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Original Research Article

Evaluation of the Biochemical Parameters and Inhibition Studies of Leaf and Stem Bark Methanol Extracts of *Diospyros Mespiliformis* on *Plasmodium Berghei (Nk*65) Cysteine Protease

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ABSTRACT

Biochemical evaluation of different fractions of leaf and stem bark methanol extracts of *Diospyros mespiliformis* and the inhibitory effects of the extracts on cysteine protease of the parasite were investigated. Aspartate aminotransferase and Alanine aminotransferase studies of the leaf methanol extract showed that, there was significant ($P < 0.05$) increase in the n-butanol and aqueous fractions. Also, significant ($P < 0.05$) increase in the serum level of Alkaline phosphatase of the infected group in all the fractions. AST ALT and ALP studies of the stem bark extract had significant ($P<0.05$) increase in the infected group of all the fractions and significant ($P<0.05$) reduction in the infected but treated with both low and high dose of the extract. Total Protein of the leaf extract had significant ($P<0.05$) increase in n-butanol and aqueous fractions while stem bark extract had significant ($P<0.05$) increase in ethyl acetate fractions of the infected group. The level of Serum Bilirubin, shows significant ($P < 0.05$) increase in the level of direct bilirubin in ethylacetate and N-butanol while stem bark shows significant ($P < 0.05$) increase in the level of direct bilirubin in all the fractions of the infected group. Stem bark extract had significance ($P<0.05$) increase in creatinine level in the normal but treated with medium dose of the extract and infected group but significance ($P < 0.05$) reduction in the infected, treated with medium dose. The mechanism of action of the leaf and stem bark methanol extracts exhibited competitive and non-competitive pattern of inhibition respectively. These plant extracts could therefore be effective therapeutic agents for the treatment and management of malaria.

Keywords: Antimalarial, Plasmodium berghei, Biochemical parameters, Inhibition studies, Cysteine protease.

INTRODUCTION

Malaria remains one of the most dreaded human parasitic diseases in the tropics and subtropics, especially the African and Asian developing/under developed nations. It is still a major cause of mortality in children (<5 years). [1] In addition to its direct health impact, malaria imposes a huge economic burden on afflicted individuals and nations, through high health care cost, missed days at work or school, and reduced economic output and productivity. [2] Despite the success recorded with the Artemisinin Combination therapy (ACT), about 80% of most malaria endemic communities still rely on traditional herbal medicines, which are often more affordable, accessible and available. [3] In view of the problems associated with antimalarial drug resistance and the use of substandard ACT's, researchers are now focusing on other alternatives, including

investigation of medicinal plants known to have antiplasmodial activity. [4,5] Cysteine proteases are so-named due to the function of a catalytic cysteine, which mediates protein hydrolysis via nucleophilic attack on the carbonyl carbon of a susceptible peptide bond. Cysteine proteases play important roles in the life cycles of malaria parasites. Cysteine protease inhibitors block haemoglobin hydrolysis and development in *Plasmodium falciparum*, suggesting that the cysteine proteases of this major human pathogen are appropriate therapeutic targets. [6] A number of older studies have supported this concept, with the demonstration that cysteine protease inhibitors have potent in vitro and in vivo antimalarial effects [7] Drug development has shown that, proteases are drug-able target and protease inhibitors, in clinical development are used to treat diabetes, thrombosis, osteoporosis, hypertension, cancer and infectious diseases. [8]

MATERIALS AND METHODS

Chemicals/reagents

All assays kits were from Randox La boratories Ltd. Ardmore, Co. Antrum UK. C hemicals and reagents used were purchased from Sigma Chemical Company St. Louis U.S.A.

Experimental animals

A total of one hundred and sixty (160) mice of both sex weighing between 18-28g were purchased from the animal house, Department of pharmacology, Ahmadu Bello University, Zaria. The animals were housed in well-ventilated cage and allowed to acclimatize under standard laboratory condition for a period of two weeks before commencement of the experiment.

Plant material and extraction

Fresh leaf and stem bark (blend) of *Diospyros mespiliformis* were collected from Zango village, Sabo LGA of Kaduna State in the month of June 2013 and authenticated in the herbarium unit of the Department of Biological sciences, Ahmadu Bello University Zaria where a voucher specimen with voucher number 901431 was deposited. The collected plants were rinsed in clean water and air dried at room temperature for two weeks. The dried leaves were pulverized into powder before being extracted.

A portion of five hundred grams (50 0 g) of the pulverized plant leaves was suspended in 2.5 L of methanol for 48 h in large amber bottles with intermittent shaking. At the end of the extraction, the crude methanol extract was filtered using Whatman No. 1 filter paper (1 mm mesh size) and then concentrated in a water bath maintained at 45°C until greenish black residues were obtained.

Fractionation of crude extract

The crude extract of D. mespiliformis was subjected to liquid-liquid partition to separate the extract into different fractions. The reconstituted extract (250ml) was placed in a separatory funnel and 250ml of ethylacetate and n-butanol solvents were added sequentially as a 1:1 (v/v) solution and rocked vigorously. [9] The sample was left standing for 30min for each solvent on the separatory funnel until a fine separation line appear which was eluted sequentially. The process was repeated thrice in order to get adequate quantity of each fraction. The ethylacetate, n-butanol as well as the aqueous residue fractions were evaporated to dryness in a water bath to obtain the three fractions (grams) respectively

Acute toxicity study

The median lethal dose (LD_{50}) was conducted in order to select a suitable dose. This was done using the method described by [10] in the initial phase, mice were divided into 3 groups of 3 mice each and were treated with the extracts at 10, 100 and 1000 mg/kg body weight orally. They were observed for 24 h for signs of toxicity, includi4ng death. In the final phase, 3 mice were divided into 3 groups of one mouse each, and were treated based on the findings in the first phase. The LD_{50} was calculated from the results of the final phase as the square root of the product of the lowest lethal dose and the highest non-lethal dose,

that is, the geometric mean of the consecutive doses with 0 and 100% survival rates were recorded.

