

ASSESSMENT OF SERUM UREA, CREATININE AND BILIRUBIN LEVELS AFTER INDUCING WISTAR RATS WITH MALARIA AND TREATED WITH ETHANOLIC EXTRACT OF ARTEMISIA ANNUA L. AND ARTEMISININ COMBINATION THERAPY (ACT)

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ABSTRACT

The assessment of serum urea, creatinine and bilirubin levels in malaria infested wistar rats treated with the crude extract of *Artemisia annua* and artemisinin combination therapy (ACT) was investigated using 24 wistar rats weighing between 180 – 220g. The rats were divided into four groups of six rats each. Group 1 served as the normal control and received 1ml of distilled water, group 2 animals were malaria induced but untreated and received 0.2ml of distilled water, group 3 animals were malaria induced and received ACT (1.142mg/kg body weight), group 4 animals were malaria induced and received the crude extract from *Artemisia annua* (300mg/kg body weight). The

administration was carried out twice a day for three consecutive days. The rats were given free access to food and water. At the end of administration, blood samples were taken through cardiac puncture into treated sample bottles for analysis. The results showed that there was a significant decrease ($P < 0.05$) in the serum urea levels of all the experimental groups when compared with the control. The serum creatinine and unconjugated bilirubin levels of the experimental groups showed no significant change ($P > 0.05$) when compared with the control. The total serum bilirubin of both the malaria untreated and ACT treated groups (3.37 ± 0.34) and (4.72 ± 0.41) respectively showed no significant change compared with the control (4.07 ± 0.63) while that of the *A. annua* treated group was significantly higher than both the control and other experimental groups.

KEYWORDS: Artemisinin, *Artemisia annua*, *Plasmodium*, serum, creatinine, bilirubin, urea, blood.

INTRODUCTION

Malaria is a life-threatening disease caused by a parasite of the genus *Plasmodium* and transmitted by infected female anopheles mosquitoes through bites or blood meal. Almost half of the world's population (3.2 billion people) is at risk of malaria. Infants, pregnant women and travelers from areas void of malaria are vulnerable to the disease when infected (Wikipedia). In spite of global efforts made to fight malaria, it remains a large burden to the population, particularly the tropical and subtropical regions (Chrubasik and Jacobson, 2010). Of all the current drugs, Artemisinin (Qinghaosu) and its derivatives possess the most rapid action against *Plasmodium falciparum* malaria (White, 1997). Chemically, artemisinin is a sesquiterpene lactone containing an unusual peroxide bridge. This peroxide is believed to be responsible for the drug's mechanism of action (Wikipedia).

Urea is produced in the liver as a waste product from the metabolism of protein and amino acid and is removed from the blood through the kidneys. Blood urea level is within the normal ranges of 6 - 20mg/100ml of blood although the reference range varies within laboratories (Deepk *et al.*, 2007). The levels of urea in blood increases following a high protein diet, it can also increase when there is a decrease in glomerular filtrate rate which suggest that there could be a renal failure, congestive heart failure or decrease blood volume (Dan *et al.*, 2011). Creatinine is a by-product of muscle activities formed from the breakdown of creatinine phosphate in muscles. Creatine is synthesized from the liver and transported to organs such as muscles and brain where it undergoes phosphorylation to form creatinine by an enzyme called creatine kinase (Allen, 2012), and is removed from the blood by the kidneys. Bilirubin which is formerly known as haematoidin is a yellowish compound formed from a normal catabolic pathway that breaks down the heme moiety of haemoglobin in the spleen, liver and bone marrow. Bilirubin is excreted in bile and urine, and elevated levels indicate disease condition. Presently, there is scarcity of information as regard the effects of ACT and/ or the extracts of *A. annua* on serum urea, creatinine and bilirubin levels. It therefore became necessary to assess the effects of ethanolic extract of *Artemisia annua* and artemisinin combination therapy (ACT) on serum urea, creatinine and bilirubin levels in malaria induced wistar rats.

