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HELIOTROPIUM UNDULATUM ATTENUATES HYDRAZINE INDUCED HEPATOTOXICITY: INVOLVEMENT OF CYTOCHROME P450 CYP2E1 AND OXIDATIVE STRESS

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ABSTRACT

Background: Hepatoprotective effect of numerous phytochemicals derived herbal origin have been reported to exhibit chemopreventive potential. The aim of the present study was to evaluate the protective role of Algerian sahara *Heliotropium undulatum* on hydrazine-hepatotoxicity. **Methods:** A model of hepatotoxicity was produced by giving rats intraperitoneally (ip) a single dose of 300 mg/kg of hydrazine (HD) after successive oral administration of the *n*-BuOH extract of *Heliotropium undulatum's* leaves (BEHUL) or Vit E (200 mg/Kg, 25 mg/Kg per day) respectively for ten days. **Results:** HD caused a marked rise in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities and exhibited an hepatic oxidative stress, the hepatic CYP2E1 plays an essential role in the hepatotoxicity of hydrazine. However, pretreatment with BEHUL restored (72.1-75.67 %) the liver function, produced a detectable decrease (76.56 %) in malondialdehyde (MDA), enhanced (71.28 %) catalase (CAT) and (79.17 %) superoxide dismutase (SOD) activities as cellular antioxidant defense, lowered (79.28 %) the CYP2E1 activity and suppressed the parenchymal hepatic lesions. **Conclusion:** The present results demonstrated that BEHUL may play an important role in the protection against HD-induced

hepatotoxicity via oxidative stress. The present study may help in explaining at least in part the mechanism of action of BEHUL that could be attributed of phyto-constituents like polyphenols.

KEYWORDS: *Heliotropium undulatum*, hydrazine, Liver, CYP2E1, oxidative stress.

INTRODUCTION

Hydrazine (HD) and its derivatives are frequently found in our environment.[1,2] They are used as raw materials and/or intermediates in many industrial syntheses.[3] Hydrazine may also be used in the treatment of tuberculosis and hypertension an unregulated supplemental therapy for advanced cancer.[4,5] Hepatotoxicity is a major adverse effect of HD,[6] the cytochrome P450/FMO (flavin containing monooxygenase)-mediated oxidation reactions generate reactive intermediates from HD.[7,8] CYP2E1-mediated metabolism of these compounds generates toxic intermediates and excessive amounts of ROS.[9,10] Elevation of hepatic CYP2E1 plays an essential role in the hepatotoxicity of hydrazine through generation of free radicals from HD.[11,12,13]

The formation of free radicals during the microsomal oxidation of hydrazine leads to the hypothesis that such free radicals are involved in the hepatotoxicity of hydrazine derivatives.[14] Exposure of humans or animals to hydrazine have been reported to result in hepatic necrosis and steatosis.[15,7] A previous report proposed that hepatic CYP2E1 plays a fundamental role in the propagation of hydrazine-induced hepatotoxicity, mainly throughout ROS generation.[8]

Hepatoprotective effect of numerous phytochemicals derived herbal origin and a broad spectrum of substances have been reported that exhibit chemopreventive potential and were widely available for the treatment of many different types of liver disorders related to ROS.[16, 17,18] The genus *Heliotropium*, (*Boraginaceae*), comprises many species were considered of high importance and have been used in folk medicine for the treatment of inflammatory disorders.[19,20] Keeping this in view, together no previous study has been performed for the biological properties of *Heliotropium undulatum*, the present study was conducted to propose for the first time *Heliotropium undulatum*, which exist in Algerian sahara as a possible phytochemical agent could contribute to the preservation of hepatic function in hydrazine-rat. In order to establish the possible protective mechanisms of *n*-

BuOH leave extract of *Heliotropium undulatum* (BEHUL), CYP2E1, antioxidant status, were evaluate in rat treated with hydrazine.

