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RESEACH ARTICLE

AMELIORATIVE POTENTIAL OF *Curcuma longa* RHIZOME EXTRACT ON CRUDE OIL SOOT-INDUCED HEPATORENAL TOXICITY IN WISTAR RATS

[1]Ibitoroko Maureen George-Opuda, [2]Adebayo Olugbenga Adegoke, [2]Nsikanabasi Peter Okon, [2] Emmanuel, Vincent Ekemini, [2]Esther Chinaemerem Ezebube

[1]Department of Clinical Chemistry, Faculty of Medical Laboratory Science, Rivers State University, Port Harcourt
[2]Department of Medical Laboratory Science, Madonna University Nigeria

Abstract

Background: Crude oil soot, a by product of incomplete combustion of fossil fuel, contains toxic compounds that can disrupt the liver and its functions. *Curcuma longa* is a medicinal plant with potential antioxidant, anti-inflammatory and regenerative effects. This study aimed to determine ameliorative potential of *Curcuma longa* rhizome extract against hepatorenal toxicity induced by crude oil soot in male Wistar rats. **Methods**: Thirty five male Wistar rats divided into seven groups namely control, soot exposed for 7, 14 and 28 days were compared with rats pre-treated with 500mg/kg of *C. longa* before exposure to soot for 7, 14 and 28 days respectively. Blood samples were collected and estimated for sodium, potassium, urea, Chloride, bicarbonate, creatinine, Alanine amino transaminase (ALT), Aspartate amino transaminase (AST), alkaline phosphatase (ALP), bilirubin and total protein using standard methods. Data were analysed using Statistical tool such as analysis of variance and Tukey multicomparism. **Results:** There was significant difference (P<0.05) in activities of ALT (U/L, AST (U/L) ALP (U/L) in Soot days 7, 14, 28, Soot and *C. longa* treated days 7, 14 and 28 compared with their respective control. The result showed significant difference (P<0.05) in concentrations of total bilirubin, total protein. urea (mmol/L), creatinine (µmol/L), sodium (mmol/L), potassium (Mmol/L), Chloride (Mmol/L) and bicarbonate (Mmol/L) in Soot exposed at days 7, 14, 28, Soot and *C. longa* treated days 7, 14 and 28 compared with their respective controls. **Conclusion**: The study showed that *Curcuma longa* rhizome extract ameliorate the hepatorenal damage caused by soot exposure.

Keywords: *Curcuma longa, Soot, liver, Renal, Ameliorative*

Introduction

Crude oil soot is the particulate matter produced during the incomplete combustion of crude oil. It is composed of a complex mixture of carbonaceous particles, Polycyclic aromatic hydrocarbons (PAHs), heavy metals, and other toxic compounds (Wang *et al*., 2018). The liver, being a vital organ responsible for detoxification and metabolism, is particularly susceptible to hepatotoxicity induced by crude oil and its combustion by-products, such as crude oil soot (Wang *et al*., 2018). The liver is one of the most important organs of the body. It performs a fundamental role in the regulation of diverse physiological processes, and its activity is related to different vital functions, such as metabolism, secretion, and storage. Its capacity to detoxify endogenous (waste metabolites) and/or exogenous (toxic compounds) substances of organisms, as well as for synthesize useful agents, has been analyzed since the 1970s by many researchers (Lin &Lu 1997; Shanani 1999; Subramoniam & Pushpangadan 1999, Adewusi & Afolayan 2010). The liver is also involved in the biochemical processes of growing, providing nutrients, supplying energy, and

*Corresponding: Adebayo Olugbenga Adegoke
Email: bayoadeghq@yahoo.com

reproducing. In addition, it aids in the metabolism of carbohydrates and fats, in the secretion of bile, and in the storage of vitamins (Ahsan *et al.,* 2009). Because of all of these functions, hepatic diseases continue to among the principal threats to public health, and they are a problem worldwide (Adewusi & Afolayan 2010, Asha & Pushpangadan 1998). Inhalation or ingestion of crude oil soot can lead to the deposition of these toxic substances in the liver, resulting in hepatotoxicity characterized by oxidative stress, inflammation, and liver dysfunction (Almeda *et al*., 2020; Wang *et al*., 2018).

