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Original Research Article

Thymol, a Dietary Monoterpene, Abrogates Hexachlorobenzene-Induced Hepatic Dysfunction Via Different Mechanisms

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Abstract

Thymol (2-isopropyl-5-methyl-phenol) is a natural monoterpene phenolic antioxidant that has anti-inflammatory and anti-apoptotic potentials in experimental studies. Literatures are scarce on effects of hexachlorobenzene-induced hepatic damage. Therefore, this study investigated the ameliorating potential of thymol (THY) on hexachlorobenzene (HCB)-induced hepatic damage as well as the effects on the antioxidant status, polyamine catabolism, inflammatory and apoptotic processes. Thirty-two adult male rats were daily treated orally by gavage for 25 days and allocated into four groups; control group received corn oil, HCB alone group (15 mg/kg b.wt), THY-treated group (100 mg/kg b.wt) and HCB + THY-treated group. The results showed that HCB significantly reduced the body weight with concurrent increase in relative liver weight accompanied by widespread histological aberrations. Furthermore, HCB-treated rats revealed increases in hepatic putrescine oxidase, spermine oxidase, and myeloperoxidase activities, tumor necrosis-α and interleukin-1β levels, caspase-3 activity, induced oxidative damage as evidenced by elevated malondialdehyde (MDA) levels and significant reduction in antioxidant enzyme activities and reduced glutathione (GSH). However, co-administration of THY with HBC abated the hepatic damage by preventing the generation and release of reactive oxygen species, improving the antioxidant system, down-regulated polyamine catabolism, inflammatory and apoptotic responses. The findings of this study revealed that HCB acts as a hepatotoxicant and thymol might be a possible future therapeutic agent for HCB-induced hepatic damage.

Keywords: Hexachlorobenzene, Thymol, oxidative stress, inflammation, polyamine catabolism.

INTRODUCTION

Environmental pollution health related problems have now become a global (Wang *et al.*, 2020). HCB is an environmental tenacious non-degradable chlorinated hydrocarbon toxic pollutant previously used as fungicide and currently as byproduct in the manufacture of chlorinated solvents (Kumar *et al*., 2013; Chalouati *et al.*, 2019) and attested to trigger multi-organ damages in humans and experimental animals particularly the kidney, liver and central nervous system (Chalouati *et al.*, 2019) probably due to its persistence and biotransformation to produce reactive metabolites especially in the liver owing to their metabolism and excretion. Though HCB is no more in use in many countries as fungicides and pesticides, it is still produced as industrial secondary product of many chlorinated solvents and in smelting industry. HCB has been detected in many samples including biological samples world-wide (European Food Safety Authority, 2006). Major routes of exposure to HCB are through diet by ingestion of contaminated food and incineration, inhalation and dermal absorption (European Food Safety Authority, 2006; Agency for Toxic Substances and Disease Registry, 2013). Continued exposure of humans and animals to HCB has been reported to cause various toxicities including hepatorenal, reproductive, blood, endocrine, neurological, nervous system, dermal, lung,

immunosuppression and even carcinogenicity (Agency for Toxic Substances and Disease Registry, 2013; 2015). HCB is lipophilic and bioaccumulates in humans and is eliminated as a cysteine conjugate of pentachlorobenzene. Hexachlorobenzene is reported to be metabolized in rats in many ways including the formation of pentachlorobenzene, tetrachlorobenzene and tri- and tetrachlorophenol (International Agency for Research on Cancer, 2001).

Exposures to hazardous chemicals including HCB induce oxidative stress, release pro-inflammatory cytokines and activate apoptotic responses (Wu *et al*., 2016) as some of the mechanisms by which they evoke their toxicities. Many metabolic processes including polyamine metabolism are critical to cellular oxidative homeostasis and their dysregulation can result in organ injury and various diseases (Madeo *et al*., 2018). Increase in polyamine catabolism produces ROS and aldehydes such as acrolein that has been linked to oxidative stress, inflammation, apoptosis and pathogenesis of many illnesses (Hussain *et al*., 2017; Alfarhan *et al*., 2020; Chang *et al*., 2022).

Currently, numerous natural products potentials are being explored for their effectiveness in improving harmful effects of environmental pollutant exposure (Abu Zeid *et al*., 2021). Thymol (THY) a dietary phenolic monoterpene found in plants such as *Thymus ciliate, Nigella sativa, Ocimum gratissimum, Thymus vulgaris, and Origanum vulgarae* is amongst these natural products (Miguel *et al*., 2015; Nagoor *et al*., 2017) with many pharmacological and biological activities including antioxidant, anti-apoptotic, anti-inflammatory, antimicrobial, cardioprotective, anticancer, antidiabetic, renoprotective, anticarcinogenic and hepatoprotective effects (Kumari *et al*., 2019; Fouad *et al*., 2022; Jafari *et al*., 2018).

Generally, humans and animals are chronically exposed to hazardous chemicals by virtue of their occupation, diet or location, the consequence of which can be detrimental to their health. Understanding the interactions and mechanisms of action of these noxious agents at cellular and molecular levels has become an intense research area that has created a lot of research interests (Nys *et al*., 2018). The impact of co-exposure of humans to fungicide and numerous herb mixtures on the liver where their metabolism and excretion normally occur is indispensable. However, despite the wide reported toxicities of HCB and abundant beneficial therapeutic effects of thymol, literature on the mitigating influence of thymol on HCB hepatic toxicity is lacking. Thus, to the best of our knowledge this is the first study to investigate the mechanistic impact of THY on HCB-induced hepatotoxicity via enhancement of antioxidant status, suppression of inflammation, polyamine catabolism and apoptosis in adult wistar rats.

MATERIALS AND METHODS

Chemicals and reagents

Hexachlorobenzene (HCB; >99% purity), thymol (99.99%), reagent kits for alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total cholesterol (CHOL), gamma glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), lactate dehydrogenase (LDH), triglycerides (TRIGS) were purchased from Randox Laboratories Limited, UK. Sorbitol dehydrogenase (SDH), glutathione (GSH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), Sodium 5,5-diethylbarbiturate-Barbitone, epinephrine, L-Arginine ≥ 98%, Chloramine-T trihydrate 98%, thiobarbituric acid (TBA), 5′,5′-dithiobis-2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), hydrogen peroxide (H_2O_2), glutathione (GSH), 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate, putrescine, spermine and epinephrine were purchased from Sigma Chemical Co. (St Louis, MO, USA). Enzyme-linked Immunosorbent Assay (ELISA) kits for tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and caspase-3 (CASP3) activity assessment were purchased from Elabscience Biotechnology Company, Beijing, China. All other chemicals were of analytical grade and were obtained from the British Drug Houses (Poole, Dorset, UK).

