Acute Study Of Histomorphological And Biochemical Changes Caused By Artesunate In Visceral Organs Of The Rabbit.

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ABSTRACT

15, 30 and 60mg/kg body weight doses of artesunate were orally administered to rabbits to determine the histologic and biochemical changes induced by the different doses of the drug for 30 days. The liver and kidney showed histological lesions of varied intensity. The alkaline phosphatase levels of the test showed no statistically significant ($P > 0.05$) increase over the control during the period of drug administration. But the aspartate and alanine transaminases showed significant ($P > 0.05$) elevations at doses 60mg/kg, and 30 and 60 mg/kg respectively. Cholesterol levels were significantly ($P < 0.05$) decreased when compared with the control. Urea and creatinine levels were significantly elevated at doses 30 and 60mg/kg, and at all doses respectively. Sodium and potassium electrolytes showed statistically significant ($P < 0.05$) increases at doses 30 and 60mg/kg, and at all doses of artesunate administration respectively. The histologic findings correlate well with biochemical observations. One should be wary of artesunate consumption in view of its toxic potential.

Key words: Enzymes, cholesterol, Urea, Creatinine, electrolytes, artesunate, histology.

Malaria is the most common killer of all the parasitic diseases affecting humans. About 2000 million people live in endemic areas. 300 million people are infected every year and most of them children. In sub-Sahara Africa, over a million die annually from the disease (WHO, 1998).

Artemisinin (Qinghaosu) is the antimalaria principle isolated by Chinese scientists from Artemisia annua leaves. It is a sesquiterpene lactone with a peroxide bridge linkage. The peroxide moiety appears to be responsible for its anti malaria activity. Artemisinin is a potent and rapidly acting blood schizontocide (katzung, 2001). Artemisinin is poorly soluble in water or oil but some derivatives have been produced: artesunate and artelinic acid, the water-soluble derivatives; artemetter and arteether, the oil soluble derivatives.

These derivatives have more potent blood schizontocidal activity than the parent. Gametocytocidal activity has recently been reported (Rang. 1999).

Several derivatives of artemisinin have been marketed especially in south East Asia. Serious concern has been raised about uncontrolled use of these drugs because at the moment they are the last resort in the combat against multi-drug resistant plasmodium falciparum malaria. The use of these drugs should be controlled and restricted to proven multi-drug resistance on severe malaria in order to preserve their efficacy (Mulenga, 1998).

In endemic areas, primary health is usually lacking familiarity of symptoms and self-diagnosis is quite common. Anti malarias are usually purchased in the open market where there is little or no control although cost is taken into account when purchasing these drugs. A survey in Zaire indicated that 92% of children presented at a hospital emergency ward had detectable levels of chloroquine or quinine in their blood at the time they were seen by a health worker (WHO,1990). Risk of toxicity, this way is high, not mainly from overdose but also misuse. Since education is poor or insufficient, knowledge of adverse effects or risk assessment and determination of any contraindications cannot be made.

This work, seeks to evaluate the toxic effect of artesunate on the liver and kidney by monitoring some relevant biochemical parameters in the visceral organs, and a histomorphological assessment of the tissues.

MATERIALS AND METHODS

Animals:

Sixteen genotobiotically reared adult rabbits, were bought from the Department of Biochemistry, University of Nigeria, Nsukka and moved to the Animal house of the College of Medicine, University of Nigeria, Enugu campus and allowed acclimatization for 4 weeks. Food and water was given ad libitum. Individual identification of the animals was by metal ear tags, Rooms were well ventilated and constantly maintained at room temperature. The Guilin pharmaceutical company, Peoples' Republic of China

supplied the drugs used in this study.

Experimental Procedure

The experimental animals were divided into four groups comprising four sex matched rabbits in each of the four groups. Those in groups 1, 2,and 3 constituted the test groups, where-as the 4th group acted as the control The animals in group 1 were given doses of artesunate 15mg/ kg daily. The animals in group 2 received 30 mg (twice the dose) while the 3rd group received 60mg/kg (twice the dose), all given orally dose daily for a period of 30 days. The 4th group received equal volume of normal saline daily.

