Australian Journal of Basic and Applied Sciences, 7(7): 22-27, 2013
ISSN 1991-8178

Protective effects of *Ageratum conyzoides L.* on erythrocytes antioxidant status induced by acetaminophen toxicity in wistar rats

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Abstract: Erythrocytes are more vulnerable to oxidative stress among eukaryotic cells because it contains no nucleus and cytoplasmic organelles with high haemoglobin concentration. Present study was aimed to investigate the antioxidant status of the erythrocytes during hepatotoxicity and protective effects of extracts of *Ageratum conyzoides* in wistar rats. Antioxidant parameters like total thiols, GSH levels, activities of G6PD, GST and SOD and oxidative damage indicator MDA level have been determined to access the antioxidant status during hepatotoxicity induced by acetaminophen (APAP) and its protection by acetone and n-hexane extracts. Significant ($p<0.05$) increased in MDA, GST activity and significantly ($p<0.05$) decreased GSH, total thiols and activities of SOD & G6PD was observed in APAP exposed group as compared to control. The pre-treatment with acetone and n-hexane extracts of *A. conyzoides* fallowed by APAP exposure significantly ($p<0.05$) increased activities of G6PD and significantly ($p<0.05$) reduced activity of GST and MDA levels as compare to APAP exposed group. Protective effects of extracts of *A. conyzoides* may be due to presence of high concentration of phenolic; flavonoids and beta-caryophyllene contents, these agents have been reported to reduce free radical generation, pro-inflammatory proteins production and induces phase II biotransformation reactions. Observation suggested that APAP exposure at high dose induces oxidative stress in erythrocytes and n-hexane extract is better in combating the oxidative stress of erythrocytes as compare to acetone extract of *A. conyzoides.*

Key words: Erythrocytes; acetaminophen; hepatotoxicity**;** *A. conyzoides*

INTRODUCTION

Cellular oxidative reactions occur continuously to provide energy, destroy invading microorganisms and biotransformation for easy elimination of endogenous/exogenous molecules. During these reactions different types of reactive oxygen species (ROS) or reactive intermediate metabolites like hydroxyl radical (OH^-), superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), nitric oxide (NO), etc are commonly formed (Valko *et al.,* 2007). To counteract the damaging effects of these ROS/intermediate metabolites mammalian cells are endowed with extensive scavenging mechanisms. It consists of enzymatic viz. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione-s-transferase (GST) and non enzymatic components like reduced glutathione (GSH), total thiols, ascorbic acid, vitamin E, selenium, carotene, etc to combat the oxidative stress in mammalian cells (Sen *et al.,* 2006; Verma *et al.,* 2011). Oxidative stress may result from over production of precursors for ROS and/or decreased efficiency of inhibitory and scavenging mechanisms of the body. The stress may then be amplified and propagated by an autocatalytic cycle of metabolic stress, tissue damage and cell death, leading to further increase in free radical production and depletion of scavenging system of body.

Erythrocytes are more vulnerable to oxidative stress among eukaryotic cells because it contains no nucleus and cytoplasmic organelles with high haemoglobin concentration. GSH maintains normal structure, elasticity and integrity of erythrocytes and sustain hemoglobin in ferrous state that is essential for carrying oxygen (Jain *et al.*, 2003). For maintenance of intracellular GSH pools, GSH continuously regenerated form oxidized GSSG with the help of reduced nicotinamide adenine dineucleotide phosphate (NADPH). Glucose-6-phosphate dehydrogenase (G6PD) is a rate limiting enzyme catalyzing the first step of the pentose phosphate pathway, which is primary source of NADPH (Zhang *et al.,* 2000; Jain *et al.,* 2003). Erythrocytes of G6PD-deficient individuals are particularly sensitive to oxidative damage, since these cells cannot generate a sufficient amount of NADPH for the regeneration of GSH from GSSG (Liu *et al.,* 1994; Huang *et al.,* 1998). Decreases in GSH concentrations and increases of intracellular oxidants impair the ability to remove the ROS and cause the oxidation of protein thiols (-SH groups) in hemoglobin and/or membrane proteins. The oxidation of hemoglobin

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to methemoglobin is then followed by the formation of Heinz bodies, which leads to premature erythrocyte destruction and hemolytic anemia (Arese and Flora, 1990; Huang *et al.,* 1998). Excess electrophilic intermediate molecules get conjugated with GSH via reaction catalyzed by GST and superoxide radicals are scavenged by SOD to combat the free radical induced damage in erythrocytes (Felix *et al.,* 2003; Verma *et al.,* 2011). If scavenging system fails to scavenge these radicals than these radicals alter the enzyme/receptor proteins by cross-linking and fragmentation of protein strands, oxidation of amino acids like cysteine, methionine in cells, peroxidation of polyunsaturated fatty acid of phospholipids in membrane and fracturing of single strand DNA leading to abnormal cellular behaviour (Sen *et al.,* 2006).

