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COMPARATIVE EVALUATION OF THE EFFECT TWO ANTI-MALARIA DRUGS (CHLOROQUINE AND ARTEQUIN) ON SOME SERUM BIOCHEMICAL PARAMETERS IN RATS

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ABSTRACT
Artemisinin combination therapies (ACT) have replaced the old drugs (like chloroquine) used as first line treatment for malaria. This current study aimed to investigate the comparative effects of chloroquine (an old drug) and artequin (an ACT drug) on serum biochemical indices in rats. Thirty-six (36) Wistar rats were randomly assigned into 2 batches. Each batch had 3 groups of 6 rats each. Group 1 was control, groups 2 and 3 respectively received artequin (1.6mg/100g bwt) and chloroquine (0.875mg/100g bwt) orally and once daily. Administration lasted for 3 and 7 days for batches 1 and 2 respectively. The biochemical analysis of the serum was carried out using standard methods. Results obtained on both days 3 and 7 showed that serum total protein and globulin concentrations in the artequin group was significantly lower ($p<0.05$) compared to control. The alkaline phosphatase concentration in the artequin group on day 7 was significantly ($p>0.05$) higher compared to control. In conclusion, administration of artequin and chloroquine at their recommended doses and duration is relatively safe. Prolonged administration of artequin could predispose to low serum proteins and globulin with accompanied elevations in ALP levels while chloroquine could increase AST level signifying hepatocellular damage.
Key Words: Anti-malaria drug, chloroquine, artequin, liver enzymes, proteins

INTRODUCTION
Efforts to tackle the scourge posed on humanity by the malarial parasites are on the increase with several medications being made available for malaria prevention or treatment. However, these efforts are met with setbacks due to resistance from these parasites. Resistance of these parasites to previous generations of medicines such as chloroquine and sulfadoxine-pyrimethamine undermine malaria control efforts. Ineffectiveness, together with some adverse effects experienced upon administration of these old drugs has prompted their replacement with new ones (Martin *et al.,* 2009; Plowe, 2005).

WHO (2016) reported that artemisinin-based combination therapies are highly effective against the malarial parasites especially *Plasmodium*

falciparum, the most prevalent and lethal malaria parasite affecting humans. One of such is Artequin (Artesunate + Mefloquine), is useful for the treatment of uncomplicated *P. falciparum* malaria (Bhatt *et al.,* 2006). Artesunate is one of the semi synthetic derivatives of artemisinin. Artemisinin is a natural anti-malarial derived from the Chinese medicinal plant *Artemisia annua.* The artemisinin derivatives are the most effective anti-malarial drugs and have been proven to be successful on multi-drug resistant *Plasmodium falciparum* (Adjuik, 2004).Mefloquine is a drug used for malaria chemoprophylaxis (González *et al.,* 2013). The use of these drugs may have side effects on the blood cells, hence, this present research work was aimed at investigating the effects of Chloroquine and Artequin on serum biochemical parameters in Albino Wistar rats.

MATERIALS AND METHODS

Experimental Animals

36 female Albino Wistar rats weighing between 100-240g were obtained from the animal house of the Physiology Department, University of Uyo, Nigeria. They were housed in well ventilated cages under room temperature (28 ± 2°C) and humidity of 85 ± 5%. The animals were exposed to a normal 12/12 light/dark cycle and allowed to acclimatize for 7 days before administrations were started. The mice had access to rodent chow and drinking water *ad libitum.* The animals were handled in accordance with internationally accepted principles for laboratory animal use and care as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

Drug Preparation and Administration

Chloroquine (150mg) manufactured by Evans Medical Plc, Agbara, Ogun State, Nigeria and Artequine (600/750mg) packaged in Nigeria by Oculus Pharmacare Limited, Nigeria for Mepha Pharma AG, Aesch, Switzerland used for this experiment were bought at Amela Pharmacy, Oron Road, Uyo. It was stored at the research room of the Department of Physiology, University of Uyo in a cold dry cabinet at controlled temperature. One tablet each of Artesunate (200mg) and Mefloquine (250mg), which constitute Artequine (450 mg), were crushed together using a glass mortar and it was dissolved in a total of 45ml of distilled water to give a concentration of 10mg/ml stock. The drug combination was administered to the animals at a

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dose of 0.6mg/100g body weight [equivalent to human (70kg) daily dose], i.e. 0.06mL of stock /100g body weight.

One tablet of chloroquine (150mg) was ground to powder and dissolved in a total of 20ml of distilled water to give a stock concentration of 7.5mg/ml. The drug was first administered at a dose of 0.857mg/100g body weight (i.e. 0.11mL of stock/100g body weight) for the first 2 days. On the 3rd day and subsequent days, 0.429mg/100g body weight (i.e. 0.06mL of stock/100g body weight) was administered. After every administration, the remaining drug was poured out and a new one was prepared for the next administration

Experimental Design

Thirty six (36) female albino wistar rats were randomly assigned into 2 batches of eighteen rats each, each batch was further divided into 3 groups of 6 rats each; group 1 was control, group 2 was artequin treated (0.6mg/100g b.w.) and group 3 was chloroquine treated (0.857mg/100g b.w.). All animals had free access to normal rat chow and drinking water. The treatment lasted for 3 and 7 days for batches 1 and 2 respectively.