The use of some plants and the consumption of different fruits have played fundamental roles in human health care. Approximately 80% of the world's population has employed traditional medicine for health care, which is based predominantly on plant materials (Adewusi & Afolayan 2010). Empirical evidence for the use of natural remedies for the treatment of hepatic diseases has a long history, and this field has become an innovative field of study, with the principal aim of analyzing the consumption of traditional fruits and medicinal plants by a great number of people and the different phytochemicals that are extracted from these foods. In general, liver-protective fruits, as well as plants, contain a variety of chemical compounds, such as phenols, coumarins, lignans, essential oils, monoterpenes, glycosides, alkaloids, carotenoids, flavonoids, organic acids, and xanthines (Bhawna & Kumar 2009). *Curcuma longa*, commonly known as turmeric, is a well-known medicinal herb with established hepatoprotective effects. Curcumin, the main bioactive compound in *Curcuma longa* extract, has demonstrated potent hepatoprotective effects in various experimental models (Liao *et al*., 2019; Zeng *et al*., 2020). Therefore, information obtained from research on the hepatoprotective effects of *Curcuma longa* extract (Liao *et al*., 2019; Zeng *et al*., 2020) and the underlying mechanisms (Jiang *et al*., 2024) informs the potential of this natural compound in mitigating hepatotoxicity induced by crude oil soot. The kidneys are important organs responsible for maintaining fluid and electrolyte balance, regulating blood pressure and filtering metabolic wastes out of the blood and because of their role in filtering and excreting waste, the kidneys are particularly susceptible to the toxic effects of environmental pollutants (Ogobuiro & Tuma, 2019).

Research on the mechanisms of action and protective effects of *Curcuma longa* extract provides valuable insights into its ability to modulate oxidative stress, inflammation, and apoptotic pathways (Jiang *et al*., 2024). These studies indicate that *Curcuma longa* extract may counteract the harmful effects of crude oil soot on the liver through its antioxidant, anti-inflammatory, and anti-apoptotic properties. *Curcuma longa*, commonly known as turmeric, is a well-known medicinal herb with established hepatoprotective effects but its use has not been reported in hepatorenal damage caused by soot components hence this study which aimed to evaluate the ameliorative potential of *Curcuma longa* rhizome extract against hepatorenal damage induced by crude oil soot in Wistar rats.

Materials and Methods

Experimental Animals

Thirty five Wistar rats weighing 130-200g were obtained from the PAMO University of Medical Sciences Central Animal Housing Facility, Port Harcourt, Rivers State Nigeria. The animals were kept under natural conditions of alternating day and night, and maintained with normal laboratory chow (Grower feed) and water *ad libitium*. The animals were acclimatized for two weeks at animal House, Madonna University, Elele, Rivers State Nigeria. The study was carried out at Medical Laboratory Science department, of Madonna University, Elele, Rivers State Nigeria.

Reagent

Aspartate amino transaminase, Alanine amino transaminase, Bilirubin and Total protein produced by Randox Limited, United Kingdom and alkaline phosphatase produced by Alkaline Phosphatase (Liquid) Reagent Set, and Pointe Scientific Inc. Belgium was purchased in Lagos and used for this study. Commercially prepared bicarbonate (HCO_2), potassium, chloride, sodium, urea and creatinine reagent made by Fortress Diagnostics Limited, United Kingdom were purchased from Lagos.

Curcuma longa Rhizome extraction procedure

The *Curcuma longa* rhizome obtained from oil mill Market, Elelewon, Port Harcourt, Rivers State, was filtered to remove dirt, and air-dried under ambient temperature and milled into a coarsely powdered form using a blender. It was macerated in 80% hydro-ethanol solution for 72 hours, Filtered and concentrated in water bath at 40ºC (Paulucci *et al.,* 2013).

Generation of soot from crude oil

Thirty milliliter (30 ml) of Bonny light crude oil obtained from Rumuekpe, Port Harcourt, River State, Nigeria was put in a stainless bowl and heated with an electric hot plate in a wooden soot chamber for 30 minutes to release the soot into the chamber. The Wistar rats were kept in the closed soot chamber to inhale the soot as it is generated (Alhikami *et al.,* 2022).

Ethical approval

Rats handling and treatments conform to guidelines of the National Institute of Health (NIH publication 85-23, (1985) for laboratory animal care and use followed institutional ethical guidelines of the Faculty of Medical Laboratory Science Madonna University with ethical approval number MAN/FMLS/00011.