Natural products of plant origin gained importance and popularity in recent years because these preparations are rich sources for naturally occurring antioxidants especially phenolic and flavonoids contents, in-spite of their safety and cost effectiveness (Hussein, 2009; Hatem *et al.,* 2010). Flavonoids are large family of polyphenolic components were found to reduce blood lipid, glucose and enhance immunity in man and animals (Atoui *et al.,* 2003). These components have appreciable antioxidant property thus the exploration of possible protections against disorders associated with the excess free radical or ROS (Atoui *et al.,* 2003; Sultana *et al.,* 2012). *Ageratum conyzoides* L. (Compositae) has been used in various parts of the world like Africa, Asia and South America as folk medicine (Gonzalez *et al.,* 1991). Phytochemical investigations on *A. conyzoides* have identified a number of secondary metabolites such us flavonoids, monoterpenes, sesquiterpenes, coumarins etc. Various investigations have verified its analgesic effect in rats (Menut *et al.,* 1993), antioxidative effect (Jagetia *et al.,* 2003), hepatoprotective effects (Ita *et al.,* 2009) and as a blood booster (Ita *et al.,* 2007). Liver is the vital organ of biotransformation of exogenous and endogenous molecules in mammals through oxidative and conjugation reactions. Any abnormality in liver functions or deficiency/consumption of inputs required for reactions adversely effects the metabolic reactions leading to excess production ROS/free radicals. Chronic hepatic diseases stand as one of the foremost health troubles worldwide, with liver cirrhosis and drug induced liver injury leading to death in developing countries (Gillette, 2000; Lewerenz *et al.,* 2003). Therefore present study was aimed to investigate the alterations in erythrocytes antioxidant system and their protection by different extracts of *Ageratum conyzoides* during hepatotoxicity experimentally induced by acetaminophen (APAP) in wistar rats.

MATERIAL AND METHODS

Collection and Preparation of plant extracts:

Whole plant of *Ageratum conyzoides L* (AC) were collected in from the R S Pura area, Jammu. The plant was identified by the curator of the Herbarium, Botanical 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 (2mm mesh size). Sieved powder of plant was used for preparation of acetone and n-hexane extracts. Extract were prepared by adding 10 gm of the plant powder in 200 ml of solvent in extract container of soxhlet extractor equipment. Extraction process was done for 24 hrs at 45-65^{0}C & semisolid viscous masses were dried at 40°C in rotary evaporator and thereafter stored in airtight containers at refrigerated conditions -20^{0}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.

Drugs & Chemicals:

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

Experimental animals:

The study was conducted on apparently healthy wistar rats of either sex weighing 150 to 250g 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.

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 methylcellulose (0.5% CMC) 1ml/rats/day and a single oral dose of acetaminophen (3g/kg bw) on the fifth day of the administration. Group-III was fed with standard drug silymarin 100mg/kg bw orally daily for seven days and received

acetaminophen at 3g/kg BW orally on the fifth day. Group IV and V received acetone and n-hexane extracts of *A. conyzoides* (200mg/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 hours after acetaminophen administration i.e. day 7) blood from all the animals were collected from infra orbital fossa in a clean, sterilized test tubes containing heparin (John *et al.,* 2011). The experimental protocol was approved by institutional ethics committee.

Assay Procedure:

The collected blood samples were centrifuged at 3000 rpm for 15 min and the plasma was harvested in clean sterile glass test tubes. The blood glutathione (GSH) and total thiols was determined by the method of Beutler *et al.,* (1963) and Motchnik *et al.,* (1994) respectively. Erythrocytes sediment obtained after harvesting of plasma was washed thrice with normal saline solution in the ratio of 1:1 on v/v basis and normal saline was gently but thoroughly mixed with RBC. The diluted erythrocytes were centrifuged for 10 min. After centrifugation the supernatant was discarded along with buffy coat and again process was repeated. After final washing 1 and 33 per cent hemolysate was prepared in phosphate buffer solution (PBS, pH 7.4). The 1 per cent hemolysate was used for the estimation of G6PD, SOD and GST as for the method described by Stocks and Dormandy (1791), Marklund and Marklund (1974) and Habig *et al.,* (1974) respectively. Whereas 33 per cent hemolysate was used for estimation of MDA levels indicative of lipid peroxidation as per the method described by Ohkawa *et al.,* (1979).

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.

