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News & Views

Adulteration of Mustard Cooking Oil with Argemone Oil: Do Indian Food Regulatory Policies and Antioxidant Therapy Both Need Revisitation?

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ABSTRACT

Consumption of adulterated mustard oil (*Brassica nigra*) with argemone oil (*Argemone mexicana*) even for a short duration leads to a clinical condition referred as epidemic dropsy. In humans, argemone oil contained in adulterated mustard oil causes oxidative stress and death of red blood cells via met-hemoglobin formation by altering pyridine nucleotide(s) and glutathione redox potential. Argemone oil contamination poses a serious threat to human health and should be checked by appropriate regulatory measures. Antioxidant therapy provides symptomatic relief and should be seriously considered for therapeutic interventions against argemone oil toxicity. *Antioxid. Redox Signal.* 9, 515–525.

CONSUMPTION OF ADULTERATED EDIBLE MUSTARD OIL WITH ARGEMONE OIL CAUSES EPIDEMIC DROPSY IN HUMANS

Consumption of adulterated mustard oil with argemone oil even for a short duration leads to a clinical condition referred as epidemic dropsy (8). The main symptoms of dropsy include nausea, vomiting, diarrhea, anorexia, dyspnea, palpitation, hyperpigmentation of body parts, burning sensation of eyes, bilateral pitting edema of lower limbs, erythema, breathlessness, tachycardia, hepatomegaly, crepitations in the lungs, and gallop rhythm (7). In severe cases, glaucoma and even death due to cardiac and respiratory failure have been reported (7, 8).

PREVALENCE OF EPIDEMIC DROPSY

A number of epidemic dropsy outbreaks have been reported in different countries, including Burma (6), Fiji Island (12), Nepal (21), and South Africa (43). From time to time a number of sporadic cases have been reported in different states of India, including Andhra Pradesh (24), Bihar (27), Delhi (41), Maharashtra (47), Madhya Pradesh (37), Rajasthan (14), Uttar Pradesh (11), and West Bengal (35). But the epidemic at New Delhi, India, in 1998 was the largest so far, affecting >3,000 people while >60 lost their lives (11).

ARGEMONE OIL/SANGUINARINE CAUSES GENOTOXICITY AND CARCINOGENICITY

Sanguinarine and dihydrosanguinarine, the interconvertible alkaloids, are the toxic etiological agents of argemone oil (38). Sanguinarine has been used in toothpastes and oral rinse products in Europe and the United States (18, 25). However, use of sanguinarine for human clinical applications is controversial, as long-term use of sanguinarine in toothpastes has been linked with the development of a premalignant condition, oral leukoplakia (17). The electrophilic nature of iminium group in sanguinarine is responsible for binding to DNA by interaction with GC rich regions (29, 44). Our recent studies suggest that argemone oil and isolated sanguinarine

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alkaloid cause DNA damage in liver, bone marrow, and blood cells in mice (1, 2). Further, studies have shown that argemone oil and sanguinarine alkaloid have carcinogenic potential in a two-stage mouse skin tumor protocol and has been implicated to cause DNA damage in the blood of dropsy patients (10).

ARGEMONE OIL/SANGUINARINE CAUSES OXIDATIVE STRESS

Normal cells contain a specific antioxidant system to protect against oxidative damage caused by reactive oxygen species (ROS). If the antioxidant system may not be able to scavenge the production of ROS, a shift is formed between the pro- and antioxidant balance, normally referred to as oxidative stress (40). Sanguinarine has been shown to possess pro-oxidant properties *in vitro* towards the production of singlet oxygen and hydrogen peroxide (28, 46). Recent studies have shown decreased levels of antioxidants, including glutathione (GSH), α-tocopherol, and total antioxidant capacity (TAC), which in turn may cause oxidative damage of lipids, proteins, and DNA in dropsy patients (10, 11).

Glutathione system (GSH, GSSG), pyridine nucleotides (NAD, NADP, NADH, NADPH), and thiol groups are involved in a variety of free radical scavenging reactions, and their redox potential plays an important role in protecting hemoglobin and erythrocyte membranes (23). Hence, there is a need to investigate GSH and pyridine nucleotide redox potentials in the red blood cells (RBC) of dropsy patients of an outbreak at Lucknow and Patna cities of India.

STATUS OF EPIDEMIC DROPSY PATIENTS OF THE PRESENT OUTBREAK

The dropsy patients in Lucknow and Patna had pedal edema, erythema, breathlessness, pain in lower limbs, diarrhea, fever along with enhanced jugular venous pressure, and hepatomegaly. The patients in Lucknow were diagnosed early for epidemic dropsy, while diagnosis of Patna patients was confirmed only after 2 months of development of the symptoms, after being admitted to King George's Medical University (KGMU), Lucknow. The mustard oil consumed by these patients was found to be contaminated with argemone oil.

RED BLOOD CELLS ARE HIGHLY VULNERABLE IN EPIDEMIC DROPSY SYNDROME

Erythrocytes are highly vulnerable to free radical-induced lipid peroxidation (LPO) reactions due to their rich content of polyunsaturated fatty acids (PUFAs) and continuous exposure to high concentrations of molecular oxygen (5). A significant ($p < 0.05$) decrease in RBC count (27%) and Hb content (33%) was found in Lucknow dropsy patients (Fig. 1). The decrease in RBC count (66%) and Hb content (61%) was relatively more in Patna patients with respect to Lucknow patients when compared to controls (Fig. 2). Formation of lipid peroxides in plasma and RBC of Lucknow dropsy patients was found to be significantly elevated (82%–119%) when compared to controls. These levels were enhanced significantly ($p < 0.05$) in Patna patients (250–261%) and the values were even significantly higher than Lucknow patients (Fig. 2). The decrease in RBC count, hemoglobin content, and increase in plasma and RBC LPO in the present study may be attributed to the production of ROS by argemone oil and/or sanguinarine (11, 28, 46).

ARE REDOX PAIRS SUCH AS PYRIDINE NUCLEOTIDES(S), GLUTATHIONE, AND THIOL GROUPS TARGET FOR ARGEMONE OIL POISONING IN HUMANS?

Erythrocytes have a variety of redox systems including GSH, GSSG, NAD, NADH and NADP, and NADPH to provide electrons in ROS detoxification reactions (23). The intracellular GSH redox system, with a high GSH level and far lower concentration of oxidized GSSG, is predominantly responsible for the protection of both Hb and RBC membranes against oxidation and hemolysis (31). A significant ($p < 0.05$) decrease in GSH content (42%) with concomitant enhancement of GSSG content (107%) was observed in RBC of Lucknow dropsy patients when compared to control subjects. Lower levels of GSH (77%) and higher levels of GSSG (223%) were noticed in Patna patients with respect to Lucknow patients when compared to control (Fig. 3). The ratio of GSSG/GSH in erythrocytes of Lucknow dropsy patients (threefold) and Patna dropsy patients (14-fold) was found to be significantly ($p < 0.05$) increased when compared to controls, which may be due to the direct utilization of GSH by GSH peroxidase to detoxify sanguinarine-induced formation of peroxides, or reduced regeneration of GSH from GSSG catalyzed by GSH reductase (GR) (11). It has been suggested that GSH also participates in transhydrogenation reactions that are involved in the formation and maintenance of thiol moieties of proteins, which in turn are essential for maintaining flexibility of the RBC membranes, Hb, and other molecules such as coenzyme A (20). In this regard, a significant decrease in thiol content (47%) was observed in RBC of Lucknow dropsy patients, which was relatively more in Patna dropsy patients (69%) when compared to controls (Fig. 4).

