**ANTIPLASMODIAL POTENTIAL AND SUBCHRONIC TOXICITY OF CYSTEINE PROTEASE INHIBITORS FROM *CALOTROPIS PROCERA***

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**Key Words**: Cysteine Protease,Inhibition, *Plasmodiun berghei*,Subchronic,*Calotropis procera*.

**Abstract.**

 Cysteine protease of plasmodium parasite play major roles in the survival of this organism inside its host.Various extraction media which include Sodium Chloride, Sodium Hydroxide, Hydrochloric acid,Sodium phosphate buffer and distilled Water were used to evaluate Leave,root,flowers,latex and stem bark of *Calotropis procera*  for  Cysteine Protease inhibitory activity against Papain enzyme.Among the extraction media Sodium phosphate buffer extract of latex of *Calotropis procera* with higher Protease inhibitory activity was concentrated by cold acetone precipitation and freeze dried as crude extract.The effect of this extract on subchronic toxicity studies in mice was carried out. Inhibition of  *Plasmodium berghei* Cysteine Protease and invivo *Plasmodium berghei* infected mice by this extract in graded doses of 20,30 and 50mg/kg body weight were evaluated.The crude extract showed inhibitory activity against Cysteine protease of *Plasmodium berghei* with IC50 value of 25.50 µg/ml . In invivo model, the highest level of parasitemia suppression of 48% was observed in 50 mg/kg body weight of the crude inhibitor extract.Subchronic toxicity of the extract at 50mg/kg body weight concentration revealed statistical significant elevation in body weight and Alanine transaminase enzyme.Total protein and glucose concentration were found to be elevated as doses increases, but no significant changes were observed in PCV,triacylglycerol,Aspartate transaminase and Alkaline Phosphotase.This result showed that crude cysteine protease inhibitor from Latex of *Calotropis procera* is active against plasmodium Cysteine Protease and in a rodent malaria model.It also sugest further investigation of the anti-malarial activities from Latex of this plant.

**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, 2012).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(Dondorp *et al* 2009),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.Cysteine protease inhibitor(CPI) in plants functions as: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).

*Calotropis procera* a plant of *Asclepiadaceae* familyhas received special attention because of its use in folk medicine (Marcia *et al,*2010). Previous reports have confirmed that this plant possesses various pharmacological properties that may be used for the treatment of inflammatory disorders such as arthritis, cancer and sepsis (Kumar *et el*,2014).This research is aimed at evaluating the anti-plasmodium properties of the cysteine protease inhibitor crude extract of *Calotropis procera*

**Method:**

**Plant Material:**The plants was collected and identified in the Department of Biological Sciences,F.U.T.,Minna., leave,latex, flowers,root and stem bark were used for the study.

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

**Parasite:***Plasmodium berghei* NK65 strain was obtained from Dept of Pharmacology,A.B.U,Zaria.

**Extraction of protease inhibitor :**

Twenty five(25g) of Fresh leave,latex, root,flowers and stem bark of *Calotropis Procera* were blended with 100 mL each of sodium chloride 15% (w/v) , sodium hydroxide 0.2% (w/v), hydrochloric acid 0.05M, phosphate buffer 0.1 M (pH 7) and distilled water as described by (Wu and Whitaker,1990). The clear supernatant obtained after homogenisation and centrifugation(10,000 rpm, 15 min, 40C) was assayed for protease inhibitor activity and protein content.

**Acetone precipitation:**

Four volumes of cold acetone was added with stirring to crude extract. After 20 min stirring, the precipitate was collected by suction filtration, washed with cold acetone. The precipitate was air dried and stored at 4°C under desiccation. Acetone powder was suspended in 50mΜ phosphate buffer, pH 7.6. This was designated as the crude acetone extract.( Rao, *et al,*1983)

**Protease inhibitor assay:**

 Protease inhibitor activity was assayed according to the method of (Wannapa,2006). An aliquots 0f 500µl from extract was pre-incubated for 10 min at 37 0c with 500 µl papain prepare in 100mM phosphate buffer, pH 6.8, with 0.3mM EDTA and 2 mM cysteine-HCl.The assays was initiated by the addition of 1ml substrate solution (0.5% (w/v) azocasein),incubated for 20 min at 37 0C, and 2 ml of trichloroacetic acid (TCA, 20% w/v) to terminate the reaction. After 20 min at room temperature, the mixture was centrifuged at 10,000g for 10 min and the absorbance of the supernatant measured at 410 nm. 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.

**Protein estimation.**

 Protein content was determined according to the method of (Bradford, 1976)

**LD50  Determination and subchronic tocxicity.**

LD50 was obtained as describe by Lorke,(1983).SubChronic toxicity was determined according to method of Aniagu, *et .al.,* (2004).Mice (20) was divided into four groups of five mice each,the first group will serve as control, given only 20ml/kg/bw phosphate buffer saline(PBS), while the remaining 3 groups,will received 20mg/kg/bw,30 mg/kg/bw and 50 mg/kg/bw respectively of crude extracts intraperitonealy (i.p) for 21 days.Weights of mice,PCV, serum (total proteins, triglycerides,Glucose, SGPT and SGOT,Alkaline phosphatase) was determined using kits(randox).

