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Hepatoprotective mechanisms of *Ageratum conyzoides* L. on oxidative damage induced by acetaminophen in Wistar rats

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ABSTRACT

Background and objectives: Drug induced hepatotoxicity is a major health issue concern by drug regulatory agencies, pharmaceutical industry and health care professionals. Present study was aimed to investigate the hepatoprotective mechanisms of n-hexane and acetone extract of *Ageratum conyzoides* on oxidative damage induced by acetaminophen (APAP) in Wistar rats.

Materials and methods: 42 Wistar rats were randomly divided into seven groups. Group I and II receive distilled water, CMC respectively. Group III was fed with standard drug silymarin, group IV and V received plant extracts only whereas group VI and VII received pretreatment with acetone and n-hexane extracts for seven days respectively and APAP was administered on the 5th day. Samples were collected on 48 h of post APAP administration and analysis was done using standard protocols.

Results: Significant ($p < 0.05$) increased in MDA, G6PD and GST and significant ($p < 0.05$) decrease in SOD, GSH, total thiols in liver tissue was observed in APAP exposed group as compared to control. The pre-exposure of acetone and n-hexane extracts of *A. conyzoides* fallowed by APAP exposure significantly ($p < 0.05$) reduce activities of G6PD, GST and MDA levels as compare to APAP exposed group, whereas total thiols and GSH levels are restored only in n-hexane extract of *A. conyzoides*.

Conclusion: Observations of present study suggested that pre-exposure of n-hexane extract of *A. conyzoides* restore the levels of total thiols, GSH and GST activity which may be responsible for reducing oxidative damage induced by APAP administration.

1. Introduction

Acetaminophen (APAP) is most widely used in therapeutics and potent hepatotoxic agent at higher doses.[1] At normal doses APAP is metabolized in hepatocytes via glucuronide and sulfate conjugations and conjugated products are more water soluble which facilitate their renal excretion. A small portion of APAP metabolized through oxidative reactions catalyzed by CYP_{450} enzymes resulting in production of a highly reactive intermediate i.e. N-acetyl-p-benzo-quinoneimine (NAPQI). NAPQI is normally rapidly conjugated with reduced glutathione (GSH) and is excreted as the cysteinyl conjugate or the corresponding mercapturic acid. During overdosing excess production of NAPQI are responsible for the depletion of GSH levels in hepatocytes leading to interaction of NAPQI to other macromolecules especially cysteine groups on different enzymatic and transporter protein.[2] Most of these alterations started from mitochondrial proteins due to excessive oxidative reactions leading to loss of energy production and disruption of ionic transport across the cell membrane.[3]

Total thiols (protein and non-protein thiols) play a crucial role in ameliorating lipid peroxidative effects of reactive intermediates due to presence of disulfide linkages (−SH groups).[4] Hepatic protein thiols (Pr−SHs) and GSH (non-protein thiol) contents are responsible for the scavenging or neutralizing the NAPQI thus reduced cellular damage. The cytotoxic effects of APAP have been attributed to depletion of Pr−SHs.[5] The covalent binding of NAPQI accounts for only a part of Pr−SHs depletion induced by APAP.[6] Evidence suggests that increased lipid peroxidation due to increased generation of reactive oxygen species (ROS) and depletion of total thiols is responsible of APAP induced hepatic damage.[7,8] Glucose-6-phosphate dehydrogenase (G6PD), a rate limiting enzyme catalyzing the first step of the pentose phosphate pathway is the primary source of reduced nicotinamide adenine dinucleotide phosphate (NADPH). NADPH principal intracellular reductant, maintain GSH levels, protect cell membrane proteins and architecture of the cell.[9] Silymarin, a potent hepatoprotective agent is a

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mixture of polyphenolic compounds commonly available in different pharmaceutical preparations.[10]

Traditionally, *Ageratum conyzoides* L. (Compositae) has been used in various parts of the world like Africa, Asia and South America as folk medicine as antispasmodic, hemostatic, anti-asthmatic and insecticide activities and for the treatment of wounds and *Staphylococcus aureus* infections.[11,12] Phytochemical investigations whole plant extract of *A. conyzoides* shows presence of high phenolic and flavonoids contents in, exploration of protections against disorders associated with the excess free radical or ROS.[13] The protection effects of the extract against gamma radiation may by scavenging reactive free radicals of oxygen molecules due to appreciable antioxidant property.[14] Thus the present study was aimed to investigate the protective mechanisms of different extracts of *A. conyzoides* in APAP induced hepatotoxicity in Wistar rats.