Experimental animals and care

Thirty-two adults male Wistar rats weighing 190 ± 220 g (10 weeks old) were used for this study and purchased from the Faculty of Veterinary Medicine, University of Ibadan. The rats were kept in transparent plastic cages and acclimatized for two weeks in a well-ventilated rat house, under a natural photoperiod of 12 h light:12 h dark and had unrestricted access to standard rat chow and water. Guidelines set by the University of Ibadan Ethical Committee and the 'Guide for the Care and Use of Laboratory Animals' published by the National Institute of Health and National Academy of Science were followed for the animal care and procedures.

Experimental design

The rats were weighed and assigned to four groups consisting of eight rats each and treated once daily for 25 consecutive days by oral gavage. Group 1 was the control group and received 2 ml/kg of corn oil. Group 2 rats were given 15 mg/kg HCB alone in corn oil. Group 3 rats were co-treated with 15 mg/kg HCB and THY 100 mg/kg and group 4 rats were dosed with 100 mg/kg THY alone. The doses and period of administration were chosen from our pilot study experiments and previously established data (Khan *et al*., 2017; Ribeiro *et al*., 2016).

Collection of sample and tissues preparation

Twenty-four hours after the termination of the experiment, rats were weighed to obtain the final body weights and were anesthetized using sodium pentobarbital (35 mg/kg i.p) prior to collection of five millilitres (5 ml) of blood samples by retro-orbital venous plexus from each rat into plain tubes and rats were humanely euthanized. The blood obtained from each rat was centrifuged at 3000 g for 10 min to obtain the sera that were store in other plain tubes and preserved at -20 ^{o}C for biochemical analyses and hepatic function tests.

Immediately the liver of each rat was removed, washed in iced cold 1.15% KCl, blotted, weighed, and homogenized separately in ice cold 0.1 M phosphate buffer (pH 7.4) by a Teflon glass homogenizer. The homogenates were then centrifuged at 10,000 g for 20 min at 4 ^{o}C and the resultant supernatants obtained were kept at -80 ^{o}C and used for biochemical assays.

Estimation of liver function markers

Serum activities of ALT (cat. no. AL8004), AST (cat. no. AS8003), ALP (cat. no. AP9762), GGT (cat. no.GT3807), G6PDH (cat. no. PD409), GLDH (cat. no. GL432) were purchased from Randox Laboratories Limited, UK while MDH (cat. no. MAK194) SDH (cat. No. MAK316) were purchased from Sigma Chemical Co., St Louis, MO, USA and evaluated by using these commercially available kits following the manufacturer's instructions.

Determination of serum paraoxonase (PON1) activity

Serum PON1 activity assay was carried out according to the methods of La Du and Eckerson, (1984) by measuring arylesterase activity using phenyl acetate as a substrate. Briefly, 10 µl of diluted serum (1:10 v/v) was added to 10 mM Tris-HCl buffer, pH 8.0 containing 2 mM $CaCl_2$ and 2 mM phenyl acetate. The rate of phenol from the reaction was determined at 270 nm at 25°C, using a continuously recording spectrophotometer. One enzyme unit was defined as the amount of enzyme that catalyzes the hydrolysis of 1µmol of substrate per minute and the results were given in U/L.

Estimation of serum purine nucleoside phosphorylase (PNP) activity

Serum PNP activity was measured by the method of Chu *et al.*, (1989). Briefly, serum was incubated with potassium phosphate buffer (22 mM, pH 7.5) containing xanthine oxidase (XO, 167 U), horseradish peroxidase (2000 U), 4-aminoantipyrine (160 mM), potassium ferricyanide (120 µM), 3,5 dichloro-2-hydroxybenzenesulfonic acid (8 mM) and inosine (12 mM). Formation of N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinone-monoimine was followed at 520 nm in a spectrophotometer (Beckman Coulter DU 800, Fullerton, CA, USA) and the change in absorbance was recorded at 165 nm every 5 s for 180 s. Results are expressed as units/mg protein. One unit of PNP activity is defined as the amount of enzyme necessary to deplete 1 µM of inosine per minute at 25°C.

Measurement of serum lipid profile

Total cholesterol (CHOL; cat. no. CH8016), triglyceride (TRIGS; cat. no. TR9769), low-density lipoprotein (LDL; cat. no. CH2645), high-density lipoprotein (HDL; cat. no. CH2645) levels in the serum of rats were estimated according to the manufacturer's instructions using commercially available diagnostic kits (Randox Laboratories Limited, UK).

Assessment of polyamine catabolism

Determination of Putrescine Oxidase (PutOX) Activity

The PutOX activity was determined according to a method described by Quash *et al*., (1972). Briefly, 0.02 ml of the liver homogenate, 0.1 ml of the NaCl-Tris HCl buffer (4mMTris, 0.14MNaCl, pH = 7.7), and 0.1 ml of the putrescine (18.3mM dissolved in NaCl-Tris buffer) were added into the reaction tube. The reaction mixture was incubated for 60 min at 37 ^{o}C in a water bath, followed by addition of 0.1 ml of 0.4% 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate, the mixture was incubated for 30 min at 25 ^{o}C, and afterwards, 0.5 ml of $FeCl_3$ was added to the mixture and 15 mins later, the absorbance was read at 660 nm against the control. The activity of PutOX was expressed in µmol/mg protein.

Determination of Spermine Oxidase (SpmOX) Activity

The activity of the SpmOX was determined according to a method described by Wang *et al*., (2003). The reaction mixture contained 0.02 ml of the liver homogenate, 0.1 ml of the NaCl-Tris HCl buffer (10 mM Tris, 0.14 M NaCl, pH = 7.2) and 0.1 ml of the spermine (17.2 mM dissolved in NaCl-Tris buffer). The reaction mixture was incubated for 4 h at 37 ^{o}C in a water bath. After the addition of 0.1 ml of 0.4% 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate, the mixture was incubated for 30 min at 25 ^{o}C, and subsequently, 0.5 ml of $FeCl_3$ was added to the mixture and after 15 min, the absorbance was read at 660 nm against the control. The activity of SpmOX has been expressed in µmol/mg protein.

Determination of serum and liver arginase activity

The arginase activity was determined by the method of Porembska and Kedra, (1975). Briefly, 100 µl of the serum or the liver supernatant was diluted with 400 µl of the distilled water followed by addition of 100 µl of 200 mM arginine solution, 100 µL of 50 mM $MnCl_2$ and 500 µl of the sodium barbitone buffers (0.1 M, pH = 9.5). The reaction mixture was incubated for 30 min at 37 ^{o}C, the reaction was stopped by the addition of 1.3 ml of 20% TCA and centrifuged for 10 min at 3000 rpm. Then, 100 µl of the supernatant was mixed with 900 µl of 10% TCA followed by 1 ml of glacial acetic acid and 500 µl of the ninhydrin reagent (containing 250 mg of

ninhydrin dissolved in a mixture of 4 ml of 6 M H_3PO_4, and 6 ml glacial acetic acid). The reaction assay mixture was heated for 30 min at 95 ºC in a water bath and cooled; the absorbance was read at 515 nm against the control (without the sample). The enzyme activity was expressed in µmol/mg protein.

