

HEPATOPROTECTIVE EFFECT OF *CYMBOPOGON CITRATUS* ESSENTIAL OILS AGAINST NEVIRAPINE-INDUCED HEPATIC DAMAGE IN WISTAR ALBINO RATS

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Abstract

Background: The use of nevirapine in the management of Human Immunodeficiency Virus (HIV) infections is greatly limited by its fatal hepatotoxicity. In sub-Saharan African, traditional medicine involving use of plant materials has been proposed to counteract liver damage, albeit with no scientific evidence. The aim of the present study was to determine the hepatoprotective effect of *Cymbopogon citratus* essential oils against nevirapine-induced hepatic oxidative damage in Wistar albino rats.

Materials and Methods: Five groups of rats received a daily oral dose of 36mg/kg nevirapine for 4 consecutive weeks. After 15 minutes, rats in three of the groups were given 200mg/kg, 400mg/kg and 800mg/kg of *Cymbopogon citratus* essential oil extract. The positive control group received nevirapine and 200mg/kg of silymarin while the normal control group received only 2ml of distilled water. Blood was used to determine the levels of liver function parameters and liver sections were used for histological studies.

Results: The results revealed that oral administration of nevirapine (36mg/kg b.w. p.o) for 4 weeks significantly ($P < 0.05$) increased levels of serum hepatic function parameters with marked tissue damage in the nevirapine group as compared to the normal control. Rats that were co-treated with nevirapine and *Cymbopogon citratus* essential oils showed a dose-dependent improvement in liver architecture and liver serum biochemistry to levels comparable to those of the positive and normal control groups.

Conclusion: Our results show that *Cymbopogon citratus* essential oils have a protective effect against nevirapine-induced alterations in liver biochemistry and hepatic tissue damage.

Keywords: nevirapine, *Cymbopogon citratus*, hepatotoxicity, Human Immunodeficiency Virus (HIV)

Abbreviations: HIV: Human Immunodeficiency Virus, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase.

Introduction

Nevirapine is a non-nucleoside reverse transcriptase inhibitor used as a first-line regimen for Human Immunodeficiency Virus (HIV) type 1 infections. The use of nevirapine for HIV management has led to a significant decline in HIV associated morbidity and mortality (Emejulu et al., 2010). In addition, nevirapine has been shown to effectively decrease the vertical transmission of HIV-1 (Parietti et al., 2011). The drug has been shown to be effective against HIV-1 but with limited potency against HIV-2 or other retroviruses (Ena et al., 2012). Nevirapine has been associated with a life-threatening hepatotoxicity that impairs its effective use (Elias and Nelson, 2013). Nevirapine-induced hepatotoxicity is common in patients with higher CD4 counts and in the first three weeks of nevirapine treatment (Patel et al., 2004). Nevirapine-induced hepatotoxicity has been reported in 10-20% of HIV patients receiving the

drug and sometimes requiring discontinuation of therapy (Bruck et al., 2008). Furthermore, the risk of nevirapine-induced hepatotoxicity is exacerbated with HIV, hepatitis B or C co-infection (Sulkowski and Thomas., 2003).

Nevirapine is metabolized in the liver leading to the creation of hepatotoxic reactive metabolites known to cause hepatic damage (Boelsterli, 2002). Nevirapine reactive metabolites cause hepatotoxicity through the depletion of glutathione, or binding to enzymes, lipids, nucleic acids leading to cellular oxidative stress (Pauli-Magnus et al., 2005). Biological systems are protected from this stress by cellular antioxidant enzymes and endogenous antioxidants such as α -tocopherol, ascorbic acid, β -carotene and uric acid (Ojo et al., 2006). However, oxidative stress may overwhelm the cellular antioxidant machinery thus necessitating chemotherapeutic intervention to manage liver disease (Ha et al., 2010). Unfortunately, conventional drugs are expensive with severe side effects and complications to human health especially when administered chronically (Koh et al., 2012).