2. Material and methods

2.1. Collection and preparation of plant extracts

Whole plant of *A. conyzoides* L were collected in from the R.S. Pura area, Jammu. The plant was identified by the curator of the Herbarium, Botany Department, University of Jammu. The fresh plant materials collected were air-dried for a period of two weeks and were pre-crushed in a mortar and later pulverized into fine powder using electric blender. The powder will be sieved through a mesh (2 mm mesh size). Sieved powder of plant was used for preparation of acetone and n-hexane extracts. Extract were prepared by adding 10 g of the plant powder in 200 ml of solvent in extract container of soxhlet extractor equipment. Extraction process was done for 24 h at 45–65 °C & semisolid viscous masses were dried at 40 °C in rotary evaporator and thereafter stored in airtight containers at refrigerated conditions −20 °C till until further uses. The extract and the reference drug were suspended in carboxy methyl cellulose (CMC) (0.5%) in distilled water separately and used for *in vivo* investigations.

2.2. Drugs & chemicals

Acetaminophen and silymarin were obtained from Sigma Chemical Company (St. Louis MO, USA). All other chemicals used in the study were analytical grade obtained either from Hi Media (Mumbai) or SD-Fine Chemicals (Mumbai).

2.3. Experimental animals

The study was conducted on healthy Wistar rats of either sex weighing 150–250 g procured from Indian Institute of Integrative Medicine (CSIR Lab), Jammu. The animals were provided standard pelleted ration and drinking water *ad libitum*. All the animals were maintained under standard managemental conditions. A daily cycle of 12 h of light and 12 h of darkness was provided to animals. Prior to start of experiment, the rats were acclimatized in the laboratory conditions for a period of more than 3 weeks. All the experimental animals were kept under constant observation during entire period of study.

2.4. Experimental protocol

42 Wistar rats were randomly divided into seven groups with 6 rats in each group. The normal control (group I) will be receiving only distilled water for seven days, Group II receive carboxy methyl cellulose (0.5% CMC) 1 ml/rats/day and a single oral dose of acetaminophen (3 g/kg bw) on the fifth day of the administration. Group III was fed with standard drug silymarin 100 mg/kg bw orally daily for seven days and received APAP at 3 g/kg bw orally on the fifth day. Group IV and V received acetone and n-hexane extracts of *A. conyzoides* (200 mg/kg, bw, orally) for seven days respectively. Group VI and VII received pretreatment of the acetone and n-hexane extracts for seven days respectively and acetaminophen was administered on the fifth day of the seven days administration. At the end of the experiment (48 h after acetaminophen administration i.e. day 7) all rats will be sacrificed after cervical dislocation and liver tissue was collected in ice cooled phosphate buffer saline (PBS, PH 7.4).[15] The experimental protocol was approved by Institutional Ethics Committee.

2.5. Assay procedure

Collected tissue was homogenized by using Teflon coated glass homogenizer and 10% homogenate (W/V) was prepared. Homogenate was centrifuged at 5000 rpm for 15 min and clear supernatant was separated in clean test tubes and stored at −20 °C till further use. All enzymatic activities were carried out within 24hr of sample collection. Different parameters like activity of G6PD were determined as method described by Deutsch[16] and malondialdehyde (MDA) level as per the method described by the Ohkawa et al[17] The levels of GSH and total thiols were determined by the method described by Beutler et al[18] and Motchnik et al[19] respectively. Whereas activities of superoxide dismutase (SOD) and glutathione-S-transferase (GST) were determined as per the method described by Marklund and Marklund,[20] Habig et al[21] respectively.

2.6. Statistical analysis

The results were subjected to analysis of variance (ANOVA) in completely randomized design (CRD) with statistical significance at $p < 0.05$ being tested using the Duncan Multiple Range Test.

3. Results

The different parameters like MDA, total thiols, GSH levels and activities of G6PD, GST and SOD have been determined in liver homogenate to postulate the possible hepatoprotective mechanisms of *A. conyzoides*. Significant ($p < 0.05$) increased in MDA levels and activities of G6PD and GST was observed in APAP exposed group as compared to control. Whereas levels of GSH, total thiols and activities of SOD were significantly ($p < 0.05$) decreased in APAP exposed group as compared to control group rats (Tables 1 and 2). The pre-exposure of acetone and n-hexane extracts of *A. conyzoides* fallowed by APAP exposure significantly ($p < 0.05$) reduce activities of G6PD, GST and MDA levels as compare to APAP exposed group and values are non-significantly differ from control values. Similarly treatment with silymarin i.e. group III also significantly ($p < 0.05$) reduces G6PD activity and significant increased activity of SOD as compared to as compared to group II except in GST activity which doesn't restore the by administration of silymarin. Silymarin treatment significantly ($p < 0.05$) increased the total thiols, GSH and MDA levels as compared to APAP exposed group (Tables 1 and 2).

