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RESEARCH ARTICLE

COMPARATIVE EFFICACY OF THE METHANOL LEAF EXTRACT OF ARTEMISIA ANNUA AND AN ARTEMISININ COMBINATION THERAPY (ACT) IN THE TREATMENT OF PLASMODIUM BERGHEI INFECTION IN MICE

Echezona V. Agbo[1], Ikenna O. Ezeh[1], Romanus C. Ezeokonkwo[1] and Arinzechukwu S. Ezema[2]

1. Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria.
2. Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria.

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Abstract

This study investigated the comparative efficacy of different doses of the methanol leafextract of Artemisia annua and a brand of ACT antimalarial drug, in Plasmodium berghei-infected mice. A total of thirty albino mice weighing between 20g and 40g were used for the study. They were randomly assigned into six groups (I-VI) of five mice each. All mice in groups I-V were infected with 1 × 10^5 Plasmodium berghei suspended in 0.2 ml of phosphate-buffered saline. Group V was left untreated, whereas group VI served as the uninfected untreated control. Groups I, II, and III were treated respectively with 250 mg/kg, 500 mg/kg, and 1000 mg/kg of Artemisia annua methanol leaf extract for 4 days. Group IV was treated with 56 mg/kg ACT antimalarial drug, every day for 4 days. Parasitaemia, packed cell volume (PCV) and haemoglobin concentration (HB), were used to assess the efficacy of treatment. The Total leucocyte count (TLC) and Differential leucocyte count (DLC) were also determined. Parasitaemia became evident in all infected mice on day 7 PI. Parasitaemia was increased in groups I and II after treatment but was significantly decreased in groups III and IV. An overall significant decrease ($P<0.05$) in haemoglobin and PCV was seen, particularly in groups I, II, and V. However, a significantincrease ($P <0.05$) in TLC was seen in all infected animals, with a sustained increase in groups I, II, and V after treatment. The infection caused a significant increase ($P< 0.05$) in absolute lymphocyte, eosinophil, and monocyte counts of all infected groups, with a sustained increase in groups I, II, and V even after treatment. It is therefore concluded that the methanol leaf extract of Artemisia annua had significant efficacy as the ACT against Plasmodium berghei infection in mice at a dose of 1000 mg/kg body weight and justified its continual use as an herbal remedy against malaria in humans.

Corresponding Author:-Echezona V. Agbo
Address:-Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria.

Introduction:-

Malaria is one of the most common human infections worldwide and a leading cause of morbidity and mortality in endemic areas (10). Its impact has surpassed that of any other infectious disease globally, and it is endemic in over 90 countries, affecting a total population of 2.4billion, which represents a significant portion of the world's population (9). According to the WHO, malaria is caused by the protozoan parasite of the Plasmodiumgenus. Between 300 and 500 million individuals are infected with Plasmodiumspp.,among these population, 1.5 to 2.7 million people, mostly children, die from the infection, Ninety percent of these deaths occur in Africa (9). In 2022, a WHO report stated that Nigeria accounted for 27% of global malaria cases and 31% of malaria deaths. Additionally, in December 2024, the BBC reported that Nigeria accounts for almost a third of those who die from malaria each year. The interactions among humans, mosquitoes, and the malaria parasite lead to malaria infection (20). The blood-feeding of infectious female Anopheles mosquitoes serves as the mode of malaria transmission (8). Five major Plasmodium species cause Malaria in humans, and they are Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Plasmodium knowlesi(18). However,Plasmodiumfalciparumaccounts for 60 – 70% of malaria deaths, being the most pathogenic among thePlasmodium species (27). Malaria is recognised as one of the diseases of economic importance to humans in Africa.

Severe malaria should be treated with highly effective drugs that have potency against the malaria parasite andare able to clear the infection within a short period without any complications (1). Antimalarial drug resistance has become a major threat to the treatment of malaria (18). Artemisinin combination therapy (ACT), in which the Artemisinin component is obtained from the plant Artemisia annua(A. annua), is currently the best therapy for the treatment of malaria (30). In Nigeria, there is still a high dependence on herbal medicine for the treatment of the disease, with a belief among some people that it is more effective than orthodox medicine (17). People in South-Eastern Nigeria use tea from boiled Artemisia annualeaves to treat malaria. A WHO report of 10th October 2019 does not support people using Artemisia plant material in any form for the treatment of malaria, citing that the herbal remedies are often insufficient to kill all malaria parasites in a patient's bloodstream, and their widespread use could hasten the development and spread of Artemisinin resistance. In this study, we compared a known Artemisinin combination therapy (ACT) Artemef® and the Artemisia annua leaf extract in the treatment of malaria in albino mice.

Materials and Methods:-

Experimental Animals

Thirty (30) adult albino mice weighing between 20g and 40g were used for the study. The micewere procured from the laboratory animal unit of the Faculty of Veterinary Medicine, University of Nigeria Nsukka. Theywere randomly assigned into six (6) groups of five (5) mice each. They were identified with bodymarkings and housed throughout the study in clean plastic cages in the laboratory animal house of theDepartment of Veterinary Parasitology and Entomology, University of Nigeria Nsukka. The micewere acclimatized for 3weeks before, and were providedclean water and proprietary mice feed ad libitumthroughout the experiment.

Plasmodium

The Plasmodiumused in the study was obtained from the National Institute of PharmaceuticalResearch and Development Abuja, Nigeria. They were then maintained by serial passage in mice which servedas the donors for inoculation of the experimental mice.

