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Hepatoprotective and cytoprotective properties of *Hyptis suaveolens* against oxidative stress-induced damage by CCl_4 and H_2O_2

Hadi Ghaffari, Behrouz Jalali Ghassam, HS Prakash*

Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore, 570006 , Karnataka, India

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ABSTRACT

Objective: To investigate capacity of *Hyptis suaveolens (H. suaveolens)* methanol extract as an antioxidant to protect against carbon tetrachloride (CCl_4)-induced oxidative stress, hepatotoxicity in Albino Wistar rats and cytoprotective effect of hydrogen peroxide (H_2O_2) induced cell death in $HepG_2$ cell line. **Methods:** Two different doses of methanol extract of *H. suaveolens* were evaluated for the hepatoprotective activity against carbon tetrachloride (CCl_4) induced hepatotoxicity in rats. Animals in Group I: served as control, group II: *H. suaveolens* (100 mL/kg b.w), group III: *H. suaveolens* (50 mL/kg b.w) + CCl_4 (1 mg/kg), group IV: *H. suaveolens* (100 mL/kg b.w) + CCl_4 (1 mL/kg) and group V: CCl_4 (1 mL/kg). Histopathologic changes of liver were also evaluated. Cytotoxicity was also determined by 3, (4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay. **Results:** Oral sigle dose treatment of CCl_4 produced a marked elevation in the serum levels of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and Lactate dehydrogenase (LDH). Histopathological analysis of the liver of CCl_4-induced rats revealed marked liver cell necrosis with inflammatory collections that were conformed to increase in the levels of SOD, GSH, GST, GR and LPO. Treatment with H_2O_2 significantly induced death of $HepG_2$ cell. Pretreatment with *H. suaveolens* methanol extract inhibited or attenuated H_2O_2 induced cytotoxicity. **Conclusions:** This study shows that *H. suaveolens* methanol extract can be proposed to protect the liver against CCl_4-induced oxidative damage in rats and protect the cells against H_2O_2-induced oxidative damage in $HepG_2$ cells. The hepatoprotective and cytoprotective effects might be correlated with its antioxidant and free radical scavenger effects.

1. Introduction

Formation of reactive oxygen species (ROS) is an unavoidable consequence in aerobic organisms during respiration. It has been shown that overproduction of unstable ROS leads to unwanted reactions with other groups or substances in the body, resulting in cell or tissue injury. In addition, numerous studies have revealed that uncontrolled lipid peroxidation is involved in the occurrence of many diseases, including Parkinson's, arthritis, myocardial infarction, Alzheimer's, cancer, cardiovascular disease, and liver damage[1,2]. Therefore, during the last few decades, human nutrition and biochemistry research focused on antioxidants derived from foods that could prevent or diminish ROS-induced damage. Many studies have suggested that natural antioxidants are efficacious to prevent oxidative stress-related liver pathologies due to particular interactions and synergisms[3, 4].

The liver regulates many important metabolic functions, and hepatic injury can distort these metabolic functions[5]. In addition, the liver is the key organ of metabolism and excretion and it is continuously exposed to xenobiotics because of its strategic placement in the body. Many hepatotoxicants such as carbon tetrachloride (CCl_4), nitrosamines, and polycyclic aromatic hydrocarbons require metabolic activation, particularly by liver cytochrome P450 (CYP) enzymes, to form reactive, toxic metabolites, which in turn cause liver injury in animals and humans[6]. CCl_4, a well-known model compound for producing chemical hepatic injury, requires biotransformation by hepatic microsomal CYP to produce the hepatotoxic metabolites,

*Corresponding author: Prof. H.S. Prakash, Department of studies in Biotechnology, University of Mysore, Manasagangotri, Mysore, 570006, Karnataka, India.
Tel/Fax: 91-821-2414450
E-mail: hsp@appbot.uni-mysore.ac.in

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trichloromethyl free radicals $^{\bullet}CCl_3$ and/or $CCl_3OO^{\bullet}$[7-9]. Trichloromethyl free radicals can react with sulfhydryl groups, such as those in glutathione (GSH) and protein thiols. The covalent binding of trichloromethyl free radicals to cellular proteins is considered the initial step in a chain of events that eventually lead to membrane lipid peroxidation and finally to cell necrosis[10,11]. High levels of ROS damage cells and are involved in several human pathologies, including liver cirrhosis and fibrosis[12,13]. Therefore, the use of compounds with antioxidant properties may prevent or alleviate many diseases associated with ROS. Because ROS formation is a naturally occurring process, mammalian cells have developed several protective mechanisms that prevent ROS formation or detoxify ROS. These mechanisms employ molecules called antioxidants as well as protective enzymes[14]. To prevent the damage caused by ROS, living organisms have developed an antioxidant defense system that includes the presence of nonenzymatic antioxidants (glutathione, uric acid, bilirrubin, and vitamins C and E) and enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)[15].