Determination of serum citrulline level

The citrulline level was determined according to the method of Knipp and Vašák, (2000). Briefly, 200 µl of the reagent A (contained 80 mM of the diacetylmonoxime and 2 mM of the thiosemicarbazide dissolved in distilled water) and Reagent B (contained 3 M H_2SO_4, 6 M H_3PO_4, and 2 mM $NH_4Fe(SO_4)_2$ dissolved in distilled water mixed in a ratio of 1:3) was mixed with 20 µl of the arginine (10 mM), and 40 µl of the serum. The mixture was heated at 95 ºC for 15 min in a water bath, cooled and the absorbance was read at 530 nm against the control. The citrulline level was expressed in µmol/mg protein.

Determination of serum hydroxyproline level

Hydroxyproline level was determined according to the method described by Reddy and Enwemeka, (1996). Briefly, 1 ml of 100 µl N NaOH was mixed with 400 µl of serum and autoclaved at 120°C for 25 minutes. The reaction mixture was cooled to room temperature and 4.5 ml chloramine-T reagent (containing 0.84% chloramine-T, 42 mM sodium acetate, 2.6 mM citric acid and 39.5% isopropanol) was added and incubated at 37 ºC for 25 min. Then, 15% 4-(dimethylamino) benzaldehyde reagent (DMAB) in 2:1 (v/v) of 500 ml isopropanol/perchloric acid mixture was added. The solution was incubated again at 65°C for 20 minutes and the absorbance of the samples measured at 550 nm.

Assessment of antioxidant and oxidative stress markers

Determination of Superoxide Dismutase (SOD) activity

The hepatic SOD activity was determine by the protocol of Misra and Fridovich, (972). 1 ml of the liver homogenate was added to 9 ml of distilled water; then 0.2 ml of the diluted sample was added to 2.5 ml of 0.05 carbonate buffer (pH 10.2). The reaction was initiated by adding 0.3 ml to freshly prepared 0.3 mM adrenaline and quickly mixed by inversion. The increase in absorbance was measured at 480 nm every 30 seconds for 150 seconds, using a reference cuvette containing 2.5 ml buffer, 0.3 ml adrenaline and 0.2 ml distilled water.

Determination of Catalase activity

The liver homogenate catalase activity was determined according to the method of Beers and Sizer as described by Usoh *et al*., (2005) by measuring the decrease in absorbance at 240nm due to the decomposition of H_2O_2 in a UV recording spectrophotometer. The reaction mixture (3 ml) comprised 0.1 ml of homogenate in phosphate buffer (50 mM, pH 7.0) and 2.9 ml of 30 mM H_2O_2 in phosphate buffer pH 7.0. An extinction coefficient for H_2O_2 at 240 nm of 40.0 $M^{-1}cm^{-1}$ (Aebi 1984) was used for the calculation. The specific activity of catalase was expressed as moles of H_2O_2 reduced per minute per mg protein.

Determination of reduced glutathione concentration

Total reduced glutathione was estimated by the method of Elia *et al*., (2003). Briefly, 0.1 ml of 10% TCA was added to the liver homogenate was mixed well for complete precipitation of proteins and centrifuged at 4,000xg for 5 minutes. To 0.5 ml clear supernatant, added 2.5 ml of 0.2 M phosphate buffer and 0.5 ml of DTNB. Read the absorbance at 412 nm against a blank containing all the reagents without the sample. A series of standards were run in a similar manner to determine the glutathione content. GSH concentrations in the samples were expressed as nmoles/mg protein.

Determination of malondialdehyde concentration

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content, as an index of lipid peroxidation by the method of Driessen *et al*., (2013). Briefly, 200 µl of each liver sample was de-proteinized using 0.5 ml trichloroacetic acid (TCA) and centrifuged at 3000 rpm for 10 minutes. Then, 0.1 ml of supernatant obtained, 1 ml of 0.75% TBA was added, boiled in water bath for 20 minutes at 100°C and then cooled on ice. The absorbance was read at 535 nm.

Measurement of protein carbonyl concentration

Protein carbonyl content was measured using DNPH as described by Sohal *et al*., (1993). The protein in the liver homogenate was first precipitated with equal volume of 10% TCA, the pellets obtained was resuspended in 0.5 ml of sample (1-2 mg), an equal volume of 10 mM DNPH in 2 N HCl and incubated for 1 hour shaking intermittently at room temperature. Corresponding blank was prepared by adding only 2 N HCl to the sample. After incubation. The precipitate was washed twice with ethanol: ethylacetate (1:1) and finally dissolved in 1 ml of 6 M guanidine HCl, centrifuged at low speed and the supernatant color was read at 366 nm. The difference in absorbance between the DNPH treated and HCl treated sample was determined and expressed as nmoles of carbonyl groups per mg of protein, using extinction coefficient of 22 $mM^{-1}cm^{-1}$.

Measurement of advanced oxidation protein products concentration

The advanced oxidation protein products (AOPP) were measured as described by Witko-Sarsat *et al*., (1998). Briefly, 100 µl of the liver homogenate was added to 50 µl of 1.16 M potassium iodide followed by 100 µl of glacial acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm. The concentration of the AOPP was calculated based on the standard curve of chloramine T (0–100 µmol/L) and expressed in µmol/mg protein.

Determination of Protein Concentration

The protein concentration in the liver homogenate was determined according to the method of Popovi´c *et al*., (2019). To 10 µl of the diluted liver homogenate was added 150 µl of the reagent C (containing 1 ml of 1% $CuSO_4$, 1 ml of 2% potassium sodium tartrate, and 98 ml Na_2CO_3 dissolved in 0.1 M NaOH) and 30 min of incubation. Then, 30 µl of the Folin reagent was added to the mixture (Folin–Ciocalteu reagent and water mixed in a ratio of 1:2) and after 20 min, the absorbance was read at 550 nm, and the proteins concentration expressed as mg protein/L.

Determination of Total Antioxidant Capacity (TAC)

The concentration of the TAC was determined by the method of Erel, (2004). In brief, 200 µl of the reagent I (acetate buffer-CH_3COONa/CH_3COOH, 0.4 M, pH = 5.8) was mixed with 20 µl of the liver homogenate and absorbance was measured at 420 nm after 30 s incubation. Afterwards, 20 µl of reagent II (ABTS, 30 mM in acetate buffer, pH 3.6) were added into each sample and the absorbance at 420 nm was measured after 5 min incubation. TAC was calculated based on the differences in the absorbance at 420 nm before and after adding the Reagent 2. The assay was calibrated with trolox and the results were expressed in terms of mM trolox equivalent per liter (mmol trolox Equiv/L).

