**Effect of *Cajanus cajan* (L) Millsp. leafy extracts on haematological function, Oxidative stress, and inflammatory markers in spleen tissue of *Plasmodium berghei*-infected Mice**

**ABSTRACTS**

**Objective:** Oxidative stress and inflammation are common complications of malaria, with the infection’s effects ranging from simple to life-threatening multiple organ failure. This study investigates the protective effects of *Cajanus cajan* leafy extracts on haematological dysfunction, oxidative stress, and inflammation in organs of *Plasmodium berghei*-infected mice. **Methods:** Forty-nine mice were randomly divided into seven groups, including normal and experimental controls. Aqueous and ethanolic extracts of *Cajanus cajan* were applied at 200 and 400 mg/kg b.w. concentrations concurrently to explore their protective effects on malaria-induced blood and spleen damage. The effects were evaluated on haematological parameters; red blood cells (RBCs), haemolobin (hg), packed cell volume (PCV), white blood cells (WBCs), Lymphocytes and monocyte levels in the blood as well as oxidative biomarkers; reactive oxygen species (ROS) and Thiobarbituric (TBARS) levels, antioxidant enzymes; superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST), inflammatory markers; pro-inflammatory tumor necrosis factor-α (TNF-α) and anti-inflammatory IL-10 mediators in spleen tissues of *Plasmodium berghei-*infected mice. **Results**: It was revealed that all treated groups improved haematological functions, restored antioxidant enzyme’s activities, suppressed oxidative and pro-inflammatory markers, and boosted anti-inflammatory marker in the spleen tissue compared to *Plasmodium berghei* untreated group. Higher protective effects were observed in *Plasmodium berghei*-infected group treated at the high dose of both extracts (CcA 400 and CcE 400). **Conclusion:** Following these findings, incorporating *Cajanus cajan* leaf extracts into malaria treatment regimens could protect against oxidative stress and inflammation in spleen tissues.

Keywords: *Cajanus cajan, leaf extracts, Spleen, biomarkers, inflammatory markers, Plasmodium berghei*

**List of Abbreviations**

PLA-B – *Plasmodium berghei*-infected mice, NC – Normal control, TNF-α – Turmor necrosis factor alpha, IL-10 – Interleukin-10, RBCS – Red blood cells, WBCS – White blood cells, PCV – Packed cell volume, Hg – Haemoglobin