Oxidative stress refers to the cytotoxic consequences of oxygen radicals: superoxide, hydroxy radical, and hydrogen peroxide (H_2O_2), which are generated as byproducts of normal and aberrant metabolic processes that utilize molecular oxygen. Oxygen radicals can attack proteins, deoxynucleic acids, and lipid membranes, thereby disrupting cellular functions and integrity. It has been demonstrated that oxidative stress plays an important role as a mediator of apoptosis[16,17], and hydrogen peroxide (H_2O_2), a byproduct of oxidative stress, has been implicated in triggering apoptosis in various cell types[18,19].

The plant *Hyptis suaveolens (H. suaveolens)* (L.) Poit. is a ethnobotanically important medicinal plant belonging to Lamiaceae family. In Indian traditional medicine, the leaves are used as stimulant, carminative and in treatment of stomach ache. Both leaves and twigs are considered to exhibit antispasmodic activity and are used as source of anti-inflammatory and antifertility agents and also as antiseptic in burns, wounds and various skin complaints[20,21].

The genus Hyptis possesses a diverse range of biological activities which have been mainly reported to be present in essential oil obtained by hydrodistillation of the leaves of *H. suaveolens*[22]. Based on the traditional knowledge and recent pharmacological studies, the aim of the present study is to evaluate the *in vitro* antioxidant activity of *H. suaveolens* collected from surrounding areas of Mysore in south India. There are no reports in the literature regarding this pharmacological effect use as a hepatoprotective and cytoprotective agent. Thus the present study was performed to evaluate the hepatoprotective activity of the methanolic extract of *H. suaveolens* against CCl_4-induced hepatic injury in rats and cytoprotective effect of *H. suaveolens* methanolic extract against the H_2O_2 induced oxidative stress in $HepG_2$ cells.

2. Materials and methods

2.1. Chemicals

Nicotinamide adenine dinucleotide phosphate reduced (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA), glutathione (GSH), oxidised glutathione (GSSG), glutathione reductase (GR), pryogallol, bovine serum albumin, 2,4-dinitrophenyl hydrazine, tetraethoxypropane, eagle's minimum essential medium (MEM), trypsin (0.1%), 3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT), fetal calf serum were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid, agarose, trypan blue (0.1%) H_2O_2, DMSO, 5, 50 dithiobis(2-nitrobenzoic acid) and other chemicals were purchased from Sisco Research Laboratories, Mumbai, India. All the chemicals used were of highest purity grade available.

2.2. Plant materials

Fresh plants of *H. suaveolens* (areal parts) were collected in the month of February 2010 from Mysore in India and were authenticated by a plant taxonomist. Healthy plants were screened and thoroughly washed to remove adhering dust and shade-dried. The dried plants were pulverized in a mechanical grinder and the coarse powder was used for further studies.

2.3. Preparation of extracts

Fifty gram of whole plant powder was serially extracted with hexane, ethyl acetate and methanol using Soxhlet apparatus. The crude extract thus obtained from this extraction flask was transferred to flash evaporator for complete evaporation. The final quantities of the hexane, chloroform, methanol and aqueous extracts obtained were 0.8, 1.85 and 5.21 g, respectively.

2.4. Animals and treatments

Inbred sixty day old adult male Wistar albino rats (200±20) g was obtained from the Laboratory Animal Centre. Appropriate guidelines of the local animal ethics committee were followed for the animal experiments. In 30 d dietary study on rats it was established that the methanolic *H. suaveolens* extract is safe to the mammalian system at the highest dose used in this study. The rats were kept in separated animal rooms with a 12 h light-dark cycle, and the temperature and humidity were kept at (23 ± 2) ℃ and 55%-65%, respectively. Commercial diet purchased and tap water was supplied ad libitum. All animals received humane care according to the guidelines in the Guideline for the Care and Use of Laboratory Animals. All the animals were acclimatized for at least one week prior to experiment and then randomly divided into five groups (8 rats per group). In multiple dose pretreatment experiment, Group I served as normal control. Group II

was a negative control and rats were treated orally with a single dose of CCl_4 (1/2 LD_{50} 1 mL/kg body weight). The rats in Group III and IV were pretreated orally with methanolic extract of *H. suaveolens* at dose of 50 and 100 mg/kg of body weight once daily for 7 d, followed by a single (oral) dose of CCl_4 (1 mL/kg b.w.) on the 7th day. Group V was a positive control and rats received only methanolic *H. suaveolens* extract at 100 mg/kg body weight for 7 d. Animals were sacrificed by anesthesia 16 h after CCl_4 administration on the 7th day, the liver perfused with saline were dissected out and processed immediately for biochemical assays.

2.5. Serum enzymes

Liver damage was assessed by estimating serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) using commercially available test kits. The results were expressed as units/liter (U/L).

