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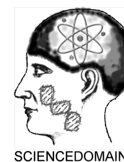

Protective Effects of *Calpurnia aurea* Seed Extract on HAART Hepatotoxicity

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Authors' contributions

This work was carried out in collaboration between all authors. Author HNM carried out all kinds of experimental parts and statistical analyses of data. Authors SD and UM supported the protocol writing and revised the manuscript. Author WE carried out the histopathology of liver and interpreted the results. Author NG designed the study, wrote the protocol, and wrote the first draft of the manuscript, managed the literature searches and provided chemical for the experiments. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The effect of hydroethanolic seed extract of *Calpurnia aurea* was evaluated against HAART induced free radical reactions in liver and liver cell damage in Wistar male albino rats.
Background: Highly active antiretroviral therapy (HAART)-correlated hepatotoxicity make difficult the management of patients infected with human immunodeficiency virus (HIV), raise medical costs, changes the prescription prototypes, and affects the principle recommendations.
Materials and Methods: Matured dried seed of *Calpurnia aurea* were collected, powdered and extracted using 70% ethanol. Preliminary phytochemical screening and *in-vitro* antioxidant properties of the extract were carried out. Thirty rats of same age and 140-200 g weight were selected and divided into five groups containing six each. The HAART and different doses of the

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Calpurnia aurea seed extract (100, 200 and 300 mg/kg) administered orally for 35 days. At the end of the experiment day the rats were fasted overnight. Then blood samples were collected by cardiac puncture for biochemical studies and there after sacrificed by cervical dislocation and liver was excised from the rats for histopathological studies. The hepatoprotective effects of the seed extract against HAART liver toxicity in rats were evaluated by monitoring the levels of alkaline phosphatase (ALP), amino transferases (AST, ALT), and histopathological analysis. In addition, the antioxidant properties of the seed extract against HAART induced alteration in rats liver antioxidant profile were evaluated by monitoring the levels of SOD, CAT, GHS, MAD and TAC analysis.

Results: Increased free radical reactions, ALP, amino transferases release and decreased antioxidant profiles were detected in HAART treated rats. The rats treated with the extract (300 mg/kg) reduce the HAART induced liver toxicity but minimum dose of extract (100 mg/kg) did not show any significant change against HAART altered parameters.

Conclusion: This study suggests that the *Calpurnia aurea* seed extract have hepatoprotective potential, thereby justifying their ethnopharmacological uses.

Keywords: Hepatotoxicity; HAART; Calpurnia aurea; antioxidant; amino transferases.

1. INTRODUCTION

The arrival of highly active antiretroviral therapy (HAART) in the management of human immunodeficiency virus (HIV) infection has significantly reduced the incidence of opportunistic infection as well as improved morbidity and mortality among HIV patients. However, along with these positive outcomes, HAART is associated with a host of unpleasant reactions such as hepatotoxicity, hyperlipidemia, hyperglycemia, and lactic acidosis. Hepatotoxicity can interrupt HIV therapy and cause an increase in morbidity and mortality [1]. Adverse effects have been reported to be associated with different classes of anti-HIV drugs, including nucleoside reverse transcriptase inhibitors, non-nucleoside analogue reverse transcriptase inhibitors, and HIV protease inhibitors [2]. Aside from host factors, several individual antiretrovirals or classes have been independently associated with hepatotoxicity, such as nevirapine, protease inhibitors, high doses of ritonavir (600 mg/day), and prolonged zidovudine or stavudine exposure [3-5]. Nucleos (t) ide-reverse transcriptase inhibitors (NRTIs) have their ability to hinder Pol-γ in mitochondrial DNA. In addition, other groups of anti-HIV drugs such as non-nucleoside reverse transcriptase inhibitors and protease inhibitors interfere with mitochondrial toxicity [6]. The mechanisms of drug-induced liver damage are not forever known, but when they are investigated mitochondrial dysfunction is frequently observed [7–9]. In addition, innate and adaptive immune responses are additional factors noticed which of importance point to the sequence and harshness of liver damage [10,11]. Detailed review mechanisms of drugs induced liver injuries focusing on pathogenesis are described elsewhere [12-15].

