**Effect of Phytocystatim from *Moringa oleifera* Leaf on some Biochemical Parameters of Mice Infected with *Plasmodium berghei* NK65**

ABSTRACT

Current drugs used in the management of Malaria encounter parasite resistance, hence the need for urgent and readily available alternative source of antiplasmodial agents. The effect of crude phytocystatin extract of *Moringa oleifera* leaf in mice experimentally infected with *Plasmodium berghei* NK 65 strain was investigated. The oral acute toxicity of the extract was determined in mice. Parasitaemia was estimated daily while Total protein, albumin, globulin, free fatty acid packed cell volume (PCV), were determined at days 5 and 10 post treatment. The results showed that the oral median lethal dose (LD50) was greater than 5000 mg extract/ Kg body weight. There was extension of life in the treated groups when compared to the negative control. The administration of the crude phytocystatin extract led to a highly significant (P<0.05) increase in Total protein, albumin, and packed cell volume levels while the globulin and free fatty acid levels decreased significantly (P<0.05). It is concluded that the crude phytocystatin extract of *Moringa oleifera* leaf has antiplasmodial activity and is effective in the management of anaemia induced by *Plasmodium berghei* in mice.

**Introduction**.

Malaria is a major cause of morbidity and mortality and it is estimated that more than half World population were at risk of malaria with an estimated 0.7-1 million deaths per year and over 106 countries are malaria-endemic(WMR, 2019).Evolution of resistance to most affordable drug such as chloroquine and gradual decline in the efficacy of artemisinine base combination therapies(ACTs) in *Plasmodium* species(Karthik *et al* 2014),with no effective vaccine in sight and resistance of vector to insecticides, necessitates the need for novel entities, ideally directed against new targets such as malarial cysteine proteases (Rosental,2011;Karthik *et al* 2014). The life cycle of malarial parasite exhibits two stages: exoerythrocytic cycle and erythrocytes life cycle. The erythrocytes life cycle was responsible for all clinical manifestations and it begins when parasites(merozoites) invade erythrocytes and develop to larger, more metabolically active form(trophozoites) followed by multinucleated schizont stages which bust out of red blood cells and reinvade the erythrocytes(Karthik *et al* 2014).Plasmodium Cysteine protease is required for the invation,rupture of erythrocytes and subsequent degradation of haemoglobin from the host(Rosental,2011).The amino acid released from haemoglobin degradation is used for the synthesis of parasite protein and hence survival of these parasite inside the host organism. Inhibition of haemoglobin degradation offers a valid target for developement of novel chemotherapeutic agents.

Phytocystatin are Cysteine protease inhibitor(CPI) found in plants Their major functions in plants includes; storage proteins, regulators of endogenous proteolysis, cell death, defence against insect and pathogens attack(Ceros and Carbonell 1993).Efficacy of synthetic peptides CPI and non protein CPI from plants in treatment of diseases caused by *Trypanosoma cruzi*, *Leishmania major,* viruses and cancer treatment has been established(Mane *et al*,2013).

 M*oringa oleifera* is a shrub plant, an angiosperm, dicot and perennial. It is also called drumstick tree, horseradish tree or Ben tree, *Moringa ol* has been proven to be useful sources of food, medicinal products, fuelwood, renewable polymer products, animal and aquaculture feeds(Lawal *et al*., 2015). In Nigeria, it is locally used as tonic and aphrodisiac, and in the treatment of intestinal worms and asthma. various parts of the tree are used therapeutically, including for treatment of rheumatism, venomous bites, and as cardiac and circulatory stimulants, cholera, scurvy, respiratory ailments, tumours and they are also applied externally to cure inflammatory swellings. Juice extracted from the leaves has antibacterial and antimalarial properties (Lawal *et al*., 2015). Anti-plasmodium properties of the cysteine protease inhibitor from crude extract of *Moringa oleifera* seed is reported.

**Method:**

**Plant Material:**The plants was collected and identified in the Department of Biolological Sciences,F.U.T.,Minna. Seeds, of *M.oleifera* was used for the study.