The rabbits were weighed weekly and weights recorded. Blood samples were taken from the marginal ear vein for enzyme and cholesterol, urea creatinine and electrolyte parameters prior to drug administration. Thereafter, similar samples were collected at day 30. Blood samples collected prior to drug administration produced enzyme and other biochemical values, which represented the baseline. Two animals from each of the test groups and two from the control group were painlessly sacrificed after 30 days. All the remaining rabbits were still living when the study terminated and were anaesthetized by intraperitoneal injection of sodium thiopentane and were exsanguinated. Necropsies were performed immediately. Representative samples of the liver were fixed promptly in 10% neutral formol-saline, histologically processed, embedded in paraffin wax, sectioned at 5 ì thick using Rotary microtome and stained by both Haematoxylin and Eosin (1886) and Gordon and sweets (1936) techniques respectively.

The results were expressed as mean ± SEM and significance of differences between control and treated as well as before and following treatment for enzyme and other biochemical parameters were determined using paired student t-test. Statistical significance was set at $p < 0.05$.

Method Of Sample Collection & Analysis

Whole blood samples were collected for the liver enzymes and serum cholesterol and urea, creatinine and electrolytes in plain test tubes. The animals were fasted overnight before collecting blood samples for serum cholesterol estimation.

The methods of Reitman and Frankel (1957) and king and kind (1954) were used for the transaminases and alkaline phosphatase respectively. The method used for the estimation of serum cholesterol was according to Zak and his colleagues (1957). The techniques of Scott and Beffac (1951), Bousnes and Taussky (1945) and Tietz (1970) were used for the determination of urea, creatinine and sodium and potassium electrolytes respectively.

RESULTS

Orally administrated artesunate at the different doses did not cause mortality or impairment of sexual activity in the animals. Rabbits receiving 15mg/kg dose of the drug showed marked dermatitis, which healed after drug administration at the end of the study period. There was no statistically significant ($p > 0.05$) loss or gain in weight at the different doses at the end of the drug administration. Gross Anatomy of the liver showed normal size, color and consistency. However, kidney showed slight cortical depressions and shrunkenness in some instances. Microscopy of the liver necropsies displayed necrosis as evidenced by nuclear pyknosis, karyolysis and karyorhexis and cytoplasmic vaculation (Fig. 1). Ductal hypertrophy, periductal cuffing by lymphocytes and mononuclear infiltatron of the parenchyma were noted. (Fig 2). Intraportal fibrosis and sclerosis was evident (Fig. 3). Bile ductal proliferation, derangement and mononuclear cell infitration of the parenchyma were noteworthy (Fig. 4). The rabbits voided dark urine which produced yellowish precipitate on drying. The kidney necropsies showed evidence of tubular hyalinization, glomerular degeneration or loss due to severe necrosis (Fig. 5). The tubules showed hyaline droplets, necrosis, vascular attenuation with congestion and interstitial degeneration (Fig. 6).

Table 1 shows the mean (mean ± SEM) values of alkaline phosphatase (ALP), aspartate transaminase (AST), alanine trasaminase (ALT) and serum cholestoral levels at all doses of drug administration. There was no statistically significant ($p > 0.05$) elevation of ALP over the control at 15, 30 and 60mg/kg doses respectively, whereas AST showed statistically significant ($p < 0.05$) increase over the control at dose 60mg/kg only. It was observed that the ALT showed statistically significant ($p < 0.05$) increase over the control at doses 30 and 60mg/kg respectively while at dose 15mg/kg the increase was merely numerical and not statistically significant ($p > 0.05$) over the control. Serum cholesterol level was statistically significantly ($p < 0.05$) decreased at all doses. Both increases and decreases were dose dependent.