MATERIALS AND METHODS

Chemicals and reagent

Hydrazine hydrochloride, ethylenediamine tetra acetic acid (EDTA), pyrogalol, thiobarbituric acid (TBA), tris-hydroxymethylaminomethane, trichloroacetic acid (TCA), pyrogallol, *p*-nitrophenol (PNP), butylated hydroxytoluene (BHT), malondialdehyde (MDA). All other reagents used in the analysis were of analytical grade and obtained from Sigma-Aldrich (USA).

Plant material

Leaves of *Heliotropium undulatum* were collected during April 2011, in Bechar province (Algeria). Identification of plant material was made by Mr. Benabdelhakem (Director of the national agency of preservation of natural resources, Bechar, Algeria). An authenticated voucher specimen, with the identification number 04/2010/BHU, was deposited at the Herbarium of the VARENBIOMOL unit research, University of Frères Mentouri Constantine1.

Extraction procedure

A quantity (2386 g) of air-dried and powdered leaves of *Heliotropium undulatum* was macerated with MeOH/H_2O (4/1) at room temperature for 48 hours three times. The crude extract was concentrated and diluted with H_2O. The remaining aqueous solution was successively extracted with $CHCl_3$, EtOAc and *n*-BuOH. The organic phases were dried with anhydrous Na_2SO_4, filtered and concentrated in vacuum to obtain the corresponding extracts. In this study, our interest was focused on the *n*-BuOH leaf extract of *Heliotropium undulatum* (BEHUL) (35g).

Animals and experimental design

Male Albino *wistar* weighing 180-240 g were housed in an air-conditioned room and had a free access to water and pellet diet. All the experimental procedures were carried out in accordance with the Institutional Animal Ethical Committee of Department of Animal Biology (University of Mentouri, Constantine). The experimental procedures adopted in this study were in strict compliance with the United States National Institutes of Health.[21]

Twenty-four male adult rat (180-210 g) were divided into four equal groups (n = 6 per group).

group I (control group), received orally1 mL of 0.9 % NaCl saline solution for ten days, group II (HD- group) received intraperitoneally (ip) a single dose of 300 mg/kg of hydrazine (HD) after successive oral administration of NaCl saline solution (1 mL) for ten days, group III (Vit E- group) received ip a single dose of 300mg/kg HD after successive oral administration of 100 mg/ kg of vitamin E for ten days. Group IV (BEHUL-group) received ip a single dose of 300 mg/kg HD after successive oral administration of 200 mg/ kg of BEHUL for ten days. All groups were sacrificed in the day 11 by decapitation. After 18 hours of HD challenge, the blood samples were collected and centrifuged at 3000 rpm for 15 minutes at 4 °C. The liver tissues were quickly excised, rinsed in ice-cold saline and used immediately or stored frozen at -80 °C until further analysis.

Assessment of liver function markers

Liver marker enzymes such as alanine aminotransferase (ALT)**,** aspartate aminotransferase (AST) were determined in the sera using the commercial assay kits. The results were expressed as an international unit/liter (IU/l).

Assessment of hepatic oxidative stress parameters andCYP2E1 activity

Preparation of liver homogenate and microsomal fraction

20 % (w/v) liver homogenation was made in buffer containing 0.1 M tris acetate (pH 7.4), 0.1 M KCl, 0.1 mM EDTA, 230 M phenylmethyosulfonyl fluoride, and 22.7 M BHT. The homogenates were centrifuged at 10000 x *g* for 10 min at 4°C. An aliquot of the supernatant was used for the measurement of antioxidant status. The remain supernatant was centrifuged at 100000 x g for 1 h at 4°C. The resulting pellet was washed with 0.15 M KCl and was re-suspended in 0.1 M phosphate buffer (pH 7.4) containing 20 % (v/v) glycerol and 1 mM EDTA and stored at 80°C for the measurement of CYP2E1 activity. The protein concentration was determined utilizing the method of Lowry *et al.*,[22] using BSA as a standard.