2.6. Antioxidant enzymes

Liver tissue was homogenised (10% w/v) in ice-cold 50 mM phosphate buffer (pH 7.4), centrifuged at 10 000 *g* for 20 min at 4 ℃ and the supernatant was used to assay the enzyme activities. SOD activity was measured using pyrogallol (2 mM) autoxidation in Tris buffer[23]. GR activity was estimated using oxidised glutathione (0.5 mM) and NADPH (2 mM) in potassium phosphate buffer (0.1 M, pH 7.4)[24]. Glutathione-S-transferase (GST) activity was assayed by the method of Warholm *et al*[25] in phosphate buffer (0.1 M, pH 7.6) containing glutathione (0.5 mM) and CDNB (0.5 mM) and change in the absorbance at 344 nm was monitored in a UV-visible spectrophotometer. All the described methods were followed from published literature and are well established.

2.7. Lipid peroxidation assay

Lipid peroxidation (LPO) in the liver tissue homogenate was measured by estimating the formation of MDA using the TBARS assay[26]. Tissue homogenate (10% w/v in 50 mM phosphate buffer, pH 7.4) was boiled in TCA (10%) and TBA (0.34%) for 15 min, cooled and centrifuged. Absorbance of the supernatant was read at 535 nm. TBARS was calculated using tetraethoxypropane as the standard.

2.8. Glutathione

A 10% (w/v) liver homogenate was prepared in 5% (w/v) trichloroacetic acid, centrifuged at 2 000 *g* for 10 min and the GSH content in the deproteinised supernatant was estimated by Ellman's reagent with a standard curve[27].

2.9. Histopathological studies

Paraffin sections were prepared and stained with haematoxylin and eosin and examined using light microscopy (×400 magnification).

2.10. Cell culture and treatments

$HepG_2$ cells are derived from human hepatocellular carcinoma. The cells were procured from National Centre for Cell Sciences. The cells were grown in 25 cm^2 flasks (Falcon, Becton, Dickinson, USA) with loosened caps, containing MEM supplemented with 10% fetal calf serum, 1% L glutamine and 50 μg/mL gentamycin sulfate at 37 ℃ in a CO_2 incubator (NuAire, Plymouth, MN, USA) in an atmosphere of humidified 5% CO_2 in 95% air. To examine possible toxic effects, HepG2 cells were treated with the *H. suaveolens* methanolic extract in a concentration ranging from 0.1 to 1 mg/mL for 24 h. Similarly, cells were treated with H_2O_2 at concentrations ranging from 50 to 200 μM for 24 h. In order to produce oxidative stress, H_2O_2 was freshly prepared from 30% stock solution prior to each experiment. Cells were pre-treated with *H. suaveolens* methanolic extract for 2 h before the addition of H_2O_2 and 24 h later the survival of cells was determined by MTT assays.

2.11. Analysis of cell viability

The assay was carried out according to the protocol described by Mossman[28]. Preliminary experiments were conducted to standardize the number of cells to be seeded onto the 96 well plates. 10×10^4 cells were seeded to each well. Exponential phase cells were used throughout the experiment. Cells were allowed to attach (for 24 h), and treated with different concentrations of drug for varying time intervals. Eight wells each served as control and blank and rest of the cells were used for different drug treatment groups. After the drug treatment, the medium containing the drug was removed, washed with PBS buffer and 100 μL of the MTT stock (1 mg/mL) was added to each of the 96 wells. After 4 h of incubation at 37 ℃ in a 5% CO_2 incubator, the solution was removed and 100 μL of DMSO was added to each well. After 5-10 min of incubation at 37 ℃, the wells were read on an ELISA plate reader (Tecan, Austia) at 540 nm wavelength. The data was recorded using the software package Magellan 6.3. The viability (%) was calculated as follows:

$$\text{Viability} = \frac{\text{Average of test wells OD} - \text{Average of blank wells OD}}{\text{Average of control wells OD} - \text{Average of blank wells OD}} \times 100$$

(OD= optical density; Test wells = treated wells)

2.12. Statistical test

All the data for animals were expressed as means ± SEM. of six observations (n = 6) and significant difference between each of the groups was statistically analyzed by Duncan's multiple range test, represented by alphabets for each level of significance. A difference was considered

significant at $P<0.05$. A one way analysis of variance and *post hoc* comparisons were used to analyze the concentration dependent effects of (1) H_2O_2 on cell viability; (2) the effect of *H. suaveolens* on H_2O_2-induced cell death Student's *t*-test was used to compare the significant difference between control and drug-treated groups. All values are presented as means ± SEM ($P < 0.05$).

3. Results

3.1. Serum enzymes

Table 1 presents the effects of *H. suaveolens* extract on CCl_4-induced hepatotoxicity of rats. The rats treated with CCl_4 alone showed serious liver damage, as revealed by a significant increase in the serum AST, ALT, ALP and LDH levels over those of the control group. Treatment with *H. suaveolens* extract attenuated the increase in AST and ALT activity with a dose-dependent effect. Treatment with high-dose *H. suaveolens* extract (100 mg/kg) showed the significant hepatoprotective effect. The results proved that supplementation of *H. suaveolens* extract could protect rats against CCl_4-induced acute liver damage.