HAART hepatotoxicity confuses the management of HIV infected patients, raises the medical costs, alters the prescription patterns, and has an impact on official treatment recommendations. Several mechanisms of liver toxicity in patients receiving HAART had been recognized. Although rare, HAART-related liver damage may have shocking consequences. Among clinical syndromes of HAART liver toxicity, allergic reactions and lactic acidosis are recognized. Among the latter, HAART-related liver fibrosis, NASH (Nonalcoholic Steatohepatitis), nodular regenerative hyperplasia, and portal hypertension are high risk diseases leading to early mortality. Prevention is the best plan to reduce the luggage of hepatotoxicity in vulnerable hosts and includes appreciation of antiretrovirals' liver safety profile. Management of hepatotoxic trial includes discontinuation of alleged causes and modified HAART regimens [16].

Medicinal plants play an important role in the lives of rural people particularly in remote parts of developing countries with few health facilities. The plants, fruits, and compounds described could offer novel alternatives to the limited therapeutic options that exist for the treatment of liver diseases. Hepatoprotective activity of phytochemicals, were related to their antioxidant potential [17,18]. *Silybum marianum* commonly known as 'milk thistle'; (family of Asteraceae) seeds contain flavonoids such as silymarin, which is recognized as a hepato-protective agent of herbal origin. It also has clinical applications in the treatment of liver related disorder such as

toxic hepatitis, fatty liver, cirrhosis, ischemic injury, and viral hepatitis via its anti-oxidative, anti-lipid peroxidative, antifibrotic, anti-inflammatory and liver regenerating effects [19-21].

Calpurnia aurea is a genus of FLOWERING PLANTS within the family of *Fabaceae*. Literature survey brings to light that, the leaf and stem of *C. aurea* has been used for different human and animal disease [22]. Mulata et al. [23] reported that the 70% ethonolic extract of *C. aurea* seed revealed the presence of tannins, flavonoids, terpenoids, saponins, steroids, glycosides, alkaloids but anthraquinones were absent, yet seeds contained more tannins and alkaloids than the leaves. This extract is an effective counter measure for the toxic haematopoietic effects of HAART [24]. The aim of the present study was to explore protective roles of hydroethanolic extract of *C. aurea* seed (CASE) in HAART-induced hepatotoxicity.

2. MATERIALS AND METHODS

2.1 Plant Materials

The *C. aurea* plants with flower and seed were collected from south Gondar, northern Ethiopia in June 2013. The plant was identified and authenticated by taxonomist of Ethiopian National Herbarium of Addis Ababa University and its voucher number is 001/2006. The seeds were washed thoroughly 2-3 times with running tap water, and dried in shade and reduced to a fine powder.

2.2 Extraction and Assay of *In-vitro* Antioxidant Activity by Spectro-photometric Method

The powdered seeds were weighed 100 g and macerated 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. Assay of *in-vitro* antioxidant activity by DPPH radical-scavenging activity of the extract was examined as previously described [25].

2.3 Acute Oral Toxicity Test for CASE

The acute oral toxicity test indicated that no visible signs of acute toxicity and mortality were observed at the dose of 300 mg/kg body weight.

2.4 Animals

Thirty adult apparently 12 weeks' old healthy male albino rats of weighing about 140 –200 g 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 EC. All the animal experiments were carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.4.1 Extrapolation of HAART dose

The humans doses of HAART drug were extrapolated to animals by the formula; Animal Equivalent Dose (AED) = Human Dose X ((Animal Km ÷ Human Km), Where Km is a correction factor reflecting the relationship between body weight and body surface area [26]. The Table 1 shows the average Km value of most frequently used laboratory animals and human adults.

Table 1. Average Km values of laboratory animals and human adult

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

Based on the above data the dosages of regimen were given for group II to V as follows Stavudine+ Lamivudine+ Nevirapine (0.11 + 0.53 + 0.7) mg/Kg administered by for 35 days.