Collection of Blood Samples

After 3 and 7 days administration, the rats were sacrificed and blood samples were taken from each rat by cardiac puncture under Chloroform anaesthesia, a modification of the method by Ohwada (1986). A quick dissection was made on the thoracic region to expose the heart. The left ventricle was pierced with a needle syringe (25 gauge) and blood obtained by slow aspiration. About 4mLs of the blood obtained were placed in plain (non-heparinized) 5mL-sample bottles and left for 2 hours for proper clotting to occur. Serum was extracted from the clotted blood using a syringe after the blood was spun at 3,000revs/min for 10 minutes.

Measurement of Alkaline Phosphatase (ALP)

This measurement of alkaline phosphatase (ALP) was modified by King *et al.,* (1951).

Principle: Phenol released by enzymatic hydrolysis from phenylphosphate under defined conditions of time, temperature and pH – is estimated colorimetrically.

Technique

Test: 1ml of buffer was mixed with 1ml of phenylphosphate substrate in a test tube placed in water bath at 37°C for 3 minutes. 0.1ml of serum was added mixed gently and incubated for exactly 15 minutes, the reaction was stopped by addition of 0.8ml of 0.5N sodium hydroxide (NaOH).

Control: In a test tube 1ml substrate was mixed with 0.8ml of 0.5N sodium hydroxide, followed by 0.1ml of serum.

Standard: 1.1ml of buffer was mixed with 0.1ml of phenol standard (1mg/100ml) and 0.8ml of 0.5N sodium hydroxide.

Blank: 1.1ml of buffer, 1.0ml of water and 0.8ml of 0.5N sodium hydroxide was mixed. To all tubes 1.2ml of 0.5N sodium bicarbonate ($NaHCO_3$) was added with 1ml of Potassium Ferricyanide solution -$K_3(Fe(CN)_6)$, mixing each tube well after each addition. The successive additions adjusted the pH to develop the color. The 0.0 of reddish –brown colors of 510 nanometer (nM), was read avoiding exposure to strong sunlight.

Calculation

Serum alkaline phosphatase (King-Armstrong Units/100ml)

$$= \frac{\text{Reading of unknown - Reading of control}}{\text{Reading of standard - Reading of Blank}} \times 100$$

Measurement of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT)

Principle: The pyruvate produced by transamination by ALT reacts with 2, 4-dinitrophenylhydrazine (DPNH) to give a brown-colored hydrazone, which is measured in the colorimeter at 510nM. The Oxaloacetate formed in the reaction with AST decarboxylates spontaneously to pyruvate which is again measured by hydrazone formation (Reitman and Frankel, 1957).

Calculations

$$\frac{T-T_B}{S-S_B} \times 67/\text{Umol/min/L for AST}$$

$$\frac{T-T_B}{S-S_B} \times 133/\text{mol/Umin/L for ALT}$$

Where T = Test

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T_B =Test Blank

S = STD

S_B = STD Blank

Measurement of Serum Protein Concentrations

The blood samples were analyzed using Biuret method.

Principle: Cupric ions in alkaline solution react with peptide bonds in proteins producing violet colour that is proportional to the amount of protein present.

Method: Test tubes were labeled to include reagent blank (Rb), standard (s) control (C_1, C_2) and test samples. Some blank test tubes were labeled to correspond with each "test tube" that is SB, C_1B_1, C_2B_2 etc. The following volumes were delivered thus:

	RB	S	C_1	C_2	SB	
Burette reagent (ml)		510	5.0	5.0	-	-
Blank Burette reagent (ml)	-	-	-	5.0	5.0	
Distilled water (µl)		100	-	-	-	-
Protein standard log/l (µl)	-	100	-	100	-	
Control or test samples (ml)	-	-	100	-	100	

It was thoroughly mixed and allowed for 10 minutes at 37°C or at room temperature for 30 minutes, spectrometer 540nm. The instrument was first set to zero with blank Burette reagent. Then the different absorbances were measured.

Measurement of Serum Albumin

Principle: Albumin binds with bromocresol green (Bch) at pH 4.2 causing a slight in absorbance of the yellow BCG dye. The blue-green colour formed is proportional to the concentration of albumin present when measure photometrically between 580 – 630nm with maximum absorbance at 625nm measured (Reinhold, 1953).

Procedure

Pipette in tubes marked	Blank	Standard	Test
Albumin reagent	1000 µl	1000 µl	1000 µl

Distilled water	10 µl	-	-
Standard	-	10 µl	-
Sample	-	-	10 µl

Each sample was properly mixed, the absorbance of standard and each sample read at 630nm against reagent blank after one minute.

Measurement of Serum Globulin

Globulin concentration will be calculated as the difference between the total plasma protein and serum albumin concentration.
Globulin = Total plasma protein – Serum Albumin.

Statistical Analysis

Data were analyzed as mean ± SEM, the data were analyzed using a one way analysis of variance (ANOVA). Significant values were processed with least significant difference (LSP). P value less than 0.05 was declared as significant statistically.