In Table 2 sodium electrolyte estimation showed statistically significant ($p < 0.05$) elevation over the

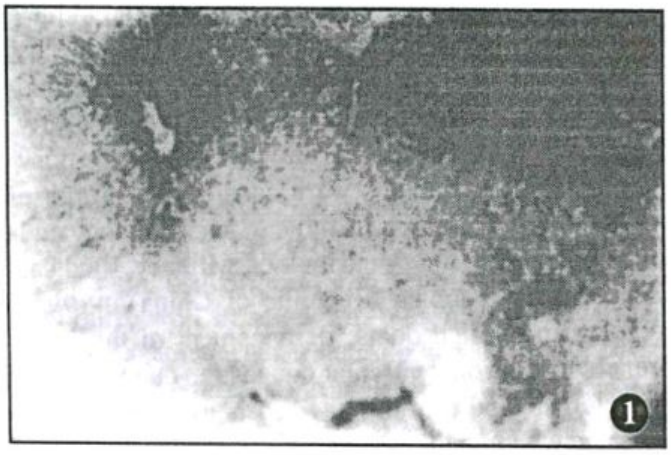
control at both 30 and 60mg/kg doses, whereas 15mg/kg dose of artesunate did not show statistically significant ($p > 0.05$) change over the control. Potassium electrolyte showed statistically significant increases over the control at all doses of the drug administration. It was also found that urea showed no statistically significant ($p > 0.05$) increase over the control at 15mg/kg dose, whereas 30 and 60mg/kg dose showed statistically significant ($p < 0.05$) elevations over the control during the treatment period. Similarly, creatinine levels were statistically significant ($p < 0.05$) over the control at different doses of the drug administration. Similarly, the increase in kidney parameters over the control values was dose dependent.

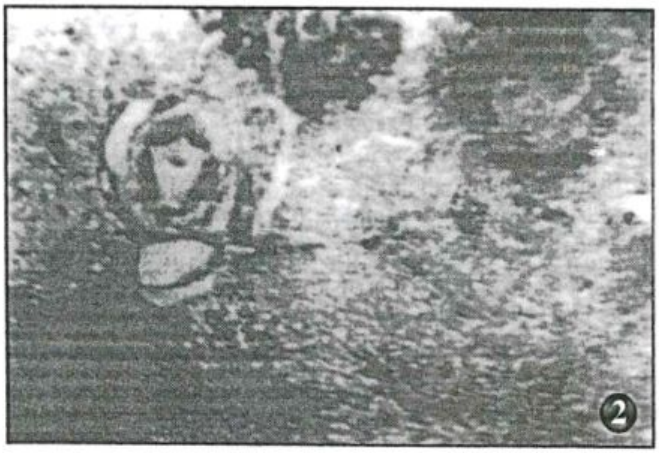
TABLE 1: Mean (Mean ± Sem) Value Profile Of Alt, Ast, Alp And **Cholesterol Levels At Day 30**

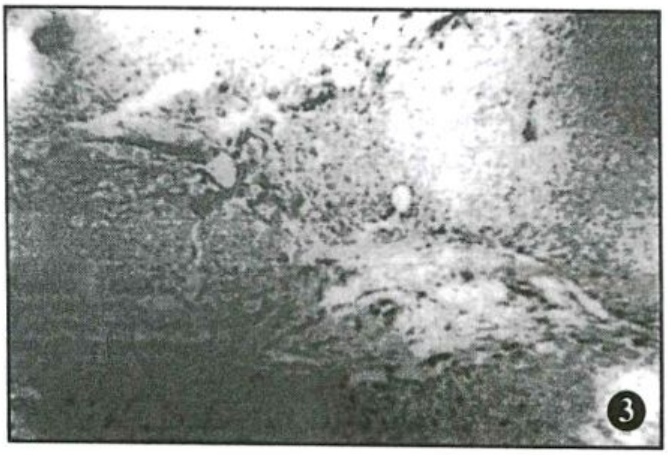
DOSE	ALP	P VALUE	AST	P VALUE	ALT	P VALUE	CHOLESTEROL	P VALUE
15 mg/kg	40.03± 3.18	0.54	13.33± 2.21	0.411	10.17±2.31	0.089	0.80±0.22	0.03
30mg/kg	38.25±6.29	0.192	15.50±420	0.226	15.50±4.66	0.034	0.60±0.28	0.01
60mg/kg	36.20±5.16	0.290	250.0±5.10	0.008	18.25±3.40	0.0034	0.42±0.09	0.0005
Control	31.87±4.18	-	12±1.0	-	7.33±1.16	-	1.41±0.24	-