Determination of CYP2E1 Activity

The CYP2E1 activity was measured by the rate of oxidation of *p*-nitrophenol to *p*-nitrocatechol in the presence of NADPH and O_2.[23] Oxidation of *p*-nitrophenol was determined using 1.5 mg of proteins in a (200 µg) of homogenate in reaction system

containing 2 mM *p*-nitrophenol in 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$ and a NADPH-generating system consisting of 0.4 mM $NADP^+$, 30 Mm isocitrate, 0.2 U of isocitrate dehydrogenase, incubated at 37 °C, and stopped after 60 min by addition of 30 µL of 20 % trichloroacetic acid. The supernatant was treated with 10 µL of 10 M sodium hydroxide. Absorbance at 546 nm was immediately measured and the concentration of 4-nitrocatechol determined (ε = 10.28 mM^{-1} cm^{-1}). Microsomal protein was assayed by the method of Lowry *et al.*,[22] using BSA as a standard

Assessment of hepatic oxidative stress parameters

Determination of lipid peroxidation level

According to the method of Ohkawa *et al.*,[24] 0.5 ml of tissue homogenate were added to 0.5 ml 25 % TCA and 1 ml of 0.67 % TBA solution. The mixture was heated for 45 min in boiling water bath. After cooling, 4 ml of *n*-butanol were added and mixed vigorously. The mixture was centrifuged for 10 min at 3000 rpm. The resultant butanol layer was separated and MDA content was determined by measuring the absorbance at 535 nm. The concentration of thiobarbituric acid was calculated by the absorbance coefficient of MDA-TBA complex 1.56 x 10^5 $M^{-1}cm^{-1}$ and expressed in µmol/mg protein.

Determination of Catalase activity (CAT)

CAT activities were determined on the basis of hydrogenperoxide decomposition.[25] Briefly, 0.1 ml of the supernatant, 0.4 ml of H_2O_2 (5.9 mM; pH 5.0) were mixed with 2.5 ml of phosphate buffer (50 mM; pH 5.0) and change in absorbance was recorded at 240 nm for one min in a spectrophotometer (Smartspeck). A change of 0.01 in absorbance for one min was used as one unit of CAT activity

Determination of superoxide dismutase (SOD)

SOD activity was measured as described by Marklund and Marklund.[26] This method is based on the ability of SOD to inhibit the autoxidation of pyrogallol. Briefly, 0.1 mL of liver homogenate was mixed with 1.5 mL of 20 mM Tris-HCl (containing 1 mM EDTA, pH 8.2), then 0.1 mL of 15 Mm pyrogallol was added. Thereafter, the change in OD per minute was determined by monitoring the increase in OD at 420 nm for 3 min for the samples. The percentage of inhibition for the samples was calculated by the aid of running a control with no sample under the same conditions. SOD enzyme activity was expressed as U/mg protein, where one unit was defined as the amount of the enzyme that inhibited the rate of pyrogallol

autoxidation by 50 %. The protein concentration was determined by the method of Lowry *et al*.,[22,27] using bovine serum albumin (BSA) as the standard.

Histopathological evaluation

Liver tissue for histopathological analysis was fixed in 10 % buffered formalin saline, processed by routine histology procedures and embedded in paraffin. Tissue sections (4-5 µm) were stained with haematoxylin-eosin (H&E400 x) and examined for possible histopathological changes.

Statistical analysis

The results were expressed as Mean ± S.D. Statistical analysis was carried out by Student's test. The obtained Results were considered statistically significant when *p* values were $P<0.05$ and high significant when p values were $P<0.01$.