**Animals:** Swiss albino mice of either sexes, ages 4-6 weeks old and weighing 20-25 g each were used.

**Extraction of Phytocystatin:**

Twenty five(25g) of seeds of *Moringa oleifera* were blended with 100 mL of phosphate buffer 0.1 M (pH 7). The clear supernatant obtained after homogenisation and centrifugation(10,000 rpm, 15 min, 40C) was precipitated by cold acetone. This was designated as the crude acetone extract of phytocystatin.( Rao, *et al,*1983)

**Phytocystatin assay:**

 Protease inhibitor activity was assayed according to the method of (Wannapa,2006). Benzyl arginine para nitroaniline( BApNA) assays was performed by adding 290µL of 50mM Tris–HCl (pH 7.6) and 200µL of 1.25mM BApNA solution to the previoustly peincubated cysteine protease enzyme (papain) and crude inhibitor extract for 10min. at 250 c. After 30 min at 370c , the reaction was stopped by adding 150µL of acetic acid (30%). The resulting color was measured by absorbance at 405nm.percent inhibitory activity calculated compare to control.

**Animals**

 Mice (25-30 g) obtained from Veterinary Physiology and Pharmacology Department, Ahmadu Bello University,Zaria were used in the study. The mice were fed with grower’s mash, water *ad libitum* and maintained under laboratory condition.. All experiments were performed according to the “Principles of Laboratory Animal Care” (NIH Publication No. 85; rev. 1985).

**Acute toxicity (LD50) study**

 Acute toxicity study was carried out using the method decribed by Lorke (1983). Briefly this study was carried out in 2 phases. In the first phase, nine mice randomly divided into three groups of three animals each were orally administer with the extract at 10, 100 and 1000 mg /kg body weight respectively. In the second phase, three mice were each, administered with the extract at 1600, 2900 and 5000 mg /kg body weight, respectively. At each phase, mice were observed for signs of adverse effects which may include restlessness and mortality for 24 h and subsequently for 14 days.

**Plasmodium inoculation**

*Plasmodium berghei* Nk 65 strain obtained from the National Pharmaceutical Research Institute , Abuja, Nigeria was used for the study. The organism maintained by serial passage in mice was inoculated into the donor mice. A heavily infected blood sample from donor mice was diluted with physiological saline to obtain inocula. Healthy mice (I - V) of 5 animals each were then inoculated with 0.2 ml of the diluted blood sample.

**Administration of the extract**

Group I animals were given 500 mg extract/kg body weight 24 h post parasite inoculation**,** group II mice were given500 mg extract/kg body weight 48 h post parasite inoculation. Group III mice received 500 mg extract/kg body weight at establishment of infection while groups IV and V mice served as the negative and positive control respectively and were not treated with the extract. Treatment was daily for 4 consecutive days.

**Therapeutic monitoring of extract**

Development of Parasitaemia in these mice was checked daily by wet blood film prepared from tail blood at x40 magnification. The number of parasite seen per field under the microscope was counted as described by Peters (1965). Total plasma Proteins and albumin concentrations were measured biuret and bromocresol green methods (Tietz, 1976), respectively on days 5 and 10 post treatment .The plasma globulin concentration was calculated as the difference between total protein and albumin concentrations. The determination of the Free Fatty Acids levels was carried out as described by Koichi (1977). Packed Cell Volume (Kelly, 1977),

**Statistical analysis**

 Results were expressed as mean ± SEM. All parameters were analyzed statistically using Student t-test. P value of < 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Acute toxicity**

No death was observed from the animals at all the doses used. The animals gained weight throughout the study duration. The only behavioral signs of toxicity observed in the mice at dose of 5000 mg /kg body weight of the extract in the course of the study were restlessness, inflammation on the mouth and nose. The oral LD50 was therefore greater than 5000 mg/kg body weight.