**INTRODUCTION**

Malaria is a parasitic infectious disease caused by plasmodium species, affecting millions of people globally, with an estimated 608,000 deaths each year. Most prevalent cases of this *plasmodium*-causing disease have been traceable to tropical and subtropical regions, where environmental factors such as seasonal rainfall, climate, and inadequate healthcare contribute to its spread. Nigeria, in particular is one of the African countries that suffers the greatest burden of malaria, with approximately 30% of this parasitic invasion (WHO., 2024). The vast numbers of malaria deaths occur among children, pregnant women, and individuals who lack acquired immunity, causing anemia and increasing their vulnerability to other diseases (WHO., 2023). The extent to which a parasitecan parasitize the host tissue determines its clinical impact, which can range from mild symptoms to life-threatening multiple organ failure and death (Clark *et al*., 1986). Despite the implementation of various control measures to eradicate this endemic disease, such as using insecticide-treated nets, applying mosquito repellents, developing vaccines, raising public awareness, managing the environment, and administering anti-malarial drugs, there is growing concern over several emergency factors. These factors include, reduced drug efficacy, high cost, and the emergence of anti-malarial drug resistance, which posed a formidable force against acquiring successful preventative strategies (Kumar and Prasad, 2024). The factors stem from an overemphasis on eliminating the malaria vectors (mosquitoes), and the parasite, rather than focusing on the critical mechanisms driving the parasite’s invasion of host tissues. This invasion triggers a cascade of mechanisms including increased oxidative stress markers, and inflammatory agents as well as decreased in the host’s antioxidant defenses. The host immunological response, marked by the systemic activation and production of pro-inflammatory agents is very crucial for malaria control (Coban *et al*., 2018). However, if these inflammatory markers are activated over time and their pathways are not regulated, their impact can result into impairment of vital organs, which overwhelm the host’s intrinsic antioxidant defenses, resulting in oxidative damage to cells and tissues (Muller, 2004; Gomes *et al*., 2022). The spleen is a crucial organs responsible for erythropoiesis (production of red blood cells), hematopoiesis (production of blood cells), and ridding the system of pathogens and damaged red blood cells. It also plays a vital role in the immune system by producing white blood cells and antibodies to help combat infections (Lewis *et al*., 2019). However, research has shown that long term exposure to malaria can lead to an enlarged spleen a condition known as splenomegaly, distorted haematological functions, which can leads to systemic inflammatory responses (Balaji *et al.,* 2020; Ferrer *et al*., 2014; Henry *et al*., 2022). The consequences may be in form of anemia, high infection susceptibility, and organ failure. Furthermore, considering the economic costs associated with drugs synthesis and their potential side effects, scientists have expressed a growing interest in harnessing the power of medicinal plants to combat malaria (Lemma *et al*., 2017). Remarkably, over 80% of local populations in many tropical countries depend on these natural remedies for effective malaria treatment (Odoh *et al*., 2018). This reliance underscores the urgent need to integrate antioxidant-rich plants as strategy to address this widespread (Ojueromi *et al*., 2022). One noteworthy example is the *Cajanus cajan* which has demonstrated potential antioxidant effects and protective characteristics against oxidative stressors (Orni *et al*., 2018). Various parts of *Cajanus cajan* have been utilized for their biological activities throughout history with keen interest in their therapeutic benefits (Gargi *et al*., 2022). Besides their uses in folklore, studies have reported various biological and pharmacological actions of *Cajanus cajan* leaves (Orni *et al*., 2018), as well as their significant amount of phytoconstituents that contributed to their antioxidant properties (Sarkar *et al*., 2009)*.* Duker-Eshum *et al*. (2004) reported that *Cajanus cajan* leaves possess hypoglycaemic, anti-sickling and anti-plasmodial properties particularly due to the antiplasmodial activities of Longistylin A, C and Betulinic acid constituent compounds against Chloroquine- sensitive *plasmodium falciparium* strains. Furthermore, research by Hassan *et al*. (2016) demonstrated an anti-inflammatory effect of *Cajanus cajan* seed extracts at different doses (200 and 400 mg/kg) in carrageenan-induced rats. Vo *et al*. (2020) studied antioxidant and anti-inflammatory activities of *Cajanus cajan* roots extracts at different ethanolic concentrations (50% and 95%) in the lipopolysaccharide-stimulated RAW 264.7 cells and reported increased superoxide dismutase and catalase activities as well as improved anti-inflammatory effects in 95% ethanolic root extracts of *Cajanus cajan*. Despite the extensive research on *Cajanus cajan* plants, information regarding the antioxidant, anti-inflammatory and haematological functions in spleen tissues remain limited. Therefore, this study evaluates the protective effects of the *Cajanus cajan* aqueous and ethanolicleaf extracts on the antioxidant’s enzyme status, inflammatory markers, and hematological profiles in spleen tissues of *Plasmodium berghei*-infected mice.

**Effect of *Cajanus cajan* leafy extracts on haematological function, oxidative stress and inflammatory markers in blood and spleen tissues of mice infected with *Plasmodium berghei***



Figure 1. Schematic representation of impact of *Cajanus cajan* leafy extracts (200/400 mg/kg) on blood and spleen tissues of *Plasmodium berghei*-infected mice that are screened on haematological profile (RBCs, Hg, PCV, WBCs, Monocytes, and Lymphocytes counts), oxidative stress (ROS and TBARS), Antioxidant enzymes activity (SOD, CAT, and GST) and inflammatory markers (TNF-α, IL-10).

2.0 **Methods**

2.1 **Sample collection and preparation**

Fresh leaves of *Cajanus cajan* (L) were sourced from a farm in Ile-Oluji, Ondo State, Nigeria, and verified by the Centre for Research and Development (CERAD) at the Federal University of Technology, Akure, Nigeria. The leaves were air-dried until they reached a constant weight and then powdered. For the extraction process, both aqueous and ethanolic extractions were performed using 500 g of milled *Cajanus cajan* leaves. Each extraction utilized 3 L of distilled water and 96% ethanol, respectively, for a duration of 24 hours. The concentrates were obtained by freeze-drying and then reconstituted for analysis, following the method suggested by Oyebanji *et al*. (2018).

**2.2 Experimental animal**

Forty-nine (49) healthy matured male mice of about 10-12 weeks age and weighing between 20-25g were obtained for this study. The animals were acclimatized for two weeks and were provided with water and a commercial diet ad libitum. The animals were handled humanely and strict animal use and care protocols were followed before the beginning of the experiment.

**2.2.1 Parasite Inoculation**

Infected *Plasmodium berghei* (NK-65 strain) procured from the Institute of Medical Research and Training (IMRAT) at the University College Hospital (UCH), Ibadan, Nigeria were used as the donor mice. The inoculation of the mice was performed in accordance with the methodology established by David *et al*. (1983). The initial assessment of parasitemia levels in donor mice was conducted prior to inoculation by cutting off the tail tip and extruding the blood into a beaker containing 0.5 ml of normal saline. The parasitemia level of the donor mice was evaluated using rapid diagnostic malaria test strips for confirmation. Mice exhibiting higher levels of parasitized blood were sacrificed, and blood was obtained through cardiac puncture. This blood was processed by incorporating 0.2 ml of parasitized blood from donor mice into 9.8 ml of normal saline to inoculate the experimental mice according to (Tarazona *et al*., 2004, Cheesbrough *et al*., 2005). After seventy-two (72) hours post-infection, all *Plasmodium berghei*-infected mice underwent treatment via oral administration of 200/400 mg/kg/b.w. of aqueous and ethanolic extracts derived from the *Cajanus cajan* leaves in conjunction with standardized drug (chloroquine) using Hassan *et al*. (2016) method. The experimental animal groupings were outlined in the following protocol:

The mice were divided into (7) seven groups of seven mice each.