Results:

The different parameters like MDA, total thiols, GSH levels and activities of G6PD, GST and SOD have been determined to access the antioxidant status in blood during hepatotoxicity induced by APAP and its protection by acetone and n-hexane extracts of *A. conyzoides*. Values are presented in table 1 and 2. Significant ($p<0.05$) increased in MDA levels and GST was observed in APAP exposed group as compared to control. Whereas levels of GSH, total thiols and activities of SOD & G6PD were significantly ($p<0.05$) decreased in APAP exposed group as compared to control group rats.

The pre-treatment with acetone and n-hexane extracts of *A. conyzoides* fallowed by APAP exposure significantly ($p<0.05$) increased activities of G6PD and significantly ($p<0.05$) reduced activity of GST and MDA levels as compare to APAP exposed group and values are non-significantly differ from control values (Table 1). Whereas levels of GSH, total thiols and SOD were not restored after pre-treatment with acetone and n-hexane extracts of *A. conyzoides* except total thiols levels which was restored in n-hexane extract treated group rats (Table 2).

Similarly treatment with silymarin i.e. in group III also significantly ($p<0.05$) increased G6PD, SOD activities and significantly reduced GST activity as compared to APAP exposed group i.e. group II. Silymarin treatment significantly ($p<0.05$) increased the total thiols, GSH and reduces MDA levels as compared to APAP exposed group rats (Table 1 & 2).

Table 1: Protective effects of acetone and n-hexane extracts of *Ageratum conyzoides* on activities the activities of superoxide dismutase (SOD), Glucose-6-phosphate dehydrogenase (G6PD), glutathione-s-transferase (GST) in erythrocyte lysate of control and experimental groups of rats.

Group	G6PD (U/L)	GST (µmole of GSH-CDNB conj. formed/ min/gm protein	SOD (Units/gm of Protein)
I. Control	$455.34^{a} \pm 43.63$	$1.05^{a} \pm 0.14$	$73.03^{a} \pm 3.37$
II. APAP	$303.56^{b} \pm 22.16$	$2.50^{b} \pm 0.12$	$54.90^{b} \pm 4.03$
III. Silymarin +APAP	$394.28^{a} \pm 41.47$	$1.16^{a} \pm 0.48$	$86.95^{a} \pm 7.38$
IV. Acetone Ext of AC	$718.43^{c} \pm 42.68$	$3.10^{c} \pm 0.19$	$106.95^{c} \pm 7.38$
V. n-hexane Ext of AC	$516.06^{a} \pm 62.20$	$3.53^{c} \pm 0.48$	$121.28^{c} \pm 9.75$
VI. Acetone Ext of AC + APAP	$526.18^{a} \pm 33.86$	$1.86^{a} \pm 0.32$	$123.65^{c} \pm 5.07$
VII. n-hexane Ext AC + APAP	$445.23^{a} \pm 76.24$	$1.14^{a} \pm 0.14$	$122.54^{c} \pm 7.47$

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, CDNB - 1-chloro-2, 4 dinitrobenzene, AC - *Ageratum conyzoides*)

Table 2: Protective effects of acetone and n-hexane extracts of *Ageratum conyzoides* on the levels of reduced glutathione (GSH), plasma total thiols and malondialdehyde (MDA) in blood of control and experimental groups of rats.

Group	GSH (n moles/ml)	Total thiols (mM)	MDA (nmoles of MDA produced / gm of protein/hr)
I. Control	72.27 ^{a} ± 6.27	3.93 ^{a} ± 0.41	2.14 ^{a} ± 0.42
II. APAP	40.05 ^{b} ± 3.80	2.42 ^{b} ± 0.12	6.24 ^{b} ± 1.37
III. Silymarin +APAP	62.63 ^{a} ± 3.17	3.83 ^{a} ± 0.30	3.34 ^{a} ± 0.70
IV. Acetone Ext of AC	148.35 ^{c} ± 9.32	1.83 ^{c} ± 0.38	6.28 ^{b} ± 1.39
V. n-hexane Ext of AC	133.58 ^{c} ± 8.79	2.33 ^{b} ± 0.18	9.41 ^{b} ± 0.62
VI. Acetone Ext of AC + APAP	53.18 ^{b} ± 6.40	2.72 ^{b} ± 0.34	2.35 ^{a} ± 0.36
VII. n-hexane Ext AC + APAP	47.29 ^{b} ± 8.37	3.65 ^{a} ± 0.69	2.16 ^{a} ± 0.52

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*)

Discussion:

APAP is one of the most commonly and widely used hepatotoxic drugs, is safe at therapeutic doses, but causes liver failure in overdoses due to excess production of highly reactive intermediate, acetyl-p-benzo-quinoneimine (NAPQI) through oxidative reactions (Mitchell *et al.,* 1973). NAPQI depleted GSH by as much as 90% and covalently binds to cysteine groups on protein, forming acetaminophen-protein adducts resulting in disturbance in functional and enzymatic proteins lead to oxidative stress and cell death. High dose of APAP is commonly used for the induction of hepatotoxicity in experimental animals (Nelson, 1990; Lewerenz *et al.,* 2003).