 **Anti-plasmodial study**

**Table 1**. Effect of crude Phytocystatin Extract from *Moringa oleifera* Leaf on Parasite count in mice infected with *Plasmodium berghei*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Group I**  | **Group II**  | **Group III**  | **Group IV**  | **Group V**  |
| Day 5  | 4.37±1.42\* | 4.17±1.42\*  | 12.40±2.31\*  | 22.75±6.56  | 0  |
| Day 6  | 80.95±0.00\*  | 128.15±20.09  | 163.23±26.78\*  | 132.95±13.15  | 0  |
| Day 7  |  90.80±4.85\* | 157.45±16.12\* | 205.29±13.16\*  | 260.13±6.71  | 0  |
| Day 8  | 137.74±8.08\*  | 98.45±7.96\* | 183.19±6.67\* | 343.85±13.32  | 0  |
| Day 9  | 168.44±4.05\*  | 167.38±12.07\*  | 211.65±11.75\*  | 452.42±13.55  | 0  |
| Day 10  | 195.94±0.00  | 137.74±8.08  | 167.05±20.80  | 0  | 0  |

 \*\* Values significantly different from the infected untreated control group (group IV) at p<0.05

**Table 2*.***Base line values of some Biochemical Parameters of Animals infected with *Plasmodium berghei.*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Groups**  | **Protein (mg/dL)**  | **Albumin (mg/dL)**  | **Globulin (mg/dl)**  | **Free fatty acid (mg/dl)**  | **PCV values**  |
| **I**  | 6.90±0.06  | 4.70±0.06 | 2.35±0.02  | 0.64±0.04  | 41±0.01  |
| **II**  | 6.95±0.02  | 4.70±0.08  | 2.20±0.04  | 0.62±0.01  | 40±0.12  |
| **III**  | 6.80±0.02  | 4.75±0.20  | 2.30±0.04  | 0.63±0.02  | 41±0.04  |
| **IV**  | 6.75±0.06  | 4.80±0.20  | 2.10±0.04  | 0.63±0.02  | 41±0.06  |
| **V**  | 6.50±0.04 | 4.15±0.02  | 2.20±0.04  | 0.62±0.02  | 40±0.01  |

**Table 3.** Effect of Phytocystatin Extract from *M.oleifera* Leaf on some biochemical parameters of mice infected with *Plasmodium berghei* on day 5 post treatment

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Groups**  | **Protein (mg/dL)**  | **Albumin (mg/dL)**  | **Globulin (mg/dl)**  | **Free fatty acid (mg/ml)** | **PCV values**  |
| **I**  | 7.34±0.02b  | 5.10±0.04 b  | 2.05±0.06 b  | 1.26±0.004 b  | 39±0.03 b  |
| **II**  | 6.70±0.02b  | 3.90±0.04 b  | 2.85±0.02 b  | 1.57±0.0 b  | 38±0.05 b  |
| **III**  | 6.15±0.02**b** | 3.55±0.10 b  | 2.50±0.12 b  | 1.83±0.00 b  | 38±0.08 b |
| **IV**  | 5.8±0.00a  | 0.15±0.02 a  | 5.65±0.02 a  | 6.2±0.00 a  | 25±0.02 a  |
| **V**  | 6.4±0.08b  | 4.40±0.00 b  | 2.20±0.00 b  | 0.62±0.04 b | 40±0.03 b  |

n=5 Values with different superscript are Significantly different at p<0.05

**Table 4.** Effect of Phytocystatin Extract from *M.oleifera* Leaf on some biochemical parameters of mice infected with *plasmodium* on day 10 post treatment

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Groups**  | **Protein conc (mg/dL)**  | **Albumin conc. (mg/dL)**  | **Globulin conc (mg/dl)**  | **Free fatty acid conc. (mg/dl)**  | **PCV values**  |
| **I**  | 6.80±0.04  | 4.10±0.04  | 2.80±0.45 | 0.96±0.004\*\*  | 37±0.09  |
| **II**  | 6.30±0.08  | 3.90±0.02  | 2.45±0.10  | 0.99±0.004\*\*  | 36±0.13  |
| **III**  | 5.70±0.00  | 3.10±0.04  | 2.70±0.04  | 1.01±0.08  | 34±0.07  |
| **IV**  | 4.90 ±0.00 | 2.80±0.02  | 2.90±0.03  | 1.05±0.07  | 24±0.17  |
| **V**  | 6.40±0.04  | 4.20±0.08  | 2.20±0.04  | 0.63±0.004\*\*  | 41±0.05  |

n=5

\*\* Significantly different from the infected untreated control (group IV) at p<0.05