3.2. Antioxidant enzymes

Hepatic antioxidant activities were variably affected in livers with CCl_4-induced damage (Table 2). After treatment with CCl_4, the activities of SOD, GR and GST activities were significantly decreased as compared with the control group. Pre-treatment of animals with different doses of *H. suaveolens* extract (50 and 100 mg/kg of body weight) significantly elevated the expression of SOD, GR and GST as compared with the group of CCl_4-treated alone. To counteract ROS and to prevent their possible damage to biological molecules, all oxygen-consuming organisms are endowed with well-integrated antioxidant systems, which include enzymes such as SOD, GR and GST.

3.3. Lipid peroxidation

Treatment with CCl_4 promoted lipid peroxidation, the extent of which was specified by the level of MDA (Table 2). Pretreatment with 50 and 100 mg/kg body weight of *H. suaveolens* extract markedly reduced the formation of MDA, indicating that the administration of *H. suaveolens* methanolic extract effectively inhibited lipid peroxidation as induced by CCl_4.

3.4. Glutathione

Administration of CCl_4 decreased the hepatic GSH level which was restored to normal level by *H. suaveolens* extract at higher doses in multiple pretreatments (Table 2). Pretreatment of *H. suaveolens* extract alone raised the hepatic GSH level which was significant with multiple doses pretreatment.

3.5. Histopathology

Histopathological observations of liver sections from the control group showed normal cellular architecture with distinct hepatic cells and sinusoidal spaces (Figure 1 I). In contrast, the CCl_4 group revealed the most severe damage

Table 1
Protective effects of the methanol extract of *H. suaveolens* (pretreatment-multiple dose) on CCl_4 hepatotoxicity serum enzymes.

Groups	AST(Units/L)	ALT(Units/L)	ALP(Units/L)	LDH(Units/L)
Control	253.5±8.2^a	93.4±2.4^a	144.3±8.2^a	1 362.4±85.6^a
CCl_4 alone	786.3±10.2^d	231.6±1.9^d	258.6±5.2^d	1 985.0±163.3^d
H. suaveolens (50 mL/kg b.w) + CCl_4	672.4±15.4^c	195.2±3.1^c	224.5±4.5^c	1 603.7±172.2^c
H. suaveolens (100 mL/kg b.w) + CCl_4	456.5±12.3^b	142.9±1.8^b	185.4±6.7^b	1 536.3±68.4^b
H. suaveolens (100 mL/kg b.w)	306.8±11.3^a	102.1±2.9^a	132.8±9.3^a	1 392.3±58.5^a

Data with different letters in the same column are significantly different at $P < 0.05$.

Table 2
Effects of pretreatment with methanol extract of *H. suaveolens* (pretreatment-multiple dose) on liver antioxidant enzymes and MDA in CCl_4 intoxicated rats ($n = 6$).

Groups	SOD^A	GSH^B	GR^C	LPO^D	GST^E
I	2.64±0.41^a	16.26±1.32^c	253.14±10.23^d	3.97±0.44^a	153.27±11.65^b
II	2.82±0.23^a	17.51±1.75^c	278.52±15.61^d	3.69±0.36^a	183.84±12.53^c
II	0.58±0.19^c	15.26±1.62^b	185.35±22.76^b	5.35±0.39^c	145.73±13.49^b
IV	1.32±0.35^b	15.94±1.23^b	198.60±21.78^c	4.83±0.32^b	163.45±13.28^{bc}
V	0.31±0.45^c	12.24±1.54^a	139.30±22.49^a	5.56±0.29^c	118.23±11.05^a

Treatments - I: control; II: *H. suaveolens* (100 mL/kg b.w); III: *H. suaveolens* (50 mL/kg b.w) + CCl_4 (1 mg/kg); IV: *H. suaveolens* (100 mL/kg b.w) + CCl_4 (1 mL/kg); V: CCl_4 (1 mL/kg). Data with different letters in the same column are significantly different at $P < 0.05$.
A: Units/mg proteins. B: μg/mg protein. C: nmoles NADPH/min/mg protein. D: nmoles MDA/mg protein. E: mole CDNB conjugate/min/mg protein.

of any of the groups; the liver sections showed massive fatty changes, necrosis, ballooning degeneration, broad infiltration of lymphocytes, and the loss of cellular boundaries (Figure 1 II). The liver sections of the rats treated with *H. suaveolens* + CCl_4 (Figure 1 III–IV) showed a more or less normal lobular pattern with a mild degree of fatty change, necrosis, and lymphocyte infiltration almost comparable to the control. Histological picture of the liver of rats pretreated only with plant extract (Figure 1 V) did not reveal degenerative signs and was comparable to that of control.