Determination of Total Oxidative Status (TOS)

The concentration of the TOS was determined by the method Erel, (2005). Briefly, the reaction 225 µl of the Reagent I (containing xylenol orange 150 µM, NaCl 140 mM and glycerol 1.35M in 25 mM H_2SO_4 solution, pH 1.75) was mixed with 35 µl of the liver homogenate and the absorbance of each sample was read spectrophotometrically at 560 nm as a sample blank. Then, 11 µl of the reagent II (containing 10 mM o-dianisidine and 5 mM $(NH_4)_2Fe(SO_4)_2$ dissolved in 25 mM H_2SO_4) was added to the mixture and incubated for about 3-4 mins. After 3 min the absorbance was recorded spectrophotometrically at 560 nm, TOS was calculated based on the differences in the absorbance at 560 nm before and after adding the Reagent 2. The assay was calibrated with H_2O_2 and the results were expressed in terms of µM H_2O_2 equivalent per liter (µmol H_2O_2 Equiv/L).

Evaluation of pro-inflammatory markers and caspase-3 activity

Determination of Myeloperoxidase activity

Myeloperoxidase activity was measured by the method of Slungaard and Mahoney (1991). The assay mixture consists of 20 µl of liver supernatant in 50mM sodium acetate buffer (pH 5.25), 10 µl of 1.6 mM 3,3'5,5'-tetramethylbenzidine (TMB) dissolved in DMSO and 70 µl of 30 mM H2O2, diluted in 80 mM phosphate buffer (pH 5.4) and the decrease in absorbance at 412 nm due to oxidation of TNB ($\varepsilon = 27{,}000\ M^{-1}\ cm^{-1}$) was measured for one min. The activity of the peroxidase was expressed as unit/mg protein.

Determination of NO concentration

The hepatic concentration of NO was estimated as total nitrate/nitrite levels using Griess reagent according to the method of Li *et al*., (2014). In brief, 50 µl aliquot of liver sample was added to 50 µl of Griess reagent (0.1 % N-(1-naphthyl) ethylenediamide dihydrochloride, 1 % sulfanilamide in 5 % phosphoric acid) and the absorbance was measured at 540 nm after 10 min. Sodium nitrite was used as the standard in this assay to generate the standard curve.

Estimation of liver levels of TNF-α and IL-1β

Protocols in the purchased ELISA kits (Elabscience Biotechnology Company, Beijing, China) were followed. Briefly, 100 µl of liver homogenate or standards were pipetted into the designated wells already pre-coated with antibody specific for rat TNF-α, IL-1β or caspas-3 and incubated for 2 h at 37 °C, covered and shake delicately to allow for binding. Unbound substances were discarded and 300 µl of washing buffer were added to each well and washed three times and after the final wash, the plate was turned upside down and blotted on clean absorbent towels. 100 µl of biotin-conjugated antibody specific for rat TNF-α, IL-1β or caspase-3 was added to the appropriate well and incubated for one hour at 37 °C with light shaking, the unbounded resultant solution from each well were discarded and the plate rewashed three times. Then, 100 µl of avidin conjugated Horseradish Peroxidase (HRP) was added to each well and incubated for 1 h at 37 °C with gentle shaking, the unbound components were washed away and 90 µl of TMB substrate solution was added to each well and incubated for 15-30 minutes at 37 °C to give a blue color proportional to the amount of TNF-α or IL-1β bound. The reaction was terminated by adding 50 µl stop solution to each well to obtain a yellowish color that was measured at 450 nm using a SpectraMax plate reader (Molecular Devices, CA, USA). All the experiments were performed in triplicate, and the results were presented in pg/ml.

Histological Analysis

Histology of the liver tissue samples from each rat were performed based on the method of Songur *et al*., (2003). Briefly, liver samples were fixed in 10% neutral phosphate-buffered formalin solution, dehydrated, blocked in paraffin. Sections of 5 µm were cut and stained with hematoxylin and eosin (H & E). All slides were examined with light microscope (Olympus BX-41 microscope, Hamburg, Germany) and photographed using a digital camera by pathologists who were blinded to control and drug-treated groups.

Statistical Analyses

Data were analyzed using GraphPad Prism version 7 Software, (Graphpad software; La Jolla, California, USA) and the results expressed as the mean ± SD of replicates. Significant differences were determined by one-way analysis of variance (ANOVA)

and followed by post hoc Tukey test and P < 0.05 was considered significant.

RESULTS

Effect of THY on the body weight and relative organ weight in HCB-exposed rats

The results in Table 1 show the influence of THY on rats exposed to HCB alone and HCB and THY. The rats exposed to HCB alone exhibited a significant ($p < 0.05$) reduction in the body weight with elevated relative weight of the liver relative to the control and THY alone rats. Co-exposure of rats to HCB and THY however, increased the body weight and decreased the relative weights of the liver compared to the control and THY alone groups while rats administered with THY alone revealed both body weight and relative organ weights values that are close to that of control group.

Table 1: Body weight gain and relative organ weights of control, HCB alone, HCB and THY and THY alone

	Control	HCB alone	HCB + THY	THY alone
Body weight gain (g)	19.25 ± 1.21	9.86 ± 0.74*	15.23 ± 0.62$^{*\#\$}$	18.98 ± 1.48$^{\#}$
Relative liver weight	3. 62 ± 0.04	5.18 ± 0.22*	4.16 ± 0.06$^{*\#\$}$	3.58 ± 0.08$^{\#}$

Values are expressed as mean ± SD; n = 8 rats. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus HCB alone; $^{\$}p < 0.05$ versus THY alone; HCB: 15 mg/kg hexachlorobenzene, THY: 100 mg/kg thymol.

Effect of exposure of rats to HCB and THY on hepatic function indices

Tables 2 depicts the results of the biomarkers of hepatic toxicities in rats treated with HCB alone, combined HCB with THY and THY alone. The serum activities of ALT, AST, ALP, GGT, G6PDH, GLDH, MDH, PNP and SDH were significantly ($p < 0.05$) increased with decreased PON 1 activity in rats administered with HCB alone relative to the control and THY alone groups. Conversely, rats co-treated with HCB and THY displayed significantly ($p < 0.05$) reduction in serum activities of these enzymes with raised PON1 activity when compared to the HCB alone group. THY alone treated rats showed values that were statistically not significant to the control values in all the hepatic biomarkers analyzed.