In sub-Saharan African, traditional medicine practices involving the use of plant materials with antioxidants have been proposed as therapeutics agents to counteract liver damage (Koh et al., 2012; Samudram et al., 2008). Some plants with reported hepatoprotective activities include; *Vitis thunbergii*, *Psidium guajava*, *Citrus limon*, *Ailanthus excels*, *Andrographis paniculata* (Dhiman et al., 2012). In Uganda, *Cymbopogon citratus* is currently being used by traditional medicine practitioners in the management of liver associated diseases. Furthermore, the plant is reported to have antioxidants which have the potential to minimize the deleterious effects of free radicals (Figueirinha et al., 2008; Ojo et al., 2006). The plant has also been shown to have hypoglycemic, hypolipidemic, anxiolytic and sedative effects (Blanco et al., 2009). However, despite the traditional use of *Cymbopogon citratus* in the management of liver disease, there is limited scientific evidence to confirm its hepatoprotective effects. We thus evaluated the hepatoprotective effect of *Cymbopogon citratus* essential oils against nevirapine induced hepatic damage in Wistar albino rats.

Materials and Methods

Plant material processing and steam distillation

Cymbopogon citratus plant materials were collected from the botanical gardens at the Natural Chemotherapeutics Research Institute (00° 20' 10"N, 32° 33' 57"E) in Uganda. Plant materials were identified and authenticated at the Makerere University herbarium with a voucher specimen number deposited (01, MA) for future reference. Essential oil extraction was done as previously described (Lucchesi et al., 2004). Briefly, plant leaves were washed with tap water, chopped, weighed and transferred in a 5 liter round bottomed flask. To the flask, 200ml of deionized water was added and mixture distilled for 3 hours using Clevenger Apparatus (Sciencesupply, Australia). The total distillation time was approximately 4 hours (including about 1 hour for the oil to start distilling). The floating essential oils of *Cymbopogon citratus* were then dried using anhydrous sodium sulphate (Sigma-Aldrich USA). The extracted essential oils were then wrapped with aluminum foil and stored in a freezer at -20°C as recommended by (Rowshan et al., 2013).

Experimental design

Thirty (30) Wistar albino rats of approximately 8 weeks of age and average weight of 175±35.3kg were purchased from the small animal breeding house at the College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University. The rats were maintained in a controlled environment under standard conditions of temperature (28 ± 2°C) and humidity with an alternating dark and light cycle for a period of two weeks to enable them acclimatize. Rats were randomly divided into six groups with six animals per group and treated as in Table 1. Rats in group 1, 2, 3, 4 and 5 received nevirapine (Cipla, India) at a daily oral dose of 36mg/kg (twice the human therapeutic dose) for 4 consecutive weeks to induce hepatotoxicity as demonstrated previously (Adaramoye et al., 2012). After a duration of 15 minutes the rats in groups 1, 2 and 3 were given 200mg/kg, 400mg/kg and 800mg/kg of *Cymbopogon citratus* essential oil extract respectively for 4 consecutive weeks by oral intubation method. Rats in group 5 (positive control) received 200mg/kg of silymarin (Shreeji Pharma, India) in 1% DMSO (Sigma-Aldrich USA) instead of the essential oil. Rats in group 6 were the normal control and only received 2ml of distilled water throughout the study period. After 4 weeks, the rats in all groups were deprived of food overnight and deeply anaesthetized with diethyl ether (Sigma-Aldrich USA) and bled by cardiac puncture.

Ethical consideration

Ethical approval for the study was sought from the Institutional Review Board at the College of Veterinary Medicine, Animal Resources and Biosecurity (Ref: SBL.S.CKD.2017). In all cases, the Organization for Economic Co-operation and Development Environment Directorate (OECD) guidelines for the testing of chemicals in laboratory animals were strictly adhered to (OECD, 1996). Rats were fed on commercially available pelleted rat chow (Nuvita, Uganda) and water provided ad libitum. At the end of the experiment, all animals were euthanized and humanely sacrificed under diethyl ether.

Table 1: Treatment administration per group

| Groups | N | Treatment (Daily for 4 weeks) |
|----------------------------|---|---|
| Group 1 | 6 | 36mg/kg of nevirapine + 200mg/kg of essential oil |
| Group 2 | 6 | 36mg/kg of nevirapine + 400mg/kg of essential oil |
| Group 3 | 6 | 36mg/kg of nevirapine + 800mg/kg of essential oil |
| Group 4 (Negative control) | 6 | 36mg/kg of nevirapine only |
| Group 5 (Positive control) | 6 | 36mg/kg of nevirapine + 200mg/kg of silymarin |
| Group 6 (Normal control) | 6 | 2ml of distilled water |

N: number of animals per group.