Group 1: Normal Control + basal diet + water (NC)

Group 2: *Plasmodium berghei-infected* with no treatment (PLA-B)

Group 3: *Plasmodium berghei* infected + Standard drug (Chloroquine) (10 mg/kg)

Group 4: *Plasmodium berghei* infected + Aqueous extract (200 mg/kg) (CcA200)

Group 5: *Plasmodium berghei* infected + Aqueous extract (400 mg/kg) (CcA400)

Group 6: *Plasmodium berghei* infected + Ethanolic extract (200 mg/kg) (CcE200)

Group 7: *Plasmodium berghei* infected + Ethanolic extract (400 mg/kg) (CcE400)

For five consecutive days, oral metal gavage was employed to administer treatments daily. In addition, blood was obtained by severing the jugular veins with a sterilized steel disposable scalpel (Shirasaki *et al*., 2012). Furthermore, all mice were sacrificed by cervical dislocations after the treatment period.

**2.2.2 Homogenates Preparation**

Mice's whole blood was obtained and preserved in ethylenediaminetetraacetic acid (EDTA) at 4°C. Subsequently, the spleen tissues were excised, kept on ice, and weighed. After washing in cold normal saline (0.9%), the tissues were homogenized in a 0.1 M sodium phosphate buffer (pH 7.4) using a Teflon homogenizer. The homogenates were centrifuged at 10,000 g for 20 minutes; the clear supernatant was collected for subsequent biochemical assays (Belle *et al*., 2004).

**2.3 Haematological Parameters Determination**

A comprehensive assessment of the mice's hematological profile was carried out using an Auto Hematology Analyzer. Blood was screened for the levels of red blood cells (RBCs), hemoglobin concentration (Hb), hematocrit (PCV), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBCs), monocyte and lymphocytes counts.

**2.4 Biochemical Studies (In Vivo)**

**2.4.1 Evaluation of oxidative markers and antioxidant enzymes in spleen of *Plasmodium berghei* mice**

The thiobarbituric acid-reactive substances (TBARS) formed during acid heating reaction in a mixture of spleen homogenate and standardized reagents were evaluated using the method described by Ohkawa *et al*. (1978). Reactive oxygen species (ROS) assay measured coloured N, N-dyethyl-paraphenyldiamine (DEPPD\*-) radicals generated from reactions between sample’s peroxides and Fe2+/Fe3+ from the reagents. This was analyzed spectrophotometrically at 505nm following Hayashi *et al*. (2007) methods. Catalase (CAT) activities were assessed according to the method described by Claiborne *et al*. (1995), which measured degradation of H2O2 following treatment of the samplewith a standard H2O2 solution and finally read at 240nm. Glutathione-S-transferase (GST) activities was assessed following Mannervik and Guthenberg, (1981), by measuring the rate at which reduced glutathione (GSH) formed conjugate with 1-Chloro-2,4-dinitrobenzene (CDNB) at 340nm. Superoxide dismutase (SOD) enzyme activity was measured based on the formazan produced during the assay mixture using method described by Prasad *et al*. (1999).

**2.4.2 Evaluation of Pro and anti-inflammatory markers in spleen of *Plasmodium berghei* mice**

The pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) as described by Paim *et al*. (2011), were measured using ELISA assay with Quatikine immunoassay kits (R&D systems, Minneapolis, MN), according to the manufacturer’s guidelines. Following the coating of microplate reader with streptavidin-HRP and conjugation with biotinylated antibody of the mice, while a chemiluminescence substrate generated was measured at 450nm (Stenken and Poschenrieder, 2015).

2.5 **Statistical Analysis**

The data from the replicates were aggregated and presented as mean ± standard deviation. One-way ANOVA and a post-hoc Tukey-Kramer test for multiple comparisons were used to determine statistical significance. This analysis used Prism software (GraphPad, version 6.0, Carlsbad, CA).

**3.0 RESULTS**

This study evaluates the spleen-protective properties of *Cajanus cajan* leafy extracts through various oxidative and inflammatory markers while considering an improved haematological profile in *Plasmodium berghei*-infected mice.