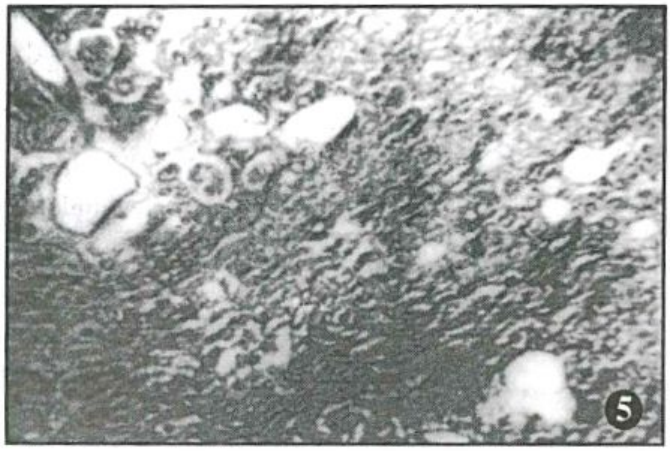
TABLE 2: Mean (Mean ± Sem) Value Profile Of Urea, Creatinine, Sodium And Potassium Electrolytes

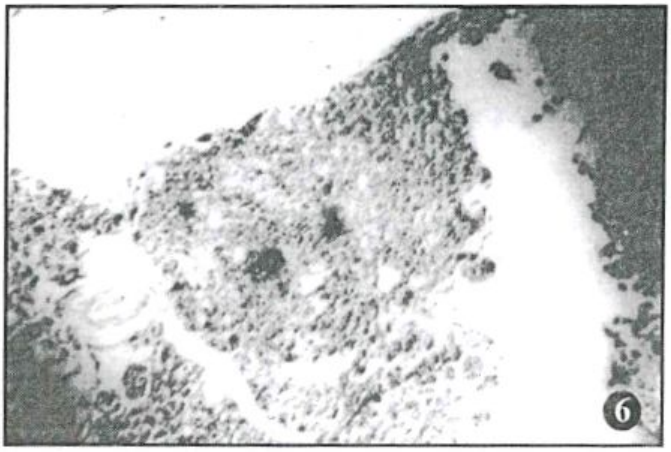
DOSE	UREA	PVALUE	CREATININE	P VALUE	NA^+	P VALUE	K^+	P VALUE
15mg/kg	6.133±0.2309	0.986	191.5±5.323	p<0.0001	143.3±5.86	0.36	7.967±0.058	p<0.0001
30mg/kg	9.05±1.109	0.0044	192.4±5.21	p<0.0001	182.5±1.915	p<0.0001	8.7±0.96	p<0.0001
60mg/kf	9.05±1.109	0.0044	193.625±3.33	p<0.0001	187.3±3.055	p<0.0001	9.63±0.86	p<0.0001
Control	6.125±0.7136	-	118±9.574	-	140±2.83	-	3.98±0.17	-