Glycolysis is the only source for NADH in the RBC, which is involved not only for the production of energy but also serves as reducing equivalents to maintain Hb in its functional [Fe (II)] state (13). Sanguinarine inhibits pyruvate oxidase, which results in an increase of pyruvic acid levels in the blood of dropsy patients (38). Our study showed that NADt content was increased (44%) significantly ($p < 0.05$) whereas decreased levels of NADH (32%) were observed in Lucknow dropsy patients as compared to controls. Increase in NADt (77%) and decrease in NADH content (59%) was relatively more in Patna dropsy patients with respect to Lucknow dropsy patients when compared to controls. The ratio of NADH/NADt was found to be decreased significantly ($p < 0.05$) in Lucknow dropsy (53%) and Patna dropsy (78%) pa-

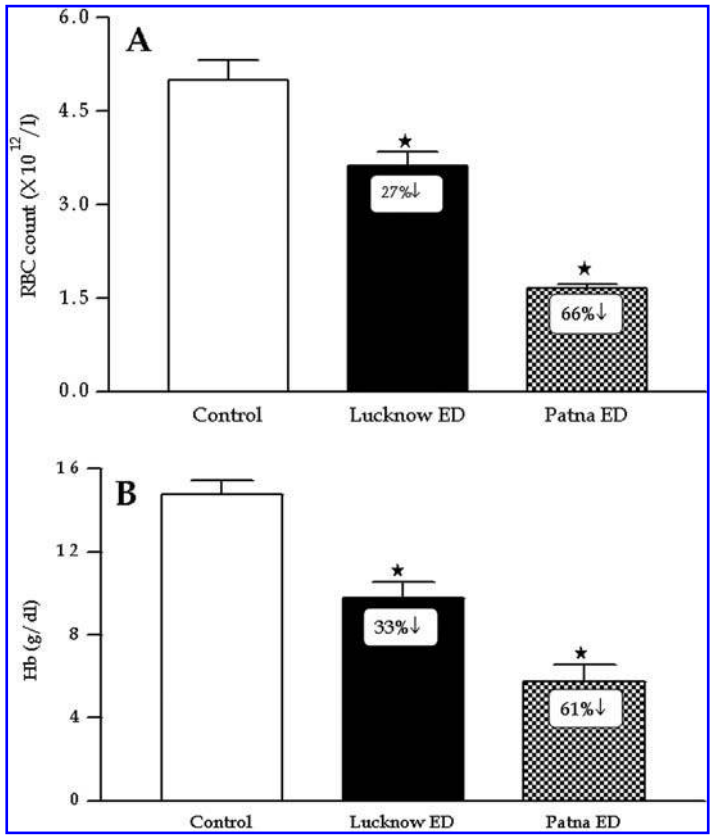

FIG. 1. RBC count and hemoglobin content in whole blood of dropsy patients. Data represent mean ± S.E of values for 14 controls, 14 Lucknow ED patients, and 2 Patna ED patients. (**A**) RBC count: control, range: 3.55–6.55 x 10^{12}/L; Lucknow ED, range: 2.15–5.21 x 10^{12}/L; Patna ED, range: 1.60–1.72 x 10^{12}/L. (**B**) Hb content: control, range: 11.40–18.60 g/dl; Lucknow ED, range: 4.60–14.70 g/dl; Patna ED, range: 4.92–6.56 g/dl. *$p < 0.05$, significance with respect to control subjects.

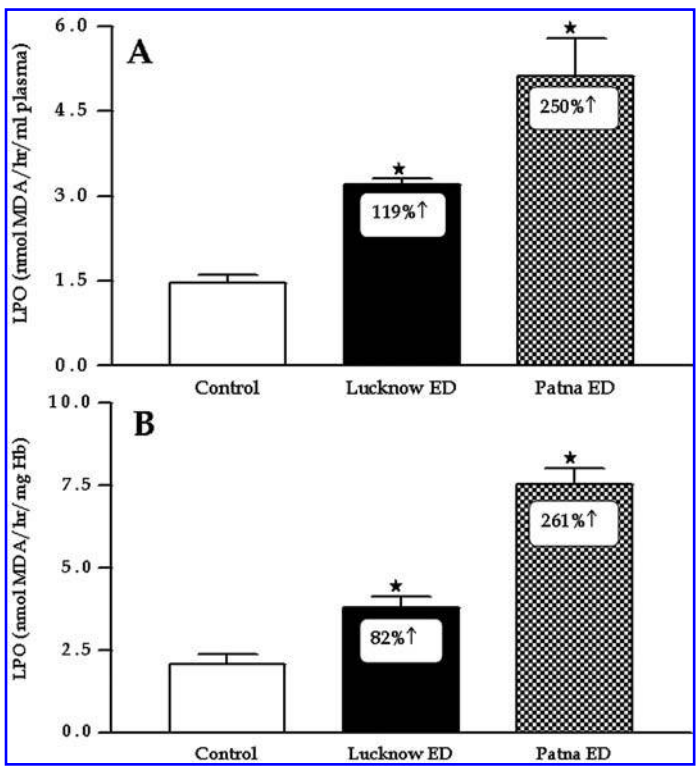

FIG. 2. Peroxidation of lipids in plasma and RBC fraction of dropsy patients. Data represent mean ± S.E of values for 14 controls, 14 Lucknow ED patients, and 2 Patna ED patients. (**A**) Plasma LPO: control, range: 0.56–2.38 nmol MDA/h/ml plasma; Lucknow ED, range: 2.55–3.87 nmol MDA/h/ml plasma; Patna ED, range: 4.43–5.78 nmol MDA/h/ml plasma. (**B**) RBC LPO: control, range: 0.92–3.64 nmol MDA/h/mg Hb; Lucknow ED, range: 2.19–7.21 nmol MDA/h/mg Hb; Patna ED, range: 7.07–8.02 nmol MDA/h/mg Hb. *$p < 0.05$, significance with respect to control subjects.