Alterations in G6PD, SOD and GST activities:

G6PD is a rate limiting enzyme catalyzing the first step of the pentose phosphate pathway which generates reducing power in the cytoplasm in the form of reduced nicotinamide adenine dineucleotide phosphate (NADPH). NADPH is require to keep the glutathione in the reduced form (GSH), which maintains cell membrane proteins in stable reduced form thus maintain the architecture of the cell (Zhang *et al.,* 2000). Inhibition of G6PD activity decreases level of NADPH, a critical cofactor for catalase and glutathione reductase that is essential for the protection against and repairs of oxidative damage (Gaskin *et al.,* 2001, Felix *et al.,* 2003). During peroxides oxidation reaction GSH is oxidized and glutathione reductase regenerates GSH by using of NADPH. NADPH is oxidized and G6PD is required for producing reduced NADPH again. Decreased activity of G6PD in APAP exposed group leads to decreased intracellular NADPH levels and makes cell very sensitive to oxidant damage. Superoxide free radical are generated during monovalent reduction of oxygen is toxic to biological systems. The major enzyme protector against superoxide in the body is superoxide dismutase which disproportionates the superoxide to hydrogen peroxide and oxygen. 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. Several authors have been reported the decreased SOD activity in paracetamol-treated animals (O'Brien *et al.,* 2000; Ahmed and Khater, 2001). Similarly GSTs are a major family of detoxification enzymes catalyzes the conjugation of the tripeptide glutathione to electrophilic centre of lipophilic compounds, thereby increasing their solubility and aiding excretion from the cell and protect the tissues from oxidative stress (Hayes and Pulford, 1995).

Alterations in total thiols and GSH levels:

The –SH groups (total thiols) a prominent role in antioxidant reactions, and also in reactions of catalysis, regulation, electron transport and those preserving the correct structure of proteins (Rokutan *et al.,* 1994) and ameliorate the lipid peroxidative effects of ROS (Prakash *et al.,* 2009). Total thiols include non-protein thiols (predominantly GSH) and protein thiols (Pr-SH- cysteine, and protein bound sulfhydryl group) are the primary site for attack of ROS or free radicals. In human alteration in level of plasma LMW thiols are being Alteration in the redox status of thiols (change in the thiol: disulfide ratio) significantly influences the structure and function of cellular and extracellular proteins (Mansoor *et al.,* 1992). In present study reduction in total thiols indicate oxidative stress during the exposure of APAP whereas level was restored during n-hexane treatment of the *A. conyzoides* which may be due to presence of excess phenolic and flavonoids content in n-hexane extract as compared to acetone extract of *A. conyzoides* (Sultana *et al.,* 2012). These flavonoids and phenolic compounds directly scavenge ROS/free radicals and also increase the antioxidant defense of the body to remove these molecules either reduced oxidative reactions or increased conjugation reactions (Kamboj and Saluja, 2008). 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 biotransformation reactions (Gill *et al.,* 1978; Ekundayo *et al.,* 1988). Similarly, n-hexane fraction of *Inula helenium* showed the highest quinine reductase and induced

glutathione-s-transferase in a dose-dependent manner due to presence of high concentration of sesquiterpenes (Lim *et al.,* 2007).

Alterations in MDA levels:

Excess reactive metabolites N-acetyl-p-benzo-quinoneimine (NAPQI) produced during oxidative reaction of APAP covalently binds to cysteine groups on protein, forming APAP-protein adducts (Mitchell *et al.,* 1973). Primary cellular targets have been postulated to be mitochondrial proteins, with resulting loss of energy production, as well as proteins involved in cellular ion control (Nelson *et al.,* 1990). If scavenging system fails to scavenge these radicals than these radicals attack the polyunsaturated fatty acid (PUFA) chain of phospholipids of biological membrane (Chow and Tappel, 1972) and free radical-induced lipid peroxidation serves to propagate and amplify oxidant-mediated damage resulting in deterioration of biological membranes (Sen *et al.,* 2006). In present study increased MDA level indicate the increased damage to cellular membrane on exposure to toxicant and similar results are also reported on exposure with fluoride and pesticides like cypermethrin, deltamethrin, etc (Kant *et al.,* 2009; Raina *et al.,* 2010).

In conclusion, present study observation suggested APAP exposure at high dose induces oxidative stress in erythrocytes and n-hexane extract is better in combating the oxidative stress of erythrocyte as compare to acetone extract of *A. conyzoides.*

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