Animal grouping

80 mice for leaf extract and 80 mice for stem bark extract were used for this determination. The mice were randomly divided into sixteen (16) groups each (leaf and stem bark extract), with five animals per group. **Group 1:** Non infected and not treated (Normal control). **Group 2:** Non infected and treated with medium dose (ethyl acetate fraction) leaf and stem bark methanol extracts (200 and 100mg/kg). **Group 3:** Non Infected and treated with medium dose (n-Butanol fraction) leaf and stem bark methanol extracts (200 and 100mg/kg). **Group 4:** Non Infected and treated with medium dose (Aqueous fraction) leaf and stem bark methanol extracts (200 and 100mg/kg). **Group 5:** Infected and administered 0.2ml normal saline (infected group). **Group 6:** Infected and treated with standard Chloroquine (5mg/kg). **Group 7:** Infected with parasite and treated with (ACT) Artemisinin Combination Therapy (10mg/kg). **Group 8:** Infected and treated with low dose ((ethyl acetate fraction) leaf and stem bark methanol extracts (100 and 50mg/kg). **Group 9:** Infected and treated with medium dose (ethyl acetate fraction) leaf and stem bark methanol extracts (200 and 100mg/kg). **Group 10:** Infected and treated with high dose ((ethyl acetate fraction) leaf and stem bark methanol extracts (400 and 200mg/kg). **Group 11:** Infected and treated with low dose ((n-Butanol fraction) leaf and stem bark methanol extracts (100 and 50mg/kg). **Group 12:** Infected and treated with medium dose ((n-Butanol fraction) leaf and stem bark methanol extracts (200 and 100mg/kg). **Group 13:** Infected and treated with high dose ((n-Butanol fraction) leaf and stem bark methanol extracts (400 and 200mg/kg). **Group 14:** Infected and treated with low dose (Aqueous fraction) leaf and stem bark methanol extracts (100 and 50mg/kg). **Group 15:** Infected and treated with medium dose (Aqueous fraction) leaf and stem bark methanol extracts (200 and 100mg/kg). **Group 16:** Infected and treated with high dose (Aqueous fraction) leaf and stem bark methanol extracts (400 and 200mg/kg).

Parasites

The chloroquine sensitive *Plasmodium berghei* used in this study was obtained from, Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria. The parasite was maintained by subpassaging into healthy mice on a weekly basis throughout the duration of this study using the method. [11,12] *P. berghei* infected red blood cells were intraperitoneally injected into the mice from the blood diluted with Phosphate Buffered Saline (PBS) so that each 0.2ml administered per kg body weight contains approximately 10^6-10^7 infected red cells

Experimental infection

A donor mouse with rising parasitemia of 20% was sacrificed and blood was drawn in heparinized syringe and diluted in phosphate buffered saline. Infection was initiated by needle passage of the parasite preparation from a donor mouse to healthy mice via intraperitoneal route. [11] Each mouse received 0.2 ml of the diluted infected blood.

Antiplasmodial studies

Test on early malaria infection (4-day suppressive Test)

The Peter's 4-day suppressive test against chloroquine sensitive *Plasmodium berghei* (NK 65) infection in mice was employed. [13]

$$\text{Average Suppression} = \left(\frac{\text{APC} - \text{APT}}{\text{APC}}\right) \times \frac{100}{1}$$

APC = Average parasitemia in the control.
APT = Average parasitemia in the test group.

Biochemical studies

Assessment of Aspartate Aminotrans ferase (AST) and assessment of Alanine Aminotransferase (ALT) were determined by the method. [14,15] Serum activity of ALP was determined by the method. [16] Serum total and direct Bilirubin was determined by

the method. [17] Total protein was determined colorimetrically according to the method. [18] Serum albumin was determined by the method. [19] Urea concentration was assessed using the method. [20] The colorimetric method was used to determine serum creatinine concentration. [21]

Isolation of cysteine protease

The malaria parasite was separated by Triton X-100 temperature-induced phase separation procedures, using the protocol [22] with slight modifications. Briefly, 0.5 % Triton X-100 in Tris-buffered saline was added to the parasites and incubated at 4^{o}C for 90 min. The supernatant was collected after centrifuging at 10,000 x g for 30 min at 4^{o}C and was layered on 6 % sucrose containing 0.06 % Triton X-100 followed by incubation at 37°C for 5 min. The cytosolic phase was collected after initial centrifugation at 900xg for 5 min at 37^{o}C and precipitated with cold acetone. The pellets from each preparation was suspended to 6 ml in 50 mM phosphate buffered saline, pH 7.2

Determination of total protein

The protein concentration was quantified according to the method described by [23]. The assay is based on the principle that the maximum absorbance of an acidic solution of Coomassie blue G-250 shift from 365nm to 595nm when binding of the dye to protein occurs. Both the hydrophobic and ionic interactions stabilize the ionic form of the dye, causing a visible color change. Coomassie Brilliant Blue G-250 (25 mg) was dissolved in 12.5 ml 95% ethanol. To this solution, 25 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 250 ml. Protein solution (0.1 ml) was pipetted in test tubes. Five milliliters of protein reagent was added to the test tube and the contents mixed by vortexing. The absorbance at 595 nm was measured after 2 min and before I hr against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown sample.

Protease inhibitor assay

Protease inhibitor activity was assayed according to the method of [24] using N-Benzoyl - DL - arginine - p - nitroanilide (BApNA). An aliquot of 500µl was preincubated for 10min at 37^{o}C with 500µl papain (0.5mg/ml) prepared in 100mM phosphate buffer, P^{H} 6.8, 0.3mM EDTA and 2mM cysteine-HCL. The assay was initiated by the addition of 1ml substrate solution (1% (w/v) incubated for 20mins at 37^{o}C, and 2ml of trichloroacetic acid (TCA, 20% w/v) was added to stop the reaction. After 20min at room temperature, the mixture was centrifuged at 10,000 for 10min and absorbance of the supernatant was measured at 410nm. 290ul of 50mM Tris-HCL (P^{H} 7.6) and 200µl of 1.25mM BApNA were added to the previously preincubated cysteine protease enzyme (papain) and incubated extract for 10min. After 30min at 37^{o}C, the reaction was stopped by adding 150µl of acetic acid (30%). The resulting colour was measured at absorbance of 405nm.

Statistical analysis

The data was analyzed by the analysis of variance (ANOVA). The results were expressed as mean ± Standard Deviation (SD) except where otherwise stated. P value less than 0.05 were regarded as significant (P< 0.05).

RESULTS

The results in table 1 and 2 shows the Effect of Different Fractions of the Leaf and Stem bark Methanol Extracts of *Diospyros mespiliformis* on Liver Function which indicates significant (P < 0.05) increase in the level of Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) in the n-butanol and aqueous fractions. Also, significant (P < 0.05) increase in the serum level of Alkaline phosphatase (ALP) in all the fractions of the infected group. AST ALT and ALP studies of the stem bark extract had significant

(P<0.05) increase in the infected group of all the fractions.