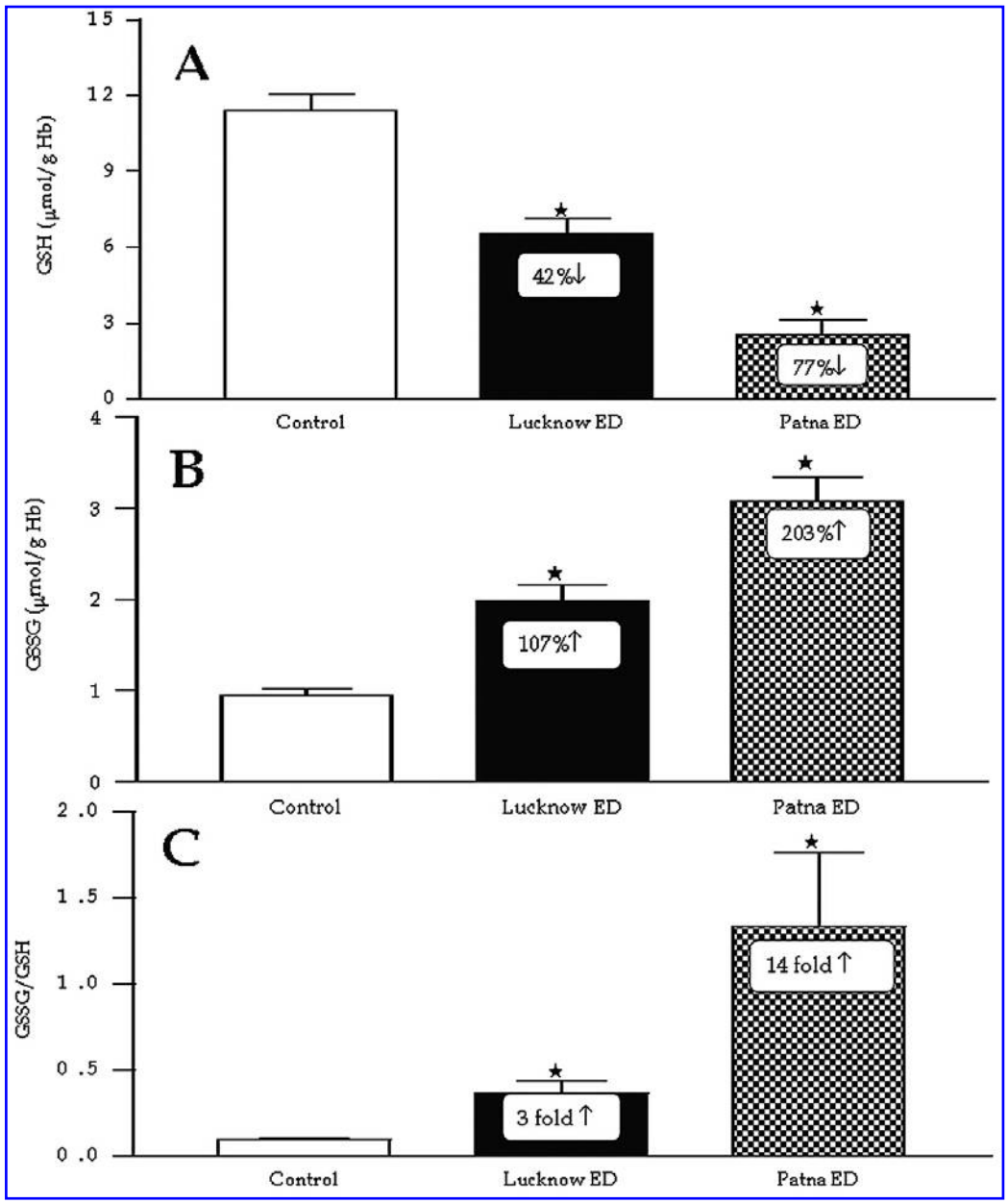

FIG. 3. GSH, GSSG, and GSSG/GSH values in RBC fraction of dropsy patients. Data represent mean ± S.E of values for 14 controls, 14 Lucknow ED patients, and 2 Patna ED patients. (**A**) GSH; control, range: 7.61–15.28 µmol/g Hb; Lucknow ED, range: 2.36–9.13 µmol/g Hb; Patna ED, range: 1.91–3.12 µmol/g Hb. (**B**) GSSG: Control, range: 0.47–1.68 µmol/g Hb; Lucknow ED, range: 0.85–2.92 µmol/g Hb; Patna ED, range: 2.81–3.35 µmol/g Hb. (**C**) GSSG/GSH: control, range: 0.031–0.131; Lucknow ED, range: 0.150–0.880; Patna ED, range: 0.90–1.75. *$p < 0.05$, significance with respect to control subjects.

tients, when compared to controls (Fig. 5), which suggests that the impairment in glycolysis pathway leads to a depleted supply of reducing equivalents. The NAD redox potential (NADH/NADt) plays an important role in the reduction of met-Hb catalyzed by met-Hb reductase, maintaining low levels of met-Hb and high concentrations of normal Hb to restore RBCs oxygen carrying capacity (13). Met-Hb formation in RBC of Lucknow dropsy and Patna dropsy patients was found to be increased by 155% and 418%, respectively, when compared to controls (Fig. 6). Hence, decrease in NADH/NADt redox potential of RBCs indicates the impairment of met-Hb reduction rate and inhibition of glycolysis in the RBC, which leads to breathlessness in dropsy syndrome.

The hexose monophosphate (HMP) shunt in erythrocytes is critical to supply the reducing equivalents, NADPH, to protect against oxidative insult through the recycling of GSH from GSSG catalyzed by GR (33). RBCs of Lucknow dropsy patients showed significant ($p < 0.05$) increase (23%) in NADPt along with decrease (33%) in NADPH concentrations as compared to control subjects (Fig. 7). The ratio of

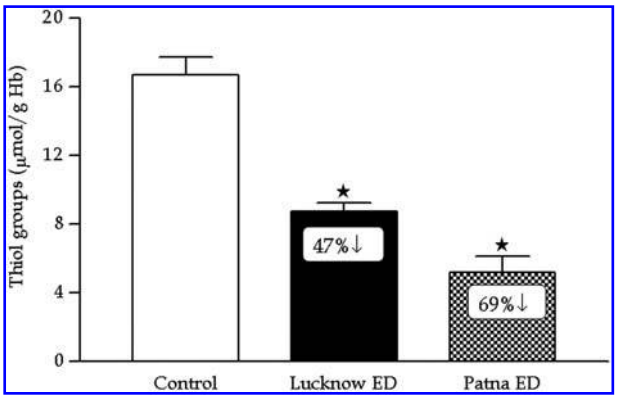

FIG. 4. Thiol content in RBC fraction of dropsy patients. Data represent mean ± S.E of values for 14 controls, 14 Lucknow ED patients, and 2 Patna ED patients. Control, range: 12.69–24.96 µmol/g Hb; Lucknow ED, range: 5.64–11.12 µmol/g Hb; Patna ED, range: 4.30–6.11 µmol/g Hb. *$p < 0.05$, significance with respect to control subjects.

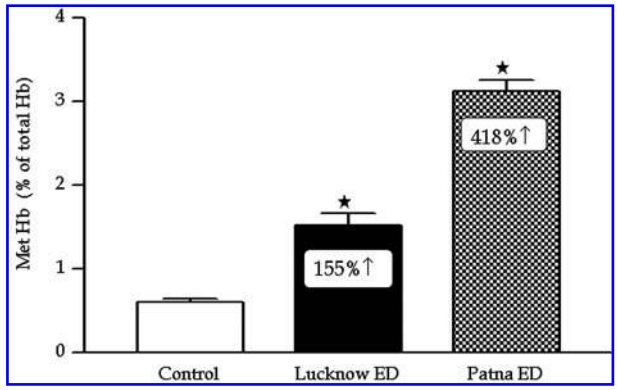

FIG. 6. Met-Hb formation in RBC fraction of dropsy patients. Data represent mean ± S.E of 14 controls, 14 Lucknow ED patients, and 2 Patna ED patients. Control, range: 0.31–0.87% of total Hb; Lucknow ED, range: 0.81–2.68% of total Hb; Patna ED, range: 2.98–3.25% of total Hb. *$p < 0.05$, significance with respect to control subjects.