RESULTS

Liver function marker enzymes

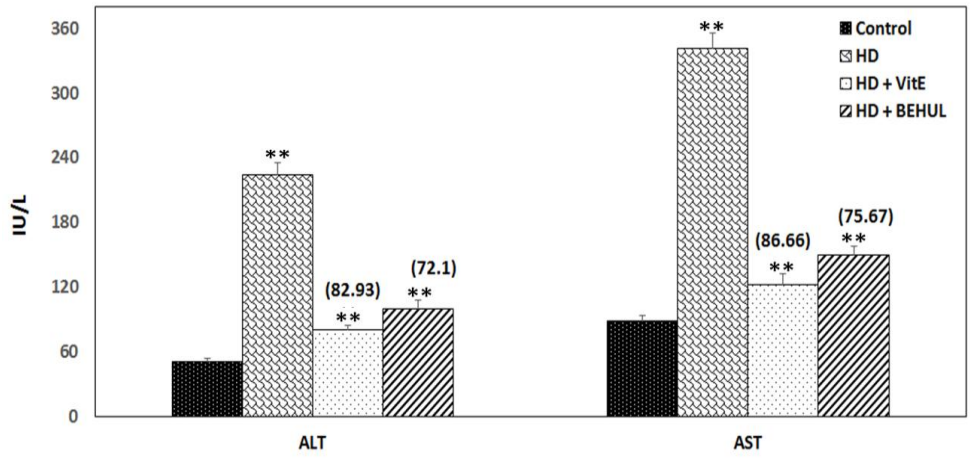

Figure 1: The effect of BEHUL (200 mg/Kg) on hepatic function markers in HD-rats. Each value is the mean ± SD (n=6); *p<0.05, **p<0.01. Values in parentheses : % of protection.

HD administration significantly increased serum ALT and AST levels by about 4.4 and 3.8 folds, respectively, as compared to control animals (Fig 1). Pre-treatment with 200 mg/kg of BEHUL significantly ($P<0.01$) restored hepatic marker enzymes (72.1-75.67 %) as compared with Vit E (82.93-86.66 %) (Fig 1).

CYP2E1 Levels

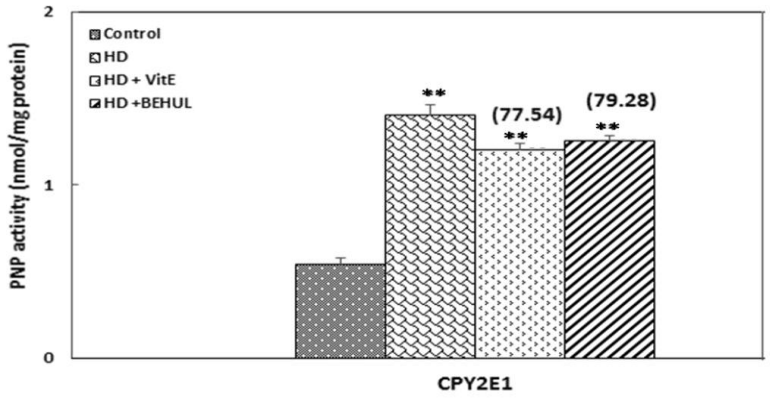

Figure 2: The effect of BEHUL (200 mg/Kg) on PNP oxidation in HD-rats. Each value is the mean ± SD (n=6); *p<0.05, **p<0.01. Values in parentheses: % of protection.

HD significantly (P<0.01) increased (3.85 folds) oxidation of PNP *p*-nitrocatechol, a representative reflection of CYP2E1 catalytic activity. Conversely, rates of PNP oxidation were significantly (P<0.01) lowest in both rats administered the BEHUL (79.28 %) or Vit E (77.54 %) (Fig 2).