MATERIALS AND METHODS

Collection and preparation of materials

ACTs, Dextrose and Distil Water: The anti malarial drug zymal® (Artemether Tablet 80mg + Lumenfantrine Tablets 480mg) manufactured by Innova CapTab, Pharmaceutical Co Ltd, 81-B EPIP, Phase-I, Jharmajri, Baddi (H.P) India. Manufacturing Lic. No.: MNB/06/394, Distilled water and 5% dextrose water was bought from Turtle Bay pharmacy in Calabar.

Plant material: The leaves of *Artemisia annua* was collected from the biotechnology farm owned and operated by Prof. Ebiamadon Andi Brisibe, of the Department of Genetics and Biotechnology, Faculty of Science, University of Calabar. It was taken to the Botany Department of the University for Identification and specimens were deposited at the department's herbarium.

Parasites: The strain of *Plasmodium falciparum* used for this study was obtained and authenticated by the Calabar office of Roll Back Malaria.

Laboratory animals: A total of 24 Inbreed adult male and female wistar albino rats weighing between 180 - 220g were used for this study, they were purchased from the animal house unit of the Department of Biochemistry, Faculty of Basic Medical Sciences, University of Calabar, and were housed in a well ventilated wooden cages in the animal house, and were fed with rat pellets (growers' marsh manufactured by Vital feeds Ltd, Lagos) and tap water *ad libitum*. The animals were acclimatized for three weeks and their body weights noted before and after the commencement of the experiment. The animals were divided into four groups, based on their weights as shown in table 1.

Innoculation: The infection of the recipient rats was initiated by injection of the parasites preparation to healthy test rats via intramuscular route as described by David *et al* (2004) and Peter and Anatoli (1998). 2ml of *Plasmodium falciparum* sample with a parasite load of 161.5 was diluted with 5% dextrose water using a dilution factor of 1:4 (Shakya *et al.*, 2012). 0.5ml per kilogram of body weight of the diluted plasmodium base solution was subsequently injected into the animals in group 2, 3 and 4 via intramuscular method (David *et al.*, 2004).

Determination of Degree of Parasitaemia: The CareStart™ Malaria HRP2 Pf (Cat #: G0141) test kit, manufactured by Access Bio, Inc. 65 Clyde Road, Somerset, NJ, 08873, USA, was used to investigate the level of infection in the groups of rats that were inoculated

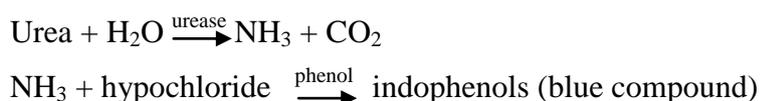
with the malaria parasites. 48 hours after inoculation, a drop of blood was collect from the tails of the infected rats and tested for the presence of *plasmodium* according to the method describe by the manufacturer.

Administration of Drug: The antimalarial drug, zymal[®] (Artemether 80mg + Lumenfantrine 480mg) tablet was used as the artemisinin combination therapy. It was powdered in a mortar, mixed with 50ml distilled water and administered as aqueous suspension by oral gavage at a dose of 1.143mg/kg body weight twice a day for three consecutive days.

Administration of Extract: 40g of the powdered *Artemisia annua* leaves was soaked in ethanol for 12 hours and filtered thereafter. The filtrate was further filtered using a watman filter paper and then concentrated by evaporation using a water bath at 40°C. The 40g of powdered *A. annua* leaves yielded 2.9g of extract. The crude extract was administered to group 4 animals at a dose of 300mg/kg body weight twice a day for three consecutive days.

Collection and preparation of tissue for analysis: After 3 days of treatment, the rats were weighed and fasted overnight. Blood samples were collected from the untreated, treated and control groups for investigation of serum urea, creatinine and bilirubin levels. The animals were anaesthetized with trichloromethane (chloroform), and were then dissected and blood samples were collected through cardiac puncture using sterile syringes into screw cap sterile test tubes.