Table 1: Effect of Different Fractions of the Leaf Methanol Extract of *Diospyros mespiliformis* on Liver Function

Treatment/ Dose	AST (IU/L)			ALT (IU/L)			ALP (IU/L)		
(n=5)	Ethyl Acetate Fraction	N-Butanol Fraction	Aqueous Fraction	Ethyl Acetate Fraction	N-Butanol Fraction	Aqueous Fraction	Ethyl Acetate Fraction	N-Butanol Fraction	Aqueous Fraction
Normal Control (NC) NC + 200mg/kg	42.66± 6.71^{c} 30.55± 1.57^{a}	42.66± 6.71^{bc} 36.03± 3.94^{ab}	42.66± 6.71^{d} 41.74± 3.86^{d}	43.61± 3.21^{b} 31.03± 5.35^{a}	43.61± 3.21^{b} 36.87± 5.21^{ab}	43.61± 3.21^{b} 40.08± 5.99^{ab}	33.07± 7.31^{ab} 26.33± 4.66^{a}	33.07± 7.31^{ab} 29.51± 6.02^{a}	33.07± 7.31^{ab} 31.51± 3.62^{ab}
IM+ normal saline	50.78± 5.42^{d}	50.78± 5.42^{c}	50.78± 5.42^{e}	56.42± 5.61^{c}	56.42± 5.61^{c}	56.42± 5.61^{c}	55.91± 14.43^{c}	55.91± 14.43^{c}	55.91± 14.43^{c}
IM+ CQ (5mg/kg)	34.20± 3.51^{ab}	34.20± 3.51^{ab}	34.20± 3.51^{c}	40.41± 2.57^{b}	40.41± 2.57^{ab}	40.41± 2.57^{ab}	30.84± 1.02^{a}	30.84± 1.02^{ab}	30.84± 1.02^{ab}
IM+ACT (10mg/kg)	34.48± 3.89^{ab}	34.48± 3.89^{ab}	34.48± 3.89^{c}	42.54± 2.27^{b}	42.54± 6.27^{b}	42.54± 10.27^{b}	35.08± 8.34^{ab}	35.08± 8.34^{ab}	35.08± 8.34^{ab}
IM + 100mg/kg	38.28± 2.29^{bc}	32.68± 9.46^{a}	28.72± 4.95^{bc}	27.62± 4.18^{a}	32.43± 8.66^{a}	30.57± 5.27^{a}	36.87± 3.84^{ab}	34.14± 3.82^{ab}	29.01± 3.71^{a}
IM+ 200mg/kg	41.00± 7.65^{bc}	30.44± 6.53^{a}	22.60± 2.33^{a}	36.11± 2.00^{ab}	40.39± 5.28^{ab}	32.29± 9.08^{ab}	41.85± 9.33^{b}	36.28± 2.70^{ab}	41.81± 11.53^{b}
IM+ 400mg/kg	49.80± 5.99^{d}	42.60± 9.83^{bc}	25.88± 3.61^{ab}	58.44± 5.20^{c}	45.41± 8.97^{b}	36.66± 10.79^{ab}	42.30± 4.07^{b}	40.20± 2.10^{b}	42.09± 7.32^{b}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different (p<0.05).
NC=Normal Control, CQ=Chloroquine, IM=Infected Mice, ACT=Artemisinin Combination Therapy.

Table 2: Effect of Different Fractions of the Stem Bark Methanol Extract of *Diospyros mespiliformis* on Liver Function

Treatment/ Dose	AST (IU/L)			ALT (IU/L)			ALP (IU/L)		
(n=5)	Ethyl Acetate Fraction	N-Butanol Fraction	Aqueous Fraction	Ethyl Acetate Fraction	N-Butanol Fraction	Aqueous Fraction	Ethyl Acetate Fraction	N-Butanol Fraction	Aqueous Fraction
Normal Control (NC) NC + 100mg/kg	42.66± 6.7^{c} 40.07± 2.1^{bc}	42.66± 6.71^{d} 32.57± 2.30^{bc}	42.66± 6.71^{b} 42.89± 7.09^{b}	43.61± 3.21^{e} 33.31± 5.51^{bc}	43.61± 3.21^{c} 36.49± 4.75^{bc}	43.61± 3.21^{b} 38.93± 4.84^{b}	33.07± 7.31^{ab} 32.67± 2.44^{ab}	33.07± 7.31^{a} 33.25± 5.75^{a}	33.07± 7.31^{a} 38.25± 6.24^{ab}
IM (Infected group)	50.78± 5.42^{d}	50.78± 5.42^{e}	50.78± 5.42^{c}	56.42± 5.61^{f}	56.42± 5.61^{d}	56.42± 5.61^{c}	55.91± 14.43^{c}	55.91± 14.43^{c}	55.91± 14.43^{d}
IM+ CQ (5mg/kg)	34.20± 3.51^{b}	34.20± 3.51^{c}	34.20± 3.51^{a}	40.41± 2.57^{cde}	40.41± 2.57^{bc}	40.41± 2.57^{b}	30.84± 1.02^{a}	30.84± 1.02^{a}	30.84± 1.02^{a}
IM+ ACT (10mg/kg)	34.48± 3.89^{b}	34.48± 3.89^{c}	34.48± 3.89^{a}	42.54± 10.27^{de}	42.54± 10.27^{c}	42.54± 10.27^{b}	35.08± 8.34^{ab}	35.08± 8.34^{a}	35.08± 8.34^{ab}
IM + 50mg/kg	33.93± 3.99^{b}	25.35± 4.25^{ab}	31.97± 5.83^{a}	35.29± 6.50^{bcd}	38.49± 6.97^{bc}	30.27± 2.26^{a}	42.09± 3.97^{b}	45.71± 3.51^{b}	39.65± 2.95^{ab}
IM+ 100mg/kg	34.51± 5.52^{b}	21.89± 5.90^{a}	38.47± 2.63^{ab}	31.85± 2.77^{b}	34.70± 2.50^{b}	38.37± 2.04^{b}	39.51± 5.67^{ab}	40.89± 2.84^{ab}	44.65± 2.41^{bc}
IM+ 200mg/kg	25.69± 5.92^{a}	30.73± 5.40^{bc}	42.97± 4.23^{b}	23.65± 1.78^{a}	26.03± 0.71^{a}	42.05± 7.37^{b}	31.81± 3.25^{a}	35.23± 2.96^{a}	49.97± 2.63^{cd}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different (p<0.05).
NC=Normal Control, CQ=Chloroquine, IM=Infected Mice, ACT=Artemisinin Combination Therapy.

Table 3 and 4 shows significant (P<0.05) increase in n-butanol and aqueous fractions in the level of Total Protein of the leaf extract while stem bark extract had significant (P<0.05) increase in ethyl acetate fractions of the infected group.

