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# Effect of *Calpurnia aurea* Seed Extract on HAART Induced Haematotoxicity in Albino Wistar Rats

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author HNM designed the study, analyses of the study performed the automated hematology auto analyzer (Sysmex KX-2IN) and statistical analyses of data. Author NG wrote the protocol, and wrote the first draft of the manuscript, managed the literature searches and provided the DPPH and TLC plates for antioxidant assay. Authors UM and SD supported the protocol writing and revised the manuscript. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aim:** To investigate the effect of *Calpurnia aurea* seeds extract on highly active antiretroviral therapy (HAART), first phase regimens (Lamivudine + Efavirenz + Zidovudine) induced heamtotoxicity in rats.
**Study Design:** Thirty adult healthy male albino wistar rats of weighing about 140-150 gms were used in the present study. They were divided into five groups six each. Group- I distilled water only; Group- II HAART drugs only; Group- III HAART drugs + 100 mg/kg of CASE (CASE: *Calpurnia aurea* Seed Extract); Group- IV HAART drugs + 200 mg/kg of CASE and Group- V HAART drugs + 300 mg/kg of CASE were administered orally for 35 day.
**Methodology:** Matured dried seeds of *Calpurnia aurea* were collected, powdered and extracted using 70% ethanol. Preliminary phytochemical screening and in-vitro antioxidant properties of extract were done. The HAART and different doses of the *Calpurnia aurea* seed extract were administered orally for thirty-five days. On 35th day, the rats were fasted overnight and the blood sample was collected by cardiac puncture after sacrificed the rats by cervical dislocation.

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**Results:** HAART did not alter the total WBC (P=0.56, 7340±500 vs 7080±1381) and platelets count (P=0.76, 751000±56059 vs 742200±11921) but significantly alter the total RBC (P=0.001, 7008000±559521 vs 8832000±142211), HCT (P=0.001, 46.56±3 vs 64.75±1) and haemoglobin (P=0.001 12.90±1.12 vs 15.42±0.49) the affected parameters were restored by CASE in dose dependent meaner. However the CASE significantly reduced the platelets counts in the experimental rats.

**Conclusion:** This report shows that CASE is an effective counter measure for the toxic haematopoietic effects of HAART. This is may be because of CASE contains phytochemicals such as tannins, flavonoids, terpenoids etc which attenuate the HAART induced hematopoietic cell death in the periphery and bone marrow or it may be promote hematopoietic functions by regulating erythropoietin. Furthermore CASE oral administration reduces the platelet count not in the dose dependant manner. The molecular mechanism of action of the drug needs further clarification.

*Keywords: Calpurnia aurea; HAART; haematotoxicity; CASE; antioxidant.*

## 1. INTRODUCTION

Antiretroviral drugs are pills for handling of infection by retroviruses, primarily human immunodeficiency virus (HIV). The *United States* of America, Food and Drug Administration (FDA) have been approved 24 antiretroviral drugs [1]. Among the 24 drugs the following drugs are available in Ethiopia since 2004: nucleoside reverse transcriptase inhibitors (*NRTIs*) (zidovudine, stavudine, lamivudine, abacavir, tenofovir and didanosine) and nucleotide reverse transcriptase inhibitors (NtRTIs) (nevirapine and efavirenz) and protease inhibitors (ritonavir and lopinavir) [2]. When numerous such drugs, typically three or more than three drugs, are taken in combination is known as highly active antiretroviral therapy, or HAART. The normal guidelines for management of HIV infection recommend the combination of three antiretroviral agents, either three reverse transcriptase inhibitors (RTIs) or two RTIs plus one protease inhibitor [3,4]. In resource-limited settings, combination HAART consisting of 2 RTIs [either zidovudine (AZT) or stavudine (d4T) along with lamivudine (3TC)] and 1 non-nucleoside reverse transcriptase inhibitor (NNRTI) [either nevirapine (NVP) or efavirenz (EFV)] are frequently used. AZT, a nucleoside reverse transcriptase inhibitor (NRTI) is one of the earliest antiretroviral agents used as a combination in some of the HAART regimens for the treatment of HIV /AIDS, and it was the first drug which was approved by the US FDA for use in HIV/AIDS. The last fifteen years observation HAART has been the most important marked reduction in HIV infection related morbidity and mortality and also answerable for a wide range of toxicities and life-threatening side-effects [5].