**3.1 Haematological profile of *Plasmodium berghei*-infected mice treated with *Cajanus cajan* leafy extracts**

A significant reduction of red blood cells (RBCs), Haemaglobin (Hg), and packed cell volume (PCV) were revealed by the untreated *Plasmodium berghei* infected mice (PLA-B) as shown in Table 1. The counts for RBCs, Hg and PCV in the PLA-B were 2.52±0.23 × 106/µL, 3.85±0.49 g/dl, and 19.7±0.07 % respectively. In contrast, the normal control exhibited counts of 5.08±0.06 × 106/µL,8.9±0.28 g/dl, and 32.7±0.30%. This data indicates an anemic condition in the untreated *Plasmodium berghei*-infected group due to parasitic infection. On the other hand, the groups treated with aqueous extracts of *Cajanus cajan* (CcA200/400) mg/kg), and ethanolic extracts (CcE200/400 mg/kg), along with the standard drug chloroquine, showed a significant improvement in RBCs levels, hemoglobin, and PCV percentages when compared to the untreated group. Notably, the CcA 200, CcA 400, and CcE 400 treatment groups demonstrated a marked increase in RBCs, hemoglobin, and PCV levels, indicating their effectiveness in restoring these blood parameters. The roles of white blood cells (WBCs) and their agranulocytes (monocytes and lymphocytes) as agents of an immune response against foreign invaders such as malaria parasites were evaluated. From table 2, the PLA-B group demonstrated a significant increase in the levels of WBCs, which measured 105.1±0.3 × 103/µL, Monocytes at 36.95±1.8 × 103/µL, and Lymphocytes at 33.50.±05 × 103/µL. In contrast, the normal control group had WBCs levels of 18.85±1.34 × 103/µL, Monocytes at 1.07±0.02 × 103/µL), and Lymphocytes at 5.47±0.61× 103/µL. In the PLA-B group, higher immune responses to infections were associated with elevated level of circulatory defensive agents, which prepared the body to combat *Plasmodium* invasion. Interestingly, the WBC and its agranulocytes (monocytes and lymphocytes) components were considerately reduced in all treated *Cajanus cajan* leafy extracts groups. Both dosages of aqueous extracts at (CcA 200/400) exhibited pronounced effects, indicating the protective benefits of *Cajanus cajan* in reducing parasitemia levels.

Table 1. **Haematological profile (RBCs, Hg, PCV, MCHC) of *Plasmodium berghei*-infected mice treated with *Cajanus cajan* leafy extracts**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Parameters | Red blood cells (RBCs) 103/µL | Haemoglobin(Hg) g/dL | Packed cell volume (PCV) % | Mean corpuscular haemoglobin conc. (MCHC) g/dL |
| NC | 5.08±0.06 |

|  |
| --- |
| 8.9±0.28 |

 | 32.65±0.80 | 27.6±0.64 |
| PLA-B | 2.52±0.23 | 3.85±0.49 | 19.7±0.85 | 22.4±0.07 |
| Chloro | 4.95±0.13 | 8.8±0.6 | 34.1±0.57 | 26.4±0.09 |
| CcA 200 | 4.70±0.02 | 10.8±0.3 | 33.6±0.57 | 32.1±0.42 |
| CcA 400 | 3.76±0.07 | 9.25±0.21 | 31.0±0.85 | 29.4±0.02 |
| CcE 200 | 3.4±0.13 | 6.3±0.14 | 28.7±0.91 | 34.8±1.13 |
| CcE 400 | 4.42±0.05 | 7.05±0.35 | 30.85±0.21 | 28.9±0.07 |

Values are means ± SEM, (n=3).

Table 2. **Haematological profile (WBCs, Monocytes, and Lymphocytes) of *Plasmodium berghei*-infected mice treated with *Cajanus cajan* leafy extracts**

|  |  |  |
| --- | --- | --- |
| **Parameters** | **White blood cells (WBCs) 103/µL** | **Agranulocytes** |
| **Monocytes** **103/µL** | **Lymphocytes****103/µL** |
| NC | 18.85±1.34 | 1.07±0.02 | 5.47±0.61 |
| PLA-B | 105.1±4.1 | 36.95±1.8 | 33.50±0.50 |
| Chloro | 28.38±0.29 | 1.97±0.18 | 4.05±0.63 |
| CcA 200 | 27.93±0.33 | 4.8±0.6 | 15.20±0.7 |
| CcA 400 | 29.93±0.74 | 9.8±0.53 | 16.23±0.33 |
| CcE 200 | 54.55±5.19 | 54.6±0.27 | 13.76±0.62 |
| CcE 400 | 51.30±0.42 | 7.18±1.0 | 16.55±0.12 |

Values are means ± SEM, (n=3)