Table 5 and 6 shows the Effect of Different Fractions of the Leaf and Stem bark Methanol Extracts of *Diospyros mespiliformis* on Serum Bilirubin. Table 5 shows significant (P < 0.05) increase in the level of direct bilirubin in ethylacetate and N-butanol while table 6 shows significant (P < 0.05) increase in the level of direct bilirubin in all the fractions of the infected group.

Table 3: Effect of Different Fractions of the Leaf Methanol Extract of *Diospyros mespiliformis* on Serum Total Protein and Albumin

Treatment/Dose	Total Protein (G/L)			Albumin (G/L)		
(N=5)	Ethyl Acetate Fraction	N-Butanol Fraction	Aqueous Fraction	Ethyl Acetate Fraction	N-Butanol Fraction	Aqueous Fraction
Normal Control	44.72±9.36^{ab}	44.72±9.36^{bc}	44.72±9.36^{b}	16.60±9.70^{a}	16.60±9.70^{a}	16.60±9.70^{a}
NC + 200mg/kg IM (infected group)	38.67±12.88^{a} 54.99±4.17^{b}	47.96±4.44^{cd} 54.99±4.17^{e}	43.65±7.21^{b} 54.99±4.17^{c}	32.00±5.10^{d} 29.00±5.70^{cd}	36.20±6.06^{b} 29.00±5.70^{b}	18.60±3.78^{a} 29.00±5.70^{b}
IM+ CQ(5mg/kg)	36.06±6.74^{a}	36.06±6.74^{ab}	36.06±6.74^{ab}	17.80±1.92^{a}	17.80±1.92^{a}	17.80±1.92^{a}
IM+ ACT (10mg/kg)	36.16±10.94^{a}	36.16±10.94^{ab}	36.16±10.94^{ab}	23.60±8.50^{abc}	23.60±8.50^{ab}	23.60±8.50^{ab}
IM + 100mg/kg	37.05±9.49^{a}	31.10±5.35^{a}	30.92±10.87^{a}	19.20±3.90^{ab}	16.80±2.68^{a}	19.00±6.69^{a}
IM+ 200mg/kg	39.56±10.42^{a}	36.44±3.28^{ab}	33.40±7.93^{ab}	23.40±3.05^{abc}	17.80±4.44^{a}	21.40±4.22^{ab}
IM+ 400mg/kg	46.40±15.14^{ab}	40.56±5.19^{abc}	39.44±5.08^{ab}	25.60±3.05^{bcd}	21.20±3.83^{ab}	22.40±7.30^{ab}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different (p<0.05).
NC=Normal Control, CQ=Chloroquine, IM=Infected Mice, ACT=Artemisinin Combination Therapy.

Table 4: Effect of Different Fractions of the Stem Bark Methanol Extract of *Diospyros mespiliformis* on Serum Total Protein and Albumin

Treatment/Dose	Total Protein (G/L)			Albumin (G/L)		
(N=5)	Ethyl Acetate Fraction	N-Butanol Fraction	Aqueous Fraction	Ethyl Acetate Fraction	N-Butanol Fraction	Aqueous Fraction
Normal Control	44.72±9.36^{ab}	44.72±9.36^{abc}	44.72±9.36^{a}	16.60±9.70^{a}	16.60±9.70^{a}	16.60±9.70^{a}
NC + 100mg/kg IM (Infected group)	43.88±12.77^{ab} 54.99±4.17^{c}	45.00±6.68^{abc} 54.99±4.17^{c}	61.72±8.16^{b} 54.99±4.17^{b}	33.60±4.45^{b} 29.00±5.70^{b}	27.80±6.38^{bc} 29.00±5.70^{c}	23.20±5.93^{ab} 29.00±5.70^{b}
IM+CQ(5mg/kg)	36.06±6.74^{a}	36.06±6.74^{a}	36.06±6.74^{a}	17.80±1.92^{a}	17.80±1.92^{ab}	17.80±1.92^{a}
IM+ ACT (10mg/kg)	36.16±10.94^{a}	36.16±10.94^{a}	36.16±10.94^{a}	23.60±8.50^{ab}	23.60±8.50^{abc}	23.60±8.50^{ab}
IM + 50mg/kg	42.52±7.59^{a}	48.40±2.73^{bc}	61.12±5.22^{b}	23.40±9.81^{ab}	24.80±9.01^{abc}	18.80±3.35^{a}
IM+ 100mg/kg	40.80±4.01^{a}	38.80±4.23^{ab}	57.48±8.26^{b}	16.80±8.14^{a}	20.80±6.61^{abc}	15.20±1.92^{a}
IM+ 200mg/kg	45.32±3.75^{ab}	54.04±10.30^{c}	57.92±6.21^{b}	29.00±10.12^{b}	26.00±8.06^{abc}	17.20±3.90^{a}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different (p<0.05).
NC=Normal Control, CQ=Chloroquine, IM=Infected Mice, ACT=Artemisinin Combination Therapy.

Table 5: Effect of Different Fractions of the Leaf Methanol Extract of *Diospyros mespiliformis* on Serum Bilirubin

Treatment/Dose	Ethylacetate Fraction		N-Butanol Fraction		Aqueous Fraction	
(n=5)	DIR BIL(g/L)	IND BIL(g/L)	DIR BIL(g/L)	IND BIL(g/L)	DIR BIL(g/L)	IND BIL(g/L)
Normal Control	4.57±0.65^{bc}	1.29±0.65^{a}	4.57±0.65^{bc}	1.29±0.65^{a}	4.57±0.65^{a}	1.29±0.65^{ab}
NC + 200mg/kg IM (Infected group)	4.80±1.15^{b} 5.75±1.25^{c}	1.21±0.24^{a} 1.89±1.57^{a}	4.42±1.07^{bc} 5.75±1.25^{c}	1.70±0.67^{a} 1.89±1.57^{a}	4.23±3.26^{ab} 5.75±1.25^{b}	2.00±1.54^{ab} 1.89±1.57^{ab}
IM+ CQ (5mg/kg)	3.45±0.87^{ab}	1.15±0.15^{a}	3.45±0.87^{ab}	1.15±0.15^{a}	3.45±0.87^{a}	1.15±0.15^{b}
IM+ ACT (10mg/kg)	3.76±1.71^{b}	2.91±1.07^{a}	3.76±1.71^{ab}	2.91±0.07^{a}	3.76±1.71^{a}	2.91±1.07^{bc}
IM + 100mg/kg	4.12±1.24^{b}	1.28±1.16^{a}	2.80±1.41^{ab}	3.04±1.34^{a}	4.35±3.73^{ab}	0.56±0.12^{a}
IM+ 200mg/kg	2.10±0.69^{a}	1.58±1.34^{a}	3.08±1.78^{ab}	2.09±1.09^{a}	3.77±1.80^{a}	2.15±0.99^{abc}
IM+ 400mg/kg	2.20±1.14^{a}	1.58±0.98^{a}	2.07±0.79^{a}	1.91±0.62^{a}	2.97±0.12^{a}	3.87±1.34^{c}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different (p<0.05).
NC=Normal Control, CQ=Chloroquine, IM=Infected Mice, ACT=Artemisinin Combination Therapy.