Evaluation of hepatic function

At the end of the experiment, about 3mls of blood were obtained using vacutainers by cardiac puncture. Blood was centrifuged at 3000 g for 15mins and the obtained serum stored at -20°C until further analysis within 6 days. Laboratory quantification of liver specific enzymes that is; alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin and total protein were quantified as previously described (Thapa and Walia., 2007). Assays were done using an automated COBAS 6000 chemistry analyzer (Roche diagnostics, USA) with machine calibration achieved by running a set of controls and standards.

Histopathological examination

Following surgical removal, the liver was immediately fixed in 10% buffered formalin for 2 days in order to prevent autolysis and petrification. Trimmed tissue sections were processed in a Histokinette (Leica, Germany) as previously described (Bancroft and Gamble., 2008). The processed tissues were embedded in molten paraffin wax, blocked and sectioned using a rotary microtome (Baired and Tatlock, London) at 5µm thickness. Sections were floated out on a water bath (Leica, German) set at 44°C, dried and fixed onto glass slides in an oven set at 52°C for 48 hours. The sections were subsequently deparaffinized and stained with hematoxylin and eosin as previously described (Suvarna and Layton., 2008). Tissue sections were then mounted using DPX (Distrene, Plasticizer and Xylene) in preparations for microscopic examination. Slides were viewed using a Carl Zeiss light microscope (Axiostar, Germany) so as to identify lesions indicative of hepatotoxicity across groups. From selected sections, photomicrographs were taken with a mounted digital camera (PowerShot, China) using ZoomBrowser EX version 2 imaging software.

Statistical Analyses

The data were analyzed using Graphpad 6.0 statistical software. Data on liver function parameters for each group was expressed as mean ± SEM. The differences between the levels of liver function parameters among the different groups were analyzed using a one way ANOVA. Comparison between groups for statistical significance was done using Tukey multiple comparison test set at a significance level $\alpha = 0.05$.

Results

Effect of *Cymbopogon citratus* essential oil on nevirapine-induced alterations in serum biochemistry.

The results revealed that oral administration of nevirapine (36mg/kg b.w. p.o) for 4 weeks was effective in inducing hepatotoxicity as observed by the significant ($P < 0.05$) increase in serum hepatic function parameters when compared with the normal control group (Table 2). Serum ALT levels significantly varied across treatment groups ($p = 0.004$, $F_{(5, 30)} = 68.3$). On comparison between groups, serum ALT levels were significantly lower in the nevirapine+800mg/kg (176.50±14.71U/I) and nevirapine+400mg/kg (223.86±23.53U/I) essential oil groups to levels comparable to those of the nevirapine+silymarin (206.82±7.33 U/I) and the normal control group (178.78±27.80U/I). Similarly serum AST levels significantly ($p = 0.0001$, $F_{(5, 30)} = 55.4$) varied across treatment groups. Comparisons across groups revealed that serum AST levels in the nevirapine+800mg/kg (365.00±59.41U/I) and nevirapine+400mg/kg (385.04±50.55U/I) essential oil groups were comparable to those of the nevirapine+silymarin (400.36±33.86U/I) and the normal control group (354.94±70.22U/I). Serum ALP levels were associated with significant variations across treatment groups ($p = 0.0001$, $F_{(5, 30)} = 63.9$). Levels of serum ALP in the nevirapine+800mg/kg (148.40±18.40U/I) and nevirapine+400mg/kg (170.00 ± 27.38U/I) essential oil groups were comparable to those of the nevirapine+silymarin (156.60±11.07U/I) and the normal control group (128.12±7.25 U/I).

Total serum bilirubin levels significantly differed across treatment groups ($p = 0.0001$, $F_{(5, 30)} = 62.6$). Levels of serum bilirubin in the nevirapine+800mg/kg (0.06±0.01mg/dl) and nevirapine+400mg/kg (0.07±0.01mg/dl) essential oil

groups were comparable to those of the nevirapine+silymarin (0.06±0.01mg/dl) but higher than in the normal control group (0.04±0.00mg/dl). Similarly, serum total protein levels differed across groups (p = 0.0001, F_(5, 30) = 37.3). Total protein levels in the nevirapine+800mg/kg (7.06±0.15g/dl) essential oil group were comparable to those of the nevirapine+silymarin (7.62±0.28g/dl) and the normal control group (7.27±0.40g/dl).