AZT is used in the first line drug combination as stavudine is more frequently associated with mitochondrial toxicity. Its use, however, is associated with haematological toxicity particularly bone marrow aplasia leading to varying degrees of cytopenias especially anemia in some patients. The mechanism of this anemia is attributed to 50-70 per cent inhibition of proliferation of blood cell progenitor cells [6,7] in a time-and dose-dependent fashion. Further, laboratory studies have also shown that AZT exhibits cytotoxicity to the myeloid and erythroid precursors in the bone marrow at drug concentrations close to those associated with the optimal antiviral effect *in vitro*. This haematological toxicity is observed in most of the patients within 3-6 months and is reversible. Female gender has been found to be a risk factor for anemia in some studies. This adverse effect of anemia from AZT limits its use in some patients. AZT has also been reported to produce pure red cell aplasia (PRCA) [8,9]. Previous studies have shown that the incidence of anemia is high in patients either acquired immunodeficiency syndrome (AIDS) or human immunodeficiency virus (HIV) infection [10,11]. HIV infection is associated with anaemia consistently adverse outcomes such as infections and neurologic weakening and progression to AIDS [11]. Earlier studies have found that the occurrence of anemia increases with progression of HIV infection. A number of other etiologic factors may also be concerned in the development of HIV-associated anemia, including, impaired erythropoietin production, blood loss from intestinal opportunistic disease, immunological myelosuppression, and micronutrient deficiencies [12]. HIV infected patients mortality was associated with low haemoglobin levels even reduce the viral load and increased CD4 cell count [11,13-15].

Research has shown that oral ingestion of plant medicinal compounds or drugs can alter the normal range of haematological parameters. These alterations could either be positive or negative [16,17]. Literature survey brings to light the different kinds of medicinal plant phytocheicals promote haematopoiesis and prevent the anaemia for some examples *Hibiscus cannabinus*, *Telfairia occidentalis*, *Ageratum conyzoides*, *Brillantasia nitens* Lindau and *Psidium guajava* [18-22].

*Calpurnia aurea* is a genus of FLOWERING PLANTS within the family of *Fabaceae*. The leaf and stem of *C. aurea* has been used for different human and animal disease [23]. In Ethiopia, traditionally, the leave of *C. aurea* is used for the treatment of syphilis, malaria, rabies, diabetes, hypertension, diarrhoea, leishmaniasis, trachoma, elephantiasis, fungal diseases and different swellings, stomach-ache, bowel, and bladder disorders [24]. Umer et al. [25] reported that the 80% methanol extract of *C. aurea* leaf revealed the presence of alkaloids, tannins, flavonoids and saponins. Hence, the present study is undertaken to investigate the anti-haematotoxic potential of hydroethanolic *C. aurea* seed extract against HAART-first phase regimen (Lamivudine + Efavirenz + Zidovudine) drugs induced haemtotoxicity in rats.

## 2. MATERIALS AND METHODS

### 2.1 Plant Materials

The *C. aurea* leaves with flower and seeds were collected from south Gondar, northern Ethiopia in June 2013. The plant has identified and authenticated by taxonomist of Ethiopian National Herbarium of Addis Ababa University and its voucher number was 001/2006.

### 2.2 Extraction

The seeds were washed thoroughly 2-3 times with running tap water, then shade dried and grinded in mixer. The grinded seeds were weighed 100 gm using an electronic balance and were macerated separately in 70% ethanol for 72 hours with mechanical shaking and it was filtered through Whatman No.1 filter paper. Then filtrate was evaporated using rotary evaporator and dried at 40ºC. The yield was found to be 17.62% w/v. Preliminary phytochemical tests were performed by standard phytochemical test procedures [26,27].