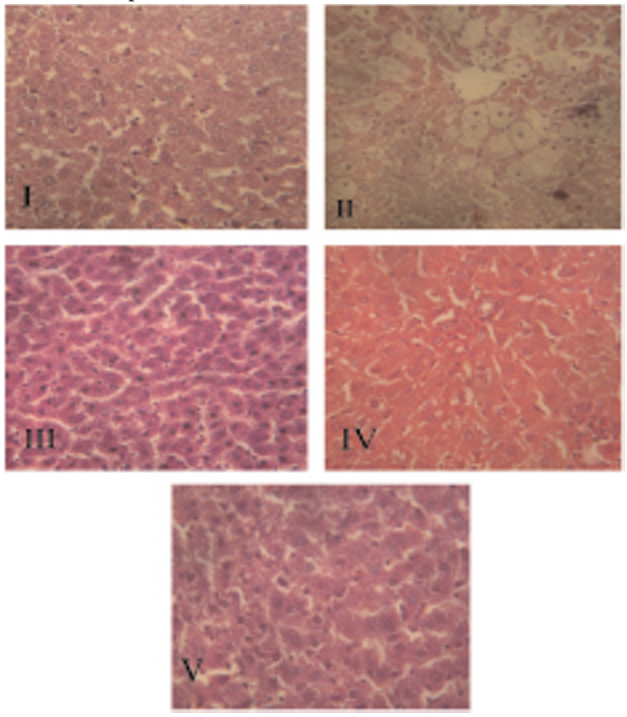

Figure 1. Histopathology of liver sections of animals. Haematoxylin and eosin staining, magnification, ×400.
Hepatoprotective effect of *H. suaveolens* methanolic extract pretreatment (single dose) on CCl_4–induced liver damage. Group I – control; Group II – CCl_4; Group III – *H. suaveolens* 50 mg/kg b.w. + CCl_4; Group IV – *H. suaveolens* 100 mg/kg b.w. + CCl_4; Group V – *H. suaveolens* 100 mg/kg b.w.

3.6. H_2O_2–induced cytotoxicity in $HepG_2$ cells

There were no significant differences in cell viability (Figure 2A) between untreated cultures and treated ones when the dose of H_2O_2 was below 50 μM. For 100 μM H_2O_2 treated groups, a significant decrease in cell viability was seen after 24 h exposure. Cell viability decreased to 35%–45% at 100 μM, and to 15%–20% at 200 μM H_2O_2 or higher. To determine the effect of *H. suaveolens* methanolic extract on H_2O_2–induced cytotoxicity, HepG2 cells were pretreated with varying concentrations of *H. suaveolens* for 2 h. 100 μM H_2O_2 was added for an additional 24 h incubation. Cell viability then was examined using an MTT mitochondrial function assay. As shown in (Figure 2B), *H. suaveolens* methanolic extract at 0.7 mg/mL significantly inhibits cytotoxicity induced by H_2O_2.