Similarly pre-exposure of n-hexane extract of *A. conyzoides* fallowed by APAP exposure causes significant ($p < 0.05$) increase in total thiols and GSH levels as compared to APAP treated group, however pre-exposure of acetone extract of *A. conyzoides* didn't restore the values reduced values of total thiols and GSH levels as compared to APAP treated group (Tables 1 and 2).

Table 1
Activities of Glucose-6-phosphate dehydrogenase (G6PD), glutathione-s-transferase (GST) and superoxide dismutase (SOD) in liver of control and experimental groups of wistar rats.

Group	G6PD (U/L)	GST (μmole of GSH-CDNB conj. formed/min/g protein	SOD (units/g protein)
I. Control	$3217.76^{a} \pm 58.64$	$10.49^{a} \pm 1.35$	$182.03^{a} \pm 6.37$
II. APAP	$8165.83^{c} \pm 726.67$	$33.64^{b} \pm 2.73$	$64.90^{c} \pm 4.03$
III. Silymarin + APAP	$3561.80^{a,b} \pm 431.69$	$38.00^{b} \pm 1.78$	$146.95^{b} \pm 8.54$
IV. Acetone ext of AC	$3177.29^{a} \pm 440.14$	$40.07^{b} \pm 4.43$	$132.95^{b} \pm 7.38$
V. n-Hexane ext of AC	$4492.73^{a,b} \pm 754.73$	$54.95^{b} \pm 5.68$	$128.21^{b} \pm 9.75$
VI. Acetone ext of AC + PAP	$4867.12^{b} \pm 170.55$	$18.60^{a} \pm 3.19$	$153.65^{b} \pm 5.07$
VII. n-Hexane ext AC + APAP	$3551.68^{a,b} \pm 191.15$	$11.40^{a} \pm 1.41$	$172.54^{a} \pm 7.81$

Values are given as mean ± SE of six animals in each groups. Values having different superscripts (a, b, c) in a column are statistically differ significantly $p < 0.05$. (APAP – Acetaminophen, AC – *Ageratum conyzoides*).

4. Discussion

Liver, an important visceral organ of body responsible for the metabolism of the endogenous and exogenous molecules and facilitate their excretion through body. Chemical induced hepatotoxicity is the major concern of health care professionals and regulatory agencies. Various studies have been shown that different extracts of *A. conyzoides* protect different biochemical and stress parameters in acetaminophen induced hepatotoxicity in animals and are safer for oral administration upto 2 g/kg.[21–23] Increased MDA levels in hepatocytes indicate the excess alleviation of reactive metabolite NAPQI by cytochrome P450 enzyme produced during the metabolism of APAP.[24] Increased reactive metabolites attack on biological membranes resulting damage to membrane as indicated in increased MDA level in present study. Initially NAPQI interacts with cellular GSH to resulting in inactivation of active metabolites. However, high doses of APAP limit the ability of GSH to detoxify NAPQI, and result in the consumption of liver GSH stores as well as other thiols.[2] Non-protein thiols (GSH) and protein thiols (Pr–SHs) are low molecular weight molecules such as reduced glutathione and cysteine, and protein bound sulfhydryl group protecting cellular functions against oxidative stress. Liu et al[25] suggested that the decrease in liver GSH could be the result of decreased synthesis or increased utilization of GSH in scavenging ROS produced during tissue injury. Increased ROS levels, resulting significant increased in aldehydic products of lipid peroxidation due to probably decreased hepatic GSH content. In the present study, the elevated GSH level in hepatocytes was observed in n-hexane exposed rats.

Table 2
Levels of reduced glutathione (GSH), malondialdehyde (MDA) total thiols, in liver of control and experimental groups of Wistar rats.