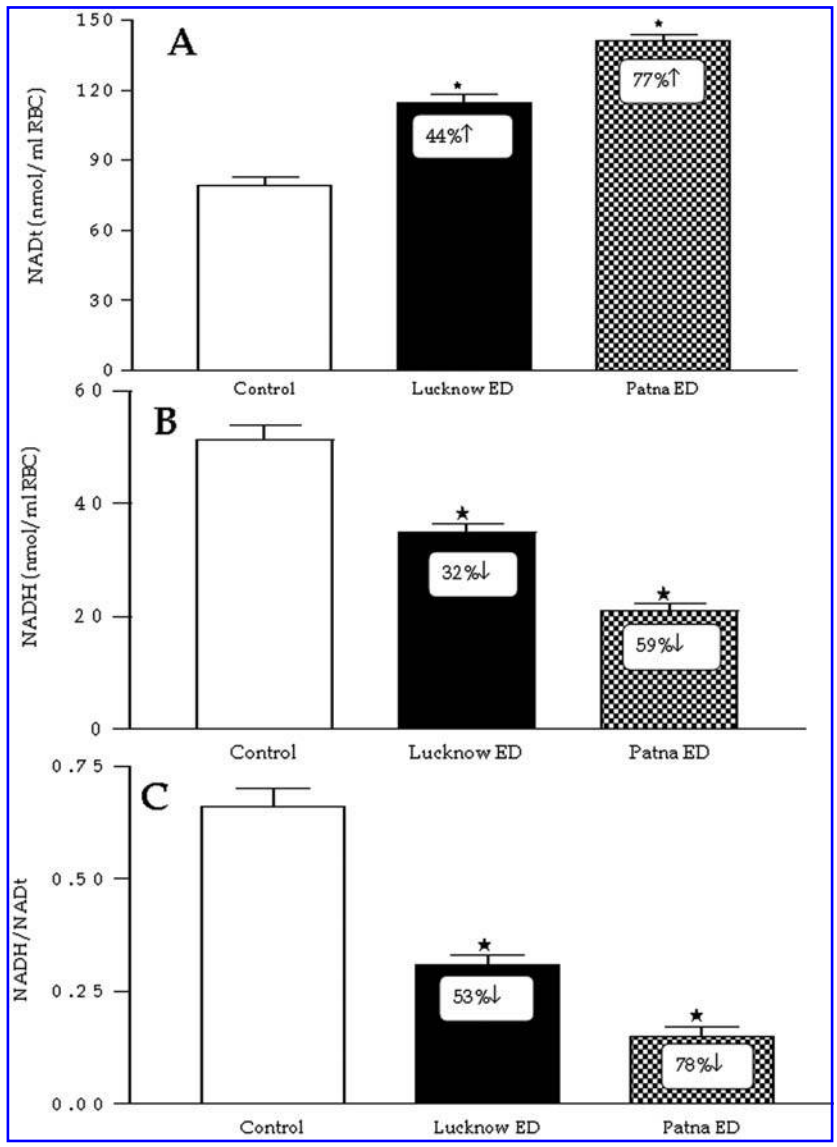

FIG. 5. NADt, NADH, and NADH/NADt values in RBC fraction of dropsy patients. Data represent mean ± S.E of values for 14 controls, 14 Lucknow ED patients, and 2 Patna ED patients. (**A**) NADt: control, range: 69.08–97.61 nmol/ml RBC; Lucknow ED, range: 93.80–136.58 nmol/ml RBC; Patna ED, range: 138.7–143.7 nmol/ml RBC. (**B**) NADH: control, range: 37.24–64.33 nmol/ml RBC; Lucknow ED, range: 26.30–46.58 nmol/ml RBC; Patna ED, range: 19.67–22.34 nmol/ml RBC. (**C**) NADH/NADt: control, range: 0.492– 0.890; Lucknow ED, range: 0.193–0.387; Patna ED, range: 0.13–0.16. *$p < 0.05$, significance with respect to control subjects.

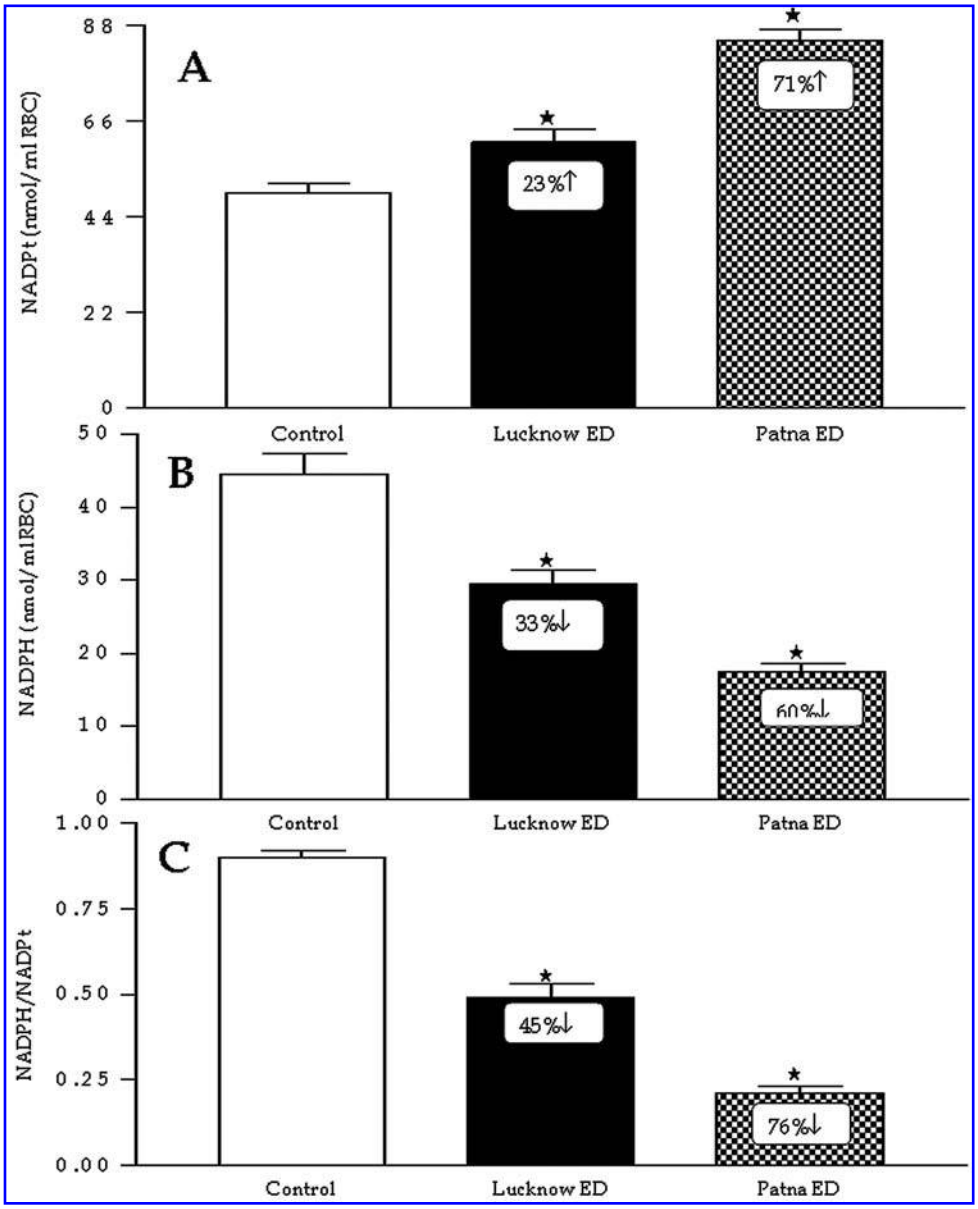

FIG. 7. NADPt, NADPH, and NADPH/NADPt values in RBC fraction of dropsy patients. Data represent mean ± S.E of 14 controls, 14 Lucknow ED patients, and 2 Patna ED patients. (**A**) NADPt: control, range: 39.30–59.62 nmol/ml RBC; Lucknow ED, range: 40.56–76.69 nmol/ml RBC; Patna ED, range: 82.12–87.25 nmol/ml RBC. (**B**) NADPH: control, range: 32.26–58.34 nmol/ml RBC; Lucknow ED, range: 21.23– 43.42 nmol/ml RBC; Patna ED, range: 16.34–18.64 nmol/ml RBC. (**C**) NADPH/NADPt: control, range: 0.66–0.97; Lucknow ED, range: 0.33–0.76; Patna ED, range: 0.19–0.23. *$p < 0.05$, significance with respect to control subjects.

NADPH/NADPt was decreased (45%) significantly ($p < 0.05$) in RBC of Lucknow dropsy patients as compared to control subjects. The depletion of NADPH content (60%) and the ratio of NADPH/NADPt (76%) were more severe in Patna patients with respect to Lucknow patients when compared to controls (Fig. 7). The decrease in NADPH content clearly shows the unavailability of NADPH to recycle GSH from GSSG, leading to accumulation of GSSG in dropsy patients, which may be due to inhibition of G6PDH, a key enzyme of HMP shunt to produce NADPH from G6P (3). Further, high levels of GSSG could inhibit the activity of G6PDH, which in turn may decrease the NADPH content (36). In addition, depletion of NADPH concentration may decrease the formation of Hb from met-Hb, as it also plays a minor role in the reduction of met-Hb by NADPH dependent met-Hb reductase (20).