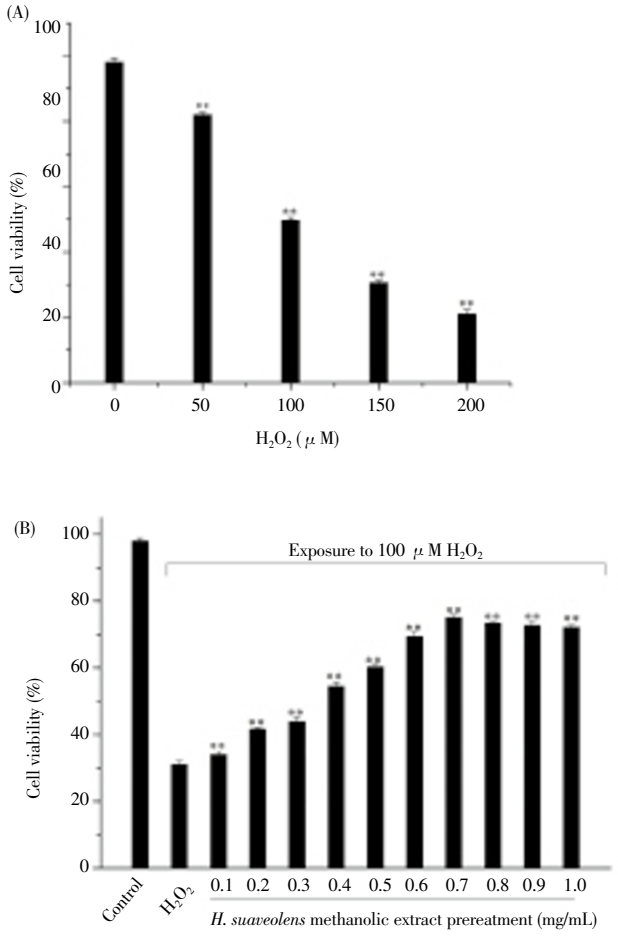

Figure 2. Cytoprotective effects of *H. suaveolens* methanolic extract against H_2O_2 induced cell death in $HepG_2$ cells. (A) Concentration–dependent effect of H_2O_2 on cell survival in $HepG_2$cells. $HepG_2$ cells were exposed to different concentrations of H_2O_2 for 24 h. Cell viability was assessed using MTT assay. Cells without treatment serve as control. $^{**}P<0.05$ *vs*. Control. (B) Cytoprotective effects of *H. suaveolens* methanolic extract on H_2O_2–induced cytotoxicity in $HepG_2$ cells. $HepG_2$ cells were treated with 100 μM H_2O_2 for 24 h. Some cells were pretreated with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mg/mL *H. suaveolens* methanol extract for 2 h prior to incubation with 100 μM H_2O_2 for an additional 24 h. $^{**}P<0.05$ *vs*. H_2O_2 alone.

4. Discussion

In the present study, administration of CCl_4 markedly raised the serum level of enzymes such as AST, ALT, ALP and LDH in rats. However, pre–treatment of the rats with *H. suaveolens* methanolic extract at 50 and 100 mL/kg b.w for 7 d prior to CCl_4 administration resulted in a significant decrease in serum AST, ALT, ALP and LDH activities. With respect to the histological examination, pre–treatment with *H. suaveolens* suppressed the acute hepatic damage and was consistent with an improvement in the serum biological parameters of hepatotoxicity. Many chemical regents such as CCl_4 could significantly increase the serum AST, ALT, ALP and LDH levels and cause serious injury to the liver. Sturgill and Lambert[29] noted that once the liver was exposed to

CCl_4, AST and ALT were released from the liver into blood, and caused the level of these serum maker enzymes to increase significantly. Valcheva-Kuzmanova *et al*[30,31] reported that natural fruit juice from Aronia melanocarpa could decrease the serum AST and ALT level of rats. Augusti *et al*[32] noted that the liver damage by CCl_4 was satisfactorily prevented by garlic oil as effectively as vitamin E. Elevation in the levels of antioxidant enzymes in CCl_4 treated animal was observed when compared to control group. Increase in GSH, GST, GR, SOD and LDH levels in the liver homogenates of CCl_4 treated group suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms. Treatment with *H. suaveolens* methanolic extract significantly prevented these changes and this may be due to its antioxidant effects. Since *H. suaveolens* methanolic extract treated groups has significantly elevated the GSH, GST, GR and SOD contents of the liver, it may also be helpful in treating the hepatotoxicity induced by other agents. These antioxidant enzymes are the first line of defense against free radical induced oxidative stress[33]. Glutathione dependent defense against xenobiotic toxicity is a multifaceted phenomenon that has been well characterized in animals. SOD is an exceedingly effective defense enzyme that converts superoxide anions into H_2O_2[34], and CAT is a haemeprotein in all aerobic cells that metabolize H_2O_2 to oxygen and water. These antioxidant enzymes are inactivated by lipid peroxides or ROS. Under normal conditions, excess free radicals are neutralized immediately by enzymatic scavengers such as SOD, GR and GST which contribute to the maintenance of a normal oxidation-reduction balance. Jayakumar *et al*[35] noted that pretreatment with ethanol extract of oyster mushroom could significantly increase SOD, GR and GST activities in the liver of CCl_4-administered rats compared to the negative group. The protective effects of *H. suaveolens* on liver result from its antioxidant activity, including stabilization in the intracellular defense systems and reductions in the lipid peroxidation products.

Lipid peroxidation is one of the major characteristics that can be included as an oxidative damage marker. Furthermore, tissues and cells are subjected to oxidative injury when there is overproduction of ROS or when antioxidant systems fail to function effectively. When the liver is damaged by some chemical toxin, hepatocytes generate a large number of free radicals, causing lipid peroxidation of the cytomembrane to produce MDA. MDA levels indirectly reflect the extent of cellular damage by free radicals and are widely used as an index of free radical mediated lipid peroxidation[36,37]. The increase in MDA and hydroperoxide levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals[38]. Opoku *et al*[39] reported that aqueous extract of Zulu medicinal plant significantly decreased MDA levels in CCl_4-induced acute liver injury in rats. In the present study, significantly elevated levels of TBARS, products of membrane lipid peroxidation, observed in CCl_4 administered rats indicated hepatic damage. Pretreatment of *H. suaveolens* methanolic extract prevented lipid peroxidation which could be attributed to the radical scavenging antioxidant constituents.

According to microscopic examinations, severe liver damage induced by CCl_4 was markedly reduced by the administration of *H. suaveolens* methanolic extract, which was in good correlation with the results of serum enzyms activities, hepatic antioxidant enzyme activities and hepatic lipid peroxidation.