Table 2: Effects of *Cymbopogon citratus* essential oil on nevirapine induced alterations in serum biochemistry

| Treatment groups | ALT (U/l) | AST (U/l) | ALP (U/l) | Bilirubin (mg/dl) | Total protein (g/dl) |
|----------------------------|-----------------------------|-----------------------------|-----------------------------|--------------------------|---------------------------|
| NVP+200mg/kg essential oil | 349.70 ± 52.37 ^b | 692.46 ± 71.14 ^b | 259.40 ± 28.77 ^b | 0.10 ± 0.01 ^b | 5.95 ± 0.20 ^{ab} |
| NVP+400mg/kg essential oil | 223.86 ± 23.53 ^a | 385.04 ± 50.55 ^a | 170.00 ± 27.38 ^a | 0.07 ± 0.01 ^a | 6.30 ± 0.19 ^b |
| NVP+800mg/kg essential oil | 176.50 ± 14.71 ^a | 365.00 ± 59.41 ^a | 148.40 ± 18.40 ^a | 0.06 ± 0.01 ^a | 7.06 ± 0.15 ^a |
| NVP (36mg/kg) | 433.92 ± 39.56 ^b | 826.20 ± 97.61 ^b | 284.80 ± 16.16 ^b | 0.12 ± 0.01 ^b | 4.11 ± 0.35 ^b |
| NVP+200mg/kg silymarin | 206.82 ± 7.33 ^a | 400.36 ± 33.86 ^a | 156.60 ± 11.07 ^a | 0.06 ± 0.01 ^a | 7.62 ± 0.28 ^a |
| Distilled water (2ml) | 178.78 ± 27.80 ^a | 354.94 ± 70.22 ^a | 128.12 ± 7.25 ^a | 0.04 ± 0.00 ^c | 7.27 ± 0.40 ^a |

Lower case letters (^{a, b}) indicate significant differences when compared across treatment groups for each parameter. Results are expressed as mean ± SEM (n = 6). NVP; nevirapine, ALT; alanine transaminase, AST; aspartate transaminase and ALP; alkaline phosphatase.

Effect of *Cymbopogon citratus* on liver histology

Histological examination of sections from the normal control group revealed the central vein with radiating hepatic cords indicating normal liver lobular architecture (plate 1). Increased hepatocyte degeneration, cytoplasmic vacuolation, enlarged hepatocytes, disintegration of hepatic cords, infiltration by inflammatory cells and central venous congestion provided histopathological evidence of tissue injury in the group that received only nevirapine (plate 2). Rats that were co-treated with nevirapine and *Cymbopogon citratus* essential oil showed a dose dependent improvement in liver architecture (plates 4, 5 and 6). At 800mg/kg essential oil (plate 6), liver architecture was comparable to that of the nevirapine+silymarin (plate 3) and normal control groups (plate 1).