Hepatic oxidative stress parameters

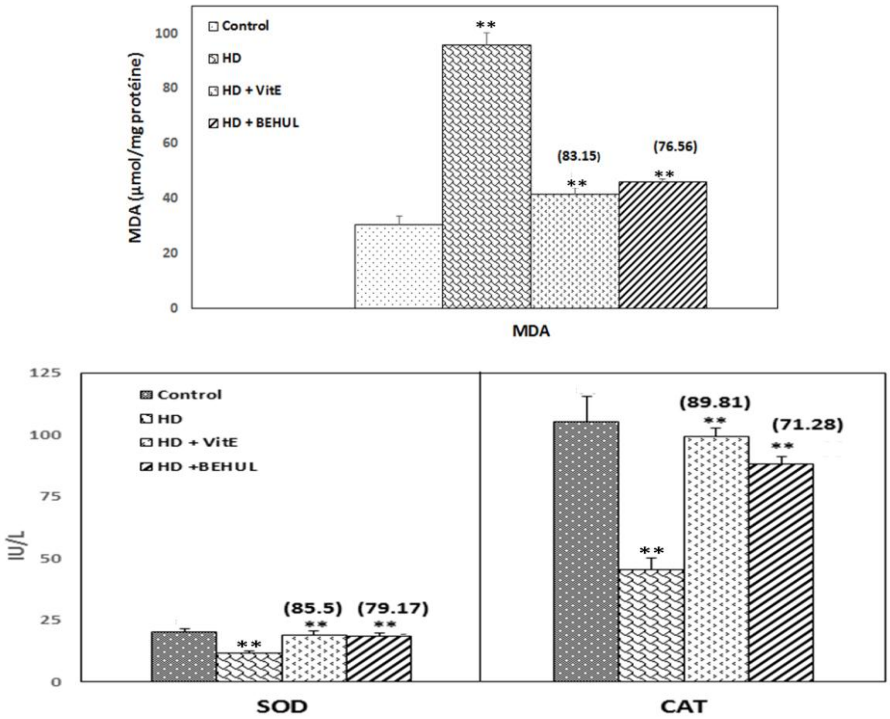

Figure 3: The effect of BEHUL (200 mg/Kg) on hepatic oxidative stress markers (MDA, SOD, CAT) in HD-rats. Each value is the mean ± SD (n=6), *p<0.05, **p<0.01. Values in parentheses: % of protection.

Administration of HD caused a significant increase ($p<0.01$) in MDA level (2 folds) and a significant ($p<0.01$) decline was observed in both CAT, SOD activities as hepatic antioxidants parameters when compared with the normal control group. However, the pre-treatment with 200 mg/kg of BEHUL significantly ($P<0.01$) reduced hepatic MDA level (76.56 %) and significantly ($P<0.01$) raised the levels of liver antioxidant parameters (CAT; 71.28 %) and, (SOD; 79.17 %) as compared to the HD-rats. Vit E as standard drug supressed (83.15 %) significantly ($P<0.01$) the hepatic MDA level and also significantly ($P<0.01$) helps in the preservation of antioxidants parameters, CAT (89.81 %) and SOD (85.5 %) (Fig 3).

Liver histopathological analysis

The histological analysis supported the results obtained from serum enzyme assays. Livers of rats-BEHUL extracts showed noticeable recovery from HD-induced liver damage, a moderate necrosis changes and a minimal inflammatory cellular infiltration.