Table 6: Effect of Different Fractions of the Stem Bark Methanol Extract of *Diospyros mespiliformis* on Serum Bilirubin

Treatment/Dose	Ethylacetate Fraction		N-Butanol Fraction		Aqueous Fraction	
(n=5)	DIR BIL(g/l)	IND BIL(g/l)	DIR BIL(g/l)	IND BIL(g/l)	DIR BIL(g/l)	IND BIL(g/l)
Normal Control	4.57±0.65^{b}	1.29±0.65^{a}	4.57±0.65^{b}	1.29±0.65^{a}	4.57±0.65^{b}	1.29±0.65^{ab}
NC + 100mg/kg IM (Infected group)	0.54±0.23^{a} 5.75±1.25^{c}	2.26±0.67^{ab} 1.89±1.57^{ab}	0.42±0.24^{a} 5.75±1.25^{c}	1.94±0.65^{ab} 1.89±1.57^{ab}	0.40±0.16^{a} 5.75±1.25^{c}	0.56±0.43^{a} 1.89±1.57^{ab}
IM+ CQ (5mg/kg)	3.45±0.87^{b}	1.15±0.15^{a}	3.45±0.87^{b}	1.15±0.15^{a}	3.45±0.87^{b}	1.15±0.15^{a}
IM+ ACT (10mg/kg)	3.76±1.71^{b}	2.91±1.07^{b}	3.76±1.71^{b}	2.91±1.07^{b}	3.76±1.71^{b}	2.91±2.07^{b}
IM + 50mg/kg	0.74±0.34^{a}	0.98±0.68^{a}	1.20±0.52^{a}	0.92±0.33^{a}	0.36±0.11^{a}	1.86±0.48^{ab}
IM+ 100mg/kg	0.48±0.30^{a}	0.82±0.16^{a}	0.38±0.08^{a}	1.10±0.21^{a}	0.34±0.17^{a}	1.16±0.15^{a}
IM+ 200mg/kg	0.56±0.24^{a}	0.66±0.53^{a}	0.48±0.15^{a}	0.94±0.42^{a}	0.38±0.08^{a}	1.86±0.91^{ab}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different (p<0.05).
NC=Normal Control, CQ=Chloroquine, IM=Infected Mice, ACT=Artemisinin Combination Therapy.

Table 7: Effect of Different Fractions of the Leaf Methanol Extract of *Diospyros mespiliformis* on Serum Creatinine and Urea

TREATMENT/DOSE (n=5)	CREATININE(mg/dl)			UREA(mg/dl)		
	Ethylacetate fraction	N-Butanol fraction	Aqueous fraction	Ethylacetate fraction	N-Butanol fraction	Aqueous fraction
Normal Control	37.00±1.54^{ab}	37.00±1.54^{ab}	37.00±1.54^{ab}	2.30±0.84^{a}	2.30±0.84^{a}	2.30±0.84^{a}
NC + 200mg/kg IM (Infected group)	40.10±4.60^{b} 44.90±5.52^{c}	42.20±5.14^{cd} 44.90±5.52^{d}	39.10±2.53^{b} 44.90±5.52^{c}	2.20±0.45^{a} 3.60±2.19^{ab}	4.20±0.76^{ab} 3.60±2.19^{ab}	3.84±1.45^{ab} 3.60±2.19^{ab}
IM+ CQ (5mg/kg)	36.90±1.64^{ab}	36.90±1.64^{ab}	36.90±1.64^{ab}	2.32±0.81^{a}	2.32±0.81^{a}	2.32±0.81^{a}
IM+ ACT(10mg/kg)	40.30±1.04^{b}	40.30±1.04^{bc}	40.30±1.04^{b}	2.56±1.17^{a}	2.56±1.17^{ab}	2.56±1.17^{ab}
IM + 100mg/kg	35.00±3.22^{a}	35.00±1.27^{a}	44.20±1.64^{c}	3.38±1.80^{ab}	3.70±2.14^{ab}	4.74±2.48^{b}
IM+ 200mg/kg	46.40±1.47^{c}	44.50±1.84^{d}	34.00±2.43^{a}	3.62±1.30^{ab}	4.64±1.40^{b}	3.62±1.60^{ab}
IM+ 400mg/kg	35.10±2.70^{a}	34.30±1.92^{a}	34.10±1.14^{a}	4.90±2.25^{b}	4.48±1.73^{ab}	4.26±1.64^{ab}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different (p<0.05).
NC=Normal Control, CQ=Chloroquine, IM=Infected Mice, ACT=Artemisinin Combination Therapy.

Table 8: Effect of Different Fractions of the Stem Bark Methanol Extract of *Diospyros mespiliformis* on Serum Creatinine and Urea

TREATMENT/DOSE (n=5)	CREATINIE (mg/dl)			UREA (mg/dl)		
	Ethylacetate fraction	N-Butanol fraction	Aqueous fraction	Ethylacetate fraction	N-Butanol fraction	Aqueous fraction
Normal Control	37.00±1.54^{ab}	37.00±1.54^{ab}	37.00±1.54^{bc}	2.30±0.84^{a}	2.30±0.84^{a}	2.30±0.84^{a}
NC + 100mg/kg IM (Infected group)	50.60±7.19^{d} 44.90±5.52^{c}	53.60±3.30^{e} 44.90±5.52^{c}	46.60±6.77^{e} 44.90±5.52^{d}	4.34±1.23^{b} 3.60±2.19^{ab}	4.60±2.88^{b} 3.60±2.19^{ab}	3.50±1.17^{ab} 3.60±2.19^{ab}
IM+ CQ (5mg/kg)	36.90±1.64^{a}	36.90±1.64^{ab}	36.90±1.64^{bc}	2.32±0.81^{a}	2.32±0.81^{a}	2.32±0.81^{a}
IM+ ACT(10mg/kg)	40.30±1.04^{b}	40.30±1.04^{b}	40.30±1.04^{bc}	2.56±1.17^{a}	2.56±1.17^{a}	2.56±1.17^{a}
IM + 50mg/kg	42.20±5.14^{bc}	35.90±3.01^{a}	32.20±1.44^{b}	3.92±1.31^{ab}	3.50±1.03^{ab}	2.92±0.73^{a}
IM+ 100mg/kg	45.20±2.64^{c}	46.40±3.23^{cd}	28.50±2.82^{a}	4.60±2.88^{b}	4.20±0.97^{b}	4.60±2.46^{b}
IM+ 200mg/kg	41.10±5.09^{b}	44.60±2.10^{c}	40.20±4.70^{bc}	4.06±1.55^{b}	3.28±0.61^{ab}	4.10±2.88^{b}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different (p<0.05).
NC=Normal Control, CQ=Chloroquine, IM=Infected Mice, ACT=Artemisinin Combination Therapy.