**Inoculation of Mice and Parasite count:**

A donor mouse with rising parasitemia of 20 % was sacrificed and blood was drawn in a Heparinised syringe and diluted in phosphate buffered saline.Infection was initiated by

0.2ml needle passage of the parasite preparation from a donor mouse to healthy mice via

intraperitoneal route.Parasitemia was monitored by microscopic Giemsa-stained thin blood smears. The number of parasitized erythrocytes in about 10-50 fields was counted twice and the average was computed to give the parasitemia of each mouse.

Percent parasitemia was calculated as:

 % Parasitiemia = Number of parasitized RBC X 100

 Total number of RB

**Preparation of parasite cysteine protease Extracts:**

 Blood from infected mice at the peak of parasitemia will be incubated with 0.01% saponin in phosphate buffer saline (PBS) at 370C for 10min to lyse RBC and this will be washed 3 times in ice cold PBS. The released parasite will be lyse using the protocol described by Amlabu *et al.* (2011)

 **Antiplasmodial Screening of extract:**

The extract from latex of the plants showing highest specific papain inhibition was use for in vivo 4-day suppressive test. Mice was maintained and bred at the animal facility of the University. Thirty six mice was group into five groups(1,2,3,4,and 5). They were infected with parasitized erythrocytes from a donor mouse with 0.2ml of of diluted(108 parasitized erythrocytes/ml) each intraperitoneally(i.p) Groups 1 and 2 serves as Negative(PBS) and positive control(10mg/kg/b.w),while groups 3,4 and 5 were treated with 20mg/kg/b.w,30mg/kg/b.w and 50mg/kg/b.w.Treatment of mice commence 2hours after infection and repeated daily for 72 hours.

 % Parasite inhibition of the extract=

 Parasitemia in negative control - Parasitiemia intreatment x100 Parasitemia in negative control group

 RESULTS:

Fig 1:Specific Protease inhibitory activity  *calotropis procera* parts with different solvents. Papain inhibitory activity was quantified by incubating papain and plant extract with 0.5% Azocasein

Values are Mean ± SD

Different letters within the same parts of plant indicate significant different(<0.05)

 Fig 2 :***Plasmodium berghei* Protease inhibtory activity of of the acetone precipitated latex of *Calopropis procera***

Papain inhibitory activity was quantified by incubating papain and plant extract with1.25mM BAPNA substrate with IC50 of 25.5µg/ml

Values are Mean ± SD(n=3)

Table1: Determination of Median lethal dose(LD50)

|  |  |
| --- | --- |
|     |   Death  |
| Plant sample | Experiment | Dose(mg/kg) | After24hrs | LD50(mg/kg/b.w) |
| *Calotropis procera* (Latex)      | Phase1 | 10 | 0/3 | 490.00 |
|    | 100 | 0/3 |
| 1000 | 3/3 |
| Phase2   | 200 | 0/3 |
| 400 | 0/3 |
| 600 | 2/3 |

LD50 was calculated as geometrical mean of lowest dose that produced motality

**FIG3:Mean Body Weight of Experimental Mice treatedwith CPI from latex of *Calotropis procera*  for 21 days**

Values are Mean ± SEM (n=5)

**Fig4: Mean** **Packed cell volume(PCV) of Experimental Mice  treated with CPI from *Calotropis procera latex*  for 21 days**

Values are Mean ± SEM

|  |
| --- |
| **Table2:Effect of Biochemical parameters on mice treated with crude CPI extract from *calotropis procera* latex** |
|   | Glucose(mg/dl) | Protein(mg/dl) | Triacylglycerol(mg/dl) | AST(U/L) | ALT(U/L) | ALP(U/L) |
| PBS(control) | 95.73±0.42a | 4.86±0.09a | 150.88±2.34a | 36.56±0.47 | 39.24±0.39a | 129.88±0.63a |
| 20mg/kg/b.w  | 93.28±0.42a | 5.44±0.22b | 145.54±1.25ab | 36.40±0.80 | 40.64±0.71ab | 128.94±0.74a |
| 30mg/kg/b.w | 103.02±2.00**b** | 4.74±0.28a | 140.66±0.67b | 37.32±.94 | 38.50±0.31a | 128.04±0.34ab |
| 50mg/kg/b.w  | 108.04±1.07**c** | 5.52±0.08b | 148.12±2.69a | 37.64±1.44 | 42.82±1.43b | 126.70±0.73b |

Values are Mean ± SEM

Values with different superscript letters within the same column are significantly different (P < 0.05)

CPI-Cysteine protease inhibitor

**Fig 5:Parasiteamia levelof the experimental mice infected with *P.berghei* and treated with crude CPI from *Calotropis procera latex***