RESULTS

Comparison of Serum Total Proteins, Albumin and Globulin Concentration

The serum total protein concentrations in the control, artequin (AQ) and chloroquine (CQ) administered groups were 8.53 ±0.78, 7.09 ±0.20 and 7.75 ±0.37 g/dL respectively on day 3. Values in AQ group were significantly ($p<0.05$) lower compared with control. Also on day 7, values obtained in AQ group (6.70 ±0.06 g/dL) reduced significantly ($p<0.05$) compared with CQ (8.01 ±0.26 g/dL) and control, figure 1.

The serum albumin concentrations on day 3 did not vary significantly among the different experimental groups. Same wise on day 7, figure 2.

On day 3, serum globulin concentration reduced significantly ($p<0.05$) in the AQ administered groups compared with control. Also on day 7, AQ group had significant ($p<0.05$) reduction in levels of serum globulin compared to CQ group and control. The control values were 5.58 ±0.80 g/dL and 4.47 ±0.65 g/dL on days 3 and 7 respectively, figure 3. There were no significant differences in the ratio of albumin to globulin among the different experimental groups both at days 3 and 7, figure 4.

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Comparison of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP) Concentration

On day 3 of the experiment, no significant differences were observed in the serum AST (IU/L) concentrations of the different experimental groups. But on day 7, values obtained for CQ group (226.18 ±20.36) were significantly ($p<0.05$) higher compared with control (167.75 ±14.26; $p<0.05$) and AQ (170.07 ±37.21; $p<0.01$) groups, figure 5.

ALT concentrations did not vary significantly among the different experimental groups on both days 3 and 7, figure 6. ALP concentration recorded after 3 days of treatment did not differ significantly among the different experimental groups. But on day 7, significant increases were observed in AQ group compared with control, figure 7.

AST/ALP ratio was significantly higher in artequin group compared with control and significantly ($p<.0.05$) lower in chloroquine group compared with artequin.

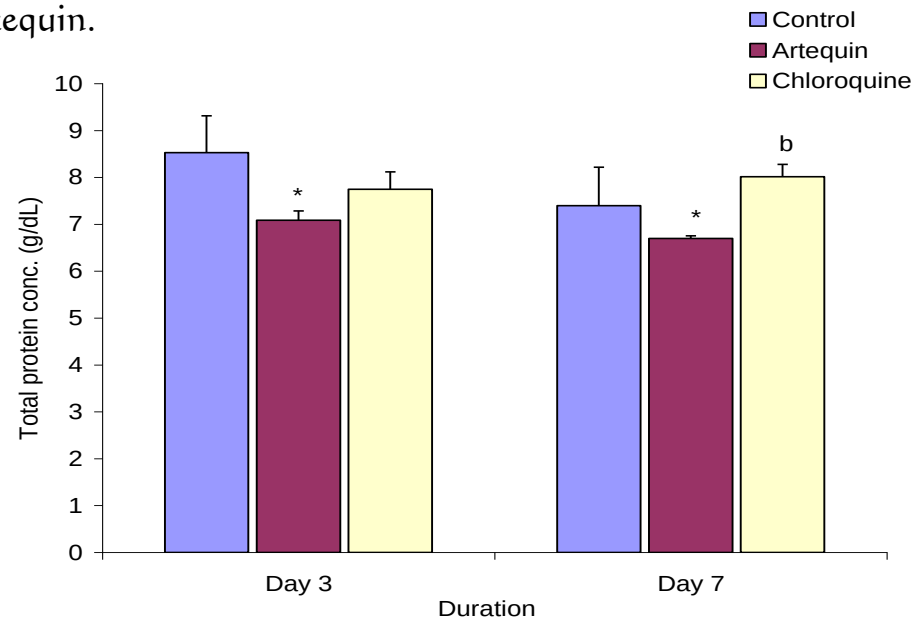

Figure 1: Comparison of total protein concentration between the different control, artequin and chloroquine treated groups at days 3 and 7.

Values are expressed as mean ± SEM, n = 6
*p<0.05 vs control
b = p<0.01 vs atequin