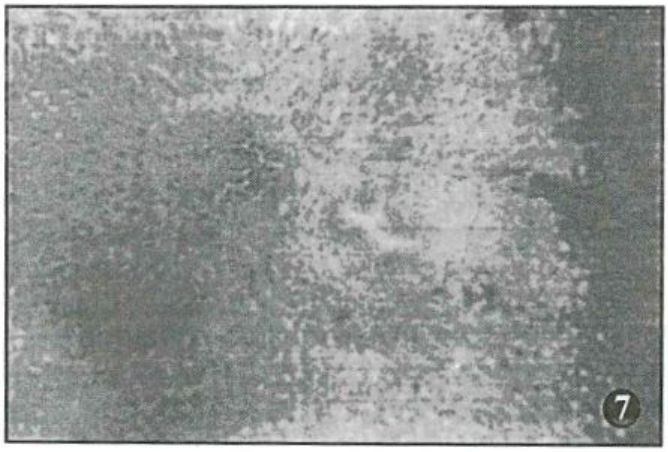












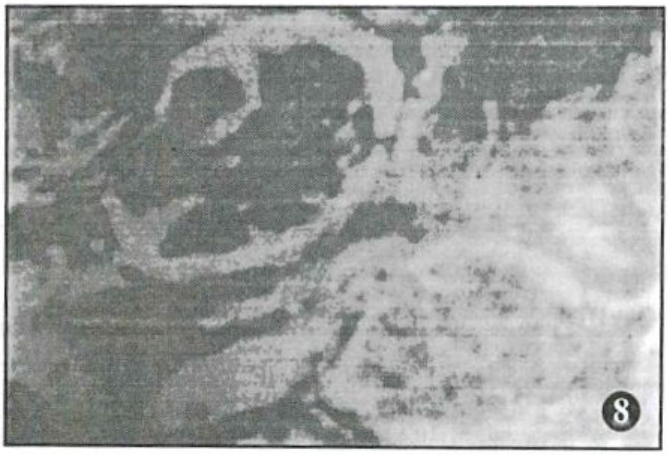


Fig 1 is a section of liver necropsy displaying necrosis as evidenced by nuclear pyknosis, karyolysis, karyorhexis and cytoplasmic vacuolation at dose 15mg/kg of artesunate. Stained by H and E technique. X100.

Fig 2 is a photomicrograph of liver section showing ductal hypertrophy, periductal cuffing by lymphocytes and mononuclear cell infiltration of the parenchyma at doses 30 and 60mg/kg. Stained by H. E. technique x 100.

Fig 3 displays features of intraportal fibrosis and sclerosis at dose 60mg/kg of artesunate administration. Stained by H&E technique. X100.

Fig 4 shows evidence of bile ductal proliferation, parenchymal derangement and mononuclear cell infiltration of the parenchyma at doses 30 and 60mg/kg. Stained by H&E technique x100.

Fig 5 is a photomicrograph of kidney necropsy section showing evidence of tubular hyalinization, glomerular degeneration or loss due to severe necrosis at 30 and 60mg/kg doses. Stained by H&E technique. X100.

Fig 6 represents kidney tubules with hyaline droplets, tubular necrosis, vascular attenuation and congestion and interstitial degeneration at doses 15, 30 and 60mg/kg of artesunate. Stained by H&E technique.x100.

Fig 7 represents the control section of liver with normal architecture.

Fig 8 shows kidney section of rabbit with normal architecture.

DISCUSSION

The current investigation has shown that artesunate caused toxicity in both the liver and kidney of rabbits. Necrosis, intraportal infiltration by mononuclear cells, hemorrhage, parenchymal degeneration, cytoplasmic vacuolation and fatty infiltration were observed in the liver necropsy sections. These findings were consistent with those reported by the Research Institute of Microbiology and Epidemiology of the Academy of Military Medical Science of the Peoples' Republic of China in 1995. Irregular and varied ducts, derangement of the liver parenchyma, chronic vascular congestion due to stasis, fibrosclerosis and intraportal fibrosis as noted in this series and which to the best of our knowledge are novel findings. The kidney, on the other hand, showed tubular necrosis, glomerular degeneration and loss possibly due to severe necrosis. Necrosis is one of the end courses of inflammatory response (Kumar et al,, 2001). Hyaline droplets and microhemorrhage were observed in the kidney section. This finding reflects the clinical observation made by Guyton and Hall (2000) and that increased blood sodium is one of the major causes of increased blood pressure bringing about alteration in renal filtration rate leading to rupture of renal vessels resulting in hemorrhage.