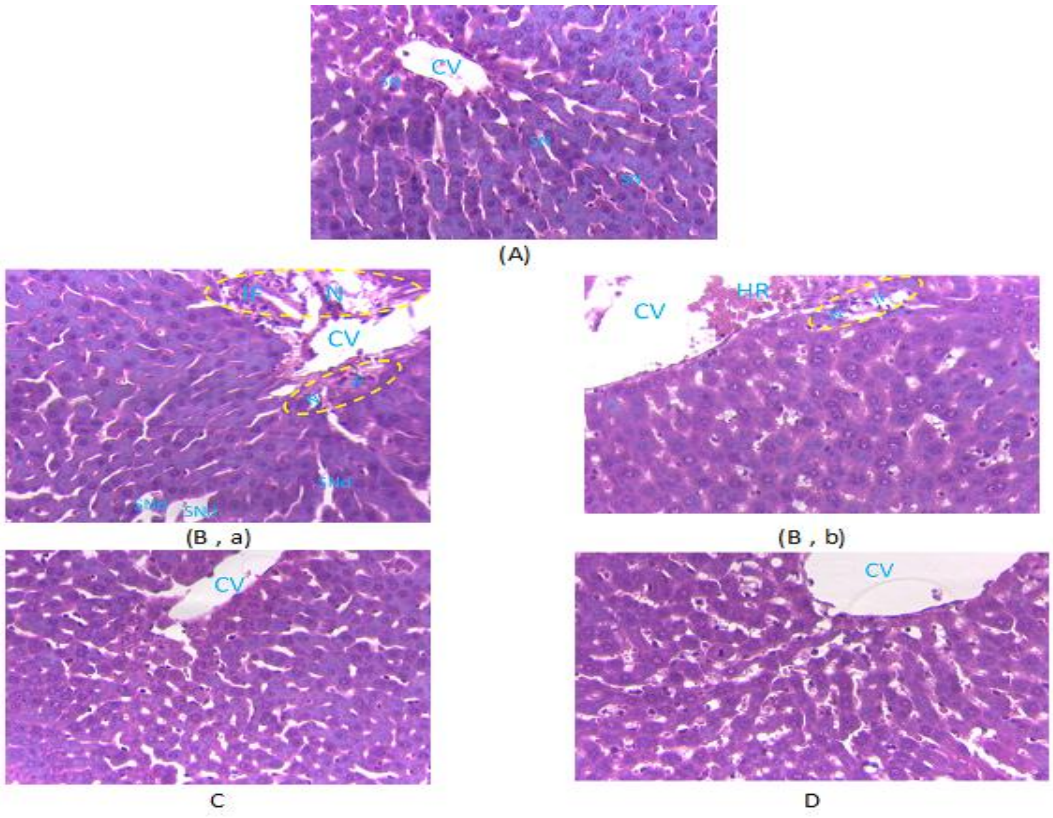

Figure 4: Micrographe of histopathological examination(H&E ×400).

A. control: normal hepatocytes with sinusoidal spaces (SN) central veins (CV), and peripheral portal triads.

B. HD-treated rats: liver architecture was disturbed with loss of radial arrangement hepatic cords and marked sinusoidal dilatations (SNd), severe degree of hemorrhages(HR) in and dilated central vein, centrilobular necrosis (N) with vacuolar degeneration and infiltration of inflammatory cell (IF), (Figures 4; Ba , Bb).

C. HD+ BEHUL-rats: showed slight degeneration in hepatic parenchyma with a moderate degree of histopathological alteration illustrated mild necrosis and inflammatory changes, with less severity reduced degree of sinusoidal and central vein dilations, ballooning, and hepatocytes necrosis were noticed.

D. HD+Vit E: A less sever histopathological changes and preserved hepatic architecture and showed a regular hepatocyte cords, minimal inflammatory cellular infiltration, with minimal congested central vein.

DUSCUSSION

It has been suggested that metabolic activation of hydrazine leads to their toxicity, various non-enzymatic and enzymatic systems such as CYP1A1, CYP1A2 and CYP2E1, were involved.[27] The present study revealed that hydrazine remarkably altered hepatocyte membrane integrity that resulted in increasing the release of ALT and AST from the cytosol.[28] Both Vit E and BEHUL improved liver function, indicating the protection of structural integrity of hepatocytic cell membrane or the regeneration of damaged liver cells.