For the first time, demonstrated the anti-oxidative effects possessed by the methanolic extract of the *H. suaveolens* through the suppression of H_2O_2-induced ROS formation against H_2O_2 insult. We used H_2O_2 as a toxic stimulus in a $HepG_2$ culture model to examine the protective role of methanolic extract of *H. suaveolens*. We chose H_2O_2 to induce cell death in our cell cultures because: 1) H_2O_2 is a precursor of highly oxidizing, tissue-damaging radicals such as hydroxyl radicals and is known to be toxic to many systems; 2) exogenous H_2O_2 can enter the cells and induce cytotoxicity due to its high membrane permeability[40]. The MTT assay is a colorimetric assay for the non-radioactive quantification of cell viability and is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. The formation of formazan is thought to take place via intact mitochondria. A decrease in number of living cells results in a decrease in the total metabolic activity in the sample. Our MTT assays demonstrated that the reduction of metabolic activity was significant, suggesting an inhibition of mitochondria function by H_2O_2 in this culture system.

In conclusion, we report for the first time, that pre-treatment with *H. suaveolens* methanolic extract was effective in the prevention of CCl_4-induced liver damage in rats. Our results show that the hepatoprotective effects of *H. suaveolens* methanolic extract may be due to several constituents with potential healthy biological properties, such as polyphenol compounds, flavonoids and terpenoids. The mechanisms of protection include the inhibition of lipid peroxidation processes and an increase in antioxidant enzyme activity, all of which resulted in the recovery of biological parameters and integrity of the tissues. Hydrogen peroxide, a free radical generator, significantly induces cell death in cultured cells and pretreatment of the cells with plant extract inhibits this process in a dose-dependent manner. The inhibitory effects of methanolic extract of *H. suaveolens* may be useful as a hepatoprotective and cytoprotective agent against chemical-induced hepatotoxicity and cytotoxicity *in vivo*.

Conflict of interest statement

The authors declare that there is no conflict of interests.

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References

[1] Qian ZJ, Jung WK, Kim SK. Free radical scavenging activity of a novel antioxidativepeptide purified from hydrolysate of bullfrog skin, *Rana catesbeiana Shaw*. *Bioresour Technol* 2008; **99**: 1690-1698.

[2] Blomhoff R. Dietary antioxidants and cardiovascular disease. *Curr Opin Lipidol* 2005; **16**: 47-54.

[3] Bhathal PS, Rose NR, Mackay IR, Whittingham S. Strain differences in mice in carbon tetrachloride-induced liver injury. *Br J Exp Pathol* 1983; **64**: 524-533.

[4] Vitaglione P, Morisco F, Caporaso N, Fogliano V. Dietary antioxidant compounds and liver health. *Crit Rev Food Sci Nutr* 2004; **44**: 575-586.

[5] Wolf PL. Biochemical diagnosis of liver diseases. *Clin Biochem* 1999; **14**: 59-90.

[6] Gonzalez FJ. The molecular biology of cytochrome P450s. *Pharmacol Rev* 1998; **40**: 243-288.

[7] Brattin WJ, Glende Jr. EA, Recknagel RO. Pathological mechanisms in carbon tetrachloride hepatotoxicity. *Free Radical Bio Med* 1985; **1**: 27-38.

[8] Williams AT, Burk RF. Carbon tetrachloride hepatotoxicity: an example of free radical-mediated injury. *Semin Liver Dis* 1990; **10**: 279-284.

[9] Brent JA, Rumack BH. Role of free radicals in toxic hepatic injury. II. Are free radicals the cause of toxin-induced liver injury? *J Toxicol Clin Toxicol* 1993; **31**: 173-196.

[10]Recknagel RO, Glende Jr. RO, Britton RS. Free radical damage and lipid peroxidation. In: Meeks RG (ed.). *Hepatotoxicology*. Florida: CRC Press; 1991, p. 401-436.

[11]Brautbar N, Williams II J. Industrial solvents and liver toxicity: risk assessment, risk factors and mechanisms. *Int J Hyg Environ Health* 2002; **205**: 479-491.

[12]Wu D, Zhai Q, Shi X. Alcohol-induced oxidative stress and cell responses. *J Gastroenterol Hepatol* 2006; **21**: S26-S29.

[13]Loguercio C. Federico A. Oxidative stress in viral and alcoholic hepatitis. *Free Radical Biol Med* 2003; **34**: 1-10.

[14]Chen XL, Kunsch C. Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases. *Curr Pharm Des* 2004; **10**: 879-891.

[15]Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007; **39**: 44-84.

[16]Hivert B, Cerrui C, Camu W. Hydrogen peroxide-induced motoneuron apoptosis is prevented by poly ADP ribosyl synthetase inhibitors. *Neuroreport* 1998; **9**: 1835-1838.