Table 2: Hepatic function indices of rats exposed to HCB alone, HCB and THY, and THY alone for 25 consecutive days

Group	Control	HCB alone	HCB + THY	THY alone
ALT (U L^{-1})	28.13 ± 1.01	46.28 ± 2.06*	24.26 ± 1.02$^{*\#\$}$	27.68 ± 1.08$^{\#}$
ALP (U L^{-1})	30.24 ± 1.22	63.22 ± 2.04*	42.32 ± 1.48$^{*\#\$}$	29.58 ± 1.96$^{\#}$
AST (U L^{-1})	42.16 ± 2.14	79.34 ± 2.65*	38.21 ± 1.66$^{*\#\$}$	41.64 ± 2.28$^{\#}$
GGT (U L^{-1})	22.12 ± 0.68	82.16 ± 3.22*	39.64 ± 1.18$^{*\#\$}$	21.56 ± 0.90$^{\#}$
G6PDH (U L^{-1})	3.56 ± 0.14	7.28 ± 1.28*	4.12 ± 0.82$^{*\#\$}$	3.52 ± 0.16$^{\#}$
GLDH (U L^{-1})	12.35 ± 0.78	33.74 ± 1.24*	24.10 ± 1.12$^{*\#\$}$	12.87 ± 0.28$^{\#}$
MDH (U L^{-1})	14.12 ± 0.86	29.32 ± 1.24*	18.14 ± 1.26$^{*\#\$}$	21.87 ± 1.93$^{\#}$
SDH (U L^{-1})	8.34 ± 0.96	20.44 ± 0.98*	12.20 ± 1.08$^{*\#\$}$	7.98 ± 0.66$^{\#}$
PON 1(U L^{-1})	112.16 ± 6.02	36.24 ± 3.12*	99.98 ± 5.24$^{*\#\$}$	113.02 ± 6.04$^{\#}$
PNP (U mg $protein^{-1}$)	10.34 ± 1.02	28.68 ± 1.02*	17.34 ± 1.28$^{*\#\$}$	10.18 ± 1.20$^{\#}$

Values are expressed as mean ± SD; n = 8 rats. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus HCB alone; $^{\$}p < 0.05$ versus THY alone; HCB: 15 mg/kg hexachlorobenzene, THY: 100 mg/kg thymol.

Effect of oral administration of HCB and THY on serum lipid profile of rats

The serum levels of CHOL, HDL, LDL and TG are presented in Table 3. Exposure of rats to HCB alone significantly ($p < 0.05$) increased the levels of CHOL, LDL and TG with a significant ($p < 0.05$) decrease in the level of HDL relative to the control and THY alone groups. Nevertheless, co-treatment of HCB and THY to rats reduced the levels of CHOL, LDL and TG, and increased the HDL level significantly ($p < 0.05$) whereas rats administered with THY alone revealed values that were similar to the control group values.

Table 3: Serum lipid profile of rats exposed to HCB alone, HCB and THY, and THY alone for 25 consecutive days

Group	Control	HCB alone	HCB + THY	THY alone
CHOL (mg dL^{-1})	1.36 ± 0.02	2.08 ± 0.12*	1.12 ± 0.02$^{*\#\$}$	1.34 ± 0.02$^{\#}$
TRIGS (mg dL^{-1})	1.10 ± 0.01	3.18 ± 0.24*	1.32 ± 0.28$^{*\#\$}$	1.08 ± 0.02$^{\#}$
HDL (mg dL^{-1})	1.08 ± 0.02	0.26 ± 0.02*	0.68 ± 0.04$^{*\#\$}$	1.12 ± 0.02$^{\#}$
LDL (mg dL^{-1})	1.26 ± 0.02	3.08 ± 1.10*	2.46 ± 0.10$^{*\#\$}$	1.22 ± 0.01$^{\#}$

Values are expressed as mean ± SD; n = 8 rats. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus HCB alone; $^{c}p < 0.05$ versus THY alone; HCB: 15 mg/kg hexachlorobenzene, THY: 100 mg/kg thymol.

Effect of THY on ARG, PutOX and SpmOX activities and levels of CIT and HYP in HCB-treated rats

Table 4 shows a significant ($p < 0.05$) increase in serum ARG activity and a decreased activity in the liver supernatant in HCB alone rats relative to the control and THY alone rats. However, co-administration of THY with HCB significantly ($p < 0.05$) reduced the serum ARG but increased the ARG activity in the liver of rats. PutOX and SpmOX activities and levels of CIT and HYP were significantly ($p < 0.05$) elevated in the liver of HCB alone treated rats compared to the control and THY alone treated groups, however co-administration of HCB with THY to rats significantly ($p < 0.05$) decreased PutOX and SpmOX activities and levels of CIT and HYP while rats administered with THY alone showed activities and levels of these markers that were comparable to the control group.

Table 4: Activities of ARG, PutOX and SpmOX and levels of CIT and HYP of rats treated with HCB alone, HCB with THY and THY alone for 25 consecutive days

Group		Control	HCB alone	HCB + THY	THY alone
ARG	Serum	4.18 ± 0.98	19.64 ± 1.02*	10.36 ± 0.68$^{*\#\$}$	4.22 ± 0.02$^{\#}$
ARG	Liver	1.94 ± 0.10	0.65 ± 0.01*	0.98 ± 0.01$^{*\#\$}$	1.88 ± 0.18$^{\#}$
PutOX	Liver	2.18 ± 0.01	6.42 ± 0.72*	3.16 ± 0.10$^{*\#\$}$	2.24 ± 0.03$^{\#}$
SpmOX	Liver	1.14 ± 0.01	4.35 ± 0.39*	2.23 ± 0.04$^{*\#\$}$	1.20 ± 0.01$^{\#}$
CIT	Liver	1.14 ± 0.01	3.84 ± 0.03*	1.52 ± 0.2$^{*\#\$}$	1.22 ± 0.01$^{\#}$
HYP	Liver	1.33 ± 0.01	2.98 ± 0.04*	1.08 ± 0.02$^{*\#\$}$	1.38 ± 0.01$^{\#}$

ARG (µmol/mg protein); PutOX (µmol/mg protein); SpmOX (µmol/mg protein); CIT (µmol/mg protein); HYP (µmol/mg protein). HCB: 15 mg/kg hexachlorobenzene; THY: 100 mg/kg thymol. n=8. Values are mean ± SD of 8 rats. $^{*}p < 0.05$ versus Control; $^{\#}p < 0.05$ versus HCB alone; $^{\$}p < 0.05$ versus THY alone.

Effects of THY on antioxidant status and oxidative markers in the liver of rats treated with HCB

Exposure of rats to HCB alone significantly ($p < 0.05$) decreased the activities of SOD, CAT and GSH level with concomitant increase in MDA, PCO and AOPP levels in the liver of rats relative to the control and THY alone groups (Table 5). HCB co-administered with THY significantly ($p < 0.05$) increased the antioxidant enzymes activities and GSH level and simultaneously decreased the levels of MDA, PCO and AOPP. Rats given THY alone showed similar activities and levels of both antioxidant and oxidative markers with control rats.

Table 5: Hepatic antioxidant status and oxidative stress markers in rats treated with HCB alone, HCB with THY, and THY alone for 25 consecutive days

Group	Control	HCB alone	HCB + THY	THY alone
SOD	12.05 ± 1.07	5.16 ± 0.28*	8.92 ± 0.42$^{*\#\$}$	11.95 ± 1.64$^{\#}$
CAT	16.15 ± 1.21	3.17 ± 0.04*	7.63 ± 0.10$^{*\#}$	16.23 ± 1.14$^{*\#}$
GSH	7.15 ± 0.16	2.88 ± 0.15*	4.78 ± 0.18$^{*\#\$}$	6.94 ± 0.74$^{\#}$
MDA	2.16 ± 0.24	7.04 ± 1.12*	3.14 ± 0.26$^{*\#\$}$	2.10 ± 0.12$^{\#}$
PCO	2.10 ± 0.01	4.12 ± 0.14*	3.06 ± 0.02$^{*\#\$}$	1.98 ± 0.02$^{\#}$
AOPP	3.10 ± 0.14	5.64 ± 0.38*	2.34 ± 0.04$^{*\#\$}$	3.04 ± 0.02$^{\#}$

SOD activity (unit/mg protein); CAT activity (µmole H_2O_2 consumed/min/mg protein); GSH concentrations (nmoles/mg protein); MDA (µmol/mg protein); PCO (nmoles of carbonyl groups/mg protein); AOPP (µmol/mg protein). HCB: 15 mg/kg hexachlorobenzene; THY: 100 mg/kg thymol. n=8. Values are mean ± SD of 8 rats. $^{*}p < 0.05$ versus Control; $^{\#}p < 0.05$ versus HCB alone; $^{c}p < 0.05$ versus THY alone.