Plant Extract

The already dried and pulverized plant material of*Artemisiaannua* was obtainedfrom Nike, in Enugu State, Nigeria. It was then extracted in theDepartment of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine,University of Nigeria, Nsukka, Nigeria, using the cold maceration method (14)with80% methanol. After extraction, the percentage yield was calculated

$$\% \text{ Yield} = \frac{\text{Weight of extract.}}{\text{Original weight of plant}} \times \frac{100}{1}$$

$$\frac{16.12\text{g} \times 100}{150\text{g}} \quad 1$$

and 10.75% was obtained.
Where weight of extract =16.12g
Original weight of plant = 150g

Experimental Design
The thirty mice were assigned into groups as follows:
Group 1: Infected and treated with Artemisia annua at 250mg/kg.
Group 2: Infected and treated with Artemisia annuaat 500mg/kg.
Group 3: Infected and treated with Artemisia annua at 1000mg/kg.
Group 4: Infected and treated with ACT (Artemeter&Lumefantrine) at 56mg/kg.
Group 5: Infected and untreated.
Group 6: Uninfected and untreated.

Infection of Experimental Animals
Each mouse in groups 1-5 was inoculated intraperitoneally with 1.0 X 10^5*Plasmodiumberghei* suspendedin 0.2ml of Phosphate buffered saline (PBS). The mice were screened from the 4^{th}day postinfection (PI) by examiningthe stained thin blood smear to establish the onset and level of parasitemia.
The efficacy of the treatments were assessed using parasitaemia, packed cell volume (PCV), hemoglobin concentration (Hb), total erythrocyte count (TEC), totalleucocyte count (TLC) and the differential leucocyte counts.

For the acute toxicity test, four groups of three mice each were administered methanol leaf extract of Artemisia annua at graded doses of 250 mg/kg, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg body weight. Each group was housed separately, and the mice were closely monitored for clinical signs of toxicity, behavioral changes, and mortality for 24 hours post-administration.
Blood was collected from the tail vein by nipping a small portion of the tip of the tail with a pairof scissors. A thin smearwas then made by milking the tail, placing a drop of blood on a cleangrease-free microscope slide, and another slide was used to make a thin smear on the slide. Thesmear was then fixed with absolute methanol and stained with Giemsa stain. The slide was allowed to dry and then examined carefully under the microscope for Plasmodium parasitized redblood cells using ×100 objective lens (oil immersion). The level of parasitemia was thenestimated by expressing the number of infected cells as a percentage of red blood cells (29).

Estimation of PCV was done using the microhaematocrit method (6). Blood wascollected from the retro-orbital plexus of the median cantus of the eye into heparinized samplebottles. Heparinized haematocrit tubes were used to collect blood from the sample bottles by capillary action up to three-quarters full. One end was sealed with plasticine and centrifuged in a microhaematocritcentrifuge (Hawksley, England) at 10,000 revolutions per minute (rpm)for 5 minutes. The PCVwas then read as a percentage using a microhaematocrit reader (Hawksley, England).

Hemoglobin concentration was determined using the cyanohaemoglobin method (21), 5mls of Drabkinsreagent was put in a clean test tube, and 20µl of blood was added to the reagent and mixed properly, the mixture was then allowed to react for 20 minutes, and the absorbance was read at 540nmwavelength against a reagent blank on a colorimeter (Lab-tech, India).
The red blood cells were counted using an improved Neuber chamber (6). Briefly, 4ml ofRBCdiluting fluid was added to test tubes in which 20µl of blood was equally added and mixedproperly. The counting chamber was charged with the diluted cells, mounted on a microscope, and allowed to settle. The cells were then viewed using x40 objective lens and counted in the four (4) edge squares and the inner square of the central square of the Neubauer chamber, and values were recorded with the aid of a tally counter. The number obtained was then multiplied by 10^4andexpressed as cells(millions) per cubic millimeter (x10^6cells/mm^3).

The white blood cells were counted using the improved Neuber chamber (6). Here380µl of WBC diluting fluid was added to the test tubes in which 20µl of blood was equallyadded and mixed properly. The counting chamber was charged with the diluted cells, mounted ona microscope, and allowed to settle. The cells were then viewed using x10 objective lens andcounted in the 4 corner squares and values were recorded with the aid of a tally counter. The numberobtained was then multiplied by a factor of 50 and expressed as cells (thousand) per cubic millimeter (x10^3cells/mm^3)

A thin blood smear was made on a grease-free microscope slide, allowed to dry and then stained with Giemsa staining technique (6). The smears werefixed in methanol for 2 minutesand then placed in atank containing a 10% diluted Giemsa stain for 45 minutes. They were then rinsed with buffered distilledwater (pH 6.8) and left to air dry. The different leucocyte proportions were enumerated and recorded. Thevalues of the different proportions were then converted to absolute numbers based on the countsrecorded for the total leucocyte count for each corresponding mouse.

Data collected were computed into means and standard error of means and analyzed using one way analysis of variance (ANOVA) using SPSSS statistical softwareversion 15.0 for windows. Meanswere separated using Duncan's multiple range test at Post hoc.Probability values of ≥ 95 were considered significant.

Results:-

clinical signs

The clinical signs observed in the mice include anaemia, emaciation, dullness, and anorexia. Wetting of the abdominal and anal region with urine was noticed in group administered with 2000mg/kg during the acute toxicity study.