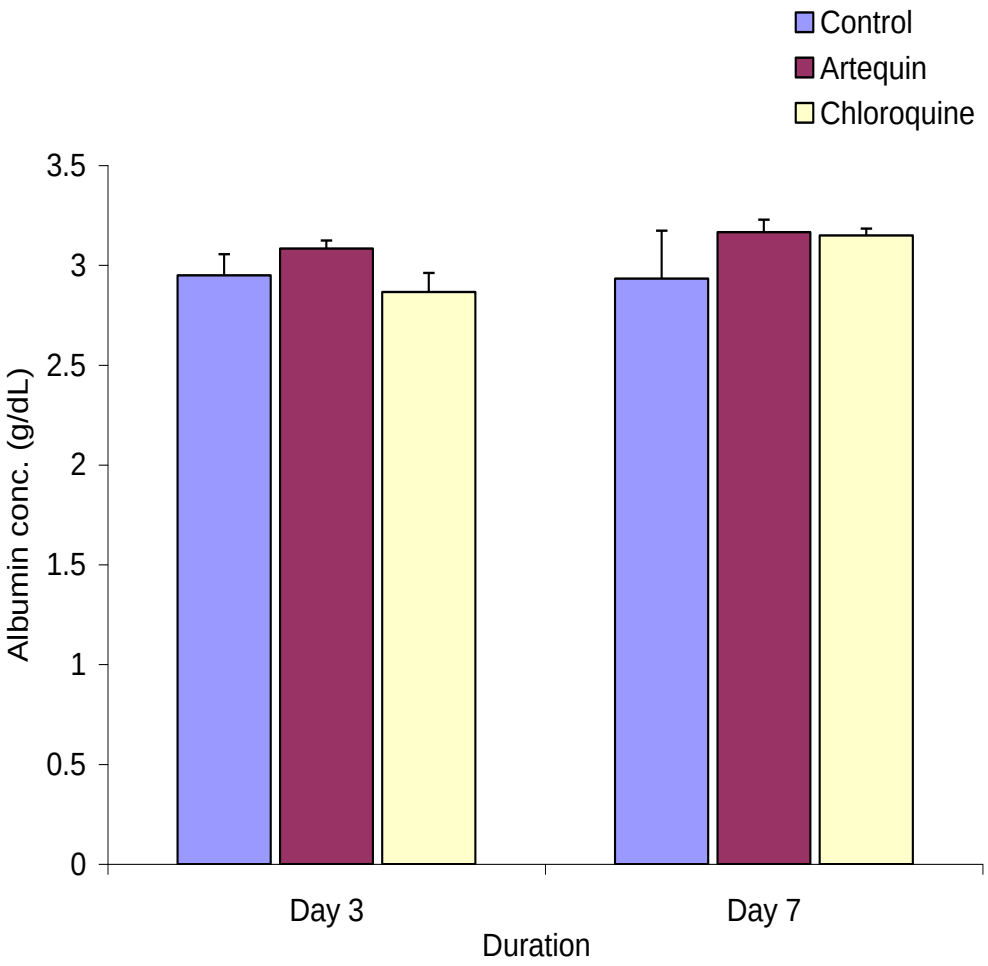

Figure 2: Comparison of albumin concentration between the different control, artequin and chloroquine treated groups at days 3 and 7.

Values are expressed as mean ± SEM, n = 6
No significant differences among groups

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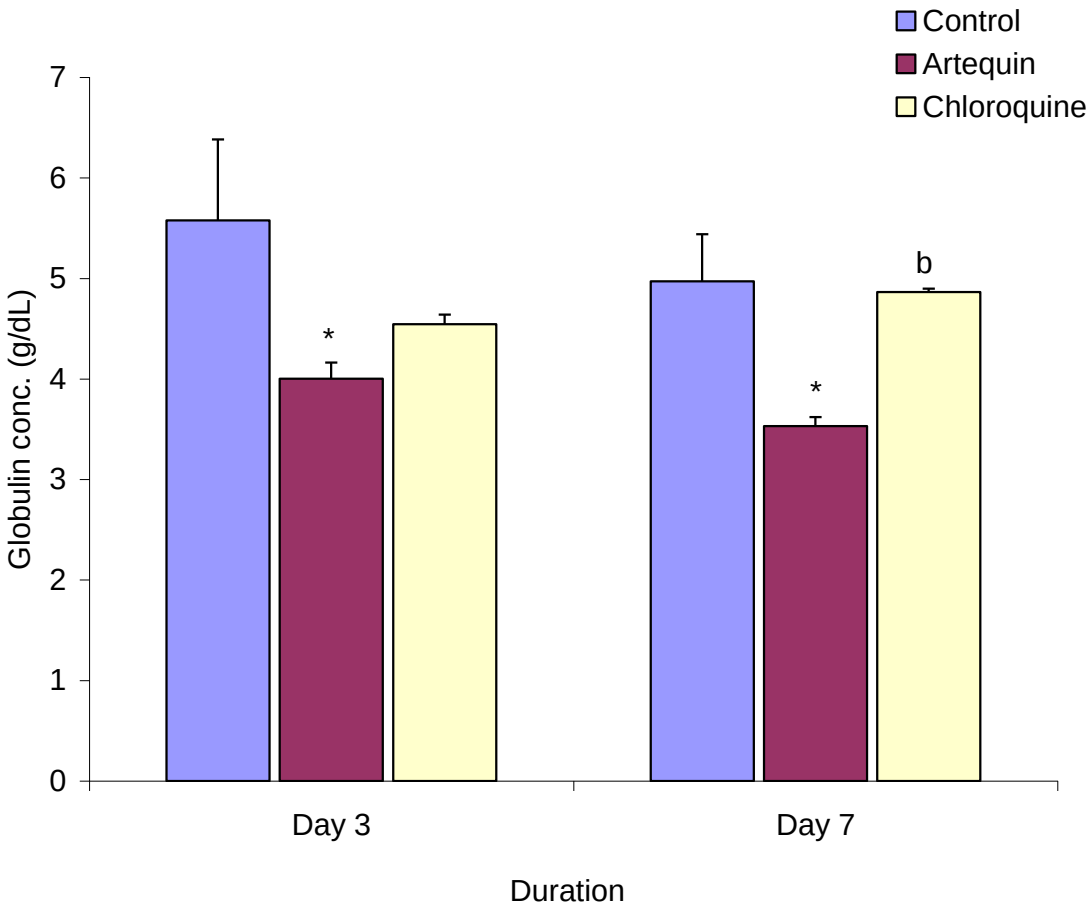

Figure 3: Comparison of globulin concentration between the different control, artequin and chloroquine treated groups at days 3 and 7.