2.4.2 Animal grouping and drug dose

Group- I normal control, given distilled water only

Group- II positive control, given 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
Group- V HAART drugs + 300 mg/kg of CASE

2.4.3 Blood sample collection and analysis

At the end of the experiment day the rats were fasted overnight, sacrificed by cervical dislocation and blood has been collected by cardiac puncture and serum was obtained. The serum was stored at -20ºC for biochemical studies.

2.4.4 Serum enzyme assay

The appropriate kits were used for the determination of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) according to Reitman and Frankel [27], alkaline phosphatase (ALP) according to Belfield and Goldberg [28]. The enzyme activity was expressed as units/liter computed directly from the absorbance values. Serum total protein was measured according to Gornall et al. [29], and Albumin according to Doumas et al. [30]. Total and direct bilirubins were determined according to Walters and Gearde, 1970 [31].

2.4.5 Liver antioxidant

Liver was homogenized (10% w/v) in ice–cold 0.1 M Tris–HCl buffer (pH 7.4). The homogenate was centrifuged at 3,000 rpm for 15 min at 4ºC and the resultant supernatant was used for assessing different oxidative stress markers. Superoxide dismutase (SOD) was determined according to Nishikimi et al. [32]. catalase (CAT) according to Aebi, [33]. reduced glutathione (GSH) according to Beutler et al. [34], malondialdehyde (MDA) according to Mihara and Uchiyama [35], total antioxidant capacity(TAC) according to Koracevic et al. [36] and Thiobarbituric Acid Reactive Substances (TBARS) according to Yagi [37].

2.4.6 Liver histopathological studies

Liver sections taken from the liver, fixed in 10% buffered formalin, dehydrated in ethanol (50–100%), cleared in xylene, and embedded in paraffin. Sections (4–5 Im thick) were prepared and then stained with hematotoxylin and eosin (H–E). The sections were examined for the pathological findings of hepatic changes.

2.5 Statistical Analysis

All 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. P less than 0.5 were considered significant.

3. RESULTS

3.1 Phytochemicals and *In vitro* Antioxidant Activity

The preliminary phytochemical analysis of 70% ethanolic extracts from the *C. aurea* seeds showed the presence of tannins, flavonoids, terpenoids, saponins, steroids, glycosides, alkaloids compounds. The extract showed significant activities in all antioxidant assays compared to the reference antioxidant ascorbic acid in a dose dependent manner. In DPPH, scavenging assay the IC_{50} value of the extract found to be 58.26 µg/ml while the IC_{50} value of the reference standard ascorbic acid was 52.92 µg/ml.

3.2 Improvement of Liver Functions in CASE Treated Rats

The activities of aminotransferase (AST and ALT), ALP levels as well as serum TP, albumin, TB and DB in the control, HAART, and different doses of CASE-HAART administered rats are presented in Table 2. Administration of HAART - induced significant increase (P≤0.001) in serum AST, ALT, ALP TB, and DB as well as significant decrease in the serum TP and albumin content as compared to control. Treatment for different doses of CASE especially the group 5 rats (300 mg/kg CASE) significantly (P≤0.01) decrease serum ALT, AST, ALP, TB and DB and significant increase in the serum TP and albumin content near to the control . The results showed that administration of the CASE shows a significant positive change in the liver function markers of HAART administered rats.

3.3 Antioxidative Activity of CASE in the Livers of HAART Administered Rats

Oxidative stress markers such as superoxide dismutase (SOD) catalase (CAT), reduced glutathione (GSH), malondialdehyde (MDA) levels and TBARS as well as total antioxidant capacity (TAC) in liver tissue of control, HAART and different doses of CASE-HAART

administered rats in Table 3. Data showed that HAART administration caused a significant decrease (P≤0.001) in SOD and CAT activities, GSH level, TAC and significant increase (P≤0.001) of TBARS as compared to control. Treatment of rats with the CASE (200,300 mg/kg body weight) significantly increased (P≤0.001) the level of SOD, CAT, GSH and TAC as compared to HAART treated group. HAART treatment significantly (P≤0.001) increased the level of MDA in the liver tissue as compared to control, MDA levels were assessed as an indicator of lipid peroxidation. Pretreatment with CASE (100, 200 and 300 mg/kg) had more or less prevented this trend, according to the amount of CASE (p<0.05). When the dose reached 300 mg/kg, the results were as good as compared to the dose of 100 mg/kg.