Research design

This research was an experimental study designed to determine hepatorenal protective effect of *Curcuma longa* rhizome extract in Wistar rats exposed to controlled levels of crude oil soot. Thirty-five Wistar rats were divided into seven groups of five rats each namely A, B, C, D, E, F and G. The control (group A) was fed normal laboratory chow (Grower feed) and water *ad libitium***,** groups B, C, and D were exposed to crude oil soot for 7, 14 and 28 days respectively. Groups E, D and F were exposed to crude oil soot and treated with 500 mg/kg of *C. longa* for 7, 14 and 28 days respectively. The *Curcuma longa* extract was administered every morning to those in the supplementation group before exposure to crude oil soot every evening for 30 minutes for the duration of the study. All experimental protocols were observed under strict supervision, the experiment lasted for 28 days, and extracts were administered through oral gavage.

Sample collection

At the end of the experiment, animals in the different groups were anesthetized using isoflurane in an enclosed container after 24-hours of the last administered dose of the aqueous extract of *Curcuma longa* rhizome. Blood were collected through cardiac puncture, and retrieved serum was used for biochemical analysis.

Determination of ALT and AST was done by monitoring the concentrations of pyruvate hydrazone formed with 2, 4 dinitrophenylhydrazine (Reitman, and Frankel, 1957).The activity of the alkaline phosphatase (ALP) was determined when Inorganic phosphate and p-nitrophenol are produced during the hydrolysis of p-nitrophenyl phosphate. The alkaline phosphatase activity is directly related to the rate of p-NPP hydrolysis, which is measured at 405 nm (Helmy *et al*., 2019). Total bilirubin was determined by Jendrassik & Grof (1938) method where bilirubin in the presence of caffeine releases albumin bound bilirubin, by the reaction with diazotized sulphanillic acid.

The Total Protein concentration was determined using Biuret reaction. This Principle relies on a chemical reaction known as the Biuret reaction. In this reaction, a reagent containing copper ions (usually copper sulfate) and an alkaline solution (usually sodium hydroxide or potassium hydroxide) is added to the serum or plasma sample to form complex ion. The amount of complex ion form is proportional to the total protein present in the specimen (Henry *et al.*, 1974).

Creatinine assay was done using Jaffe Slot Method. Principles showed that creatinine and picric acid react to generate a dark yellow complex in an alkaline media, The amount of complex produced is inversely related to the sample's creatinine concentration at 492nm (Bartels and Bohmer, 1972). The Principle of Chloride assay showed that Mercuric ions and chloride ions react to generate a soluble, non-ionized molecule, which will thiocyanate ions from non-ionized mercuric thiocyanate. The ferric ions and released thiocyanate ions react to create a color complex that absorbs light at 480 nm. The correlation between the chloride content and color production is linear. Bicarbonate was determined using Liquid stable Method. Oxaloacetate is produced when phosphoenolpyruvate reacts with HCO3- in the

presence of phosphoenolpyruvate carboxylase. Oxaloacetate is further oxidized to malate by malate dehydrogenase, NADH+ H^+ is converted to NAD+. 405 or 415 nm are used to measure the absorption. NADH oxidation results in a decrease in absorbance which is proportional to serum/plasma bicarbonate concentration (Segal and Am, 1955). Urea assay was carried out using Modified Berthelot method. Urease hydrolyzes urea to create ammonia and carbon dioxide. In the presence of GLDH, the ammonia generated reacts with 2 - oxoglutarate and NADH to form glutamate and NAD. The reduction in absorbance introduced by the drop in NADH concentration per unit time is proportional to the level of urea (Chaney and Marbach, 1962).Estimation of sodium was done using monoliquid method (Tietz, 1983). The principle of the test is based on sodium's potential to interact with particular chomogens and increase absorbance when sodium ion concentration rises in the sample material. .Estimation of potassium was done using colorimetric Method (Henry, 2001) where Potassium ions and sodium tetraphenylboron react to form a turbid suspension of potassium tetraphenylboron. The relationship between the potassium concentration and turbidity depth is linear.

Statistical analysis

Data obtained were subjected to statistical analysis using the statistical package for social sciences version 22 using statistical tools such as analysis of variance (ANOVA) and Post Hoc.

RESULTS

There was significant difference (P<0.05) in activities of ALT (U/L)(F= 45.859,p=0.000), AST (U/L))(F= 26.503,p=0.000) and ALP (U/L))(F= 3.415,p=0.016). The total bilirubin concentration (Umol/L) showed significant difference (P<0.05) in mean (F=29.724,p=0.000) as well as total protein concentration (g/L) (F=18.116,p=0.000).