Values are expressed as mean ± SEM, n = 6
*p<0.05 vs control
b = p<0.01 vs atequin

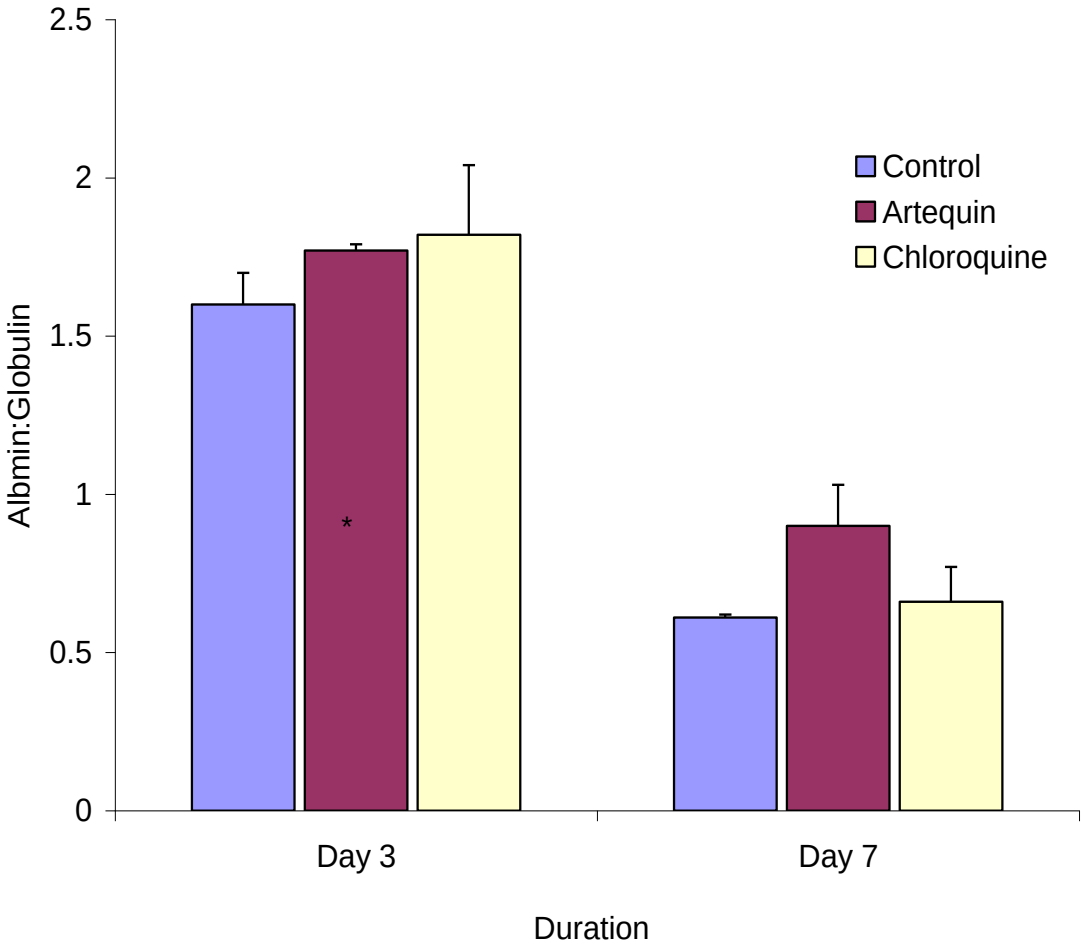

Figure 4: Comparison of albumin to globulin ratio between the different control, artequin and chloroquine treated groups at days 3 and 7.