Production of dark urine by the animals and the

appearance of yellowish precipitate on drying was observed. These findings were in concord with the findings of Oduola et al (1998), Heppner and Ballou (1998) and Davis, T.M (1997). Production of dark urine, blood stasis, minor cardiac changes and drug fever constitutes the side effects of artesunate on laboratory animals.

Biochemically, the present study showed elevated levels of liver enzymes, AST and ALT consequent upon artesunate administration. The significant increase in AST and ALT and derangement of sinusoidal reticular meshwork is indicative of damage to the liver. This finding is in agreement with the work of Adam et al (2001), price (`1999), White et al (1992), Chung et al (1998) and WHO (1981). The detailed mechanisms by which enzymes are released from the cytosol and mitochondria of hepatocytes into the blood stream are unknown. Clinical observations and experimental studies, however, have shown that subtle membrane changes are sufficient to allow passage of intra cellular enzymes to the extracellular space. Cell damage or death increases membrane permeability causing cystolic enzymes to spill into the sinusoids and from there into the peripheral blood. Hepatotoxicity is caused by an increased level of hydroxyl and peroxide radicals, which are induced by artesunate treatment. Astesunate administration causes increases in type "O" xanthine oxidase levels (Isamah et al, 1996). Xanthine oxidase is produced by the activation of non-lysosomal proteases such as calpain, which induces the conversion of xanthine dehy drogenase "D" type to xanthine oxidase. "O" type facilitates the production of superoxide ions (Dykens et al, 1987). The type "O" oxidase can mobilize iron from ferritin by a mechanism largely dependent on the generated superoxide ions.

The released iron catalyses the formation of oxo-radicals especially hydroxyl and peroxide radicals through the Fenton mechanism (Morris et al, 1995). The hydroxyl species is the most biologically toxic of most free radicals and it is known to lack a specific enzyme based defense system. The Oxo species invariably caused the various toxic effects on the liver as observed in our study. The study has also demonstrated that artesunate administration at 15, 30 and 60mg/kg doses induced statistically significant decrease ($p < 0.05$) in serum cholesterol levels of the liver and kidney respectively. The fall in serum cholesterol levels, in this case, was dose dependent. Numerous population studies have linked elevated concentration of total cholesterol or low- density lipoprotein (LDL)- Cholesterol in serum with increased incidence of atherosclerotic events (Goldstein et al, 1973; keys, 1975).

It has been further shown that the clinical complications of atherosclerosis could be diminished and life prolonged when plasma lipids are lowered by hypocholesterolemic agents (Lipid Research Clinics program, 1984a; Lipid Research Clinics program, 1984b; Helsinki Heart study, 1987). Many drugs with proven hypocholesterolemic activity are available clinically to ameliorate cases of individuals with premature arterosclerosis and those with other risk factors such as hypertension or diabetes mellitus (Brown and Goldstein, 1992). Our finding, in this case, suggests that artesunate therapy could be useful in arterosclerosis treatment but its overall toxicity constitutes a source of worry.

The significant increases in urea and creatinine levels of test over the control are suggestive of renal compromise.

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REFERENCE

Adam sel; Al Qarani A A; Elhag, E A (2001). Effects of various levels of dietary artemisia leaves on rats. Laboratory Animals; 34 93) : 307-312.

Bousnes:, R N; and Taussky, HH (1945) Determination of serum and urine creatinine. J. Biol Chem. 158:581.

Brown, S B; Goldstein, J L. Drugs used in the treatment of hyper lipo proteinemias in:

Gilman A.G, Rall TW, Nies AS, Taylor P (Eds), Goodman and Gilman's pharmacological Basis of the therapeutics 1992 Vol 1, 8th ed McGraw- Hill Inc New York pp 874 - 894.

Chung S.Y; Das R: O. on C.T (1998) Treatment of severe falciparum malaria with artesunate and mefloquine. Singapore Medical Journal; 39(5): 208-210.