Table 1: Liver Function Parameters in Rats Treated with Soot and *C. longa*

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	Total bilirubin (Umol/L)	Total Protein (g/L)
Control	26.63 ± 0.49	90.66±1.20	52.23±3.94	12.24±0.27	5.59±0.70
Soot day 7	44.55± 2.49[a]	120.83±6.69[a]	54.32 ± 3.22	15.02±1.25	3.54±0.78
Soot day 14	55.47± 4.30[a]	133.08±5.34[a]	51.15 ± 2.10	21.03±3.32	3.71±0.31
Soot day 28	60.37± 5.44[a]	145.84±3.64[a]	57.67 ± 1.52	24.50±0.72[a]	2.40±0.22[a]
Soot and *C. longa* day 7	35.12 ± 1.10[a,b,c, d]	113.13 ±3.20[a, c, d]	53.03 ± 1.13[d]	14.19±2.37[d]	5.42±0.57[c,d]
Soot and *C. longa* day 14	41.95 ± 3.67[a,d]	123.42±4.90[a,d]	53.57 ± 0.63	14.82±0.96[d]	4.19±0.22[d]
Soot and *C. longa* day 28	45.97 ± 3.31[a]	121.24± 13.70	51.80 ± 2.07	21.78±0.96[a,b]	4.52±0.51[d]
F	45.859	26.503	3.415	29.724	18.116
P	0.000	0.000	0.016	0.000	0.000

a= significant when compared with Control b= significant when compared with soot day 7
c= significant when compared with soot day 14 d= significant when compared with soot day 28
e= significant when compared with soot and *C. longa* day 7 f= significant when compared with soot and *C. longa* day 14

The result of the study showed that soot significantly increased (P<0.05) AST (U/L) from 90.66±1.15 in control to 133.25±11.72 while *C. longa* supplementation reduced it to 119.26±9.03. The ALT (U/L) was significantly increased (P<0.05) from 26.63±0.49 in control by soot to 53.46±7.90 while *C. longa* supplementation reduced it to 41.01±5.37. Soot significantly increased (P<0.05) Bilirubin (Umol/l) from 12.24±0.27 in control to 20.18±4.51 while *C. longa* supplementation reduced it to 16.93±3.90. The Protein (g/l) was significantly decreased (P<0.05) from 5.59±0.70 in control by soot to 3.21±0.76while *C. longa* supplementation increased it to 4.71±0.70. There were no significant changes in ALP (U/l) activities of control, soot and Soot and *C. longa* respectively.

Table 2: Comparative analysis of *C. longa* rhizome extract on liver function in soot exposed rats

Groups	AST (U/L)	ALT(U/l)	ALP (U/l)	Bilirubin (Umol/l)	Protein (g/l)
Control	90.66±1.15	26.63±0.49	52.23±3.94	12.24±0.27	5.59±0.70
Soot	133.25±11.72 [a]	53.46±7.90 [a]	54.38±3.51	20.18±4.51 [a]	3.21±0.76 [a]
Soot and *C. longa*	119.26±9.03[a,b,]	41.01±5.37 [a, b]	52.80±1.49	16.93±3.90 [a]	4.71±0.70[b]
F	28.608	29.634	1.284	6.452	21.535
P	0.000	0.000	0.295	0.006	0.000

a= significant when compared with Control b= significant when compared with soot

There was significant difference in Urea (Mmol/L)(F=303.135,p=0.000), Creatinine (µmol/L) (F=4.493, p=0.004), Sodium (Mmol/L)(F=18.122, p=0.000), Potassium(Mmol/L) (F=12.216, p=0.000), Chloride (Mmol/L) (F=12.391,p=0.000) and Bicarbonate(Mmol/L)(F=8.924,p=0.000) of Wistar rats exposed to Soot exposure and those administered with *C. longa* compared with their respective controls as shown in table 3 below