Effect of thymol on hepatic TAC and TOS in HCB-treated rats

Figure 1 depicts the impact of HCB and THY on liver's total antioxidant capacity and total oxidative stress. Hexachlorobenzene administered to rats significantly ($p < 0.05$) decreased the TAC level and markedly increased the TOS level relative to the control and THY alone groups. Treatment with THY and HCB however, significantly ($p < 0.05$) increased TAC level and decreased TOS level. Rats administered with THY alone showed levels of TAC and TOS that were similar to the control rats.

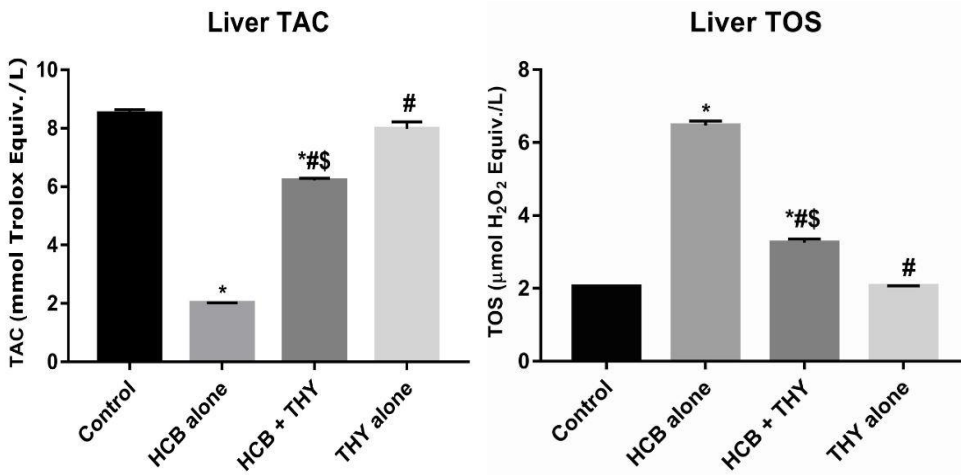

Fig 1: Effect of thymol on hepatic TAC and TOS levels in HCB-treated rats. n=8. Each bar represents mean ± SD of 8 rats. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus HCB alone. $^{\$}p < 0.05$ versus THY alone. HCB: 15 mg/kg hexachlorobenzene; THY: 100 mg/kg thymol

Thymol inhibits pro-inflammatory biomarker and activates caspase-3 in HCB-treated rats

Rats treated with HCB alone exhibited significant ($p < 0.05$) increase in the hepatic MPO activity, the levels of NO, TNF-α, IL-1β and caspase-3 activity relative to control and THY alone groups (Figures 2). However, HCB co-treatment with THY to rats significantly ($p < 0.05$) decreased the activities and levels of these markers of inflammation and apoptosis in rats' liver compared to the HCB alone rats. Rats administered with THY alone showed activities and levels of the markers similar to the control rats.

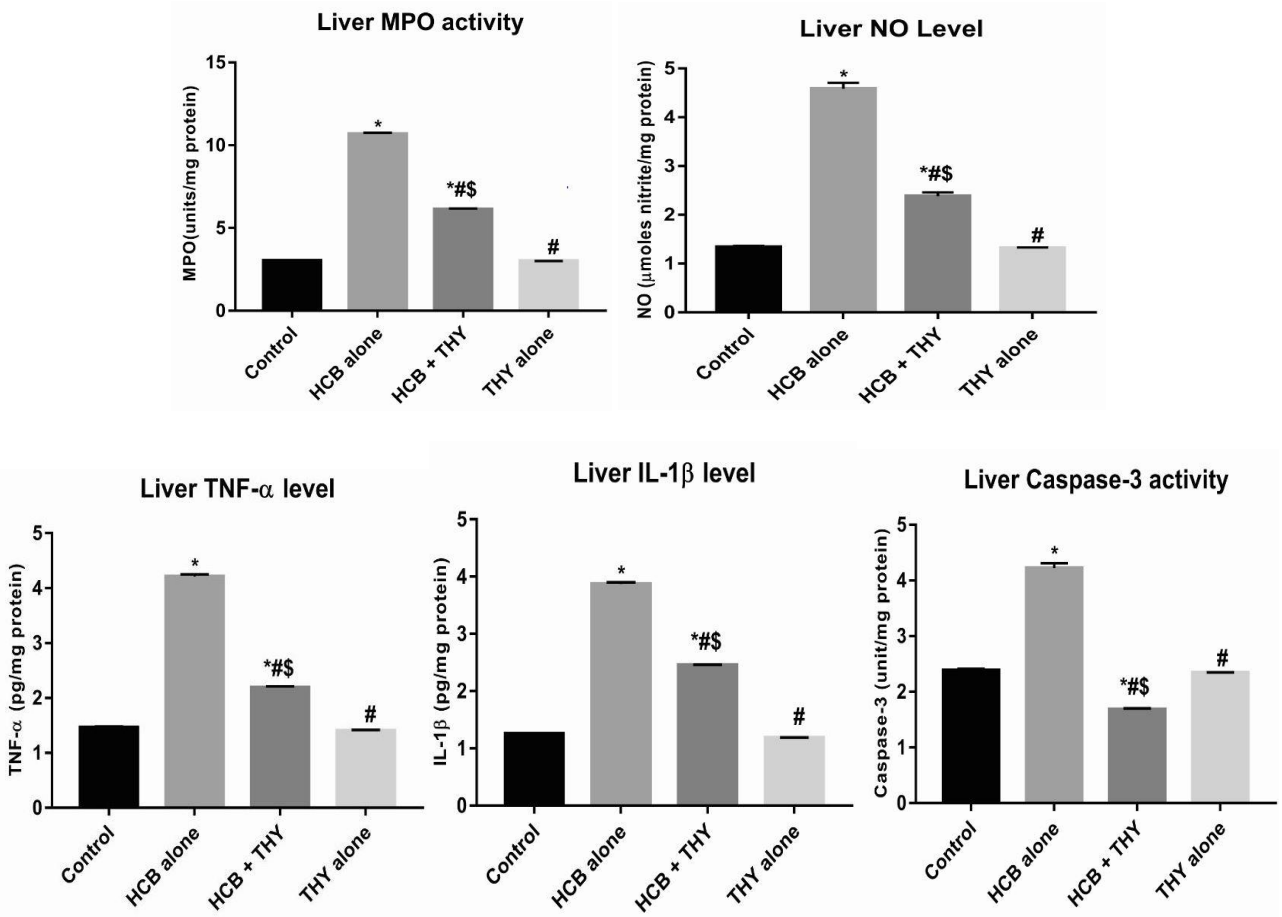

Fig 2: Effect of thymol on hepatic MPO activity and levels of NO, TNF-α, IL-1β and caspase-3 activity in HCB-treated rats. n=8. Each bar represents mean ± SD of 8 rats. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus HCB alone. $^{\$}p < 0.05$ versus THY alone. HCB: 15 mg/kg hexachlorobenzene; THY: 100 mg/kg thymol

Effect of THY on histological changes in the liver of rats exposed to HCB.