**3.2 Oxidative stress biomarkers evaluation in spleen homogenates**

The study evaluated the effect of *Cajanus cajan* leaf extracts on oxidative biomarkers in mice infected with *Plasmodium berghei* spleen. The biomarkers assessed were reactive oxygen species (ROS) and Thiobarbituric acids (TBARS), both indicators of oxidative stress. The untreated infected group (PLA-B) exhibited a significant increase in ROS levels (1413.0 ± 0.4 µmol/L) and MDA (22.90 ± 1.24 µmol/L), as shown in Figures 2a and 2b. This finding suggests that *Plasmodium berghei* infection induces oxidative stress, likely as a result of the parasite's metabolic activities and the inflammatory response of the host. In contrast, the normal control (NC) group and all other treated groups showed a significant reduction in ROS and TBARS levels compared to the untreated group. Notably, a higher dose of *Cajanus cajan* extract (400 mg/kg) further decreased ROS and MDA levels more effectively than the lower dose (200 mg/kg). This higher dose proved slightly more effective than the NC and conventional drug treatments. Overall, these results demonstrate a dose-dependent antioxidant effect of *Cajanus cajan* leaf extract.



*Figure 2a. Reactive oxygen species (ROS) level in the splenic tissue homogenates of Plasmodium berghei-infected (PLA-B) mice treated with Chloroquine (Chloro), Cajanus cajan aqueous extracts (CcA200, CcA400) and Cajanus cajan ethanolic extracts (CcE200, CcE400), (n=7). Mean values with bar\* significantly different (p<0.05) compared to the normal control (NC), bar# significantly different (p<0.05) compared to the PLA-B*



*Figure 2b. Thiobarbituric acid reactive substance (TBARS) level in the splenic tissue homogenates of Plasmodium berghei-infected (PLA-B) mice treated with Chloroquine (Chloro), Cajanus cajan aqueous extracts (CcA200, CcA400) and Cajanus cajan ethanolic extracts (CcE200, CcE400), (n=7). Mean values with bar****\**** *significantly different (p<0.05) compared to the normal control (NC), bar****#*** *significantly different (p<0.05) compared to the PLA-B.*

**3.3 Antioxidant effects of *Cajanus cajan* leafy extracts on spleen homogenate of *Plasmodium berghei*-infected mice**

The evaluation of antioxidant enzyme activities in the spleen of *Plasmodium berghei-*infected mice (PLA-B) reveals significant findings regarding the compromised antioxidant defense mechanism resulting from malaria infection. The results presented in Figures 3a-c show significantly lower activities of superoxide dismutase (SOD) (128.68 ± 0.12 µm/min/mg), catalase (CAT) (0.018 ± 0.03 µ/g), and glutathione-S-transferase (GST µ/g) (34.3) in untreated infected mice (PLA-B). This decline illustrates the oxidative stress induced by *Plasmodium berghei* infection, which can lead to increased levels of reactive oxygen species (ROS) and subsequent cellular damage. In contrast, the normal control group, along with the groups treated with *Cajanus cajan*, exhibited increased activities of SOD, CAT, and GST antioxidant enzymes. Notably, the high-dose (400 mg/kg) aqueous and ethanolic treated groups not only restored the enzymatic antioxidant defense system and effectively mitigated the oxidative damage typically associated with malaria-infected tissues.

**Table 3: Total phenols and flavonoids content of *Cajanus cajan* aqueous extracts at different concentration**

|  |  |  |
| --- | --- | --- |
| Sample | Total phenol(mgGEA/g) | Total Flavonoid(mgQE/g) |
| CcA (0.1mg/mL) | 4.145098±0.45 | 0.747934±0.003 |
| CcA (0.2mg/mL) | 8.961765±0.12 | 1.454545±0.03 |

Values are means ± SEM, (n=3)



*Figure 3a. Superoxide dismutase (SOD) enzyme activities in the splenic tissue homogenates of Plasmodium berghei-infected (PLA-B) mice treated with Chloroquine (Chloro), Cajanus cajan aqueous extracts (CcA200, CcA400) and Cajanus cajan ethanolic extracts (CcE200, CcE400), (n=7). Mean values with bar\* significantly different (p<0.05) compared to the normal control (NC), bar# significantly different (p<0.05) compared to the PLA-B.*



*Figure 3b. Catalase (CAT) enzyme activities in the splenic tissue homogenates of Plasmodium berghei-infected (PLA-B) mice treated with Chloroquine (Chloro), Cajanus cajan aqueous extracts (CcA200, CcA400) and Cajanus cajan ethanolic extracts (CcE200, CcE400), (n=7). Mean values with bar\* significantly different (p<0.05) compared to the normal control (NC), bar# significantly different (p<0.05) compared to the PLA-B.*