Table 3: Renal Function Parameters in Rats Treated with Soot and *C. longa*

Groups	Urea (Mmol/L)	Creatinine (µmol/L)	Sodium (Mmol/L)	Potassium (Mmol/L)	Chloride (Mmol/L)	Bicarbonate (Mmol/L)
Control	17.05±1.74	35.90±0.42	116.20±61.8	29.70±0.82	51.77±7.15	34.47±3.64
Soot day 7	48.47±3.08[a]	49.65±0.29[a]	136.40±82.00	53.33±0.28	68.22±4.90	40.16±2.89
Soot day 14	56.73±2.25[a]	54.93±1.08	147.00±67.00	53.25±0.11	73.28±2.15	43.35±2.50
Soot day 28	61.56±0.62[a]	67.00±2.50	148.30±43.70	52.85±0.72	71.63±3.90	46.20±2.43[a]
Soot and *C. longa* day 7	31.48±1.12 [a,c,d]	39.70±0.80	128.20±46.30 [c,d]	42.80±0.41	64.35±3.73	37.75±3.41
Soot and *C. longa* day 14	49.93±1.71 [a,b,c,d,e]	42.40±0.27	128.00±63.90 [d]	41.53±0.21[b ,c]	62.67±2.58 [c]	37.65±1.86 [d]
Soot and *C. longa* day 28	52.06±0.59 [a,d,e]	62.15±0.40 [a,b,f]	136.00±33.40 [c,d]	46.20±0.47[a]	68.66±1.44	37.78±0.86[d]
F	303.135	4.493	18.122	12.216	12.391	8.924
P	0.000	0.004	0.000	0.000	0.000	0.000

a= significant when compared with Control b= significant when compared with soot day 7
c= significant when compared with soot day 14 d= significant when compared with soot day 28
e= significant when compared with soot and *C. longa* day 7 f= significant when compared with soot and *C. longa* day 14

The soot exposure significantly increased (P<0.05) urea concentration (Mmol/L) of 17.05±0.90 to 55.60±1.73 while *C. longa* reduced it to 44.49±2.80. The Creatinine concentration was significantly increased by soot from 35.90±0.21,to 57.18±0.47 while administration of *C. longa* reduced it to 48.10±0.33. Soot exposure increased potassium concentration (Mmol/L) from 2.97±0.41 to 5.31±0.12 while administration of *C. longa* reduced it to 4.35±0.12. Soot significantly increased chloride (Mmol/L) and bicarbonate (Mmol/L) from 51.77±3.57 and 34.47±1.81 to 71.04±1.18 and 43.24±1.01 respectively. Administration of *C. longa* reduced it to 65.23±1.05 and 37.73±0.60 respectively as shown in table 4 below.

Table 4: Comparative analysis of *C. longa* rhizome extract on renal function in soot exposed rats

Groups	Urea (Mmol/L)	Creatinine (µmol/L)	Sodium (Mmol/L)	Potassium (Mmol/L)	Chloride (Mmol/L)	Bicarbonate (Mmol/L)
Control	17.05±0.90	35.90±0.21	116.16±3.09	2.97±0.41	51.77±3.57	34.47±1.81
Soot	55.60±1.73[a]	57.18±0.47[a]	143.90±2.13[a]	5.31±0.12[a]	71.04±1.18[a]	43.24±1.01[a]
Soot and *C. longa*	44.49±2.80[a,b]	48.10±0.33	130.55±1.68[a,b]	4.35±0.12[b]	65.23±1.05[b]	37.73±0.60[b]
F	38.740	4.143	29.753	38.951	29.010	17.246
P	0.000	0.028	0.000	0.000	0.000	0.000

a= significant when compared with Control b= significant when compared with soot

Discussion

The result showed that rat exposed to soot caused increased in alanine amino transaminase (ALT), aspartate amino transaminase (AST), alkaline phosphatase (ALP) activities, Bilirubin concentration and total protein concentration when compared to control. This is suggestive that soot can cause hepatocellular injury (Almeda *et al*., 2020; Wang *et al*., 2018). However, this crude oil sooth-induced hepatotoxicity is initiated physiologically by a cascade of hepatotoxic events resulting in oxidative stress, inflammation, and perturbation of liver function (Almeda *et al*., 2020; Wang *et al*., 2018). It has been shown that exposure to crude oil soot has been associated with increased lipid peroxidation and reduced antioxidant enzyme activity in the liver, indicating oxidative stress-induced damage (Almeda *et al*., 2020; Wang *et al*., 2018). Therefore, pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), which have been found to be upregulated in the liver following exposure to crude oil soot, suggesting an inflammatory response that provokes the cascade of heightened enzymatic reactions that indicate peak biochemical responses to the function tests (Almeda *et al*., 2020; Wang *et al*., 2018). Howbeit, these has been implicated to be the reason behind the hepatotoxicity of the sooth induced pollution. The finding of this study is in agreement with the findings of Almeda *et al*. (2020) and Wang *et al*. (2018) who from their study revealed that crude oil sooth causes the destruction of the liver. The reason behind this damage according to these authors is the increase peroxidation of the lipid and by extension reducing enzymatic activities that acts as antioxidant in the liver. Such damage is thus cause by oxidative stress resulting reactive oxygen species (ROS) stimulate by the pollution.