3.4 Histopatological Observations

Histopathology of the normal control rat liver (Fig. 1) shows preserved cytoplasm, no vacuolation, no lymphocyte infiltration (inflammation), and no area of necrosis.

However, histopathology of HAART received rat liver (Fig. 2), shows chronic inflammation, necrosis, lymphocyte infiltration (inflammation), sinusoidal dilation and cytoplasmic vacuolation.

Histopathology of group – III rat liver (Fig. 3), shows focal lymphocyte infiltration (inflammation), and sinusoidal dilation.

Histopathology of group–IV rat received 200mg/kg CASE + HAART (Fig. 4), shows sinusoidal dilation and cytoplasmic vaculation but lower than group-II and Group – III rats.

Histopathology of group–V rats received 300 mg/kg CASE + HAART (Fig. 5), depicts generally no abnormal features and the cords of hepatocytes were distinct, sinusoids were well demarcated, no vacuolation, no lymphocyte infiltration (inflammation), no significant area of inflammation and necrosis.

4. DISCUSSION

The administration of HAART to the rats resulted in marked elevation of serum enzymes ALT, AST, ALP and bilirubin. Membrane disintegration of hepatocytes with subsequent release of AST, ALT and ALP, among others, is one of the consequences of HAART -induced lipid peroxidation [38]. Among the liver specific enzymes, mostly alanine aminotransferase considered very responsive for pointers of hepatotoxic as well as hepatoprotective or curative effects of different compounds.

Table 2. The influences of the different dose of CASE on the levels of serum ALT, AST, ALP, total protein, albumin, total and direct bilirubin in HAART administered rats

Groups	ALT U/L	AST U/L	ALP U/L	Total protein (g/dl)	Albumin (g/dl)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
Control	30.02±3.94[a]	26.72±3.29[a]	91.14±6.56[a]	8.71±0.14[a]	4.70±0.11[a]	0.40±0.01[a]	0.11±0.01[a]
HAART	48.48±10.52[b]	41.43±4.92[b]	131.40±10.62[b]	7.32±0.23[b]	4.31±0.26[b]	0.56±0.08[b]	0.17±0.03[b]
HAART+ 100 mg CASE	45.60±14.49[b]	40.36±4.92[b]	129.24±8.56[b]	7.52±0.42[b]	4.36±0.02[b]	0.52±0.03[b]	0.14±0.02[b]
HAART+ 200 mg CASE	37.00±2.12[c]	32.56±4.23[c]	103.33±8.56[c]	7.93±0.32[c]	4.48±0.17[c]	0.45±0.01[c]	0.13±0.02[c]
HAART+ 300 mg CASE	31.80±2.75[a]	28.62±5.32[a]	94.34±4.60[a]	8.32±0.22[c]	4.57±0.12[ca]	0.43±0.02[c]	0.12±0.01[c]

Table 3. Effect of the different dose of CASE on the levels of SOD, CAT, GSH, MDA, TBARS, TAC, in liver homogenate of HAART administered rats

Groups	SOD U/g	CAT U/g	GSH mg/g	MDA nmol/g	TBARS nmol/g	TAC µmol/g
Control	16.17±0.73[a]	1.84±0.02[a]	44.86±0.98[a]	374.93±13.01[a]	8.32±1.31[a]	49.86±1.16[a]
HAART	7.22±0.41[b]	072±0.035[b]	24.39±0.76[c]	835.06±19.14[b]	15.42±2.12[b]	39.33±1.02[b]
HAART+100 mg CASE	10.86±0.74[c]	1.51±0.033[c]	29.17±1.59[d]	482.44±12.74[c]	14.32±2.32[b]	43.80±1.16[c]
HAART+200 mg CASE	12.99±0.43[d]	1.65±0.027[d]	38.64±1.13[e]	458.79±10.46[c]	10.23±1.21[c]	47.80±0.91[a]
HAART+300 mg CASE	14.04±0.49[d]	1.75±0.038[a]	40.41±1.41b[e]	451.09±9.12[c]	9.23±1.72[ac]	48.46±1.24[a]