*Figure 3c. Glutathione-S-transferase (GST) enzyme activities in the splenic tissue homogenates of Plasmodium berghei-infected (PLA-B) mice treated with Chloroquine (Chloro), Cajanus cajan aqueous extracts (CcA200, CcA400) and Cajanus cajan ethanolic extracts (CcE200, CcE400), (n=7). Mean values with bar\* significantly different (p<0.05) compared to the normal control (NC), bar# significantly different (p<0.05) compared to the PLA-B.*

**3.4.1 Pro-inflammatory biomarker in spleen homogenates of *Plasmodium berghei*-infected mice**

The investigation into pro-inflammatory mediators within the spleens of experimental mice yields striking results, as illustrated in Fig. 4a. The untreated *Plasmodium berghei*-infected (PLA-B) mice exhibited a dramatic elevation in tumor necrosis factor alpha (TNF-α) levels, soaring to 88.00 pg/mg, an alarming increase compared to the normal control (NC) group, which recorded a mere 45.00 pg/mg, as depicted in Fig. 4a. Intriguingly, administration of *Cajanus cajan* leafy extracts at various doses resulted in a significant and measurable reduction in TNF-α levels in the spleen. The data powerfully highlight the potential therapeutic benefits of *Cajanus cajan* in modulating inflammatory responses in the context of malaria infection.



*Figure 4a.Tumor necrosis factor alpha ( TNF-*α) *levels in the splenic tissue homogenates of Plasmodium berghei-infected (PLA-B) mice treated with Chloroquine (Chloro), Cajanus cajan aqueous extracts (CcA200, CcA400) and Cajanus cajan ethanolic extracts (CcE200, CcE400), (n=7). Mean values with bar\* significantly different (p<0.05) compared to the normal control (NC), bar# significantly different (p<0.05) compared to the PLA-B.*

**3.4.2 Anti-inflammatory biomarker in spleen homogenates of *Plasmodium berghei*-infected mice**

The results for the anti-inflammatory mediator IL-10 in the spleen of experimental mice are presented in Figure 4b. Untreated *Plasmodium berghei*-infected (PLA-B) mice exhibited significantly decreased levels of IL-10 (9.00 pg/mg) compared to the normal control (NC), which showed levels of 24.19 pg/mg. Interestingly, all the groups treated with *Cajanus cajan* leaf extracts demonstrated a significant increase in IL-10 levels in the spleen.



*Figure 4b. Interleukin-10 (IL-10) levels in the splenic tissue homogenates of Plasmodium berghei-infected (PLA-B) mice treated with Chloroquine (Chloro), Cajanus cajan aqueous extracts (CcA200, CcA400) and Cajanus cajan ethanolic extracts (CcE200, CcE400), (n=7). Mean values with bar\* significantly different (p<0.05) compared to the normal control (NC), bar# significantly different (p<0.05) compared to the PLA-B.*

**4.0 Discussion**

This study assesses the hematological profile and oxidative stress biomarkers in relation to immune responses in *Plasmodium berghei*-infected mice. It utilizes varying dosages of aqueous and ethanolic extracts from the leaves of *Cajanus cajan*, with an emphasis on their impact on blood and spleen tissues. Several studies have highlighted the significant negative impact of *Plasmodium* invasion on the overall health of blood and spleen based on their specific roles (Wang *et al*., 2021, Ghosh *and* Stumhofer, (2021)). *Plasmodium* selectively parasitizes red blood cells (RBCs) to the point the cell ruptures to release new active parasites that rapidly invade additional RBCs in a new cycle. As the cycle continues, more RBCs are destroyed, and a worsening anemic is prevalent (Autino *et al*., 2012, Mavondo and Mzingwane, (2017)). The spleen serves several important roles during malaria infection. First, it removes damaged and parasitized RBCs (pRBCs) from the circulation, and second, the spleen serves as major site for erythropoiesis and hematopoiesis (Ghosh *et al*., 2021). Lastly, it is responsible for synthesizing T and B cell responses that target specific pathogens created in different parts of the white pulp of the spleen (Thakur *et al*., 2020). Each of these functions is crucial for the control of malaria, and all are modulated following infection with *Plasmodium* parasites. In recent years, substantial progress has been made in our understanding of the events leading to the architectural changes to splenic tissue that accompany malaria infection (Almutawa *et al*., 2023). These include splenomegaly, white pulp hyperplasia (overproduction of cells), lymphocyte destruction, and prominent migration of macrophages into the white pulp regions. Interestingly, significant congestion of both RBCs and parasitized RBCs was also noted in the red pulp, particularly in fatal cases of malaria, and varying degrees of necrosis were observed (Ojueromi *et al*., 2024). Anemia, a common occurrence in malaria, is a direct consequence of the parasitization of red blood cells. A condition characterized by decreased hematocrit levels, called Packed Cell Volume (PCV), is affected by various factors such as increased hemolysis, inflammatory responses, and changes in hematopoiesis in the bone marrow (Paulson *et al*., 2020). As a result, the diminished capacity that leads to reduced erythropoiesis ultimately causes a decline in the quantity of healthy red blood cells in circulation (Zivot *et al*., 2018). Considering the blood tissues as the primary target of *Plasmodium* parasites, this study revealed an anemic state in the untreated *Plasmodium berghei*-infected (PLA-B) group, prevalence often experienced in high parasite load (Ozojiofor *et al*., 2021; Kotepui *et al*., 2015). PLA-B group established lower levels of the following blood parameters; Hg, RBCs, and PCV. The reduced levels in Hg, RBCs, and PCV are consistent with previous studies by Shaganuwan and Onyeyili, (2012), which documented variations in erythrocyte size, area, and volumes in different species of animals under different diseased states including malaria. However, the levels of these parameters in infected mice increased after being treated with *Cajanus cajan* extracts at different dosages. The elevated hemoglobin, RBCs, and PCV levels observed in *Cajanus cajan* extracts treated groups), support the notion of their anti-plasmodial properties attributed to their indispensable bioactive constituents that combat Plasmodium invasion (Ajaiyeoba *et al*., 2013; Havyarimana *et al*., 2023). Interestingly, the mean corpuscular hemoglobin concentration (MCHC), a test indicating the proportion of RBCs occupied by hemoglobin, revealed normochromic results in *Cajanus cajan* treated groups and hypochromic in the PLA-B group. This indicates an active regeneration of red blood cells that potentially serving to offset the depletion of RBCs. The observed improvement in anemia among the infected mice after treatment with *Cajanus cajan* extracts suggests a significant therapeutic effect.