However, the result further showed that administration of *C. longa* rhizome extract reduced the alanine amino transaminase (ALT), aspartate amino transaminase (AST), alkaline phosphatase (ALP) activities, Bilirubin and total protein concentrations when compared with soot exposed groups suggesting that *C. longa* can ameliorate the damage caused by soot in the experimental animals. This finding is in agreement with the findings of Liao *et al*. (2019) and Zeng *et al*. (2020) who studied *Curcuma longa* for its antioxidant, anti-inflammatory, and hepatoprotective effects in various experimental models. This hepatoprotective actions is achieve by modulating multiple cellular signalling pathways involved in oxidative stress, inflammation, and apoptosis (Zeng *et al*., 2020).

Liao *et al*. (2019) investigated the protective effects of *Curcuma longa* extract against oxidative stress-induced liver damage in hepatoma cells. The study demonstrated that *Curcuma longa* extract effectively attenuated oxidative stress markers and protected liver cells from damage. Similarly, Zeng *et al*. (2020) investigate the hepatoprotective effects of curcumin, the main bioactive compound in *Curcuma longa* extract, in type 2 diabetic rats with non-alcoholic fatty liver disease. The results revealed that curcumin treatment significantly reduced liver injury by reducing hepatic lipid accumulation and improving mitochondrial function.

This hepatoprotection is brought to bear by the active ingredient of the *Curcuma longa* which is curcumin. Curcumin possesses diverse pharmacological properties, including antioxidant, anti-inflammatory, anti-fibrotic, and anti-apoptotic effects (Jiang *et al*., 2024). In the context of this study, *Curcuma longa* extract has been shown to possess potent antioxidant properties, which can counteract the excessive production of reactive oxygen species (ROS) and lipid peroxidation in the liver courtesy of crude oil sooth (Jiang *et al*., 2024). Curcumin exerts its antioxidant effects through multiple mechanisms, including scavenging free radicals, enhancing endogenous antioxidant enzyme activity, and preserving cellular redox balance (Jiang *et al*., 2024).

The result showed that rats exposed to soot caused increased in urea, creatinine, sodium, potassium, chloride and bicarbonate when compared to control. This is suggestive that soot can cause renal damage. Soot accounts for over one- quarter of the total hazardous pollution in the air. Soot has been a serious concern for human health due to its direct and broad impact on the kidney. In earlier times, health professionals associated long term exposure to soot to increase the risk of renal damage (Ye *et al.,* 2021).

However, the result further showed that administration of *Curcuma longa* rhizome extract reduced the urea, creatinine, sodium, potassium, chloride and bicarbonate

when compared with soot exposed groups, suggesting that *C. longa* can ameliorate the damage caused by soot which is in sync with Ceja-Galicia *et al*. (2023) and Trujillo *et al.* (2013) in terms of the renoprotection bestowed by the active ingredient in curcumin. The result showed dose dependent increase in urea, creatinine, sodium, potassium, chloride and bicarbonate while administration of *C. longa* caused dose dependent decrease in urea, creatinine, sodium, potassium, chloride and bicarbonate. This is suggestive that exposure caused dose dependent damage.

Conclusion

The study showed that soot caused hepatorenal damage by causing increase to the potassium, Urea, creatinine and liver enzymes. It also caused increases in bilirubin with decrease protein suggestive of hepatic damage. Administration of *C. longa* reduced some of the effect caused by soot. This is suggestive that *C. longa* can ameliorate the damage caused by soot.

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Conflict of Interest:

The authors declare that no known conflict of interest or personal relationships that have influenced the work reported in this paper.

Authors' contributions

I.M.G-O.: Conceptualized the study, Design, A.O.A.: Supervision. Project administration, N.P.O.: wrote, reviewed and edited the manuscript original draft preparation. E.V.E.: Methodology, data analysis. E.C.E.: Laboratory analysis. All the authors have read and approved the final version of the manuscript.

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