### 2.3 *In-vitro* Antioxidant Activity by Spectrophotometric Method

The DPPH radical-scavenging activity of the test extracts was examined as previously described [28]. Different concentrations (31.25-1000 µg/ml) of each extract were added, at an equal volume, to methanolic solution of DPPH (100 mM). The mixture was allowed to react at room temperature in the dark for 30 minutes. Vitamin C was used as standard controls. Three replicates were made for each test sample. After 30 minutes, the absorbance (A) was measured at 517 nm and converted into the percentage antioxidant activity. $IC_{50}$ values denote the concentration of sample which is required to scavenge 50% of DPPH free radicals. The $IC_{50}$ values were calculated from the linear regression curves, where the abscissa represented the concentration of the test plant extracts and the ordinate the average percent of scavenging capacity from three replicates. $IC_{50}$ value (concentration of sample where absorbance of DPPH decreases 50% with respect to absorbance of the control) of extracts were determined [29]. The higher the antioxidant activity, the lower $IC_{50}$ value.

### 2.4 Animals

Thirty adult (12 weeks' old) healthy male albino rats weighing 140-150 gms were used in the present study and housed in polypropylene cages and maintained standard laboratory conation. They were provided with standard pellet rat diet supplied by Kality Animal Nutrition Production Ltd., Addis Ababa Ethiopia, and water *ad libitum.* The research protocol was approved by the Research & Ethics Review committee (DRERC) of the department of Medical Biochemistry, Addis Ababa University with approval number SOM/BCHM/012/2013 E.C. All the animal experiments were carried out according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

### 2.5 Extrapolation of HAART Dose

The human doses of HAART drug were extrapolated to animals by the formula; Human Equivalent Dose (HED in mg/kg) = Animal Dose (mg/kg) × (Animal Km ÷ Human Km), Where Km is a correction factor reflecting the relationship between body weight and body surface area [30]. Table 1 shows the average Km value of most frequently used laboratory animals.

**Table 1. Average Km value of laboratory animals**

| | |
|---|---|
| Mouse | 3 |
| Rat | 6 |
| Guinea pig | 8 |
| Rabbit | 12 |
| Dog | 20 |
| Human adult | 37 |

Based on the above Km value, the HAART drugs were calculated per weight of the rats. So that, the four experimental groups (II, III, IV, & V) were treated with (Lamivudine=0.53 mg/kg, Efavirenz= 0.7 mg/kg, and Zidovudine = 0.11 mg/kg) administered orally for 35 days.

## 2.6 Animal Grouping and Drug Dose

Group- I Normal control, given distilled water only
Group- II Positive control, given HAART drugs only
Group- III, Given HAART drugs + 100 mg/kg of CASE (CASE: *Calpurnia aurea* Seed Extract)
Group- IV, Given HAART drugs + 200 mg/kg of CASE
Group- V, Given HAART drugs + 300 mg/kg of CASE

## 2.7 Blood Sample Collection and Analysis

After 35 days of treatment, the rats were fasted overnight, sacrificed by cervical dislocation and blood was aseptically collected by cardiac puncture. The blood samples were taken for haematological analysis in heparinzed vials. The haematological parameters were analyzed by automated haematology auto analyser (Sysmex KX-2IN) following the manufacturer's guideline.

## 2.8 Statistical Analysis

The data was expressed as mean±SEM. Statistical significance between the groups were tested using one-way ANOVA followed by Dennett's post-hoc test. A *P* less than 0.5 were considered significant.

## 3. RESULTS

## 3.1 Phytochemical

The preliminary phytochemical analysis of 70% ethanolic extract of *C. aurea* seed showed the presence of tannins, flavonoids, terpenoids, saponins, steroids, glycosides, alkaloids compounds.

## 3.2 *In-vitro* Antioxidant Activity

The percentage inhibition in *C. aurea* seed extract and standard ascorbic acid (vitamin C) Vs concentration shows that the antioxidant activities of 70% ethanol extract of *C. aurea* seed and the standard vitamin C was found to be positively correlated with the % inhibition. The $IC_{50}$ (Inhibition Concentration at 50%) value of *C. aurea* extract were calculated as 88.46 µg/ml while that of Ascorbic Acid was 51.41 µg/ml from their corresponding regression curves.

## 3.3 Haematological Profile

The WBC total count shows that no significant difference between HAART administered rats (group- II) as compared with the normal rats (Group- I).This finding is against the pervious finding of Kayode et al. [31] they are reported that acute administration of lamivudin and efavirenz increased total WBC count 40% and 43% respectively and Osonuga et al. [32] reported that HAART administered rats showing significant decrease WBC total count. However, the WBC count mean value shows an increase ($p<0.05$) in group IV and V, those receiving HAART and 200 and 300 mg/kg CASE compared with the normal rats (Group- I). Only Group- V shows statistically significant increase ($p<0.05$) in LYM# compared with the normal rats (Table 2).