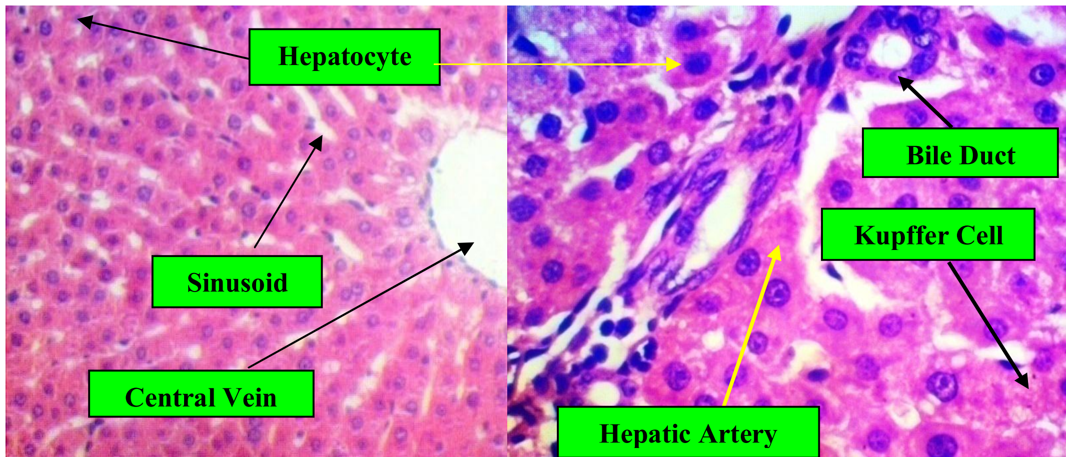

Fig. 1. Histopathology of normal control rat, (Group- I) (20X and 40X)

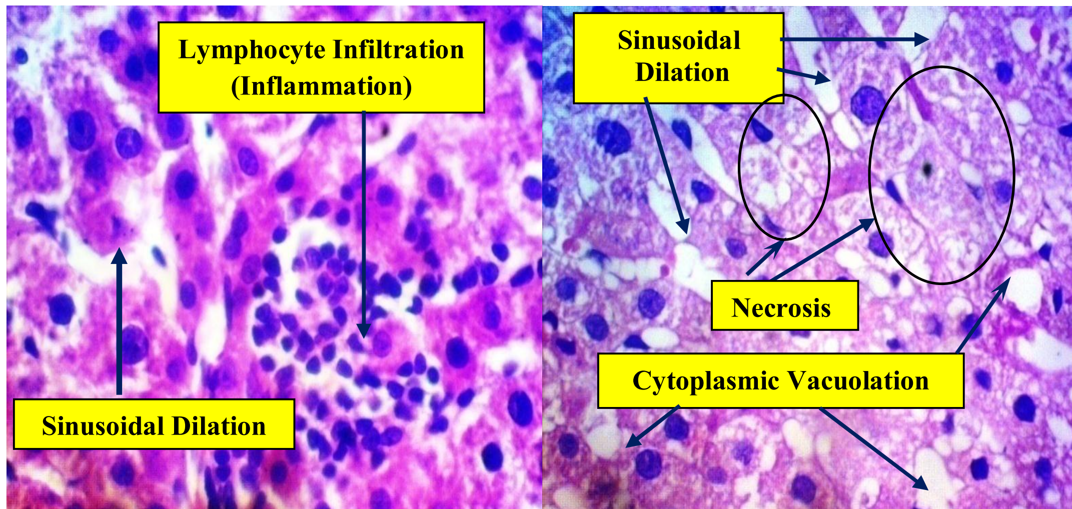

Fig. 2. Histopathology of HAART received rat, (Group- II) (40X)