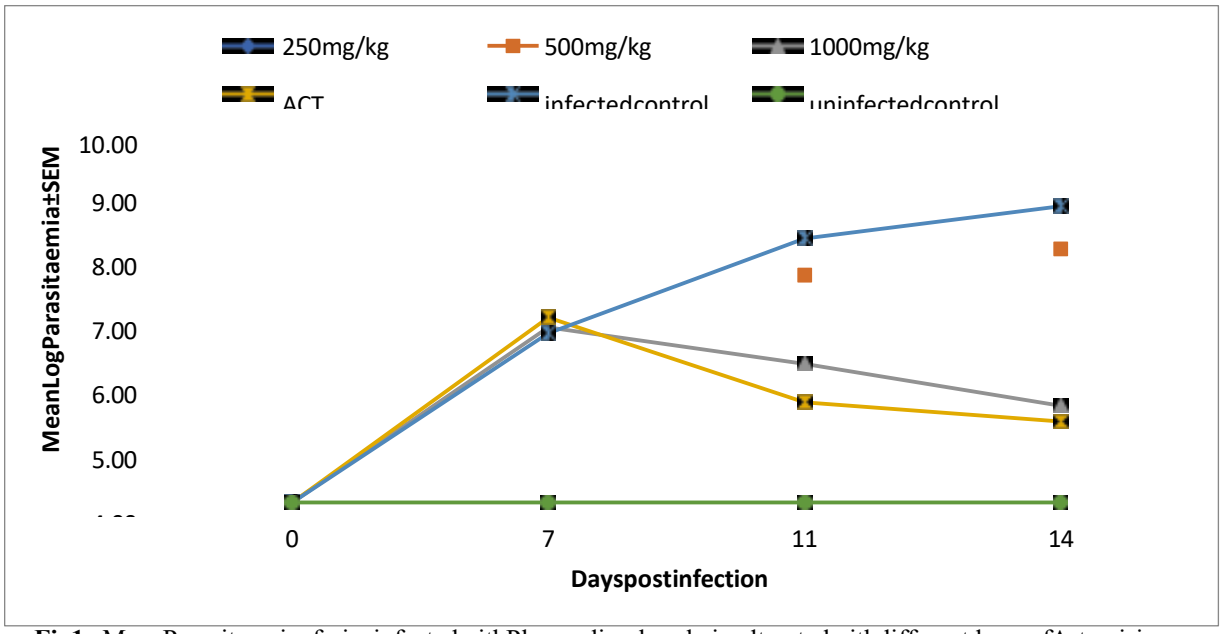

Fig1:-MeanParasitaemiaofmiceinfectedwithPlasmodiumbergheiandtreatedwithdifferentdosesofArtemisiaannua

Fig 1 shows the mean parasitaemia. Parasitaemia became evident in all infected mice 7 dayspost infection. On day 10 post infection, there was an increase in parasitaemia in mice in groups 1, 2 and 5. On day 14 post infection, mice in groups 3 and 4 showed significantly lower ($P<0.05$) parasitaemia than groups 1, 2 and 5 with group 5 (infected control) having the highest parasitaemia

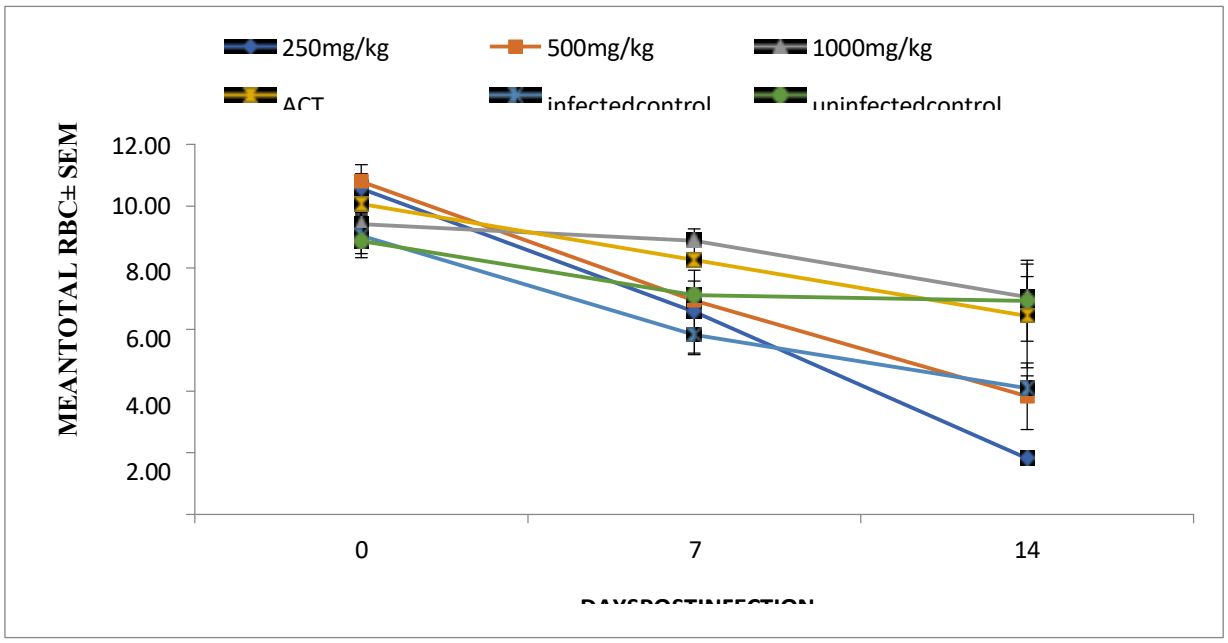

Fig2:-MeantotalErythrocytecountofmiceinfectedwithPlasmodiumbergheiandtreatedwithdifferent doses of Artemisia annua.