RBC, MCV, MCH and MCHC mean values found significantly decreased ($p<0.05$) in HAART administered group II rats. Previous study showed that the toxic effects of antiretroviral drugs lead to anemia. These could be as a result of these drugs interfering with the progenitor cells of the bone marrow leading to suppression of their activity [33]. The affected parameters are restored by CASE in dose dependent manner. However the platelet count is not altered by HAART, but CASE receiving rats are significantly reduced the platelet count.

## 4. DISCUSSION

This study revealed a significant alterations total RBC, HCT and haemoglobine level in positive control (Group II) rats that the primary regime of

HAART induced anemia. Several antiretroviral drugs, most importantly zidovudine and other nucleoside reverse transcriptase inhibitors (NRTIs), are known to cause anemia in adults and children [34-37]. Maternal highly-active antiretroviral therapy containing zidovudine reduces mother-to-child HIV transmission but may increase the risk for infant anemia through breast milk [38]. Zidovudine is a well-known drug causing haematotoxicity [39,40]. Zidovudine use is yet not without adverse effect mainly bone marrow aplasia foremost to varying degrees of cytopenias (blood cell deficiencies) mostly anemia. This calls for adequate evaluation and monitoring of patients on this drug. Its major side effect; which is anemia limits its use in some patients [41]. Highly active antiretroviral drugs (zidovudine + Lamivudine ) cause suppression of the bone marrow cell precursors, leading to haematotoxicity. Lamivudine induced anemia is more readily attributed to red cell aplasia (RCA). Which is an unusual disorder in which maturation occurs in the formation of erythrocytes [42].

Evaluation of haematological parameters can be used to determine the degree of harmful or helpful effects of foreign compounds as well as plant extract on blood [43]. The outcome of this study showed that the CASE shows salubrious effects on HAART induced haemototoxicity. The salubrious effects are indications that the animals recovered from haemototoxicity. In the Traditional Chinese medicine, Radix *Astragali* is commonly used as a health food supplement to reinforce the body vital energy. Previous study also showed that the *Astragali* Radix extract could improve haematopoietic functions by regulating erythropoietin (EPO) expression. EPO is an erythrocyte-specific hematopoietic growth factor produced by kidney and liver [44]. Flavonoids, including formononetin, ononin, calycosin, and calycosin-7-O-β-d-glucoside, are considered to be the major active ingredients within Radix *Astragali*. These four flavonoids can induce the expression of EPO [45]. Pervious study in *C. aurea* leaves [25] and present study in *C. aurea* seed contains flavonoids, it may be promotes the EPO synthesis and restored the HAART affected parameters.

HAART drugs amplify chemically reactive species in blood, possibly by producing more oxidized metabolites deriving from the interaction between reactive oxygen species (ROS) and infected cell biomolecules [46]. This is supported by some biochemical mechanisms, such as mitochondrial interference, following treatment with HAART-AZT [47-49]. RBCs are prone to oxidative stress being the first cells in the body because they are extremely vulnerable to oxidative injure due to the high concentration of iron in haemoglobin and oxygen the chief cradle of the oxidative process. RBCs have a rich polyunsaturated fatty acid (PUFA) chains in the plasma membrane and they are highly susceptible to oxidation [50]. This molecular mechanism taken to account for reduction of the total RBC count in positive control (Group II) rats. The CASE treated groups (IV and V) significantly increase the total RBC count may be related to its antioxidant activity. The phytochemical results confirm that the CASE contains tannins and flavonoids which are powerful antioxidant phytochemicals. Tannins do not function solely as primary antioxidants (i.e., they donate hydrogen atom or electrons), they also function as secondary antioxidants. Tannins have the ability to chelate metal ions such as $Fe^{2+}$ and interfere with one of the reaction steps in the Fenton reaction and thereby retard oxidation [51]. The inhibition of lipid peroxidation by tannin constituents can act via the inhibition of cyclooxygenase [52]. Flavonoids act as antioxidants by ‘mopping up’ free radicals in cells, thereby limiting the oxidative damage. They are well known to reduce lipid-peroxidation and lipoxygenase enzyme activities. They apply these antioxidant properties as free radical scavengers, chelators of divalent cation [53,54]. Moreover, the high-level of ROS can damage the haematopoietic reconstitution capacity of haematopoietic stem cells (HSCs). Thus, the application of antioxidant intervention in the *in-vivo* mobilization of bone marrow haematopoietic stem cells may be effective against the negative effects of ROS on bone marrow haematopoietic stem cells. Antioxidant intervention may also better protect the haematopoietic reconstitution capacity of HSCs [55].