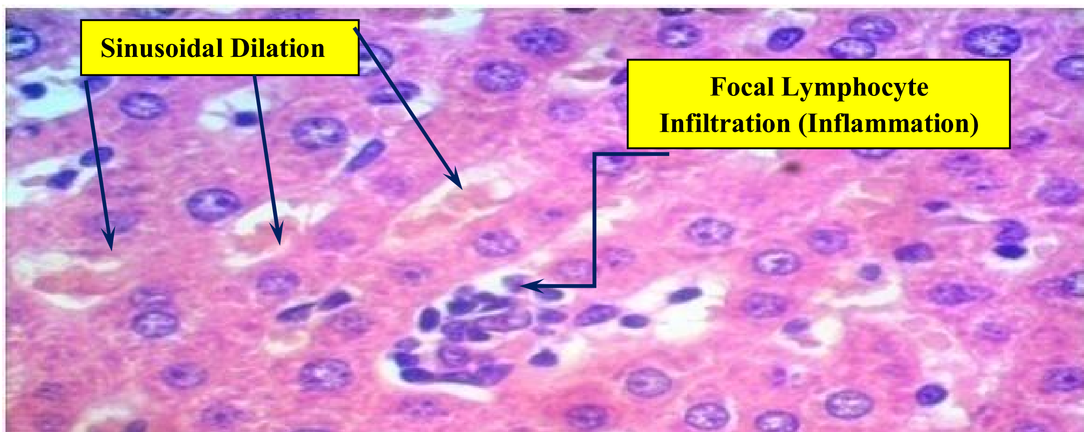

Fig. 3. Histopathology of 100 mg/kg CASE + HAART received rat liver (Group – III) (40X)

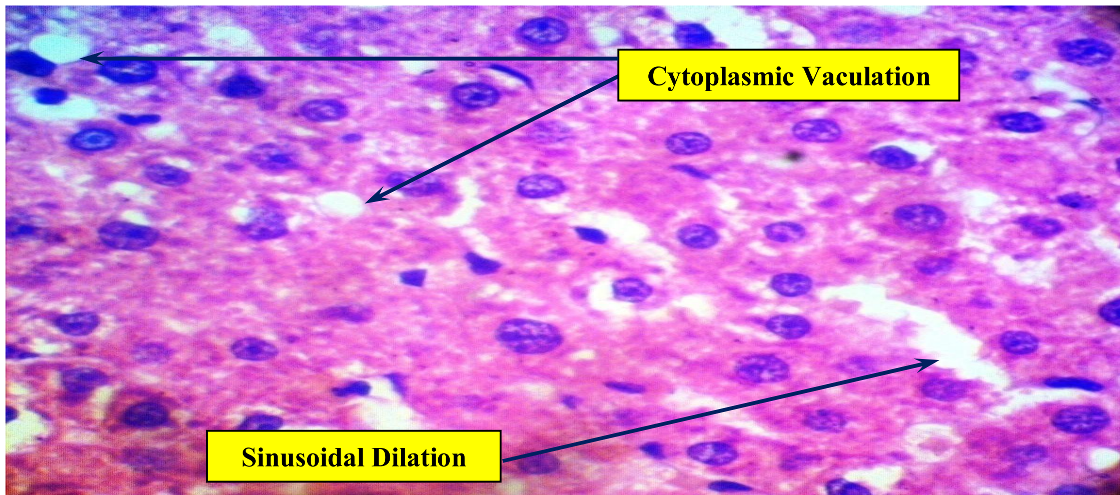

Fig. 4. Histopathology of 200 mg/kg CASE + HAART received rat liver (Group – IV) (40X)