Estimation of serum urea, creatinine and bilirubin levels: the serum urea concentrations was assessed using randox assay kit (urease-berthlot method) as described by weatherburn (1964). This involves the hydrolysis of urea to ammonia and carbon IV oxide in the presence of an enzyme called urease. Here, the ammonia whose concentration is proportional to the initial concentration of urea in the sample is quantitated photometrically by berthelot's reaction,



The serum creatinine concentration was estimated using randox assay kits as described by the manufacturer (Henry, 1974). This is based on the interaction of creatinine with picrate in an alkaline solution to form a coloured complex which is read colorimetrically at 520nm.

Serum bilirubin level was assessed using randox assay kits as described by the manufacturer (Jendrassik and Grof, 1938). This was done using a colorimetric method as described by Jendrassik and Grof (1938). In this method, direct (conjugated) bilirubin in the sample reacts with diazotized sulphanilic acid in an alkaline medium to form a blue coloured complex whose colour intensity is proportional to the bilirubin concentration in the sample. The total bilirubin is determined by the reaction of the specimen sample with diazotized sulphanilic acid in the presence of caffeine, which releases albumin bound bilirubin. Unconjugated or indirect bilirubin was determined by subtracting the conjugated (direct) bilirubin levels from the total bilirubin result.

Statistical analysis: Data was expressed as mean \pm standard error of mean. The data obtained were analyzed statistically using one-way analysis of variance (ANOVA) at a 95% (0.05) probability level.

RESULT

Table 2 shows the results of serum urea (mmol/l), creatinine (mmol/l), bilirubin (mmol/l).

Effect of treatment on serum urea level: the results presented in table 2 showed that the serum urea level of animals in the malaria untreated group (5.48 ± 0.43), ACT treated group (5.52 ± 0.78) and *A. annua* (4.22 ± 0.43) were all significantly lower ($P < 0.05$) when compared with the control (8.65 ± 0.52). However, the *A. annua* treated group showed lower serum urea content than both the malaria untreated and ACT treated groups.

Effect of treatment on serum creatinine: from the results obtained, the serum creatinine levels for both the malaria untreated group (41.37 ± 2.49), ACT treated group (43.13 ± 2.28) and *A. annua* treated group (44.35 ± 2.65) showed no significant changes ($P > 0.05$) when compared with the control group (43.53 ± 2.22).

Effect of treatment on serum bilirubin: the results obtained from the research also showed that the total serum bilirubin levels for the malaria untreated group (3.37 ± 0.34) and ACT treated group (4.72 ± 0.41) showed no significant change ($P > 0.05$) when compared with the control (4.07 ± 0.63). The *A. annua* treated group was significantly higher ($P < 0.05$) compared with both the control, malaria untreated and ACT treated group but the ACT treated group is significantly higher than the malaria untreated. More so, the conjugated bilirubin (CB) levels of group 3 (3.10 ± 0.36) and group 4 (4.68 ± 0.40) showed a higher significant difference when

compared with the control (2.37 ± 0.66) at $P < 0.05$ while that of group 2 (1.63 ± 0.26) was significantly lower ($P < 0.05$) when compared with the control. The *A. annua* treated group was significantly higher ($P < 0.05$) compared with both the control, malaria untreated and ACT treated group. The ACT treated group is significantly higher than the malaria untreated group. This implies that both the ACT treated and *A. annua* treated groups show a higher conjugated bilirubin levels than the malaria untreated group at $P < 0.05$. The unconjugated bilirubin levels of both group 2 (1.63 ± 0.26), group 3 (1.62 ± 0.50) and group 4 (2.23 ± 0.71) showed no significant change ($P > 0.05$) when compared with the control (1.70 ± 0.52). The *A. annua* treated group shows a higher unconjugated bilirubin than both the malaria untreated and ACT treated group.