HAART did not alter the White blood cell count in the present study. However in CASE treated groups (IV and V) WBC count increase in a dose dependent manner compare with both positive (Group II) and negative control (Group I). The effect of low dose of CASE did not alter the WBC count. The phytochemical result of CASE reveals that presence of cardiac glycosides. The cardiac glycosides have an anti-inflammatory property and are attributed to cause increase in WBC count [56].

**Table 2. Haematological profile of normal control, and HAART and CASE administered albino wistar rats**

| Haematology parameters | Group - I (Dis. Water) | Group - II (HAART) | Group – III (100 mg/kg CASE + HAART) | Group – IV (200 mg/kg CASE +HAART) | Group – V (300 mg/kg CASE +HAART) |
|---|---|---|---|---|---|
| WBC per µL | 7080±1381.087[a] | 7340±1500.599[a] | 7360±639.218[a] | 7740±1319.318[b] | 9060±1556.792[c] |
| LYM% | 71.376±1.670[a] | 73.18±2.126[a] | 77.38±3.717[a] | 72.86±2.642[a] | 76.60±2.762[b] |
| LYM# per µL | 5300±474.342[a] | 5384±521.973[a] | 5580±1231.828[b] | 5560±860[b] | 7120±1358.823[c] |
| RBC per µL | 8832000±14221.111[a] | 7008000±55952.224[b] | 7146000±158472.999[b] | 7160000±28360.834[c] | 7536000±25611.161[d] |
| HCT (PCV) % | 64.75±0.633 | 46.56±3.793[b] | 48.58±1.928[b] | 51.172±1.248[c] | 63.992±1.810[d] |
| HGB g/dL | 15.42±0.498[a] | 12.90±1.126[b] | 13.50±0.532[b] | 14.244±0.293[c] | 15.80±0.192[d] |
| MCV per fL | 73.102±0.857[a] | 66.306±0.240[b] | 66.42±0.788[b] | 67.86±0.409[b] | 72.82±0.991[c] |
| MCH per pg | 21.66±0.309[a] | 19.90±0.315[a] | 19.942±0.122[a] | 21.112±0.178[a] | 21.28±0.185[a] |
| MCHC per g/dL | 32.06±0.426[a] | 27.244±0.227[b] | 27.34±0.163[b] | 31.57±0.247[c] | 31.94±0.615[c] |
| PLT per µL | 751000±56059.7895[a] | 742200±119212.164[a] | 507200±92496.162[b] | 414000±13309.576[c] | 598600±56772.881[d] |
| MPV per fL | 7.698±0.230[a] | 7.82±0.073[a] | 8.06±0.112[a] | 7.72±0.211[a] | 7.94±0.181[a] |

The significant decrease of platelet count recorded in all CASE treated groups compared with both positive (Group II) and negative control (Group I) not in dose dependent manner. These data are in agreement with the data reported by Tohti et al. [56]. These decreases might be attributed to the toxic effect of the CASE because of the fact that it contain saponins and cardiac glycosides.

## 5. CONCLUSION

This report shows that oral administration of CASE is an effective counter measure for the toxic hematopoietic effects of HAARTM may be CASE phytochemicals such as tannins, flavonoids, terpenoids, etc attenuate the HAART induced hematopoietic cell death in the periphery and bone marrow or it may be promotes hematopoietic functions by regulating erythropoietin. Furthermore CASE oral administration reduces the platelet count not in the dose dependant manner. The molecular mechanism of action of the drug needs further clarification.

## CONSENT

It is not applicable.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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