Values are expressed as mean ± SEM, n = 6

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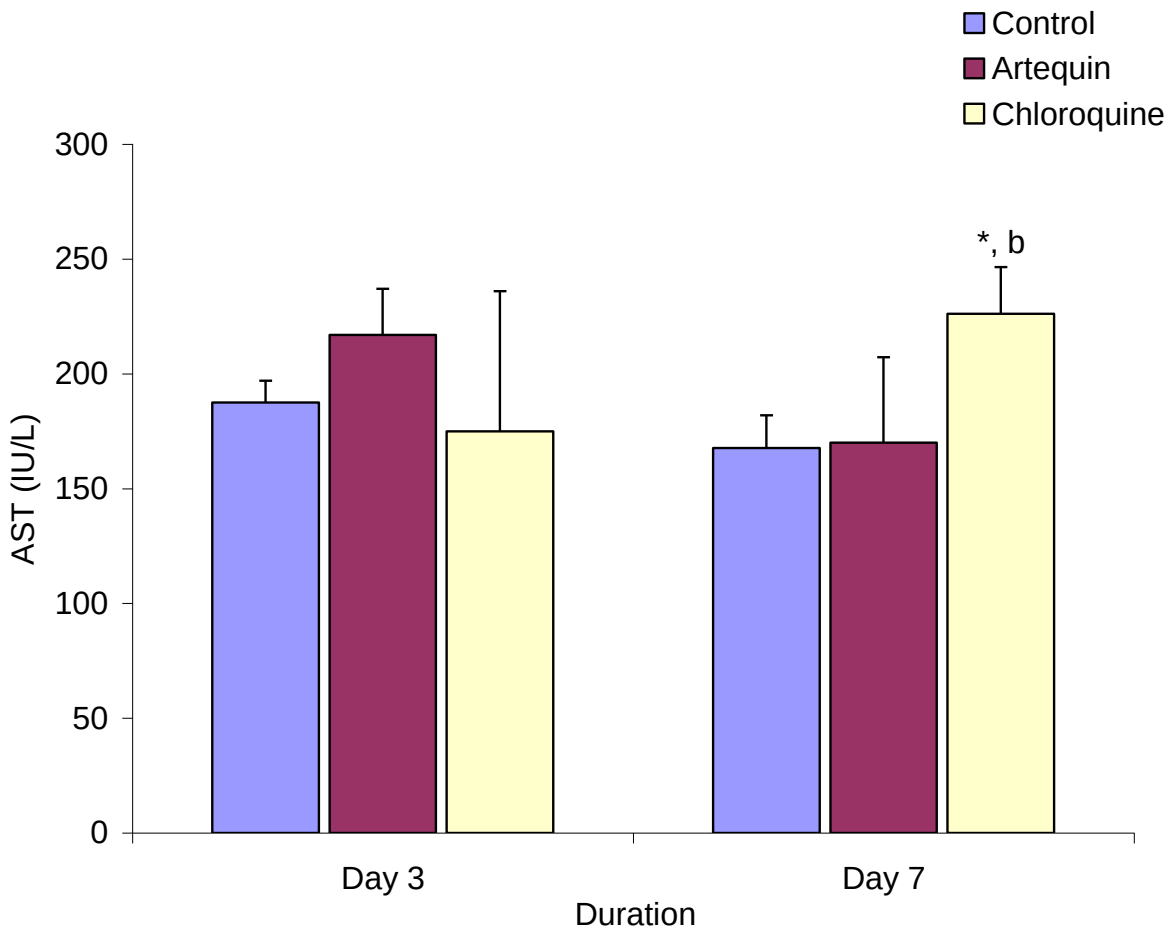

Figure 5: Comparison of aspartate aminotransferase concentrations between the different control, artequin and chloroquine treated groups at days 3 and 7.

Values are expressed as mean ± SEM, n = 6
*p<0.05 vs control
b = p<0.01 vs atequin

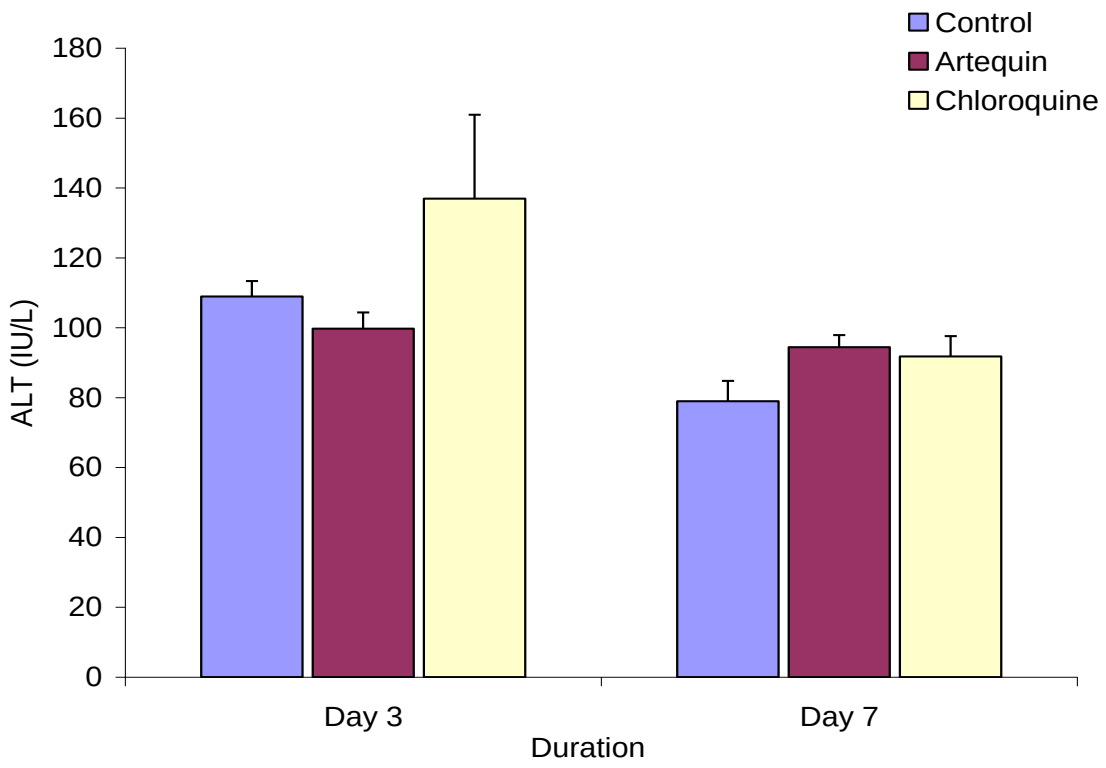

Figure 6: Comparison of alanine aminotrasnferase concentrations between the different control, artequine and chloroquine treated groups at days 3 and 7.