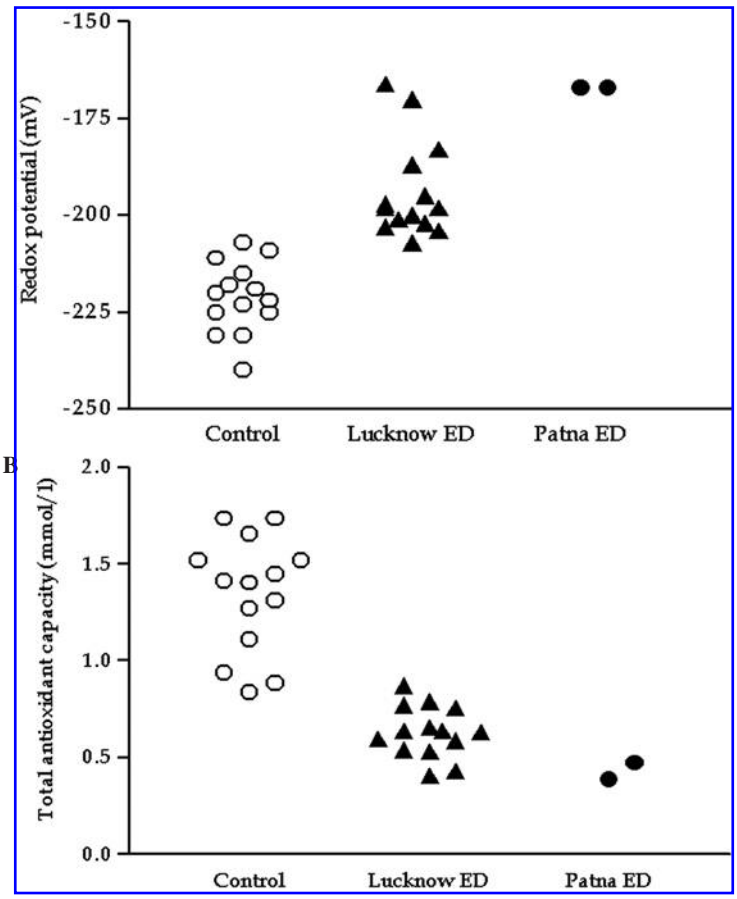

FIG. 8. Redox potential values for glutathione pool in RBC and total antioxidant capacity in plasma of dropsy patients. Data represent mean ± S.E of 14 controls, 14 Lucknow ED patients, and 2 Patna ED patients. (**A**) Redox potential values: control [−221 ± −2.45 (range: −207 to −240)]; Lucknow ED [−194 ± −3.36 (range: −166 to −207)] + 28 mV; Patna ED [−167 (range: −167 to −167)] + 54 mV. (**B**) TAC: control [1.28 ± 0.11 (range: 0.73–1.74)]; Lucknow ED [0.63 ± 0.04 (range: 0.41–0.87)] 50% ↓; Patna ED [0.43 ± 0.04 (range: 0.39–0.47)] 66% ↓.

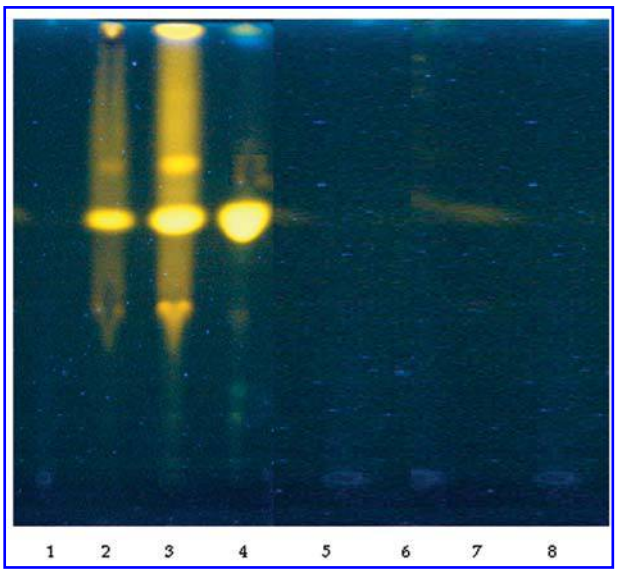

FIG. 9. HPTLC profile of sanguinarine extracted from plasma of dropsy patients. *Lane 1*, control; *lanes 2 and 3*, Patna ED patients; *lane 4*, standard sanguinarine; *lanes 5–8*, Lucknow ED patients. Sanguinarine was extracted from plasma of dropsy patients as described in Materials and Methods. The extracted samples were analyzed by HPTLC using butanol:acetic acid:water (4:1:2) as mobile phase. The plate was viewed under dark room at 365 nm.

GLUTATHIONE REDOX POTENTIAL IS A PRIME SOURCE FOR THE EXISTING PRO-OXIDANT ENVIRONMENT

Redox potential (E_h) of GSH/GSSG pool in RBC represent different components of oxidative stress and provides a parameter for pro- and antioxidant events (22). The Eh values for RBCs are more positive in Lucknow and Patna dropsy patients (−193 and −167 mV; pro-oxidant environment) than controls subjects (−221 mV; pro-reducing environment) and supports the concept that erythrocytes of patients are more in oxidative environment. A significant ($p < 0.05$) reduction (50%) in total antioxidant capacity (TAC) was found in the plasma of Lucknow dropsy patients as compared to control subjects. The reduction in TAC was found to be more in Patna dropsy patients (66%) with respect to Lucknow dropsy patients (50%) when compared to controls (Fig. 8), which may be due to the antioxidant action to scavenge sanguinarine-induced production of free radicals or due to nonavailability of reducing equivalents to their oxidized forms.

Sanguinarine alkaloid was found to be present in Patna dropsy patients ranging from 7.15 to 25.7 μg/ml plasma (lanes 2 and 3 of Fig. 9). The spot of dihydrosanguinarine was also observed in plasma of Patna dropsy patients. Interestingly, the plasma of a patient where sanguinarine content was higher (25.7 μg/ml), showed a metabolite spot at Rf 0.69, However, Lucknow dropsy patients showed neither the pres-

ence of sanguinarine/dihydrosanguinarine spots nor the metabolite spot (Fig. 9, lanes 5–8).

CONCLUSIONS AND DIRECTIONS

The ROS-dependent damage of erythrocyte membranes and Hb along with decreases in GSH, NADH, NADPH, and thiol contents is responsible to enhance pro-oxidant environment and alterations in plasma TAC leading to the development of normocytic anemia in dropsy patients having breathlessness (Fig. 10). Enhancement of the pro-oxidant environment in Patna patients as compared to Lucknow patients may be due to the fact that antioxidant therapy including α-tocopherol was not given to Patna patients. Hence, antioxidant therapy may be beneficial to ED patients and needs detailed clinical trials in future. Although Indian legislation clearly suggests that mustard oil should be absolutely free from argemone oil contamination (34), however, unscrupulous traders adopt unethical