Table 1: Experimental protocol

Experimental group distribution of wistar albino rats during treatment with anti malarial drug (ACT) and extract from *Artemisia annua* leaves.

Groups	Numbers of rats	Treatments
1.	6	Normal control
2.	6	Malaria infected and untreated
3.	6	Malaria and ACT treated
4.	6	Malaria infected and <i>Artemisia annua</i> treated

Table 2: Baseline values for serum urea, creatinine and bilirubin levels.

The effect of artemisinin combination therapy (ACT) and *A. annua* extract on the serum urea, creatinine and bilirubin levels of malaria infested wistar rats.

Groups	Urea (mmol/l)	Creatinine (mmol/l)	Total bilirubin (mmol/l)	Conjugated bilirubin (mmol/l)	Unconjugated bilirubin (mmol/l)
1.	8.65 ± 0.52	43.53 ± 2.22	4.07 ± 0.63	2.37 ± 0.66	1.70 ± 0.52
2.	5.48 ± 0.43^a	41.37 ± 2.49	3.37 ± 0.34	1.63 ± 0.26^a	1.63 ± 0.26
3.	5.52 ± 0.78^a	43.13 ± 2.28	4.72 ± 0.41^b	$3.10 \pm 0.36^{a,b}$	1.62 ± 0.50
4.	$4.22 \pm 0.36^{a,b,c}$	44.35 ± 2.65	$6.92 \pm 0.53^{a,b,c}$	$4.68 \pm 0.40^{a,b,c}$	2.23 ± 0.71

Values expressed as mean \pm SEM, n=6, $P < 0.05$

a significantly different from control (group 1) at $P < 0.05$

b significantly different from group 2 at $P < 0.05$.

c significantly different from group 3 at $P < 0.05$.

DISCUSSIONS

From the results obtained, the decrease in the serum urea levels of the experimental groups may be an indication that there was an impaired renal function or over hydration following

malaria infection. Urea nitrogen is a waste product gotten from the breakdown of protein in the body. It is usually excreted by the kidney in urine, decrease serum urea levels could also suggest that less protein is being broken down by the liver and this occurs when the liver is affected as reported by Omotuye *et al.*, (2008) and Izunya *et al.*, (2010) that artesunate, a derivative of *A. annua* could exert some hepatotoxic effect.

Serum creatinine levels of malaria untreated, ACT treated and *A. annua* treated groups showed no significant difference when compared with the control. The increased total bilirubin in group 4 may indicate an impairment of the liver functions. This suggests that the extract may have the ability of increasing the breakdown of the Red blood cells infested by the plasmodium parasites. Bilirubin is produced when the old red blood cells are broken down by the liver. It's normally low in the blood, so its elevation may indicate certain diseases like impairment of the liver functions, blocked bile ducts, etc. it is responsible for the colour of stool and urine. The total bilirubin of both group 2 and 3 showed no significant difference when compared with the control. But ordinarily, bilirubin level rises in malaria untreated patients. A significant increase in the conjugated bilirubin levels was recorded in both group 3 and 4 while that of group 2 was significantly lower when compared with the control, which implies that both *A. annua* extract and ACT could impair bilirubin excretion. No significant change was recorded in the unconjugated bilirubin level of both group 2, 3 and 4 compared with the control. Conjugated bilirubin is virtually absent from the serum of healthy individual because of the rapid secretion of bile which aids its excretion. The breakdown of heme from haemoglobin produces unconjugated bilirubin which is transported to the liver where it is conjugated with UDP glucuronyltransferase making it water soluble and to be easily excreted.

CONCLUSIONS

The use of medicinal plants in the treatment of various ailments has become rampant and has led to an extensive research on various plant species and their therapeutic properties, including comparing these natural plants with their synthetic forms. In this research, the *A. annua* extracts seems to have an increased ability to selectively break down red blood cells infested by plasmodial parasites, thereby elevated the serum bilirubin levels. More research on this property of the ethanolic extract of *A. annua* is recommended.

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