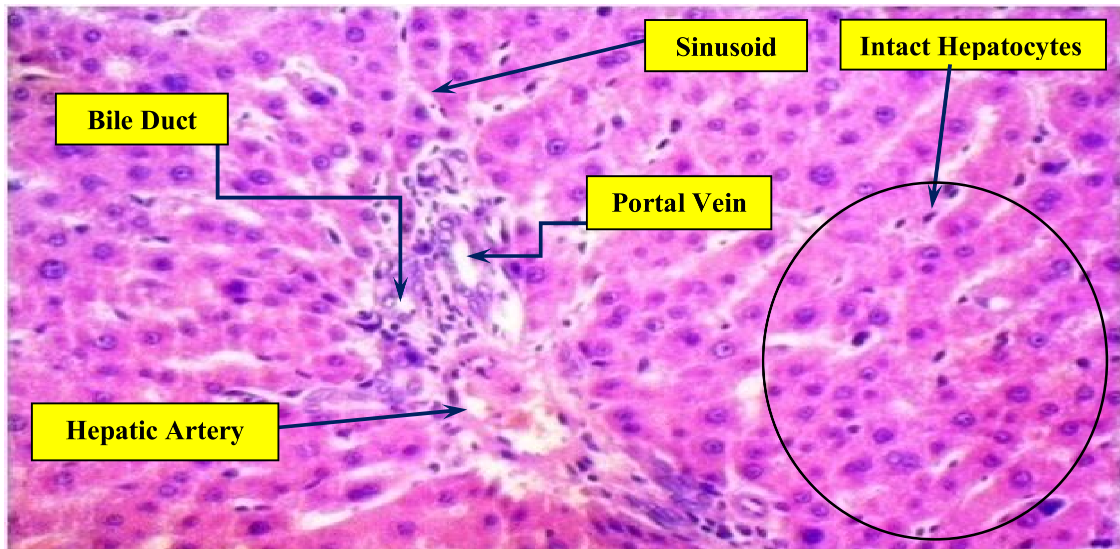

Fig. 5. Histopathology of 300 mg/kgCASE + HAART received rat, (Group – V) (20X)

The activities of these enzymes are used to assess the functional status of the liver and as the biochemical markers of liver damage [39]. Both AST and ALT levels rises in the plasma due to toxic compounds that promotes the liver cell necrosis. Decreased levels of transaminases indicate reduces the hepatocytes necrosis and safety of hepatocytes against damage caused by hepatotoxin. Normal liver functions are demarcated by the balanced activities of serum marker enzymes AST, ALT, ALP and bilirubin as well as albumin level [40]. Liver toxicity caused by antiretroviral therapy can be inflicted through several mechanisms. The pathogenesis often remains enigmatic. Five categories are proposed: direct mitochondrial inhibition, direct cell stress, hypersensitivity reactions, immune reconstitution in the presence of viral hepatitis co-infection, and disturbances of lipid/sugar metabolism and steatosis [41]. Some antiretrovirals or classes may be toxic for the liver through different pathways, a feature which is characteristic of drug-induced hepatotoxicity in general [42]. All or several members in three antiretroviral classes can cause disturbances in lipid and sugar metabolism, which seem to be contributors to a not well-defined steatohepatitis syndrome. Nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease

inhibitors (PIs) [31]. Mitochondrial liver toxicity leading to steatosis and lactic acidosis, which is secondary to mitochondrial RNA depletion by NRTI use, is particular to that class [43]. Hypersensitivity reactions with liver involvement are common to NNRTIs but are possible also for specific drugs in other classes [44-50].

Since phenolic and flavonoid compounds are documented as the bases of the antioxidant activity of plant extracts, we determined the phenolic and flavonoid content of CASE. Several studies have demonstrated the protective effects of herbals against experimentally induced liver injury. In addition, a number of herbals show promising activity including silymarin against liver cirrhosis. Silymarin, a reference drug, is a combination of flavonoids and polyphenols. Silymarin has membrane-stabilizing and antioxidant behaviors, it endorses hepatocyte regeneration, decreases inflammatory reactions, and inhibits fibrogenesis [51]. Therefore, the CASE was chosen for evaluation of the hepatoprotective activity in HAART induced hepatotoxicity in a rat model and it was found to have good hepatoprotective effects evidenced by suppression of HAART-induced oxidative stress in the liver of rats, and attenuating the morphological changes caused by HAART. Moreover, the correlation between antioxidant and hepatoprotective activity was also examined. The results suggested that the possible mechanism of this activity might be due to free radical-scavenging and antioxidant activity of the CASE.