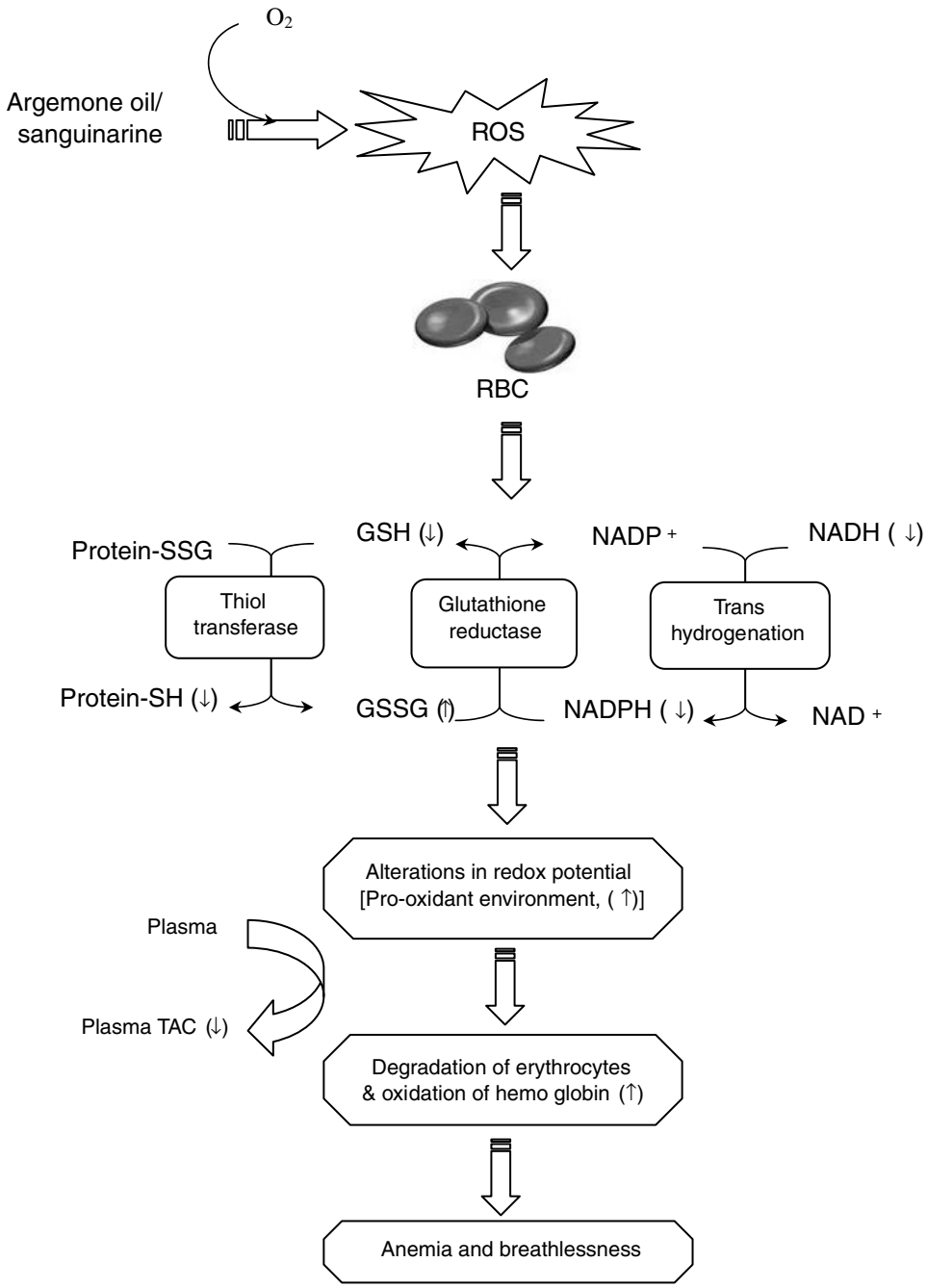

FIG. 10. Mechanism(s) of producing pro-oxidant environment due to alterations in glutathione and pyridine nucleotides leading to decrease of total antioxidant capacity, which in turn causes damage of erythrocytes and hemoglobin in epidemic dropsy patients.

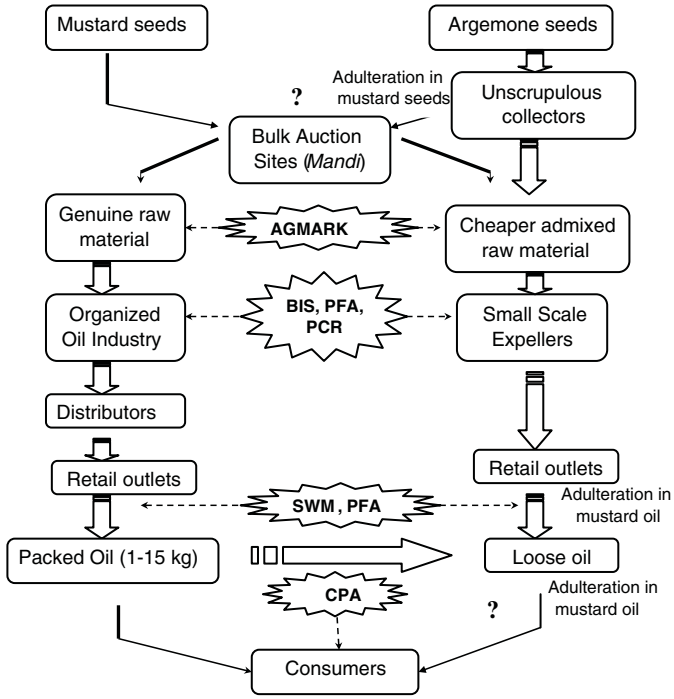

FIG. 11. Various steps from oil seeds to retail selling of mustard oil in Indian markets. Steps that are regulated by government acts are marked with *dotted arrows in asterisks.* Possible adulteration of mustard oil is mentioned at various steps before reaching consumers. Apart from different government acts regulating the genuine mustard oil, two additional steps (marked with *question mark*) may be introduced as amendments in the legislation so as to curtail this menace, which are mentioned in the Conclusion. AGAMRK, Agricultural Produce Marking and Grading Act; BIS, Bureau of Indian Standards; CPA, Consumer Protection Act; PCR, Packaged Commodities Rules; PFA, Prevention of Food Adulteration Act; SWM, Standards of Weights and Measures Act.

practices for economic gains in loose mustard oil (Fig. 11), which is consumed by lower socioeconomic groups. Hence, the regulatory authorities should enforce the banning of the sale of loose mustard oil and at the same time suggest the oil industry to prepare tamper-proof small packing (~100–200 g), so that the commodity is within the purchase capacity of lower strata.

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ABBREVIATIONS

ED, epidemic dropsy; GSH, glutathione reduced; GSSG, glutathione oxidized; Hb, hemoglobin; LPO, lipid peroxidation; MDA, malondialdehyde; NADt, nicotinamide adenine dinucleotide total; NADPt, nicotinamide adenine dinucleotide phosphate total; Met-Hb, met-hemoglobin; ROS, reactive oxygen species; TAC, total antioxidant capacity.

APPENDIX

1. β-Nicotinamide adenine dinucleotide phosphate reduced (NADPH), nicotinamide adenine dinucleotide phosphate oxidized (NADP), nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide oxidized (NAD), nicotinamide, glutathione reduced (GSH), glutathione oxidized (GSSG), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), thiobarbaturic acid (TBA), malondialdehyde (MDA), O-pthalaldehyde (OPT), *N*-ethyl maleimide (NEM), disodium ethylene diamine tetraacetic acid (Na_2 EDTA), tris (hydroxymethyl) aminomethane, glucose-6-phosphate (G6P), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), phenazine ethosulfate (PES), and sanguinarine (>98%) were purchased from Sigma Chemical Co, St. Louis, MO. Alcohol dehydrogenase (ALDH) from baker's yeast was procured from SRL Pvt Ltd, Mumbai, India. Glucose-6-phosphate dehydrogenase (G6PDH) from yeast was obtained from Boehringer, Mannheim, Germany. Absolute alcohol was obtained from Bengal Chemicals and Pharmaceuticals, Calcutta, India. Hemoglobin and total antioxidant capacity assay kits were procured from Accurex Biomedical Pvt Ltd. Mumbai, India and Randox Laboratories Ltd, Ireland, UK, respectively. Vacutainer tubes were obtained from Becton Dickinson Co, NJ. Water was purified by a Millipore system (Milli-Q Corp, Billerica, MA) and had a resistivity >18 M W cm^{-1}. All the other chemicals used were of the highest purity available from commercial sources.

2. During February 2005, an outbreak of epidemic dropsy was reported in Rajajipuram, Lucknow, Uttar Pradesh, India. Fourteen patients from different families of same locality were admitted to King George's Medical University (KGMU), Lucknow, and were given symptomatic treatment along with antioxidant therapy including α-tocopherol, based on our earlier clinical findings (26). Two patients from Patna (Bihar, India) were admitted to KGMU during December 2004 with symptoms of epidemic dropsy for the past 2 months and had under gone symptomatic treatment only. Lucknow patients were of different age groups including children, adolescents, and adults of both sexes, while Patna patients were husband and wife (37 and 47 years). By seeking the history of the patients and the records at KGMU, it was confirmed that all patients had consumed mustard oil adulterated with AO as the samples of edible oil consumed by these patients were found to contain argemone alkaloids using ITRC argemone oil detection kit (9).

3. Fourteen dropsy patients from Lucknow outbreak and 2 patients from Patna outbreak and 14 healthy volunteers were involved in this investigation. Blood was collected from Lucknow dropsy patients at KGMU after 1 week of antioxidant therapy and from 2 Patna patients after 2 months of appearing symptoms, in vacutainer tubes containing sodium citrate as anticoagulant and brought to the lab within an hour in ice-cold condition. A portion of blood was used for erythrocyte count and hemoglobin concentration and the other portion of blood was used

for preparation of RBC fraction (4). Briefly, blood was centrifuged at 2,500 *g* for 15 min at 4°C and erythrocytes were separated from plasma and buffy coat. The erythrocyte-rich precipitate was washed three times with saline (3:1, vol/vol) and directly used for the assay of lipid peroxidation (LPO), hemoglobin oxidation, oxidized and reduced GSH, oxidized and reduced pyridine nucleotide content, and thiol groups. Plasma was used for the assay of LPO, total antioxidant capacity, and sanguinarine content.