Fig 2 shows the mean total erythrocyte count. On day seven post infection, there was a significantly higher ($p < 0.05$) total erythrocyte count in groups 3 and 4 than other groups, with group 5 (infected control) showing the lowest total erythrocyte count. On day 14 post infection, mice in groups 3, 4 and 6 had significantly higher ($P<0.05$) total erythrocyte count than those in groups 1, 2 and 5

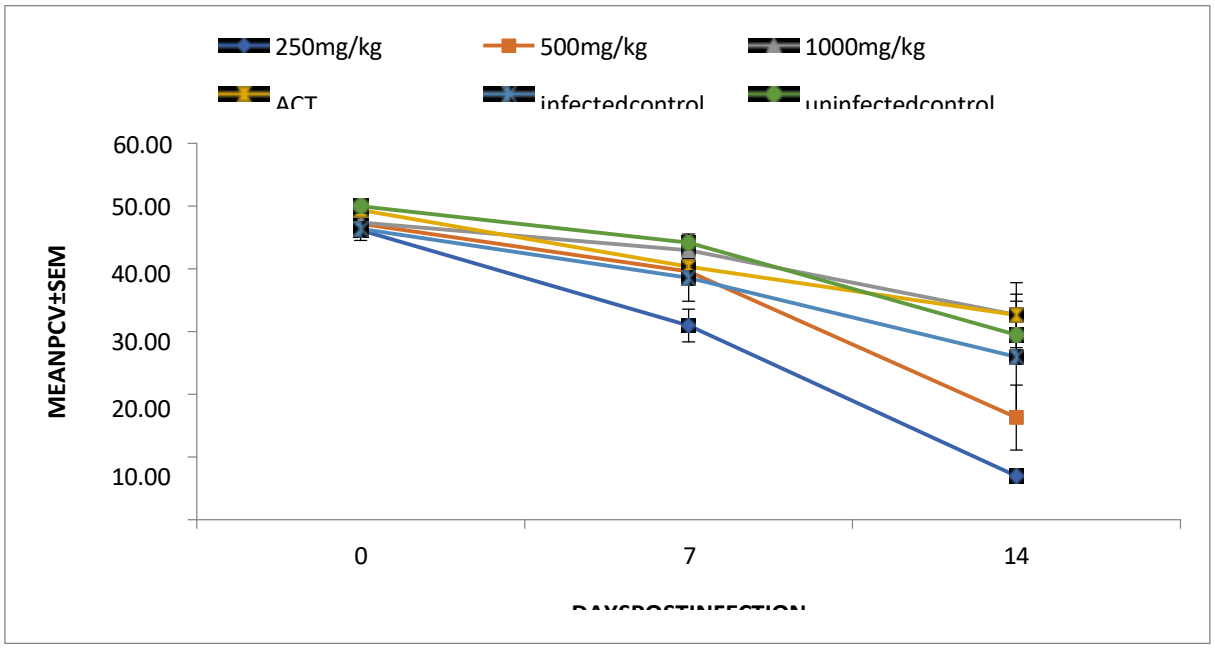

Fig3:-MeanPCVofmiceinfectedwithPlasmodiumbergheiandtreatedwithdifferentdosesof Artemisia annua.

Fig 3 shows the mean PCV. At day 7 post infection, there was a higher PCV in mice in group 6 (uninfected treated) than mice in group 1(250mg/kg). On day 14 post infection, mice in groups 3 and 4 had a significantly higher PCV ($P<0.05$) than mice ingroups 1 and 2

