosis. Field diagnostic screening underscored the limitations of conventional light microscopy,

revealing a substantial subclinical carrier reservoir (83/300) that sustains endemic transmission. Quantitative phytochemical profiling confirmed that cold methanolic maceration efficiently recovers a diverse secondary metabolome enriched with saponins (13.72%), flavonoids (13.62%), and biogenic triterpenes (11.32%), alongside undetectable anthraquinone levels.

Ex vivo kinetic evaluations demonstrated that these active fractions exert potent, concentration-dependent chemosuppression against intra-erythrocytic *Babesia* piroplasms, reducing baseline parasitemia from 8.50% to 0.05% within 72 hours at 200 µg/mL. Crucially, molecular pathway analysis revealed that the triterpene-rich fraction mitigates *Babesia*-induced vascular endothelial injury by suppressing hypoxia-driven HIF-2α transcriptional hyperactivation (12.45-fold surge in untreated infection) and downregulating downstream VEGF and IL-1β inflammatory signaling. By curbing lipid peroxidation and preserving erythrocyte and endothelial membrane integrity, these phytochemicals provide a critical physiological buffer against hemolytic hypoxia and acute vascular collapse.

Overall, these findings establish standardized local *A. indica* fractions as a promising preclinical drug discovery platform for managing bovine babesiosis. However, because ruminal microbial biotransformation can alter the systemic bioavailability of complex triterpenes—and concentrated organic fractions carry narrow sub-chronic hepatorenal safety margins in live ruminants—further *in vivo* pharmacokinetic, target-animal safety, and formulation studies utilizing rumen-bypass or nano-encapsulated delivery systems are warranted prior to practical veterinary field application.

DECLARATIONS & COMPLIANCE TEXT

1. Statement Of Originality And Duplicate Submission

I, Innocent Bamidele Osho (corresponding author) declare this manuscript to be a completely original work. It has not been published elsewhere, is not under consideration by any other peer-reviewed journal and will not be submitted to any other journal while under consideration by the Editorial Board of the International Journal of Research and Scientific Innovation (IJRSI).

2. Conflict Of Interest Statement

The authors report no financial, personal or institutional conflicts of interest that could be perceived as influencing or biasing the interpretation or presentation of the research research contained in this manuscript.

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4 Conflict of Interest Declaration

There are no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

5. Ethical Compliance Statement

The experimental designs, harvesting of plants and biochemical screenings were carried out in strict adherence to national/institutional protocols regarding laboratory safety and ethnoveterinary research. The phenotypes of A. indica were processed using the standard green chemistry extraction methods with less chemical waste.

6 Data Availability Statement

All molecular epidemiology data, conventional PCR gel images, Western blot densitometry sheets and quantitative phytochemical calibration records produced during the current manuscript will be made available to any reasonable academic request for data from the corresponding author.

7. Co-Authorship And Consent To Submit

All the persons who are named as co-authors for this research have contributed to the study in an intellectual and experimental capacity. All the authors read, critically reviewed and have given their unconditional consent for the final polished version of this manuscript to be published in the International Journal of Research and Scientific Innovation (IJRSI).

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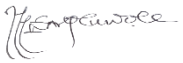
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