The incursion of the *Plasmodium* parasite into the host initiated the synthesis of certain markers associated with oxidative stress, which may impair cell membranes and their functions, especially in certain organs like red blood cells, and spleen. During a peak parasitemia stage, spleen organ’s structure changes dramatically with white pulp component highly disorganized as a result of spontaneous splenic rupture, hematoin, and splenic enlargement and pathology in response to chronic malaria process (Del Portillo *et al*., 2012). The host's immune system also responds by augmenting the production of white blood cells to combat the infection, leading to inflammation and further damage to tissues. If left untreated, severe cases of malaria can result in organ failure and even death. In the quest of establishing cellular/tissue oxidative stress, specific and sensitive biomarkers like reactive oxygen species (ROS) and thiobarbituric acid (TBARS) have been widely evaluated in spleen tissues to check the involvement of oxidative stress in such tissues (Ashok *et al*., 2016, Liguori *et al*., 2018). Studies had established the oxidative stress and inflammation as major contributory factors in the pathogenesis of many diseases (Liguori *et al*., 2018, Lugrin *et al*., 2014). Excessive ROS often results in the activation of redox-sensitive kinases cascade and transcription factors, increasing the expressions of factors associated with an inflammatory response. According to this study, the *Plasmodium berghei-*infected (PLA-B) group that received no therapy shows significantly higher ROS and TBARS levels. This suggests that *Plasmodium berghei* infection causes the generation of oxidative stress within the spleen tissue, presumably as a consequence of the parasite’s metabolic processes and the host’s inflammatory responses. Nevertheless, the remaining treated groups exhibited a notable decrease in ROS and MDA levels, indicating a dose-dependent antioxidant effect of *Cajanus cajan* with CcA 400 mg/kg exhibiting highly antioxidative potentials. This finding aligns with the report by Offor *et al*. (2019), which highlighted the ameliorative effects of *Cajanus cajan* leaf aqueous extracts on markers of kidney disease.

Antioxidant plays a crucial role in regulating both the generation and metabolic transformation of reactive oxygen species (ROS) in cells and tissues, helping to prevent oxidative damages (Liu *et al*., 2018). The mechanisms by which antioxidants act include: direct reaction with the ROS via activities of antioxidant enzymes, inhibition of oxidant enzymes, and interaction with redox signaling pathways. Antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and other glutathione-dependent enzymes serve as first line of defense against cellular damages from ROS (Kapoor *et al*., 2019). Reports on all parts of *Cajanus cajan* plant were documented as good anti-oxidative agents for all kinds of diseases (Gargi *et al*., 2022; Orni *et al*., 2018). It is worth noting that spleen homogenates from the groups treated with *Cajanus cajan* leafy extracts showed signs of antioxidant effects. This was likely because the *Cajanus cajan* leafy extracts increased activities of SOD, CAT, and GST enzymes, which in turn reduced the oxidatively stressed states in the spleen tissues caused by the invasion of malaria parasites. However, counter results were recorded in the spleen homogenates of the PLA-B group with a decrease in the activities of these endogenous antioxidant enzymes which indicates the deleterious effects of ROS on this tissue. Interestingly, the *Cajanus cajan* leafy extracts effectively counteracted the damaging effects of oxidative stress caused by malaria parasite invasion by enhancing the activities of the endogenous antioxidant enzymes in the spleen tissues according to Vo *et al*. (2020). This highlights the potential use of *Cajanus cajan* leafy extracts as a therapeutic strategy for mitigating the effects of malaria and other diseases marked by oxidative stress.