Davis, T.M.E (1997). Anti malarial drugs and Glucose Metabolism. Br J. Clin Pharmacol; 44:1-7.

Ehrlich P (1986). Staining of tissue general structures. Z, Wiss Mikr., 3:150.

Goldstein JL; Schrott H.G; Hazzard WA; Bierman EL; Motulsky AG (1973). Hyperlipidemia in coronary heart disease 11. Genetic analysis of lipid vessels in 176 families and delineation of a new inherited disorder, combined hyperlipdemia .J Clin Invest; 52:1544-1568.

Gordon H; Sweets H. H (1936) Demonstration of reticulin fibres- Amer J path; 12: 245.

Guyton M, and Hall, C E. (2000)Medical Physiology. 257-280.

Heppner D.G; Ballou W.R (1998) Malaria in 1998; Advance in diagnosis, drugs and vaccine development. Trop. And Travel Assoc. Dis;5:8-9.

Helsinki Heart Study (1987). Primary prevention trial with gemfibrozil in middle- aged men with dyslipidemia New Engl. J Med; 317: 1237-1245.

Katzung BG. Basic and Clinical Pharmacology 8th ed. New York; Lange Medical Books McGrown- Hill, P 892.

King, EJ; and Kind, PRN (1954) Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrene .J Clin path 7: 322-326.

Keys A(1975). Coronary heart disease, the global picture. Arterosclerosis; 22: 149- 192.

Kumar V; cotran, RS; Collins T. Robbins (1999). Pathologic basis of disease 6th ed. W.B saunders company, Phitadephia,440-459.

Lipid Research Clinics Program (1984a) The lipid Research Clinics coronary primary prevention trial result 1. Reduction in incidence of coronary heart disease. JAMA; 251:251-364.

Lipid Research Clinics program (1984b). The lipid Research clinics coronary primary prevention trial Result II. The relationship of reduction in incidence of coronary heart disease to Cholesterol lowering. JAMA; 251:365-374.

Morris, C J; Earl, J.B; Trenam, C W; Blake, DR (1995). Reactive oxygen species and iron A dangerous partnership in inflammation. Int. J. Biochem Cell Biol; 27:109-122.

Mulenga M(1998). Facing drug resistance: therapeutic option for treatment of uncomplicated plasmodium falciparum malaria in adult Zambians. Journal of Medicine and Health Science; 2(1):11.

Oduala, A. M; Sowunmi A; Milhous, WK; Brewer T.G; Kyle D E; Gerena L; Rossan et al (1998). Invitro and in vivo reversal of choroquine resistance in plasmodium falciparum with promethazine. AM.J. Trop. Med. Hyg; 58(5):625-629.

Price R (1999). Adverse effects in patients with acute falciparum malaria treated with artemisinin derivatives. AM J. Trop. Med. Hyg; 60(4):547-555.

Rang H.P, Dale MM; Ritter JM. Pharmacology. 4th ed. London: Churchill Livingston 734, 1999.

Reitman, S; and frank, S (1957) Transaminases. AM. J. Clin Path, 28:56

Scott, NS; Beffac, MIL (1951). Diacetyl monoxime method for urea estimation. AMJ Chem. Path. 21 (21): 275 Tietz.

Electrolytes in fundamentals of clinical chemistry. 2nd ed. W.B. Saunders company Philadelphia, 1970 PP 612-621.

White NJ; Waller D; Gawley J (1992) Comparison of artemether and chloroquine for severe malaria in Gambian children. The Lancet; 339: 317-321.

World Health Organization report on the 4th meeting of the scientific group on the chemotherapy of malaria (Beijing) WHO Geneva 1981.

WHO (1990) Practical chemotherapy of malaria. Tech. Rep. Ser. 805 WHO Geneva.

WHO (1998). Malaria chemotherapy. Tech. Rep. Ser. WHO Geneva.

Zak B; Slatkis, A; and Boyle, AJ (1957) serum cholesterol estimation. J. Lab-Clin. Med 4:486.