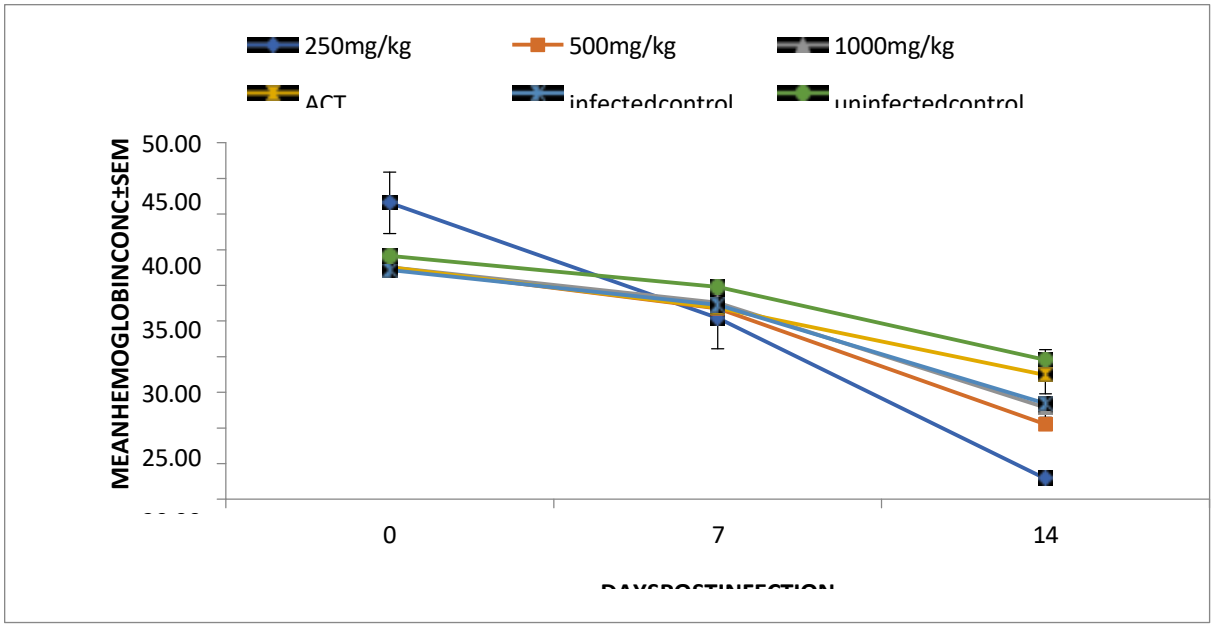

Fig4:-
MeanHaemoglobinconcentrationofmiceinfectedwithPlasmodiumbergheiandtreatedwithdifferentdosesofArtemisiaannua.

Fig 4 shows the mean haemoglobin concentration. On the day of infection, mice in group 1 had the highest haemoglobin concentration than mice in other groups. On day 7 post infection, mice in group 1 showed the lowest haemoglobin concentration while group 6 (uninfected control) showed the highest. On day 14 post infection, there was a significantly higher ($P<0.05$) haemoglobin concentration in groups 4 and 6 than those in group 1, 2, 3 and 5 with group 1 showing the lowest haemoglobin concentration.

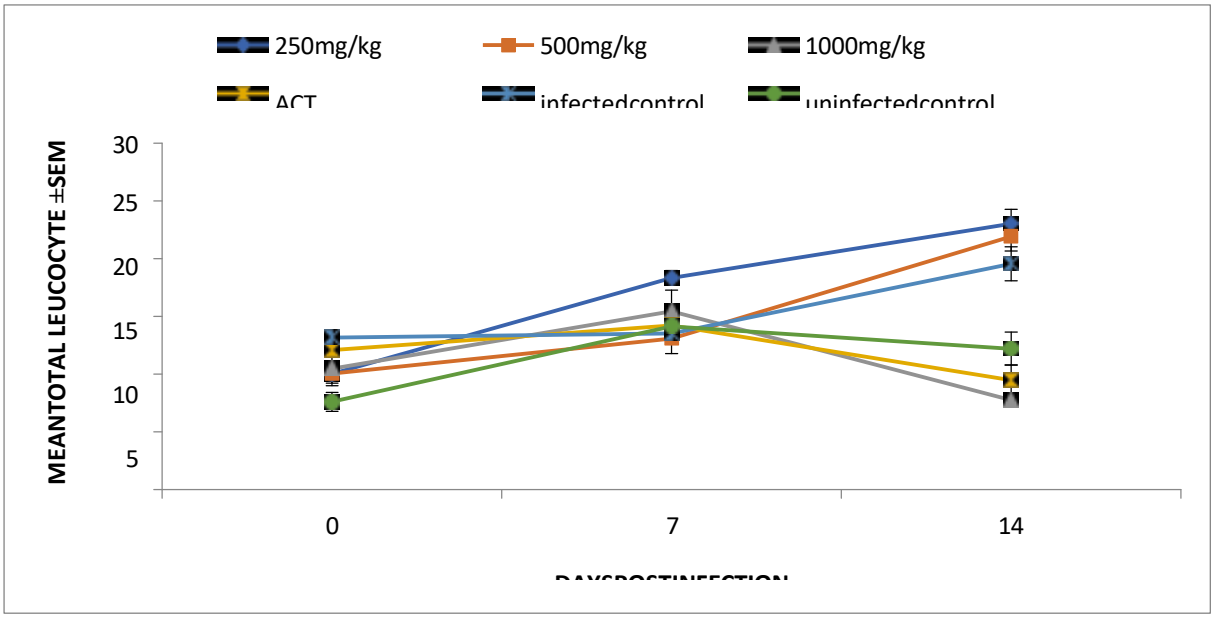

Fig5:-MeanTotalLeucocytecountofmiceinfectedwithPlasmodiumbergheiandtreatedwithdifferent doses of Artemisia annua.

Fig 5 shows the mean total leucocyte count (TLC). At day 7 post infection, mice in group 1 (250mg/kg) showed higher TLC than other groups, with group 2 (500mg/kg) showing the lowest TLC. On day 14 post infection, mice in groups 1, 2 and 5 had significantly higher (P<0.05) TLC than those in groups 3, 4 and 6.