Table 7 and 8 indicate significance (P<0.05) increase in creatinine level in the normal but treated with medium dose of the extract in the stem bark extract.

Figure 1 and 2 shows the Lineweaver-Burk Plot Showing the Inhibitory Effects of Leaf and Stem bark extracts of *Diospyros mespiliformis* on *Plasmodium berghei* cysteine protease. The Leaf and Stem bark extracts exhibited competitive and non-competitive patterns of inhibition respectively.

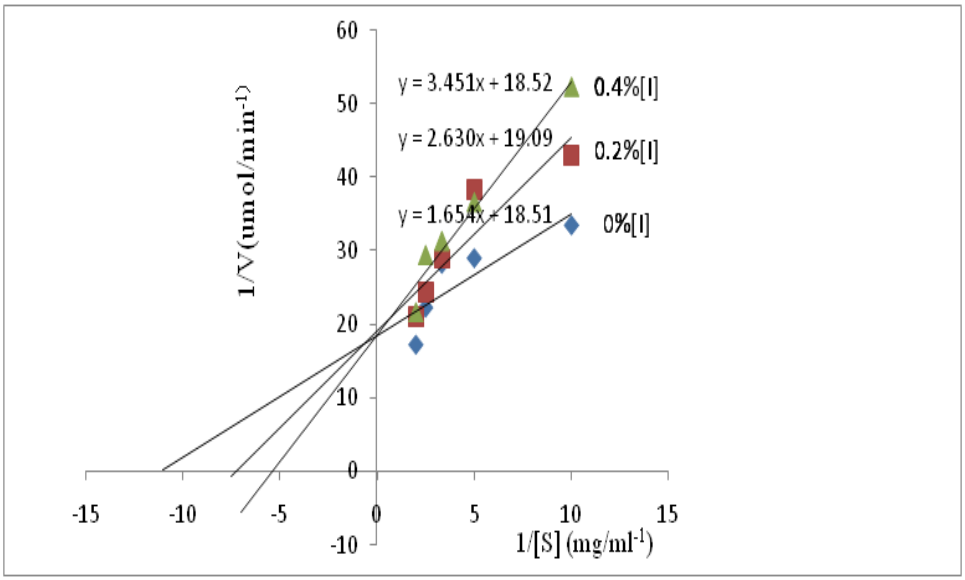

Figure 1: Lineweaver-Burk Plot Showing the Inhibitory Effects of Leaf extract of *Diospyros mespiliformis* on *Plasmodium berghei* cysteine protease (Competitive Pattern of Inhibition).

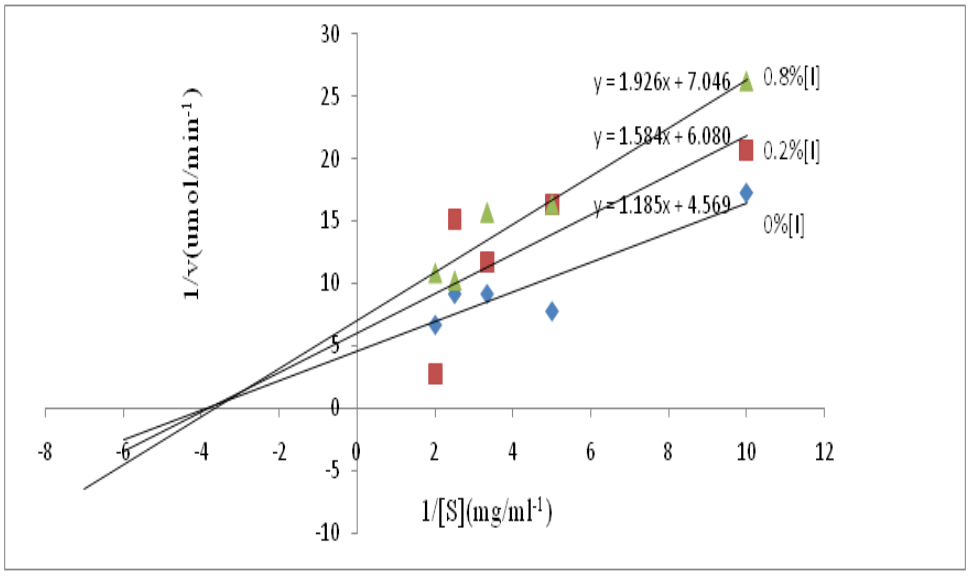

Figure 2: Lineweaver-Burk Plot Showing the Inhibitory Effects of Stem-bark extract of *Diospyros mespiliformis* on *Plasmodium berghei* cysteine protease (Non-Competitive Pattern of Inhibition)

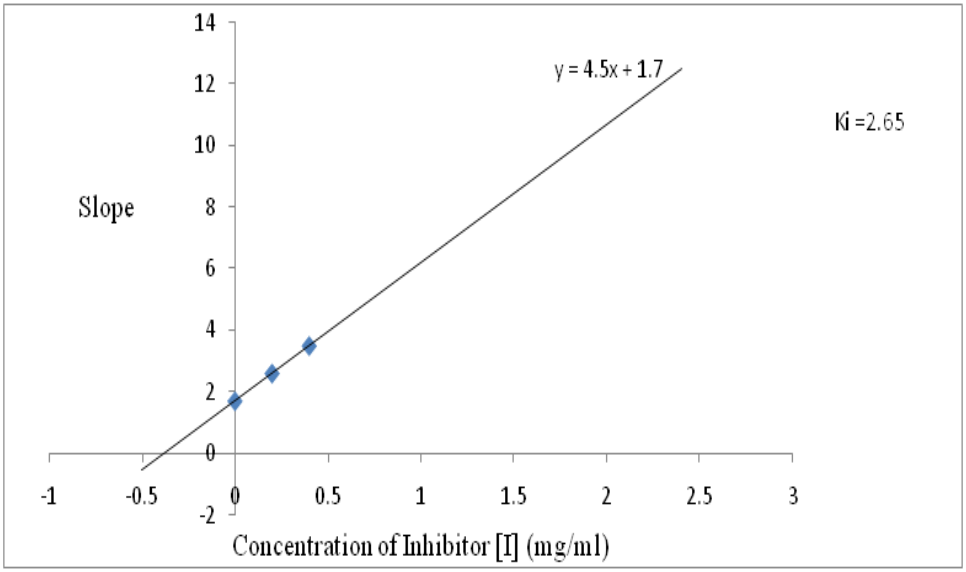

Figure 3: Secondary plot for the determination of Ki for Diospyros mespiliformis (Leaf extract) cysteine proteases.