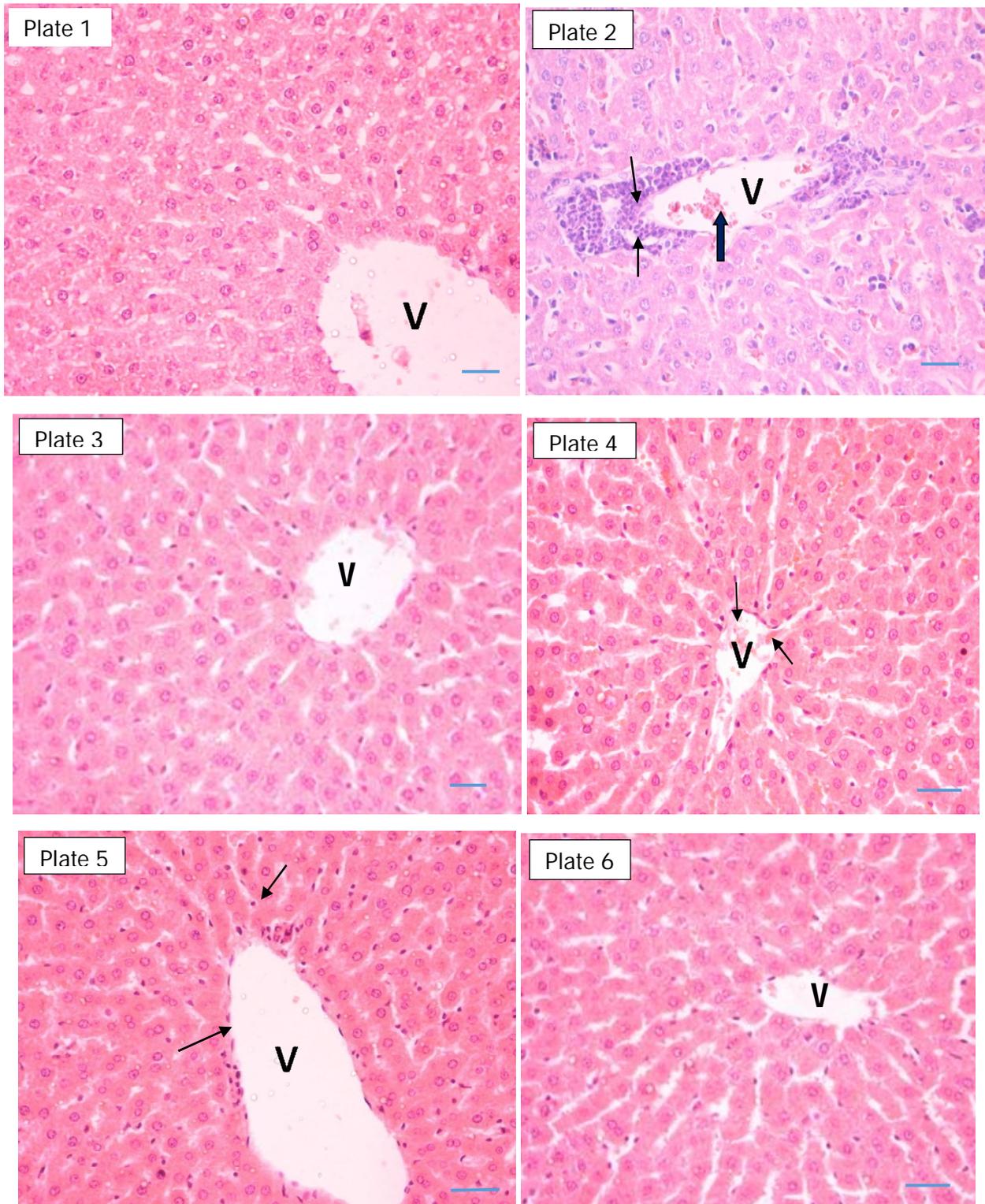


Figure 1: Transverse section through the liver showing histopathological changes. Plate 1 (normal control group) showing normal liver lobular architecture, note the central vein (V) with radiating hepatic cords that are very discernible. In plate 2 for animals that received only nevirapine note Infiltration by inflammatory cells (thin arrows), disintegration of hepatic cords and central venous congestion (thick arrow). An improved liver architecture comparable to that of the positive control (niverapine+silymarin group, plate 3) is seen in plate 4 (nevirapine+200mg/kg), plate 5 (nevirapine+400mg/kg) and plate 6 (nevirapine+800mg/kg) in a dose dependent manner. Scale bar 1s 25µm.

Discussion

In this study, we evaluated the hepatoprotective effect of *Cymbopogon citratus* essential oils against nevirapine-induced hepatic damage in Wistar albino rats. Hepatotoxicity was induced by daily oral administration of nevirapine at 36mg/kg (twice the human therapeutic dose) for 4 consecutive weeks. The resultant hepatotoxicity was confirmed by a significant elevation in the level of liver function parameters and liver tissue damage in the intoxicated group relative to the normal control. Our results are consistent with the findings of Adaramoye et al. (2012) where hepatotoxicity was confirmed at the same dosage. Nevirapine is metabolized in the liver by cytochrome P₄₅₀ enzymes that mediate oxidative phase-1 drug metabolism leading to the creation of hepatotoxic reactive metabolites. Nevirapine reactive metabolites cause hepatotoxicity by exerting initial cell stress through depletion of glutathione, or binding to enzymes, lipids, nucleic acids and other cell structures including inhibiting hepatocellular function (Pauli-Magnus et al., 2005). However, oxidative stress may overwhelm cellular antioxidant defense mechanisms (Ha et al., 2010).