[17]Clement MV, Ponton A, Pervaiz S. Apoptosis induced by hydrogen peroxide is mediated by decreased superoxide anion concentration and reduction of intracellular milieu. *FEBS Lett* 1998; **440**: 13-18.

[18]Kitamura Y, Ota T, Matsuoka Y, Tooyama I, Kimura H, Shimohama S, et al. Hydrogen peroxide-induced apoptosis mediated by P53 protein in glial cells. *Glia* 1999; **25**: 154-164.

[19]Skaper SD, Floreani M, Negro A, Facci L, Giusti P. Neurotrophins rescue cerebellar granule neurons from oxidative stress-mediated apoptotic death: selective involvement of phosphatidylinositol 3-kinase and the mitogen-activated protein kinase pathway. *J Neurochem* 1998; **70**: 1859-1868.

[20]Shirwaikar A, Shenoy R, Udupa AL, Shetty S. Wound healing property of ethanolic extract of leaves of *Hyptis suaveolens* with supportive role of antioxidant enzymes. *Indian J Expt Biol* 2003; **41**: 238-241.

[21]Shenoy C, Patil MB, Kumar R. Wound healing activity of *Hyptis suaveplens* (L.) Poit (Lamiaceae). *Int J Pharm Tech Res* 2009; **1**: 737-744.

[22]Peerzada N. Chemical composition of the essential oil of *Hyptis suaveolens*. *Molecules* 1997; **2**:165-168.

[23]Marklund S, Marklund G. Involvement of the superoxide anion in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 1974; **47**: 469-474.

[24]Calberg I, Mannervik B. Glutathione reductas. *Methods Enzymol* 1985; **113**: 484-490.

[25]Warholm M, Guthenberg C, Bahr CV, Mannervik B. Glutahtione transferase from human liver. *Methods Enzymol* 1985; **113**: 499-504.

[26]Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; **95**: 351-358.

[27]Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; **82**: 70-77.

[28]Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55-63.

[29]Sturgill MG, Lambert GH. Xenobiotic-induced hepatotoxicity: mechanisms of liver injury and methods of monitoring hepatic function. *Clin Chem* 1997; **43**: 1512-1526.

[30]Valcheva-Kuzmanova S, Borisova P, Galunska B, Belcheva A. Hepatoprotective effect of the natural fruit juice from *Aronia melanocarpa* on carbon tetrachloride-induced acute liver damage in rats. *Exp Toxicol Pathol* 2004; **156**(3): 195-201.

[31]Thirumalai T, David E, Therasa SV, Elumalai EK. Restorative effect of Eclipta alba in CCl4 induced hepatotoxicity in male albino rats. *Asian Pac J Trop Dis* 2011; **1**(4): 304-307.

[32]Augusti KT, Anuradha SP, Smitha KB, Sudheesh M, George A, Joseph MC. Nutraceutical effects of garlic oil, its nonpolar fraction and a ficus flavonoid as compared to vitamin E in CCl_4 induced liver damage in rats. *Indian J Exp Biol* 2005; **43**: 437-444.

[33]Ozden S, Catalgol B, Gezginci-Oktayoglu S, Arda-Pirincci P, Bolkent S, Alpertunga B. Methiocarb-induced oxidative damage following subacute exposure and the protective effects of vitamin E and taurine in rats. *Food Chem Toxicol* 2009; **47**: 1676-1684.

[34]Reiter RJ, Tan D, Osuna C, Gitto E. Actions of melatonin in the reduction of oxidative stress. *J Biomed Sci* 2000; **7**: 444-458.

[35]Jayakumar T, Ramesh E, Geraldine P. Antioxidant activity of the oyster mushroom, Pleurotus ostreatus, on CCl_4-induced liver injury in rats. *Food Chem Toxicol* 2006; **44**: 1989-1996.

[36]Mansour MA. Protective effects of thymoquinone and desferrioxamine against hepatotoxicity of carbon tetrachloride in mice. *Life Sci* 2000; **66**: 2583-2591.

[37]Subash S, Subramanian P. Protective effect of morin on lipid peroxidation and lipid profile in ammonium chloride-induced hyperammonemic rats. *Asian Pac J Trop Dis* 2012; **2**(3): 103-106.

[38]Amresh G, Zeashan H, Gupta RJ, Kant R, Rao CV, Singh PN. Gastroprotective effects of ethanolic extract from *Cissampelos pareira* in experimental animals. *J Nat Med* 2007; **61**: 323-328.

[39]Opoku AR, Ndlovu IM, Terblanche SE, Hutchings AH. *In vivo* hepatoprotective effects of *Rhoicissus tridentata* subsp. cuneifolia, a traditional Zulu medicinal plant, against CCl_4-induced acute liver injury in rats. *S Afr J Bot* 2007; **73**: 372-377.

[40]Halliwell B. Reactive oxygen species and the central nervous system. *J Neurochem* 1992; **59**: 1609-1623.