Values are Mean ± SEM

CPI-Cysteine protease inhibitor

Table 3:Four days suppressive test of crude CPIextracts

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|

|  |
| --- |
| *Calotropis procera* (latex) |
|   |  %Parasitaemia |  %Suppression |
| Infected+PBS(negative control) | 14.56±0.80 |   |
| Infected+artesunate 10mg/ml(positive control) | 1.82±0.34 | 87.50±0.24c |
| Infected+20mg/kg/b.wCPI | 8.82±.86 | 39.42±0.42a |
| Infected+30mg/kg/b.w CPI | 7.62± 0.62 | 47.67±0.52b |
| Infected+50mg/kg/b.w CPI  | 7.57±0.35 | 48.00±.32b |

 |

Values are Mean ± SEM

Values with different superscript letters within the same column are significantly different (P < 0.05)

TABLE 4:Effect of crude CPI extract on the body weights of *P. berghe*i infected mice

|  |  |
| --- | --- |
|  | Body weight(g) |
|   | Pre treatment | post treatment |
| Normal Control(PBS) | 26.20±2.48 | 35.00±1.87a |
| Inhibitor control(IC) | 27.80±1.30 | 37.60±1.14 a |
| Infected+PBS(negative control) | 28.00±1.58 | 19.50±2.12 b |
| Infected+artesunate | 24.20±1.92 | 33.40±2.88 a |
| Infected+20mg/kg  | 27.00±1.58 | 32.40±4.77 a |
| Infected+30mg/kg  | 28.60±3.64 | 31.80±4.91 a |
| Infected+50mg/kg  | 29.00±1.58 | 33.60±6.22 a |

Values are Mean ± SEM

Values with different superscript letters are significantly different (P < 0.05)

TABLE 5:Effect of crude CPI extract on the Packed cell volume(PCV) of *P. berghe*i infected mice

|  |  |
| --- | --- |
|  | PVC(%) |
|   | Pre treatment | post treatment |
| Normal Control(PBS) | 50.60±2.07 | 52.40±3.84b |
| Inhibitor control(IC) | 47.60±2.07 | 48.60±7.07 ab |
| Infected+PBS(negative control) | 43.80±7.85 | 33.00±6.69 a |
| Infected+artesunate | 49.20±1.92 | 50.00±2.19 b |
| Infected+20mg/kg  | 46.00±3.16 | 46.40±4.63 ab |
| Infected+30mg/kg  | 47.60±2.07 | 45.00±2.23 b |
| Infected+50mg/kg  | 50.60±2.07 | 52.40±3.84 b |

Values are Mean ± SEM

Values with different superscript letters within the same column are significantly different (P < 0.05)

**Fig 5:Percentage survival of the experimental mice infected with *P.berghei* and treated with crude CPI from *Calotropis procera latex***

**Discussion.**

Among all solvent used, phosphate buffer had the highest specific inhibitory activity for crude CPI from latex of  *calotropis procera*(fig.1) and this was cold acetone precipitated and used for toxicity and invivo antimalarial activity.

The crude CPI from latex extract showed inhibitory activity against Cysteine protease of *Plasmodium berghei* with IC50 value of 25.50 µg/ml(fig2). Crude CPI produced a dose dependent 4-day chemosuppressive effect at the various doses used in the study corresponding to suppression of 39.42%,47.67%,48.00% by 20 mg/kg, 30mg/ kg and 50mg/kg crude CPI respectively (table3). All infected mice treated with 20,30 and 50mg/kg extract survived much longer than untreated group (fig5), infected but untreated grp animals was all dead by day 11 of treatment. Only mice treated with 50mg/kg survived for 25days of treatment period with parasites persisting in circulation,grp treated with stantard drug had parasites cleared completely on 5th day of treatment. Untreated mice parasite count increased and pcv decreased markadly until the death of the animal, this may be as a result of destruction of red blood cells by the invasion of parasite which may lead to erythrophagocytosis. Several in vitro and in vivo studies using inhibitors such as peptidyl fluoromethyl ketones, vinyl sulfones, aldehydes and nonpeptidic inhibitors inhibits cysteine protease of *Plasmodium*  and cure mice infected with *Plasmodium berghei* parasite(Rosenthal,2011;Mane *et al*,2013).Similarly(Karthik et al,2014) demonstrated serine protease inhibitor isolated from Marine Actinobacteria that cure *Plasmodium berghei*  infected mice.

 Subchronic toxicity effect of crude CPI extract on mean body weight and packed cell volume(PCV) presented in figure3 and 4 shows no significant diffence(p<0.05) in both parameters in respect of treatment groups compare to control.

some biochemical parameters in mice admistered extract( table2).shows Alanine transaminase,Total protein and glucose concentration were found to be elevated as doses increases, but no significant changes (p<0.05)were observed in PCV,triacylglycerol,Aspartate transaminase and Alkaline Phosphotase compared to control. Crude cysteine protease inhibitor from Latex of *Calotropis procera* is active against *plasmodium berghei* Cysteine Protease , with considerable invivo antiplasmodial activity in a rodent malaria model and low toxicity of extract in the animals.We are currently involve in the purification of this inhibitor from the plant using Hydrophobic chromatography.

**Aknowledgement.**

We are grateful to University board of research(UBR),Federal University of Technology,Minna for supporting this work and Mal.Y.Y.Pala of Veterinary Teaching Hospital for his support.

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