We evaluated the hepatoprotective effect of *Cymbopogon citratus* essential oils against nevirapine-induced hepatic damage. Our results showed that animals receiving concurrent administration of nevirapine and *Cymbopogon citratus* essential oils were associated with significantly lower levels of serum AST, ALT and ALP in a dose dependent manner. Our results are in agreement with the findings of Thabrew et al. (1987) which revealed that serum levels of AST, ALT and ALP return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes. Results from this study suggest that the essential oils of *Cymbopogon citratus* could inhibit the nevirapine induced hepatic oxidative damage thereby inhibiting the leakage of ALT, AST and ALP into the blood stream. The significant increase in serum bilirubin among nevirapine intoxicated rats was significantly decreased in rats co-administered with nevirapine and the different concentrations of the essential oils of *Cymbopogon citratus*. These results are in agreement with the findings of Samudram et al. (2008) that reported a marked elevation in bilirubin in the serum of CCl₄ intoxicated rats as compared to those administered with a bi-herbal ethanolic extract. Biochemical restoration of the serum level of bilirubin may be due to inhibitory effects of the essential oils on cytochrome P450 or/and promotion of its glucuronidation (Cavin et al., 2001). Our results further showed that administration of nevirapine significantly decreased the level of total protein in the nevirapine intoxicated rats. This marked decrease in the serum protein level may be a result of the impairment of protein synthetic activity during stress conditions (Rao et al., 1995). Protein levels were significantly increased in groups co-treated with the different concentrations of essential oils.

Histopathological studies of the liver demonstrated that nevirapine intoxication was associated with fatty degeneration, cytoplasmic vacuolation, infiltration by inflammatory cells, enlarged hepatocytes, compression of sinusoids, disintegration of the hepatic cords and central venous congestion. Findings from this study reveal that the increase in the serum levels of these enzymes is secondary to liver dysfunction and is also associated with the damage to the structural integrity of the liver. Our findings are in agreement with a study by Adaramoye et al. (2012) who observed that nevirapine caused a significant elevation of liver enzyme activity resulting into severe liver damage. Liver histopathology was less evident in groups receiving the essential oils of *Cymbopogon citratus* in a dose dependent manner. At 800mg/kg of *Cymbopogon citratus* essential oils, liver architecture was comparable to that of the normal and positive control groups thus suggesting that essential oils were more effective at relatively higher dose. These findings are in agreement with previous work utilizing *Cymbopogon citratus* aqueous extracts on CCl₄ induced hepatotoxicity (Koh et al., 2012). However, our study has a limitation that liver function and liver histology parameters were taken at one point in time. Without baseline data from another point in time, it is a bit tricky to consider that all the effects are due to *Cymbopogon citratus*. Future studies could consider looking into this.

Studies investigating the phytochemical constituents of *Cymbopogon citratus* indicate the plant to have high profiles of saponins, sesquiterpenes, lactones, steroids and flavonoids (Oloyede, 2009). It is therefore likely that the hepatoprotective effect against nevirapine induced toxicity might be associated with the presence of these phytochemicals. Indeed, work by Natarajan et al. (2006) demonstrated that flavonoids exhibit anti-oxidative activity and are effective scavengers of superoxide anions (Robak Gryglewski., 1988). The possible mechanism responsible for the protection of nevirapine induced liver damage by the essential oils of *Cymbopogon citratus* may be through free radical scavenging thus intercepting radicals that may be involved in nevirapine metabolism by microsomal enzymes. Therefore, by trapping oxygen related free radicals, the essential oils could hinder their interaction with polyunsaturated fatty acids hence abolishing the enhancement of lipid peroxidative processes (Upadhyay et al., 2001).

Conclusion

We have demonstrated that *Cymbopogon citratus* essential oils have a protective effect against nevirapine-induced alterations in liver biochemical profiles and hepatic tissue damage. Structural elucidation of the bioactive compounds and their pharmacokinetics would aid future pharmacological research.

Conflict of Interests: Authors have declared no conflict of interest.

Authors' contributions: CDK and MA conceived the hypothesis. JK, KI, AT and GT designed the study. JK, MA, SPW and GT conducted the experiments. KI, CDK and AT analyzed the data. MA, CSK, KI, AT, SPW, JK and GT wrote the manuscript. All authors read and approved the final version of the manuscript.

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