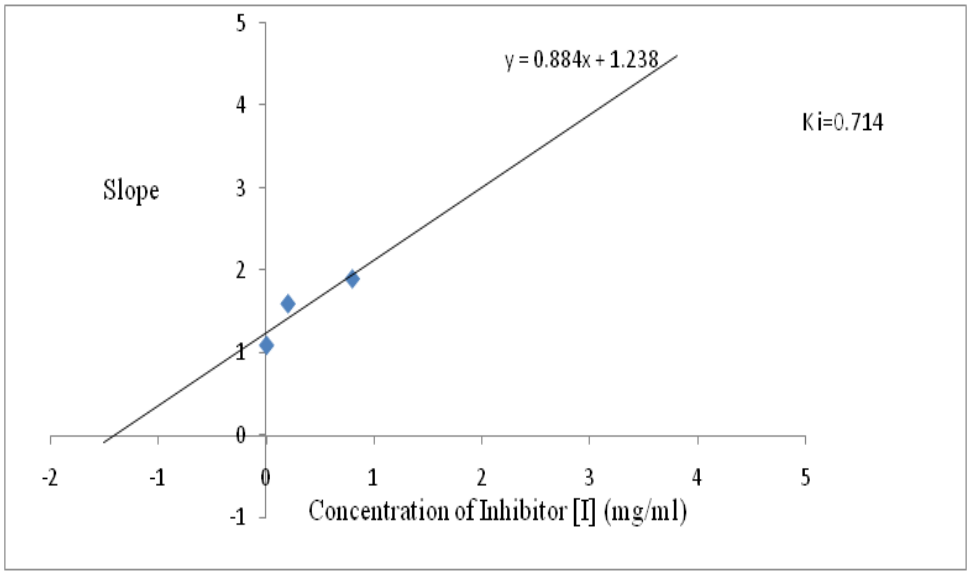

Figure 4: Secondary plot for the determination of Ki for Diospyros mespiliformis (Stem Bark extract) cysteine proteases

Figure 3 and 4 indicates competitive and non-competitive patterns with K_i values of 2.65M and 0.174M in the leaf and stem bark methanol extracts respectively.

DISCUSSION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. [25] Assessment of liver can be made by estimating the activities of serum ALT, AST and ALP which are enzymes originally present at higher concentration in cytoplasm. [26] The infected normal saline group showed significant ($p<0.05$) increase compared to other groups. The significant elevation ($p<0.05$) of serum transaminases (AST ALT and ALP) obtained in mice is noteworthy as these enzymes are indices of hepatic injury although ALT is more liver specific. Enzymes are sensitive indices of cellular injury and are elevated above normal from tissue leakage before changes are noted with clinical and histological tests. [27]

The level of total protein in the infected normal saline group showed significant ($p<0.05$) increase and significant reduction in the normal group as well as the treated group. Decrease in serum proteins could generally be early indications of liver damage renal failure or nutritional deficiency. [28] In the intestine, conjugated bilirubin may be metabolized by colonic bacteria, eliminated or reabsorbed. Metabolism of bilirubin into urobilinogen followed by reabsorption of urobilinogen accounts for the yellow color of urine as we urinate which is a downstream product of urobilinogen. The stem bark methanol extract showed significant ($p<0.05$) increase in the infected normal saline group. Elevated bilirubin may also be due to possible obstruction in the flow of the bile within the liver (hepatic jaundice) or in the bile duct (post jaundice). [29] However, the elevation of bilirubin levels in the infected normal saline group of stem bark extract, may be as a result of haemolytic anemia that may be associated with oxidative damage to red blood cells or internal haemorrhage thus, leading to elevated bilirubin level (prehepatic jaundice) since bilirubin is an intermediate product in heam (either from haemoglobin or myoglobin) breakdown in the liver. [30] This is in agreement with the commonly accepted view that serum levels of transaminases, bilirubin and serum total proteins returned back to normalcy with the healing of hepatic parenchyma cells which lead to the regeneration of hepatocytes. [31]

Creatinine is a break down waste product formed in the muscle by creatine phosphate metabolism. Creatine is synthesized in the liver, passes into the circulation and is taken up almost entirely skeletal muscle for energy production. Creatinine retention in the blood is evidence of kidney impairment. Urea is the main end product of protein catabolism. Amino acid deamination takes place in the liver, which is also the site of urea cycle, where ammonia is converted into urea and excreted through urine. [32] It represents 90% of the total urinary nitrogen excretion. Urea varies directly with protein intake and inversely with the rate of excretion. From the present study, it is evident that significant elevation in serum creatinine levels can be attributed to the damaged of nephron structural integrity. [33] However, the significant reduction in urea and creatinine levels in the normal control group can be attributed to the curative properties of the leaf and stem bark methanol extracts, of *Diospyros* mespiliformis on the glomerular and tubular cells which may improve renal function in kidney disease. [34] The mechanism of inhibition exhibited competitive and non-competitive patterns with K_i values of 2.65M and 0.174M in the leaf and stem bark methanol extracts respectively.

CONCLUSION

The results showed that, Diospyros mespiliformis leaf and stem bark methanol extracts possess antimalarial activity. This has scientifically validated the traditional

use of *Diospyros mespiliformis* in the management and treatment of malaria.