HAART may induce (i) oxidative stress (ii) decrease in free radical scavenging protection, or (iii) a failure to restore oxidative damage. Hepatotoxins initially damage the centrilobular region of liver where they are in high levels of cytochrome P450 oxidases that mediated their conversion to toxic electrophilic metabolites. These toxic metabolites can covalently bind to proteins lipids, DNA forms adducts followed by reactive oxygen species production (ROS), lipid peroxidation, releases of pro-inflammatory cytokines, glutathione depletion and cell death [52]. Therefore, free radical-scavenging is the most significant way to protect the liver against hepatotoxicity induced by HAART. In the present study in the CASE, tannins and flavonoids have been identified. Tannins have strong antioxidant properties and have ability to chelate metal ions such as Fe^{2+}, unlike primary antioxidants they donate hydrogen atom or electron [53]. The prevention of membrane lipid peroxidation by tannin can act via the inhibition of cyclooxygenase [54,55]. Flavonoids are able to inhibit D-galactosamine and CCl_4-induced hepatotoxicity in experimental models due to their potent anti-oxidant or free radical scavenging properties [56]. This active principle may account for the pharmacological properties of CASE.

It was observed that, rats which received HAART treatment, had decreased glutathione, albumin and increase in oxidative stress. Glutathione is a well known to reduce substrates and plays an important role in defense mechanisms of oxidative damage. In GSH redox cycle, glutathione, act as a direct endogenous scavenger of hydroxyl radicals, involved in detoxification of toxic substances in the liver [57]. HAART reduces GSH synthesis, increased GSH use, or limited intracellular decrease of its oxidized form (GSSG) [58]. In liver GSH deficiency may be impaired in reducing ability, immune function, protein biosynthesis, accumulations of lipid peroxidation products and detoxification capacity. Reduced detoxification ability in the liver may lead to the accumulation of toxic metabolites in liver cell leading to liver damage [59]. In the present study, the activities of hepatic GSH and antioxidant enzyme in HAART-treated rats were markedly weakened. The administration of CASE possesses potent hepatoprotective activity *in vivo*, which might be due to restoration of the GSH level and therefore the amount of glutathione available in the cell for HAART detoxification. In mice, acute silymarin treatment, increases the hepatic GHS, via direct effect on the metabolism of sulfur containing amino acid in liver cell. Silymarin increased the amount of metabolites generated from homocysteine in the transsulfuration pathway (cystathionine, cysteine, and glutathione), elevated the activity of cystathionine β-synthase, while down-regulated cysteine dioxygenase. It was concluded that Silymarin enhances hepatic glutathione generation by elevating cysteine availability via increment in cysteine synthesis and an inhibition of its catabolism to taurine [60]. Moreover, Gnanasekaran et al. [61] reported that pretreated with an aqueous leaf extract of *Tridax procumbens* in cultured mouse hapatocytes had significantly higher hepatocellular GSH levels. This active principle may account for the pharmacological properties of CASE.

Therefore, it is suggested that the salubrious effects of CASE against HAART-induced liver

damage in rats, may be due to its antioxidative properties. Further safety and efficacy studies are needed to elucidate its mechanism of action, which CASE itself reacts with the reactive oxygen species or boosting the antioxidant enzyme and GSH production.

5. CONCLUSION

In the present study, *Calpurnia aurea* seed extract (CASE) obsessed heptoprotective activity against HAART -induced liver injury in rats may be due to its free radicals scavenging and antioxidant activity, resulting from the presences of some flavonoids and tannins. However, further detailed studies are required to specify exact role of the potential active constituents of the CASE.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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