Values are expressed as mean ± SEM, n = 6
No significant differences among groups

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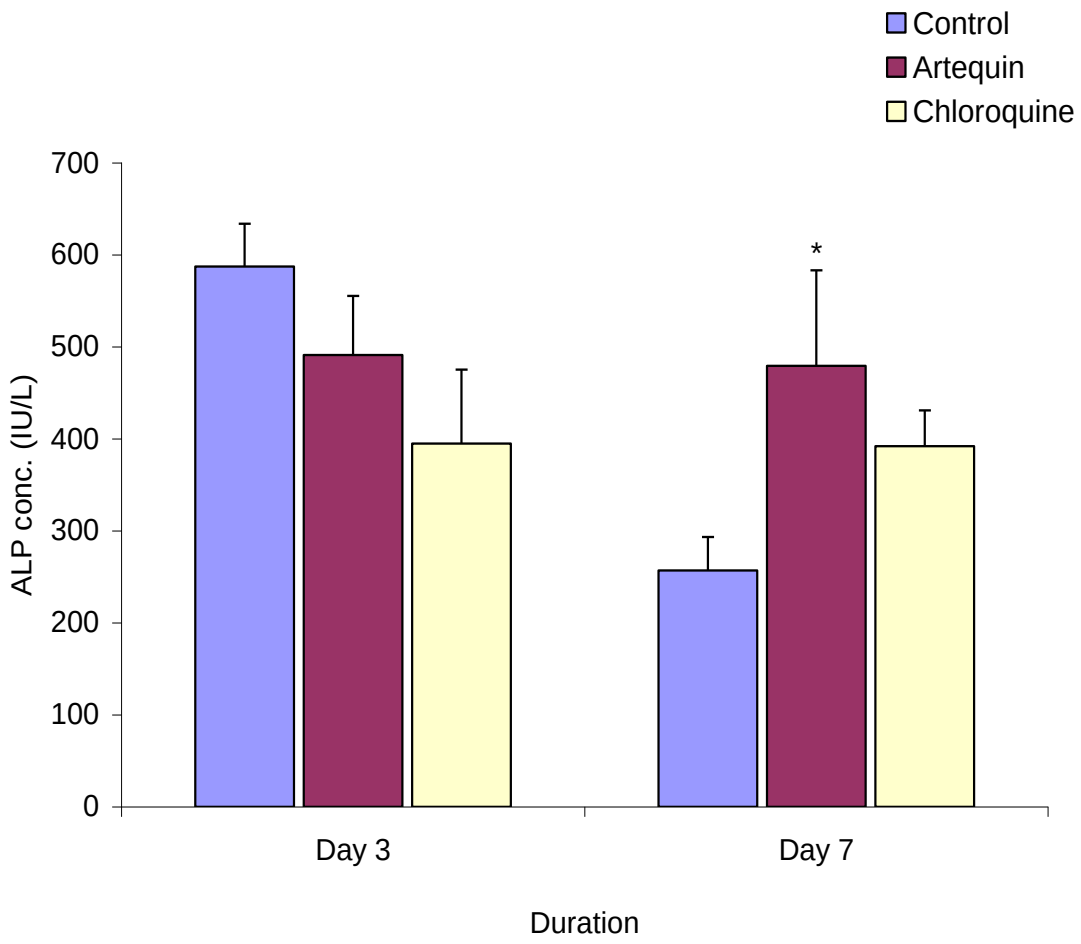

Figure 7: Comparison of alkaline phosphatase concentration between the different control, artequin and chloroquine treated groups at days 3 and 7.