4. RBC count and hemoglobin content in whole blood samples were carried out on Automatic Full Digital Cell Counter (MS9, Melet Schloesing Laboratories, Cergy-Pointoise, France) and expressed as number of cells $\times 10^{12}$/l and g/dl, respectively (45). Hemoglobin content of the RBC fraction was estimated by using an Accurex kit following the instructions given by the manufacturer, and these values were used for the calculation of LPO, GSH, GSSG, and free sulfhydryls in erythrocytes.

5. The extent of LPO in plasma and RBC fraction was assayed by measuring the formation of malondialdehyde (MDA) and expressed as nmol MDA/hr/ml (plasma) or nmol MDA/hr/mg Hb (RBC) using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (32). Thiol content was assayed in the RBC fraction using Ellman's reagent and expressed as μmol/g Hb (15).

6. The content of GSH and GSSG in RBC was assayed according to the modified method of Hissin and Hiff (19) using OPT as fluorescent probe, which allows binding of GSH to form highly fluorescent derivative. Briefly, 0.2 ml RBC pellet was diluted with 0.8 ml distilled water and precipitated with 10% TCA (1 ml). Supernatant was used for GSH and GSSG determination. For GSH assay, a 4 ml reaction mixture consisted of 3.25 ml phosphate-EDTA buffer (0.1 *M*, pH 8.0), 0.25 ml OPT (1 mg/ml methanol), and 0.5 ml supernatant. Fluorescence of GSH-OPT adduct was determined after 15 min, using excitation and emission wavelengths of 350 and 450 nm (slit widths 5 and 10 nm), respectively. For GSSG assay, NEM was used to prevent oxidation of GSH to GSSG during the assay, which allows the formation of stable complex with GSH. After incubating 0.5 ml of supernatant with NEM (0.04 *M*) for 30 min, GSSG was measured using the procedure outlined above (for GSH assay), except that 0.1 *N* NaOH was employed as diluant rather than phosphate-EDTA buffer. The quantification of GSH and GSSG was performed by comparing the standard GSH and GSSG fluorescence and expressed as μmol/g Hb. The ratio of GSSG and GSH was calculated by using specific values in the individual samples.

7. Redox potential of GSH/GSSG pool ($2GSH \rightarrow GSSG + 2e^- + 2H^+$) in erythrocytes was calculated using Nernst equation: $E_h\ (mV) = E_0 - [(RT/nF)\ \log\ (thiol^2)/(disulfide)]$, where E_0 is the respective standard potential (−264 mV) for the redox couple at pH 7.4, R is the gas constant ($8.314\ Jk^{-1}mol^{-1}$), T is the absolute temperature (310 K), n is number of electrons transferred, (2) and F is the Faraday constant (96485 J/V) (39).

8. The content of pyridine nucleotides were determined by spectrophotometric cycling assay with some minor modifications (48). In brief, 20 μl of RBC suspension was mixed with 1.98 ml of alkaline extraction solution containing nicotinamide (10 μ*M*), $NaHCO_3$ (20 μ*M*), and Na_2CO_3 (100 μ*M*). The reaction mixture was frozen immediately and thawed quickly in a room temperature water bath. The mixture was again chilled to 0°C followed by centrifugation at 14,000 *g* for 1 min at 4°C, and the supernatant was separated. For measurement of reduced form of nucleotides, the oxidized forms of NAD and NADP were destroyed by incubating 700 μl supernatant at 60°C for 30 min and again chilled to 0°C. Unheated supernatant was used for the assay of total NAD (NADt) and NADP (NADPt). In brief, a volume of 100 μl of heated (NADH) or unheated (NADt) supernatant was added to 800 μl of recycling buffer, containing 100 μmol Tris-Cl (pH 8.0), 2 μmol PES, 0.5 μmol MTT, and 0.2 mg ALDH. The mixture was incubated for 5 min at 37°C, and 100 μl of ethanol (6 *M*) was added as substrate. The rate of increase of absorbance was measured at 570 nm over a 5-min period. For the assay of NADPH and NADPt, 1.3 IU/ml of G6PDH and G6P (1 mmol) was used as enzyme and substrate instead of ALDH and ethanol. The rate of increase in absorbance was measured at 570 nm over a 90-sec period and expressed as nmol/ml RBC.

9. The oxidation of hemoglobin in erythrocytes was evaluated by measuring the formation of met-hemoglobin (Met-Hb) (16). In brief, RBC fractions (20 μl) were lysed with distilled water (1.98 ml). A portion of this lysate (600 μl) was mixed with 400 μl phosphate buffer (0.5 *M*, pH 6.1) and centrifuged at 15,000 *g* for 5 min to sediment debris. The absorbance of supernatant fraction was measured at 630 nm before (S1) and after (S2) conversion to cyanomethemoglobin by the addition of 50 μl of 10% potassium cyanide (KCN). The difference between S1 and S2 represents the absorbance due to Met-Hb. Another portion of supernatant was used to measure total hemoglobin levels, after converting to Met-Hb by potassium ferricyanide before (T1) and after (T2) addition of KCN. The Met-Hb formation was calculated by using the formula $[100(S1-S2)]/[10(T1-T2)]$ and expressed as percentage of total Hb.

10. TAC was assayed in plasma by commercially available kit according to the method of Miller *et al*. (30). In brief, 1 ml chromogen solution containing 610 μ*M* 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) and 6.1 μ*M* peroxidase (metmyoglobin) was incubated with 0.2 ml H_2O_2 (250 μmol/l) to produce the cation radical $ABTS^+$. This radical has a relatively stable blue-green color and has the absorption maxima at 600 nm. Antioxidants in the sample (20 μl) cause suppression of this color production to a degree, which is proportional to their concentration. The results were calibrated using a standard curve of Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchro-man-2-carboxylic acid) a water-soluble vitamin E analogue and expressed as mmole equivalents of Trolox concentration per liter plasma (m*M*).

11. Sanguinarine was extracted from plasma and analyzed using high performance thin layer chromatography (HPTLC). In brief, plasma (1 ml) was digested with 2 *N* HCl (1 ml) at 90°C for 30 min, followed by centrifugation at 3,000 rpm for 20 min. Supernatant (~2 ml) fraction was separated and extracted with 2 ml chloroform: acetic acid (3:1.5, vol/vol). The mixture was allowed to separate aqueous and organic phase at room temperature for 30 min. Upper acid layer was separated and filtered through filter (0.22 μ, Millipore) and dried on sand bath. The residue was dissolved in methanol and spotted onto a precoated silica gel plate (E. Merck, Mumbai, India). The plate was run using butanol, acetic acid, water (4:1:2, vol/vol/vol) as a mobile phase and the content of sanguinarine in plasma was calculated using pure sanguinarine as standard and expressed as μg/ml plasma.

12. All results were expressed as the mean ± standard error (SE), as indicated in the tables. Statistical comparisons were made by analysis of variance with rank ordering (42) employed to calculate the significance of difference between the means of healthy controls and the epidemic dropsy patients. A value of $p < 0.05$ was used as the level of significance.

REFERENCES

1. Ansari KM, Chauhan LK, Dhawan A, Khanna SK, and Das M. Unequivocal evidence of genotoxic potential of argemone oil in mice. *Int J Cancer* 112: 890–895, 2004.
2. Ansari KM, Dhawan A, Khanna SK, and Das M. *In vivo* DNA damaging potential of sanguinarine alkaloid, isolated from argemone oil, using alkaline Comet assay in mice. *Food & Chem Toxicol* 43: 147–153, 2005.
3. Babu CK, Khanna SK, and Das M. Glutathione redox paradigm in blood, a biomarker of oxidative stress in Epidemic Dropsy patients. Presented in *"International Conference on Antioxidants & Free Radicals in Health-Nutrition & Radio Protectors and VI Annual Conference of Society of Free Radical Research in India"* at St. John's National Academy of Health Sciences, Bangalore, India, January 10–12, 2005.
4. Beutler E. Red blood cell metabolism. In: *Rossi's Principles of Transfusion Medicine,* 3rd ed, edited by Simon TL, Dzik WH, and Snyder EL. Philadelphia: Lippincott Williams & Wilkins, 2002, pp. 43–49.
5. Chiu D, Lubin B, and Shohet SB. Peroxidative reactions in red cell biology. In: *Free Radicals in Biology,* Vol. 5, edited by Pryor WA, New York, NY: Academic Press, 1982, pp. 115–160.
6. Dalal KR. Report on the investigation of an outbreak in Rangoon in the year 1924. *Indian Med Gaz* 64: 19–21, 1929.
7. Das M and Khanna SK. Clinico-epidemiological, toxicological, and safety evaluation studies on argemone oil. *Crit Rev Toxicol* 27: 273–297, 1997.
8. Das M and Khanna SK. Epidemic dropsy. *Natl Medical J India* 11: 207–208, 1998.