Inflammation refers to the body’s first response orchestrated by the immune system in response to infections. This intricate process involves the isolation, clearance, and destroying injurious agents from host tissues. Central to this response is a group of signaling molecules known as cytokines, which act as messengers, mediators, and regulators of the immune and inflammatory responses (Kanny *et al*., 2019, Van *et al*., 1992). At low concentrations, cytokines bind to specific receptors on target cells, activating intracellular second messenger systems. This activation triggers the expression of various pro-inflammatory genes, ultimately leading to the enhanced production of transcriptional factors. One such cytokine mediator, tumor necrosis factor-α (TNF-α), plays a crucial role in amplifying response. It activates the critical transcription factor nuclear factor-kappa β (NF-kβ), a key player in the signal transduction cascade that can induce cellular death ((Byun *et al*., 2021, Kulyar *et al*., 2021). In this study, a compelling evidence of inflammation indicated by increased levels of TNF-α, a pro-inflammatory mediator capable of heightening inflammation in tissues was provided. This significant association is particularly evident in the spleen tissue of the PLA-B group. It is believed that activated macrophages, monocytes, lymphocytes and a variety of other immune cell types are crucial players in the intricate network of cytokine production, which is essential for immune response regulation (Kelly *et al*., 2018, Cavaillon *et al*., 1994). As mentioned, the PLA-B group exhibited significantly elevated counts in monocytes and lymphocytes which directly correlate with the increased TNF-α observed in this group. The elevated presence of these immune cells (monocytes, and lymphocytes) not only supports but actively enhances TNF-α production, underscoring the importance of their contribution in this context. In contrast, the treatment with *Cajanus cajan*-leaf extracts led to a noteworthy decrease in TNF-α level compared to the PLA-B group, providing strong evidence of its potent suppressive effect on pro-inflammatory mediators in spleen tissues. Additionally, Patel *et al*. (2014), establishing a connection regarding bioactive compounds isolated from *Cajanus cajan* leaves and the inhibition of pro-inflammatory markers, reinforcing these extracts significance in managing inflammation. The same biological mechanisms that initiate the inflammatory response are also responsible for activating the body’s anti-inflammatory defenses. These processes are effective for regulating immune response and preventing excessive inflammation that could lead to tissue damage. A key player in this regulatory network is interleukin-10 (IL-10), a potent anti-inflammatory mediator. IL-10 acts by inhibiting the synthesis of various pro-inflammatory cytokines including TNF- α and others (Lin *et al*., 2007, Chung *et al*., 2001), thereby helping to restore homeostasis in spleen tissues. Intriguingly, *Cajanus cajan* leaf extracts demonstrated a significant increase in IL-10 levels across all treated groups against reduced IL-10 levels exhibited by the PLA-B group, indicating their potential therapeutic role in inflammation management. Finally, the incorporation of *Cajanus cajan* leaf extracts into malaria treatment regimens offer protective benefits against oxidative stress and inflammation, which are common complications associated with malaria.

**5.0 Conclusion**

This study presents a remarkable efficacy of *Cajanus cajan* leafy extracts in significantly enhancing various parameters of the haematological profile, and boosting the activity of antioxidant enzymes. Notably, these extracts not only suppress the production of inflammatory cytokine (TNF- α) but also promote the activation of vital anti-inflammatory mediator (IL-10) in the spleen of mice. Considering the various doses and extracts tested, CcA 400 and CcE 400 demonstrated the most protective effects across all treated groups. These findings suggest that *Cajanus cajan* leaf may serve as a as potential spleen protective agents, warranting further consideration and investigation in therapeutic applications.

**Availability of data and materials:** All the necessary supporting data is incorporated within this manuscript.

**Compliance with ethical standards**

**Ethical statement:** All animal procedures were conducted according to the guidelines of the Committee for the Ethical Use of Research Animals, Centre for Research and Development FUTA.

**Clinical trial:** Not applicable to this manuscript

**Ethical approval and consent to participate:** *Cajanus cajan* leaves were collected from a farm in Ile-Oluji, Ondo State, Nigeria. No special licence or authourization is required to obtain the plant materials for this research work. All animal procedures were conducted according to the guidelines of the Committee for the Ethical Use of Research Animals, Centre for Research and Development, Federal University of Technology, Akure.

**Consent to publish:** All authors agreed to publish this manuscript

Disclaimer (Artificial intelligence)

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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