Values are expressed as mean ± SEM, n = 6
*p<0.05 vs control

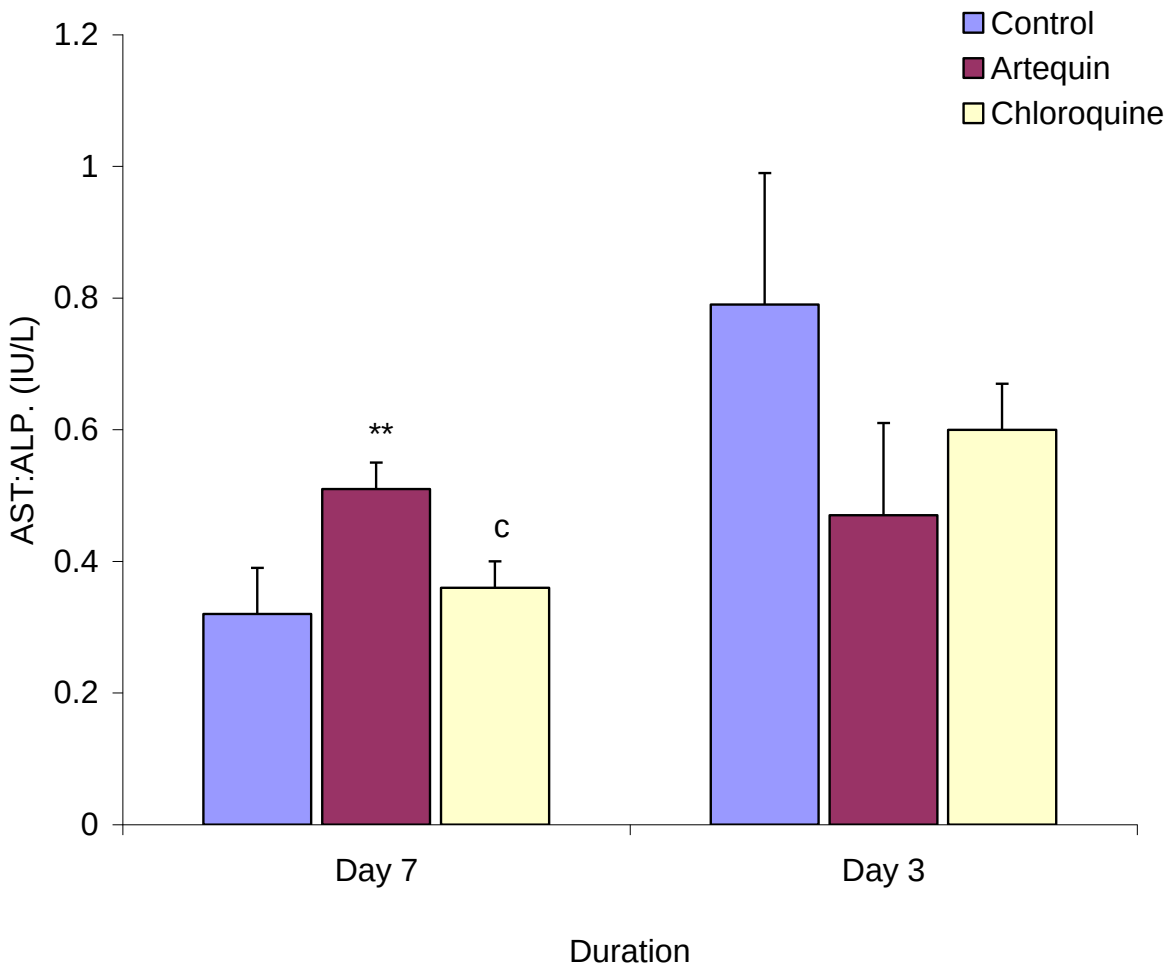

Figure 8: Comparison of the ratio of asparate aminotransferase to alkaline phosphatase between the different control, artequin and chloroquine treated groups at days 3 and 7.

Values are expressed as mean ± SEM, n = 6
**p<0.01 vs control
c = p<0.001 vs artequin

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DISCUSSION

The present study reveals significant reductions in total protein and globulin concentrations in artequin group after 3 days of administration with further reductions after 7 days of administration. Rats treated with artequin were also observed with elevated ALP levels after 7 days of treatment but not at 3 days. Previous reports have it that, total protein concentration is decreased during diarrhea, hemorrhage (blood loss), burns, pregnancy, malnutrition, prolonged starvation, cirrhosis of liver and chronic infections like (chronic hepatitis or nephritis). It is also documented that globulin levels are decreased following emphysema, acute hemolytic anaemia, glomerulonephritis and hypogammaglobulinaemia, (Sembulingam and Sembulingam, 2006).

The decrease in total protein and globulin levels in artequin administered group could be due to the negative impact of Artequin on the liver cells and blood parameters (i.e. the tendency of Artequin to cause anaemia). Reports by Omotosho *et al.*, (2014) in their research showed decrease in total protein concentrations following treatment with Chloroquine and artesunate.

Administration of Artequin and chloroquine did not impact adversely on the serum enzyme concentrations after 3 days of administration. But after 7 days of administration, AST levels increased significantly in Chloroquine group and ALP increased significantly in Artequin group compared with control. The results indicate that administration of Chloroquine and Artequin at the recommended duration of 3 days would not perturb the liver integrity adversely, but its prolonged administration may cause some damage to the liver cells leading to increase synthesis of the serum enzymes.

Enzymes are proteins that act as catalyst in the body stimulating various chemical changes and metabolic reactions and tend to be highly specialized for other individual tasks (Wongsrichanalai, 2002). A member of these enzymes may increase in quantity in the blood (serum) as a result of a specific illness, disease or accelerated cell destruction, especially involving the liver where they are majorly synthesized.

CONCLUSION

In conclusion, administration of Artequin and Chloroquine at their recommended duration (3 days) is relatively safe. But upon prolonged administration, Artequin (and not Chloroquine) could predispose the body to low serum proteins and elevated ALP levels, while both drugs could cause liver cell damage when taken beyond the recommended duration of 3 days.

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