Hydrazine is oxidized by cytochrome P450 to form some hepatotoxic intermediates that bind to cellular macromolecules.[7,29] The hepatic microsomal CYP450 system, mainly CYP2E1, is considered a central role player in the ROS generation pathway, either directly or as a by-product of its metabolic activities through the generation of reactive metabolites.[30,31] A marked decrease in the CYP2E1 level was declared in the BEHUL-rat. The protective effects polyphenol through CYP450 isozymes inhibition have been reported by several studies. Ho *et al.*[32] declared the Inhibition of human CYP3A4 activity by grapefruit flavonoids, Brantley *et al.*[33] motioned two flavonolignans from milk thistle (*Silybum maria-num*) as CYP2C9 inhibitor, Gurley et al.[34] revealed that supplementation with goldenseal (*Hydrastis canadensis*) inhibits human CYP3A activity *in vivo*, Piver *et al.*[35] motioned the inhibitory effect of resveratrol of CYP3A, CYP1A and CYP2E1 activities, Breinholt et al.[36] reported that one of the active constituents in garlic, diallyl sulfide, is a potent inhibitor of the phase I enzyme CYP2E1, thus resulted in the reduction in the ROS level, Khan *et al.*[37] revealed the protective effect of rutin on CYP2E1. Flavonoids can interact with CYP450 enzymes by

inhibition biosynthesis of the enzymes or the catalytic activity. It may occur at a transcriptional or gene translational level as well as direct modulation of CYP450 activity.[38] The inhibition effect seems to be related to the flavonoid structure where the number and position of hydroxyl-groups determine the binding mechanism to the enzyme and thereby the level and mode of inhibition. According to the literature, flavonoids possessing many hydroxyl groups are more potent CYP450 inhibitors *in vitro*.[32,39] HD which is the product of cytochrome P450 metabolizing activity induce oxidative stress.[31] The cytotoxicity mediated by CYP 2E1 has been found to be closely related to its oxy-radical producing ability, leading to lipid peroxidation,[9,10] the present study oxidative stress in HD-treated rats was evidenced by the increased accumulation of lipid peroxidation. Free radicals mediated peroxidation of membrane lipids might have resulted in loss of membrane integrity and membrane damage, that may resulted in ALT, AST enzymes release into circulation.[40] One of the consequences of oxidative stress is lipid peroxidation or ROS formation induced by hydrazine have been confirmed earlier by the findings of Sarich *et al*.[5] metabolism of HD and its derivatives may induce cellular toxicity either by covalent binding to tissue macromolecules or by initiating an autoxidative process such as lipid peroxidation *in vivo*.[41]

The present study shows that the changes in lipid peroxidation in rats treated with hydrazine were also accompanied by a concomitant decrease of both CAT and SOD activity. CAT and SOD which are mutually supporting front line antioxidant enzymes, were inactivated by the presence of lipid peroxides.[42,43] Inhibition of these enzymes results in enhanced sensitivity to free radical induced cellular damage.[40] SOD plays an important role in scavenging toxic intermediates of incomplete oxidation, which is regarded as one of the most primary antioxidant enzymes in the enzymatic defense mechanism.[44] Both CAT and SOD, the first line of defense against oxidative damage, may scavenge the superoxide anion radical ($O_2^{\bullet}$) and may decompose hydrogen peroxide (H_2O_2) that result in the tissue protection from highly reactive hydroxyl.[42] Both CAT and SOD activity was significantly elevated in the liver of mice administered with BEHUL when compared with Vit E treated groups. In the present study the leakage of large quantities of enzymes into the blood stream was associated with centrilobular necrosis in parenchymal hepatic, these histopathological observations concurred with biochemistry analysis mentioned in hydrazine-rats. The hepatoprotective effect of *Heliotropium undulatum* leaves extract that was associated with lowering the CYP2E1 level, with preservation of CAT and SOD activity and with prevention hepatic parenchyma, may be due to presence of phyto-constituents like polyphenols. Pervious phytochemical studies

showed the richness of *Heliotropium* species in various secondary metabolites such as triterpenoids,[45] flavonoids.[19] Flavonoids and other phenolic compounds have been reported as antioxidant.[46,47] The antioxidant activity of phenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers.[48,49,50]

CONCLUSION

Our finding suggest that *Heliotropium undulatum* may protect liver from hydrazine toxicity by inhibiting the CYP2E1 activity, by enhancing the SOD and CAT activity and by ameliorating the altered parenchymal hepatic. These effect may be due to presence of phytochemical constituents like polyphenols.

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