Figure 3 shows the representative photomicrographs of the liver of control, rats exposed to HCB alone, HCB co-treated with THY and THY alone. Histological examination of the liver of the control rats' tissues showed intact structural architectures. The liver of rats exposed to HCB alone showed disruption of the hepatocytes arrangements, necrosis and inflammatory cells infiltration (red arrows). But, rats co-treated with HCB and THY showed mild inflammatory cells infiltration, restoration and amelioration of the structural damage. The liver of rats given THY alone showed structural organization similar to that of the control group.

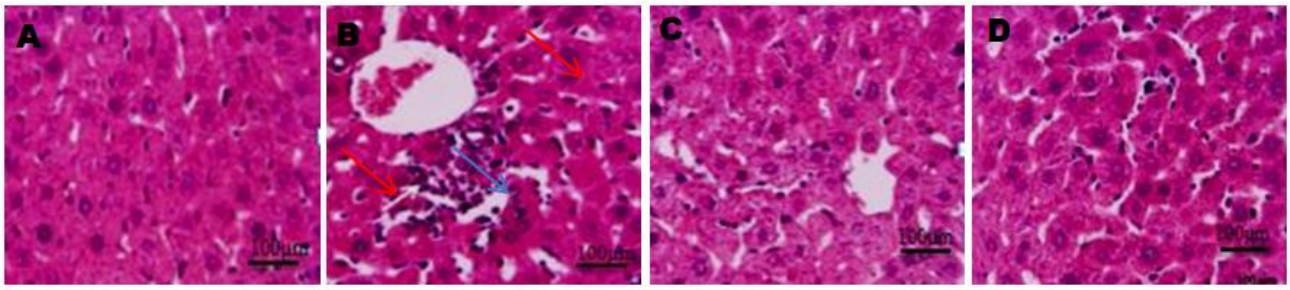

Fig 3: Representatives photomicrograph of liver of (A) Control, (B) HCB alone, (C) HCB + THY and (D) THY alone rats. Control liver showed normal intact structural arrangements. Liver of HCB alone rats showed inflammatory cell infiltration (red arrow), distension of sinusoids (blue arrow) and central vein hemorrhage; .iver of rat co-treated with HCB + THY showing few inflammatory cells and improved architectural arrangements while THY alone treated group showing near to normal architecture. (H & E X 100); Scale bar: 100 µm

4. DISCUSSION

The results of the present study revealed that HCB induced ROS, RNS and free radical formations that enhanced polyamine catabolism, inflammation and activation of caspase-3 as well as depletion of antioxidant system in the hepatic tissues of rats. Additionally, our results demonstrated the therapeutic potential of THY in HCB-induced hepatic toxicity in rats as evident in the restoration of the histopathological changes in the liver and subsequent inhibition of oxidative stress, polyamine catabolism, inflammation and activation of caspase-3. Moreover, administration of THY alone to normal rats had no effect on hepatic functions, structural architectures and also no significant effects on body and relative liver weight.

The present study results obtained from biochemical liver functions tests with histological analysis corroborate occurrence of hepatic damage in rats as shown by centrilobular degeneration, hypertrophic hepatocytes and inflammatory cells in the liver of the HCB alone rats. But THY co-administered with HCB to rats restored the hepatic damage thus, authenticating the biochemical results on the mitigating effect of THY on HCB-induced hepatic damage in rats.

The relative organ weight is a vital index of atrophy or hypertrophy and swelling. Elevation of relative organ weight indicates inflammation whereas a decrease may be associated to cellular shrinking (Moore and Dalley, 1999). The decrease in body weights of rats accompanied by increased relative liver weight in HCB alone treated rats in this study indicates organ toxicity. Our results are similar to the previous reports (National Toxicology Program, 2021). However, HCB and THY co-administered rats showed increased body weight and decreased relative organ weight, suggesting improvement by THY administration.

Evaluation of liver enzyme activities in the serum provides a valuable tool for assessing the effect and nature of liver injury. Elevated serum activities of liver enzymes such as ALP, ALT, AST, GGT and G6PDH may imply hepatocyte membrane damage with resultant loss of membrane integrity (Mostafa, 2018; Sadegi *et al*., 2019). While these biomarkers are used to evaluate overall hepatic damage, a few of them are not explicit and their discharge into the blood circulation after hepatic injury are delayed, thus specific biomarkers that can be detected early during hepatic damage were assessed. GLDH, MDH SDH are linked to raised transaminases specifically ALT in a wide range of hepatic damages (Church *et al*., 2019), whereas PNP, a glycosyltransferase involved in purine salvage pathway (Fasull and Endres, 2015) and PON1, an aryl esterase with paraoxonase activity that protects LDL and HDL from oxidation (Entedhar *et al*., 2018) show early signs of hepatic damage. The increased serum levels of ALP, ALT, AST, GGT, G6PDH, GLDH, MDH, SDH and PNP with simultaneous decrease in PON 1 in HCB alone treated rats in this present study, indicates cholestasis, hepatocyte necrosis, hepatocellular and hepatobiliary damages that disrupt metabolic, synthetic, excretory, transport and other functions of the liver. This may be due to hepatocyte membrane lipid peroxidation resulting from free radical generated by HCB metabolism and subsequently their leakage into the blood stream. The result of this study agreed with the earlier reports (Ichrak *et al*., 2013; Chalouati *et al*., 2019). The decline in the activities of these hepatic biomarkers in rats co-exposed to HCB and THY in this study suggests that THY

protected the liver against injury probably by overpowering the generation of free radicals that caused hepatocytes membrane damage by donating the hydroxyl group in its structure to the HCB metabolites formed.

The liver is a major organ in lipid metabolism and for maintenance of energy level of the cell, therefore damage to hepatocytes will affect lipid metabolism in the liver. The elevation in serum levels of CHOL, TRIGS and LDL accompanied by a decline in HDL level in the HCB alone treated rats in this present study may signify damage to hepatocyte membrane resulting to leakage of these membrane constituents into the blood stream. Our results agreed with the earlier report (Khan *et al*., 2017). The decrease in CHOL, TRIGS and LDL with resultant increase in HDL in the serum of rats co-treated with HCB and THY is suggestive of the protective ability of THY on the hepatocyte membrane damage.