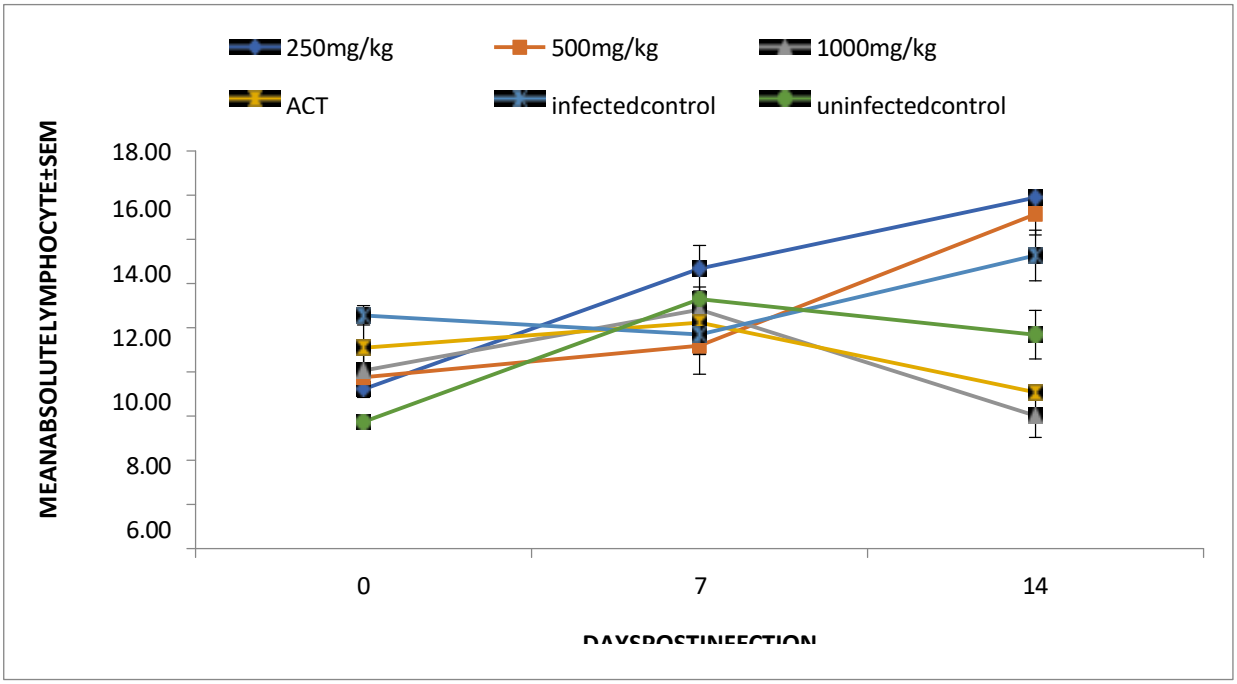

Fig6:-MeanLymphocyteofmiceinfectedwithPlasmodiumbergheiandtreatedwithdifferentdosesofArtemisiaannua

Fig 6 shows the mean lymphocyte count. On day 7 post infection, there was no significant difference (P>0.05) in the absolute lymphocyte count in mice in all the groups (group 1-6). On day 14 post infection, there was a significantly higher (P<0.05) lymphocyte count in groups 1, 2 and 5 with the highest in group 1 than in groups 3, 4 and 6.

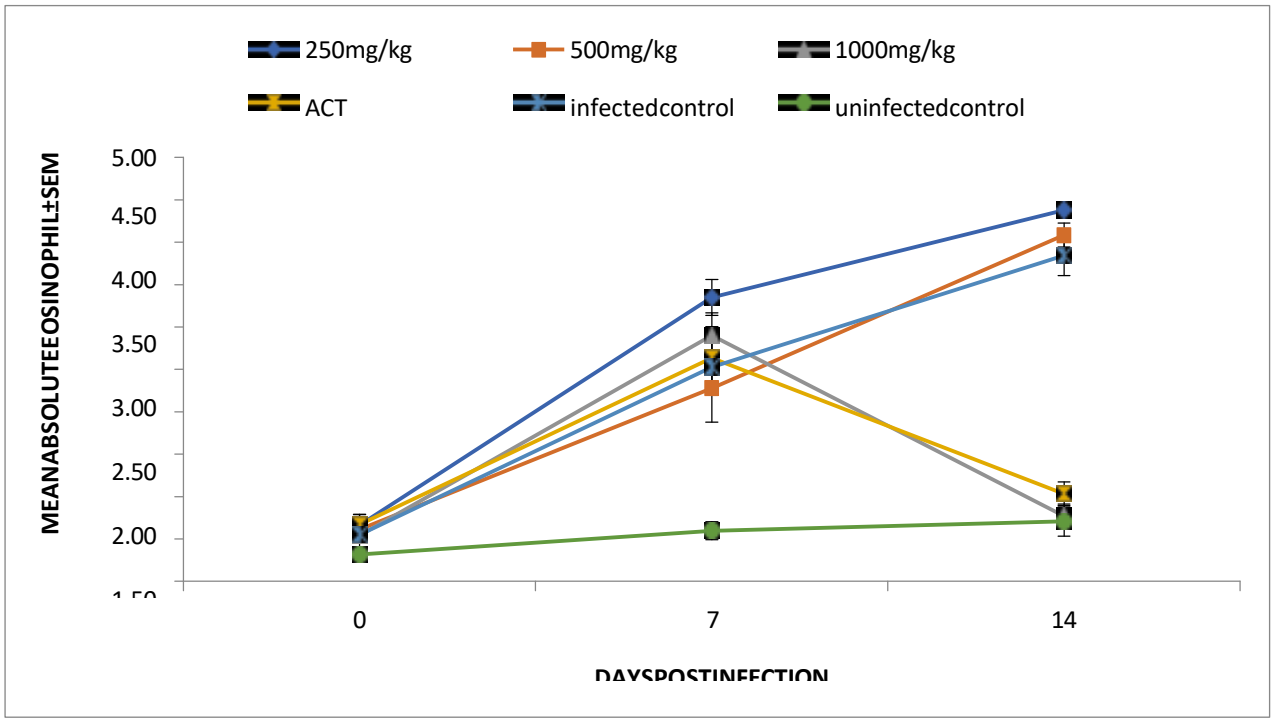

Fig7:-MeanEosinophilofmiceinfectedwithPlasmodiumbergheiandtreatedwithdifferentdosesofArtemisiaannua.