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REFERENCES

1. Fidock DA, Rosenthal PJ, Croft SL, Brun R, Nwaka S. Antimalarial drug discovery: efficacy models for compound screening. Nature Reviews Drug Discovery. 2004: 3(6):509-20.
2. Malaney P, Spielman A, Sachs J. The malaria gap. The American journal of tropical medicine and hygiene. 2004: 71 (2):141-6.
3. Etkin NL. The co-evolution of people, plants, and parasites: biological and cultural adaptations to malaria. Proceedings of the Nutrition Society. 2003:62(02):311.
4. Ajaiyeoba EO, Abiodun OO, Falade MO, Ogbole NO, Ashidi JS, Happi CT, Akinboye DO. In vitro cytotoxicity studies of 20 plants used in Nigerian antimalarial ethnomedicine. Phytomedicine. 2006; 13 (4):295-8.
5. Barrett AJ, Grubb A. The disuphide bridges of human cystatin C (trace) and chicken cystatin. FEBS Lett. 2001: 170: 370-374.
6. Rosenthal PJ. Antimalarial drug discovery: old and new approaches. Journal of Experimental Biology. 2003; 206(21):3735-44.
7. BaillySingh A. Selection of cysteine protease inhibitor resistant malaria parasites is accompanied by amplification of falcipain genes and alteration in inhibitor transport. J. Biol. Chem. 2004: 279: 35236-35241.
8. Abbot D, Andrews RS. An Introduction to chromatography 2nd ed. Longman press, London. 1970: 72-78.
9. Lorke D. A New Approach to Practical Acute Toxicity Testing. Arch. Toxicol. 1983: 53:275-289.
10. Peter IT, Anatolic, VK. The Current Global Malaria Situation. In Malaria Parasite Biology, Pathogenesis, and Protection. ASM Press. WDC, USA, 1998: 11-22.
11. David AF, Philip JR, Simon RC, Reto B, Solomon N. Antimalarial Drug Discovery: Efficacy Model for Compound Screening. Nature reviews. 2004: 3:509-520.
12. Peter G, Chin, William, Joseph S, Lunn, Joel B. Contacos. "The effect of cycloguanil pamoate (CI-501) against a chlorguanide-resistant Chesson strain of Plasmodium vivax." The American journal of tropical medicine and hygiene. 1965: 14(6): 922-924.
13. Reitman, Frankel. Colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. American Journal of Clinical Pathology. 1957: 26:56-60.
14. Gella FJ, Olivella T, Cruz PM, Arenas J, Moreno R, Durban R, Gomez JA. A Sample Procedure for Rooutine Determination of Aspartate Aminotransferase and Alanine Aminotransferase with Pymdoral Phosphate. Clin Chim Acta. 1985: 153:241-247.
15. Rosalki SB, Foo A, Burlina A. Multicentre evaluation of Iso ALP testkit for measurement of bone alkaline phosphatase activity in serum and plasma. Clinical Chemistry. 1993: 39: 648-652.
16. Jendrassik L, Grof. In-vitro determination of total and direct bilirubin in serum. J. Biochem. 1983: 299:81-88.
17. Tietz NWC. Protein Metabolism. In Fundamentals of Clinical Chemistry. W.B. Saunders Company, Philadelphia, London, Toronto; 1970: 181-191.
18. Doumas BT, Watson WA, Biggs HG. Albumin Standards and the Measurement of Serum Albumin with Bromocresol Green. Din Chim Acta. 1971: 31:87-96.
19. Fawcett JK, Scout JE. A rapid and precise method for the determination of urea. J. Clin. Pathol. 1960: 13:156.
20. Bartels H, Bohmer M. A kinetic method for measurement of serum creatinine. Med. Lab. 1973: 26(9):209-215.
21. Amlabu E, Nok AJ, Inuwa HM, Akin-Osanaiye BC, Haruna E. Cysteine protease from the malaria parasite, Plasmodium berghei-purification and biochemical characterization. Journal of Cell and Molecular Biology. 2011; 9(2):43-9.
22. Bradford, Marion M. "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Analytical biochemistry.1976: 72(1): 248-

254.
23. Khantikul N, Butraporn P, Kim HS, Leemingsawat S, Tempongko MS, Suwonkerd W. Adherence to antimalarial drug therapy among vivax malaria patients in northern Thailand. Journal of Health, Population and Nutrition. 2009:4-13.
24. Venkatalakshmi P, Senthamaraiselvi V (2012). Anti ulcer effect of basella alba leaf extract in aspirin induced albino rats. Ijpsr, 3(8):2539- 2542.
25. Reham A, Mohamed, Reham S, Ramadan, Lamiaa AA. Effect of Substituting Pumpkin Seed Protein Isolate for Caseinon Serum Liver Enzymes, Lipid Profile and Antioxidant Enzymes in CCl4-intoxicated Rats. Adv. Biol. Res. 2009: 3(1-2):09-15.
26. Obatomi DK, Jigam AA, Mogbo SCJ, Afolarin FI. Variation in Urinary Enzyme Excretion in Hypertensive Patients. Med Sci. Res.1995: 5:110-111.
27. Sood R. Rext book of Medical laboratory Technology. 1st ed. Jaypee Brothers Medical Publishers New Delhi, India. 2006: 609 - 672.
28. Emeka EJI, Obioa O. Effect of a long term consumption of a diet supplemented with leaves of Gongronema latifolium Benth on some biochemical and histological parameters in male albino rats. J. Biol. Sci. 2009: 9(8):859-865.
29. Bruxvoort K, Goodman C, Kachur, SP, Schellenberg, D. How patients take malaria treatment: a systematic review of the literature on adherence to antimalarial drugs. PloS one, 2014: 9(1):23-35.
30. Chavan Sandeep D, Patil Sandeep B, Naikwade NS. Biochemical and Histopathological Studies of Butea Monosperma (Lam) Taub Leaves on Paracetamol-Induced Hepatotoxicity in Albino Rats. J. Pharm. Res. 2012: 5 (8):4006-4008.
31. Sati P, Pandey A, Rawat S, Rani A. Phytochemicals and antioxidants in leaf extracts of Ginkgo biloba with reference to location, seasonal variation and solvent system. J Pharm Res. 2013: 7(9):804-809
32. Khan MR, Siddique F. Antioxidant effects of Citharexylum spinosum in CCl4 - induced nephrotoxicity in rat. Exp. *Toxicol. Patholog.* 2012: 64:349-355.
33. Chumbhale Deshraj S1, Chaudhari SR1, Upasani CD. Antibacterial and Antifungal Activity of Stem Bark of Abutilon Indicum (Linn.) Sweet 2. IJPRD. 2015: 7(05):023 -027.
34. Hunt L, Gupta-Wright A, Simms V, Tamba F, Knott V, Tamba K, Heisenberg-Mansaray S, Tamba E, Sheriff A, Conteh S, Smith T. Clinical presentation, biochemical, and haematological parameters and their association with outcome in patients with Ebola virus disease: an observational cohort study. The Lancet Infectious Diseases. 2015: 15(11):1292-9.

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