 I Animals in the group received 500 mg extract/kg body weight 24 h post parasite infection

 II Animals in the group received 500 mg extract/kg body weight 48 h post parasite infection

 III Animals in the group received 500 mg extract/kg body weight at the establishment of infection

 IV Mice in the negative control were infected with the parasite but were not treated with the extract

 V Mice Positive controls were neither infected nor treated with the extract. Treatment was daily for 5 consecutive day

The crude extract of phytocystatin reduce the parasitaemia level significantly time dependently (P<0.05) in all the treated groups when compared with the negative control throughout the duration of study(Table 1).

There was a significant (p<0.05) time dependent increase in total protein, albumin and globulin levels in all the treated groups on days 5 and 10 post treatment of the study when compared with the infected untreated control (Tables 2 - 4). The free fatty acid levels decreased significantly (p<0.05) in all the treated groups on days 5 and 10 post treatment of the study when compared with the infected untreated control as shown (Tables 2 - 4). The PCV value increased significantly (p<0.05) in the treated groups and the positive control on days 5and 10 post treatment when compared to the negative control. The absence of mortality at dose level of 5000 mg extract/kg body weight shows that the Phytocystatin extract of *M.oleifera* is non-toxic in mice(Lorkes, 1983) and is therefore safe for use. The results obtained from our study on the anti-plasmodial effect of phytocystatin extract from seed of *M.oleifera* showed that the extract possesssuppressive effects on plasmodium parasites, since it slowed down the rate of proliferation of the parasite. This may be due to the ability of the extract to inhibit prostaglandin biosynthesis as has been reported by Tijani *et a*l. (2008). Previous work by Allison (1978) has shown that inhibitors of prostaglandin synthesis reduces parasitaemia and shortens the time to parasite wave remission. There have been reports of decreased plasma albumin concentrations in several protozoan infections (Anosa, 1988) and this has been linked to either plasma expansion, proteinuria (Bruiju, 1987). The decrease in serum total protein could be attributed to a decrease in serum albumin probably from decreased hepatic biosynthesis. This may be explained that, malaria cause by plasmodium parasites induced an elevation of serum transaminases. This may indicate that *plasmodium* infection causes reduction in liver function which includes protein synthesis(Adah *et al*. 1993).The extract elevated the albumin level in the treated groups which may suggest the ability of the extract to prevent hepatocellular damage caused by the *plasmodium* infection. The elevated albumin level may account for low level of free-fatty acid observed in all the treated groups because free fatty acid normally does not exist in free state in the blood but complexed to serum albumin (Catherine *et al*., 2006). It has been reported that the free fatty acid attains haemolytic level only when the serum albumin binding capacity is exceeded (Tizard *et al*., 1978).It has earlier been shown that severe haemolytic crisis that occurs in most protozoan infections is caused by surface active lipids which are cytolytic and free fatty acid has been grouped among such agents especially linoleic acid (Tizard *et al*., 1978).The significant increase in packed cell volume (PCV) values of all infected and treated mice irrespective of time of treatment may in part be due to the ability of the extract to reduce the level of circulating surface active agents such as the free fatty acids especially linoleic acid that has been implicated in surface of lyse red blood cells. Previous work by Bisalla *et a*l. (2007) reported a PCV value of 23% in animals infected with *Plasmodium berghei* which is consistent with our results as the PCV values of the infected untreated mice reduced to 25%. However, the decrease in PCV values of the extract treated groups was insignificantly different from the positive control, it is therefore suggested that the extract prevented lysis of the red blood cell induced by circulating free fatty acids level. It is evident that *Moringa oleifera leaf* reduced rate of proliferation of parasitaemia in all the extract treated groups. In addition it prolonged the survival of infected mice by reducing the accumulation of free fatty acids in circulation implicated in lysis of red blood cell leading to anaemia which cause eventual death from malaria infection. It is therefore effective in the management of malaria related anemia.

**Conclusion**

This study has demonstrated that phytocystatin from *Moringa oleifera leaf* possesses antimalarial as well as anti-haemolytic effects in plasmodium berghei infected mice

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