Fig 7 shows the mean eosinophil count. On the day of infection, there was no significant difference (P>0.05) in the eosinophil count of mice in all the groups (groups 1-6). On day 7 post infection, mice in group 6 (uninfected control) had the lowest eosinophil count while mice in group 1 had the highest eosinophil count. On day 14 post infection, the eosinophil of mice in groups 3, 4 and 6 were significantly lower (P<0.05) than those in group 1, 2 and 5.

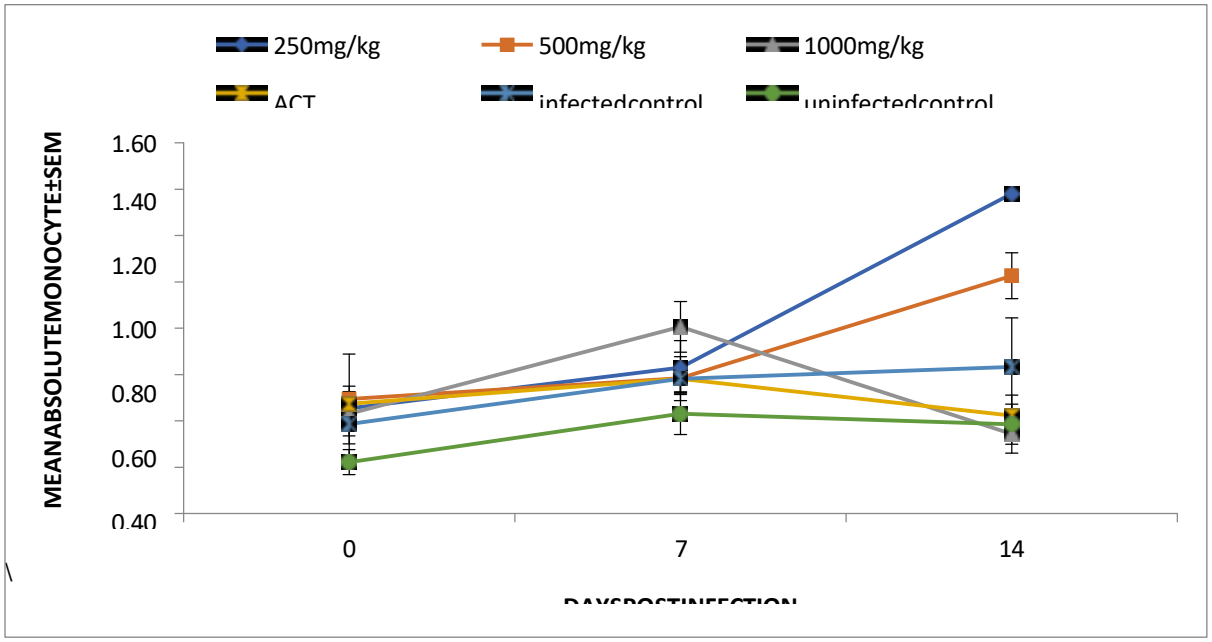

Fig8:-MeanMonocyteofmiceinfectedwithPlasmodiumbergheiandtreatedwithdifferentdosesof Artemisiaannua.

Fig 8 shows the mean monocyte count. On day 7 post infection, mice in group 3 had a significantly higher monocyte count than those in group 6 (uninfected control). On day 14 post infection, mice in group 1 (250mg/kg) had a significantly higher ($p < 0.05$) monocyte count than all other groups of mice, while those in group 3 had the lowest count.