Conversion of L-arginine by arginase to kick-start production of polyamines such as spermidine and spermine ensures polyamine homeostasis in the cell. However, during inflammation, L-arginine metabolism is switched to form CIT and NO via inducible nitric oxide synthase (iNOS) resulting in PAs degradation to maintain their equilibrium. Increase catabolism of cellular PAs by PutOX and SpmOX, escalates generation of cytotoxic ROS, H_2O_2, acrolein and cellular oxidative stress (Fratini *et al*., 2019; Igarashi *et al*., 2018). Acrolein, a highly reactive unsaturated aldehyde covalently combines with amino acid residues in proteins to modify their functions and induce tissue damage or apoptosis (Uemura *et al*., 2017). In the current study, the deceased arginase activity in the liver accompanied by an increase in serum arginase activity with simultaneous increase PutOX and SpmOX activities and CIT level in the liver of rats administered with HCB alone denotes increased PAs catabolism. This helps the organ to maintain cellular balance and reduced arginase activity with simultaneous increase in nitric oxide level in the liver indicates the switching of the arginine metabolism to citrulline synthesis (Maksymchuk and Konovchuk, 2017). The decreased CIT level, PutOX and SpmOX activities with increased ARG activity in the liver of rats accompanied by the diminution in serum ARG activity following co-treatment of HCB with THY indicates the alleviating effects of THY on HCB-induced PAs catabolism and generation of ROS. Furthermore, accumulation of HYP has been linked to liver fibrosis characterized by excessive accumulation of extracellular matrix (ECM) (Cordero-Espinoza and Huch, 2018). Elevated HYP content in HCB-treated rats in this study suggests possible fibrogenesis associated with liver necrosis and inflammation, however, administration of HCB with THY diminished HYP content suggesting anti-fibrotic effect of THY through subjugation of inflammation.

Amongst the many mechanisms by which HCB induced its multi-organ toxicities is by generation of ROS that induce oxidative damage by attacking cellular biomolecules resulting in oxidative stress (Akhigbe and Ajayi, 2020; Kapucu, 2021). Interestingly, enzymatic and non-enzymatic antioxidant systems that counter the effects of the ROS are abundant in hepatic cells. The vital antioxidant systems that counter the effects of ROS include SOD, CAT and GSH (Roh *et al*., 2018). In this present study, the decrease in the activities and level of these antioxidant cascades in the liver of rats may imply that administration of HCB to rats perturbed the antioxidant defense system through the generation of ROS and the inability of the antioxidant system to effectively scavenged and neutralize the ROS. Our result agreed with the previous reports (Chalouati *et al*., 2019) and that of Khan *et al*., (2017) who reported an increase in GSH level contrary to what we obtained. Interestingly, co-exposure of rats to HCB and THY elevated the antioxidant defense cascade in the liver of the rats confirming the reported antioxidant capability of THY (Nagoor *et al*., 2017).

Lipids and proteins oxidation by ROS produce many oxidative products such as MDA, AOPP and PCO that can react with cellular biomolecules to cause cellular distresses (Zińczuk *et al*., 2020). MDA is often used as a biomarker of lipid peroxidation and PCO as an early biomarker of protein linked oxidative stress diseases (Sadeghi *et al*., 2019). HCB itself is not very reactive, but its metabolites tetrachlorobenzoquinone (TCBQ) and others when react with lipid or protein produced lipid oxidative products and AOPP (Van Ommen *et al*., 1986). Additionally, TAC gives total free radical scavenging activity and information about the concentration of individual antioxidants whereas TOS assess the total capacity of a natural system to counter cellular oxidative damage (Choromańska *et al*., 2020). In this current study, the increased levels of MDA, PCO, AOPP and TOS with reduced level of TAC following oral administration of HCB suggests that HCB and its metabolites caused oxidative damage to the liver of rats. The declined levels of these oxidative damage markers, TOS level with elevated level of TAC in the liver of rats co-administered with HCB and THY indicates that THY successively stabilized the peroxyl radicals and protein adduct produced by terminating lipid peroxidation and protein oxidation chain reactions (Kumari *et al*., 2019; Popovic *et al*., 2019).

Inflammation occupies a vital position in the creation and progression of organ damage (Rahimi *et al*., 2019). Damage to hepatic tissues enhances infiltration of neutrophils and release of pro-inflammatory cytokines such as TNF-α and IL-1β and MPO (Liang *et al*., 2018). Additionally, activation of the classical NF-κB inflammatory signaling pathway by ROS under various pathological conditions leads to the release of many pro-inflammatory cytokines and subsequent aggravation of the inflammatory injury to the liver (Temel *et al*., 2020). TNF-α is a critical mediator that regulates organ injury by controlling the flow of inflammatory process (Sharifi-

Rigi *et al*., 2019) and its elevated level can increase the secretion of other cytokines, such as IL-1*β* and other inflammatory mediators such as NO leading to aggravated inflammation. Excessive superoxide radicals formed can react with NO to form peroxynitrite ($OONO^-$) to cause tissue nitrosative damage to lipid, nucleic acids and proteins by protein carbonyl formation, dimerization and nitrosylation of amino acids and thiols (Klimiuk *et al*., 2021). MPO is a systematic crossroads between inflammation and oxidation process in a cell (Mostafa, 2018). Taking together, the elevated levels of TNF-α, IL-1β and NO with MPO activity in the liver of rats exposed to HCB alone suggest occurrence of inflammation in this organ. Moreover, reduction in the NO, TNF-α and IL-1β levels, and MPO activity recorded in the liver of rats co-treated with HCB and THY show amelioration of inflammation by THY.

Downregulation of antioxidant system and up-regulation of polyamine catabolism lead to excessive availability of ROS and subsequent damage to DNA, proteins and lipids to produce oxidative-induced apoptosis (Ghosh, 2019). Similarly, TNF-α which regulates organ injury by direct cytotoxicity via caspase-dependent activation can also trigger the apoptotic pathway. In our present study, the increased activation of caspase-3 in the liver of HCB alone treated rats indicates involvement of apoptosis in HCB-induced hepatic injury. The diminution of the hepatic caspase-3 activity in HCB and THY treated rats suggest anti-apoptotic effects of THY.

Overall, the results of the present study clearly show that THY treatment of rats alleviated the hexachlorobenzene-induced hepatic injury via mechanisms involving suppression of oxidative stress, polyamine catabolism, inflammation and activation of caspase-3 in the liver of rats. Thus, suggesting that thymol can be a potential therapeutic agent for hepatic damage associated with hexachlorobenzene exposure.

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Authors' contribution

TAS, EJA and EOF conceived the study. TAS, MS, GCP and TMD conducted the experiments. TAS, MS and GCP analyzed the results and TAS and EOF wrote the manuscript. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper**.**

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