Group	GSH (*n* moles/ml)	Total thiols (mM)	MDA (*n* moles of MDA produced/gm of protein/h)
I. Control	$315.12^{a} \pm 29.93$	$27.04^{a} \pm 1.14$	$9.58^{a} \pm 0.59$
II. APAP	$187.42^{c} \pm 15.62$	$19.24^{c} \pm 0.82$	$23.65^{c} \pm 0.72$
III. Silymarin + APAP	$213.45^{b} \pm 17.31$	$25.95^{a} \pm 0.72$	$17.51^{b} \pm 2.80$
IV. Acetone Ext of AC	$163.46^{c} \pm 16.03$	$26.61^{a} \pm 0.43$	$15.82^{b} \pm 1.10$
V. n-hexane Ext of AC	$86.33^{c} \pm 5.66$	$29.63^{a} \pm 0.41$	$15.99^{b} \pm 1.95$
VI. Acetone Ext of AC + APAP	$80.93^{c} \pm 3.54$	$17.80^{c} \pm 0.73$	$11.24^{a} \pm 0.50$
VII. n-hexane Ext AC + APAP	$234.70^{b} \pm 18.41$	$21.65^{b} \pm 1.99$	$10.08^{a} \pm 0.69$

Values are given as mean ± SE of six animals in each groups. Values having different superscripts (a, b, c) in a column are statistically differ significantly $p < 0.05$. (APAP – Acetaminophen, AC – *Ageratum conyzoides*).

The loss of Pr–SHs leads to initiation of lethal injury caused by acute oxidative changes due to excessive NAPQI radicals. Such depletion is presumed to be a direct oxidation of thiol groups of amino acids with the formation of protein–protein disulphides.[26] APAP exposure in present study significantly reduced total thiols and GSH levels. This may be due to the covalent binding of NAPQI accounts for only a part of Pr–SHs depletion induced by paracetamol, while the remainder is said to result from direct oxidation of Pr–SHs, presumably by NAPQI itself.[5,6] Administration of n-hexane extract of *A. conyzoides* significant increase in level of total thiols in APAP treated animals which may be partially responsible for the protective mechanism by scavenging reactive metabolites NAPQI of APAP. Phenolic compounds present in the extract may implicit to induce the cellular antioxidant system by approximately increasing cellular glutathione concentration. Further, flavonoids have modulatory activity on γ-glutamylcysteine synthase in both cellular antioxidant defenses and detoxification of xenobiotics.[27]

NADPH is the principal intracellular reductant produced during pentose phosphate pathway and serves as a cofactor for glutathione reductase responsible for the production reduced glutathione (GSH) form oxidized glutathione. NADPH is also cofactor for catalase to maintain enzyme in an active state.[28,29] G6PD is a rate limiting enzyme of this pathway, hence normal activity of G6PD is essential for the protection against and repairs of oxidative damage maintains, cell membrane proteins stable and architecture of the cell.[9] Increased activity in present study during the APAP exposure may be compensatory mechanism of body to produces more GSH to neutralize the excess reactive intermediates. Pre-exposure of n-hexane extract of *A. conyzoides* fallowed by APAP restore the activity of G6PD which helps in protection against oxidative damage.

GST catalyzes the conjugation GSH via reaction of the sulphydryl group with electrophilic centers in a wide variety of substrates. The induction of GST in present study could be a defensive mechanism to counter-balance the oxidative insult by utilizing endogenous GSH thus helps in excretion of intermediate metabolites from the cells and protects the tissues from oxidative stress. In the present study reduce activity of SOD in liver have been observed in APAP treated rats. SOD is major enzymes responsible for scavenging of O_2^- and other superoxide radicals to H_2O_2 and H_2O. SOD is the first line of defense against the action of. Superoxide radicals are produced in mitochondria and endoplasmic reticulum as a result of auto-oxidation of ETS (electron transport system). Several authors reported the decrease in SOD activities in paracetamol-treated animals.[30,31] These superoxide radical are produced during monovalent reduction of oxygen is toxic to living tissues. Decreased SOD activity in APAP treated group in present study is suggestive of excess free radical generation or reactive metabolite formation which impairs natural defense mechanism of this enzyme. Restoration of SOD activity in pre-exposed group helps in protecting the oxidative damage induced by APAP. Phytochemical investigations n-hexane and acetone extracts of *A. conyzoides* shows the presence of flavonoids, phenolic, tannin and non-tannin contents in higher concentrations.[32,33] Monoterpenes and sesquiterpenes are also present in the high concentrations in leaf and stem of the *A. conyzoides* and the major sesquiterpenes are beta-caryophyllene have been reported to reduces free radical generation and pro-inflammatory proteins production and induces phase II of biotransformation reactions.[34,35]

In conclusions, present study show that APAP administration significantly increased in MDA, G6PD and GST and significant decrease in SOD, GSH, total thiols in hepatic tissues of wistar rats indicative of oxidative damage in hepatocytes. The pre-exposure of n-hexane extracts of *A. conyzoides* reduces the damage induced by APAP administration significantly which may be due to presence of higher phenolic and flavonoids contents as compared to acetone extract.

Conflicts of interest

All authors have none to declare.

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