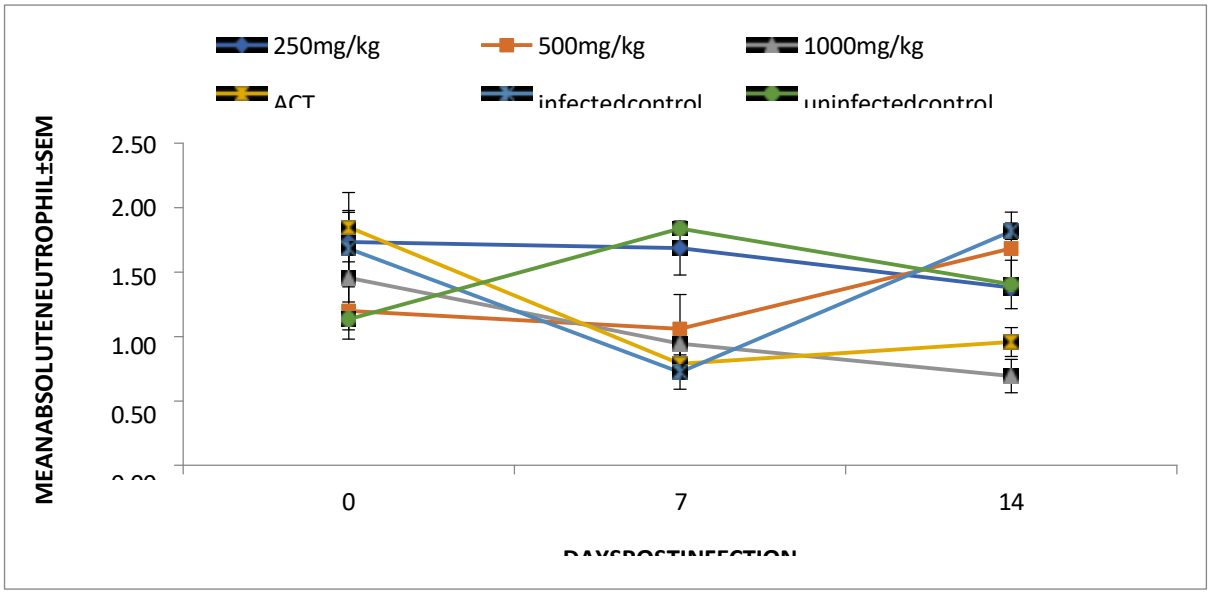

Fig9:-MeanNeutrophilofmiceinfectedwithPlasmodiumbergheiandtreatedwithdifferentdosesofArtemisiaannua.

Fig 9 shows the mean neutrophil count. On day 7 post infection, mice in groups 1 and 6 had significantlyhigher ($p < 0.05$) neutrophil count than those in all othergroups. On day 14 post infection, mice in group 5 had a significantly higher neutrophil count than mice in groups 1, 3, 4, 5 and 6.

Survivability
The mice in groups 3, 4 and 6 all survived but those in groups 1, 2 and 5 all died.

Discussion:-

Following the infection of the albino mice (Groups 1-5) with Plasmodium berghei, an average pre-patent period of 4 days was recorded. A similar pre-patent period has been recorded in mice elsewhere (23). The parasitaemia and anaemia observed in the infected groups were typical of Plasmodium infection (25)(31). Parasitaemia was cleared in group 3, following the administration of 1000mg/kg of the methanol leaf extract of Artemisia annua, and group 4 following the administration of 56 mg/kg of ACT. The parasitaemia in group 1(250mg/kg), 2(500mg/kg), and 5(infected control) continued to increase, which suggested a failure of the extract to inhibit the multiplication of the parasite in vivo at those doses.
The significant reduction in the mean total erythrocyte count and haemoglobin concentration in groups 1(250mg/kg), 2(500mg/kg), and 5(infected control) was thought to be as a result of continued increase in parasitaemia and destruction of red blood cells as also reported by (31).
Leucocytosis was observed in all infected groups, as previously reported by (24)(5). Leucocytosis persisted in the groups treated with 250mg/kg, 500mg/kg, and the infected control as the study progressed, but the group treated with 1000mg/kg and 56 mg/kg ACT showed a return of leucocytes to the pre-infection values. This suggested that the extract didstop the leucocytosis caused by the multiplication of the parasite at the low and medium doses, but had an effect on the parasite when the medium dose was doubled at 1000mg/kg, an effect similar to and comparable with that of the ACT.Leucocytosis in malaria infection is the result of the immune response to the parasite invasion of the red blood cells, causing an increased proliferation of leucocytes in response to the infection (5). Leucocytosis had been reported to be associated with the severity of, and mortality seen in malaria patients (13).

There was no significant change in the mean lymphocyte count on day 7 post-infection, but lymphocytosis was noticed as the infection progressed. There was a significant increase in lymphocyte count in groups 1(250mg/kg), 2(500mg/kg) and the infected control, which has also been recorded by (26)(22).
Eosinophilia was noticed in all infected groups, which returned to the normal baseline level pre-infection in group 3 (1000mg/kg) and group 4 (ACT). Eosinopenia has been reported in malaria infection (19)(11), but cases of Eosinophilia have also been reported in malaria (12)(16). The activation of the eosinophils is believed to be a result of the inflammatory and immune response to the parasite (12).

Monocytosis seen in malaria has been reported by (3) (10) to be due to activation of the innate immune response to the malaria parasite, phagocytosis of infected cells/parasite, Cytokine secretion, and antigen presentation. This report agrees with the findings from the study, which showed monocytosis in the infected group, but was only reversed in the groups treated with 1000mg/kg and ACT, suggesting the clearance of the Plasmodium parasite from the bloodstream, leading to the return of the mean absolute monocyte count to near pre-infection values.
Neutrophil function in malaria is understudied (4)(2). Some researchers have reported Neutropenia to be found in acute and severe malaria (7)(28), while some have reported cases of Neutrophilia (15). From the study, there was mild neutropenia in all infected groups except group 1(250mg/kg), which was gradually returning to normal count in all infected groups. There was no significant difference in the neutrophil count in group 1, while neutrophilia was observed in group 6 (uninfected control), which returned to normal on day 14. These differences in the mean neutrophil count call for further studies into neutrophils in malaria infection.

Conclusion:-

From the study, it was concluded that the methanol leaf extract of Artemisia annua was efficacious against Plasmodium berghei infection in mice at 1000 mg/kg. at this dose, the extract had a comparable efficacy to Artemef®. The efficacy of Artemisia annua was dose-dependent, showing the highest observed activity at 1000mg/kg body weight.

Declaration of Conflicting Interest

The authors declare no potential conflict of interest with respect to the research, authorship, and publication of this article.

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