9. Das M and Khanna SK. Management of epidemic dropsy caused by argemone oil. In: *Health and Dietary Aspects of Mustard Oil*, edited by Bhatnagar AK, Singh HB, and Prasad S. MRPC, New Delhi, 2001, pp. 139–149.
10. Das M, Ansari KM, Dhawan A, Shukla Y, and Khanna SK. Correlation of DNA damage in epidemic dropsy patients to carcinogenic potential of argemone oil and isolated sanguinarine alkaloid in mice. *Int J Cancer* 117: 709–717, 2005.
11. Das M, Babu K, Reddy NP, and Srivastava LM. Oxidative damage of plasma proteins and lipids in epidemic dropsy patients: alterations in antioxidant status. *Biochim Biophys Acta* 1722: 209–217, 2005.
12. Deva Sangayam AD. An epidemic of dropsy among Indians in Fiji. *Indian Med Gaz* 62: 506–508, 1927.
13. Duncan JR, Prasse KW, and Mahaffey EA. Erythrocytes. In: *Veterinary Laboratory Medicine: Clinical Pathology,* edited by Duncan JR, Prasse KW, and Mahaffey EA. Ames, Iowa: Iowa State University Press, 1994, pp. 3–35.
14. Dutta AK, Joshi CK, and Dutta P. Epidemic dropsy outbreak in Rajasthan. *J Indian Med Assoc* 83: 268–271, 1985.
15. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 82: 70–77, 1959.
16. Evelyn K and Malloy H. Micro determination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. *J Biol Chem* 126: 655–662, 1938.
17. Eversole LR, Eversole GM, and Kopcik J. Sanguinaria-associated oral leukoplakia: comparison with other benign and dysplastic leukoplakic lesions. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 89: 455–464, 2000.
18. Frankos VH, Brusick DJ, Johnson EM, Maibach HI, Munro I, Squire RA, and Weil CS. Safety of sanguinaria extract as used in commercial toothpaste and oral rinse products. *J Can Dent Assoc* 56S: 41–47, 1990.
19. Hissin PJ and Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* 74: 214–226, 1976.
20. Jaffe ER. The formation and reduction of methemoglobin in human erythrocytes. In: *Cellular and Molecular Biology of Erythrocytes*, edited by Yoshikawa H and Rapoport SM. Baltimore, MD: University Park Press, 1974, p. 345.
21. Jha N, Chaturvedi S, Yadav BK, Karki DB, and Rai BK. Field investigation of an outbreak of epidemic dropsy in eastern Nepal. *Indian J Public Health* 43: 87–88, 1999.
22. Jones DP. Redox potential of GSH/GSSG couple: assay and biological significance. *Methods Enzymol* 348: 93–112, 2002.
23. Kehrer JP and Lund LG. Cellular reducing equivalents and oxidative stress. *Free Radic Biol Med* 17: 65–75, 1994.
24. Krishnamachari KA and Satyanarayana K. Epidemic dropsy in Andhra Pradesh. *Indian J Med Res* 60: 741–746, 1972.
25. Kuftinec MM, Mueller–Joseph LJ, and Kopczyk RA. Sanguinaria toothpaste and oral rinse regimen clinical efficacy in short- and long-term trials. *J Can Dent Assoc* 56S: 31–33, 1990.
26. Kumar A, Hussain F, Das M, and Khanna SK. An outbreak of epidemic dropsy in the Barabanki District of Uttar Pradesh, India: a limited trial for the scope of antioxidants in the management of symptoms. *Biomed Environ Sci* 5: 251–256, 1992.
27. Lal SB. Epidemic dropsy in Bihar. *Indian Med Gaz* 86: 64–71, 1951.
28. Maiti M and Chatterjee A. Production of singlet oxygen by sanguinarine and beriberine. *Curr Sci* 68: 734–736, 1995.
29. Maiti M. Molecular aspects on the interaction of sanguinarine with B-form, Z-form and H^L-form of DNA structures. *Indian J Biochem Biophys* 38: 20–26, 2001.
30. Miller NJ, Rice–Evans C, Davies MJ, Gopinathan V, and Milner A. Method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin Sci* 84: 407–412, 1993.
31. Nemeth I, Orvos H, and Boda D. Blood glutathione redox status in gestational hypertension. *Free Radic Biol Med* 30: 715–721, 2001.
32. Ohkawa H, Ohishi N, and Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95: 351–358, 1979.
33. Pandolfi PP, Sonati F, Rivi R, Mason P, Grosveld F, and Luzzatto L. Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. *EMBO J* 14: 5209–5215, 1995.
34. The Prevention of Food Adulteration (PFA) Act of India, 1954: Rules and Notifications, 26th edition, Eastern Book Co., Lucknow, India, 2005.
35. Rai Chaudhuri MN and Chauduri RN. Epidemic dropsy in West Dinajpur. *Bull Calcutta Sch Trop Med* 13: 37–38, 1965.
36. Rodriquez–Segade S, Ramos–Martinez JL, and Freire M. Reversal effect of oxidized glutathione on the inhibition of glucose-6-phosphate dehydrogenase by NADPH. *Biochem J* 231: 805–806, 1985.
37. Sandhu HS, Tiwari SC, Naik G, and Bose C. An epidemiological study of an outbreak of epidemic dropsy in Itarsi (MP). *Indian J Public Health* 26: 10–15, 1982.
38. Sarkar SN. Isolation from argemone oil of dihydrosanguinarine sanguinarine: toxicity of sanguinarine. *Nature (London)* 162: 265–266, 1948.
39. Schafer FQ and Buettner GR. Redox state of the cell as viewed though the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 30: 1191–1212, 2001.
40. Sies H. Oxidative stress: oxidants and antioxidants. *Exp Physiol* 82: 291–295, 1997.
41. Singh NP, Anuradha S, Dhanwal DK, Singh K, Parkash A, Madan K, and Agarwal SK. Epidemic dropsy-a clinical study of the Delhi outbreak. *J Assoc Phys India* 48: 877–880, 2000.
42. Snedecor GW and Cochran WG. One way classification: analysis of variance. In: *Statistical Methods,* 8th ed, edited by Snedecor GW and Cochran WG. Ames, Iowa: Iowa State University Press, 1989, pp. 217–236.
43. Steyn DG. Poisoning with the seed of *Argemone mexicana* (Mexican poppy) in human beings; Indian epidemic dropsy in South Africa. *S Afr Med J* 24: 333–339, 1950.
44. Stiborova M, Simerek V, Frei E, Hobza P, and Ulrichova J. DNA adduct formation from quaternary benzo[c]phenanthridine alkaloids sanguinarine and chelerythrine as revealed by the ^{32}P- postlabeling technique. *Chem Biol Int* 140: 231–242, 2002.
45. Tietz NW. (Ed). *Clinical Guide to Laboratory Tests*. 2nd ed. Philadelphia: WB Saunders, 1990, pp. 166–167.
46. Tuveson RW, Larson RA, Marley KA, Wang GR, and Berenbaum MR. Sanguinarine, a phototoxic H_2O_2 producing alkaloid. *Photochem Photobiol* 50: 733–738, 1989.
47. Wadia RS, Relwani GS, Batra RK, Ichaporia RN, and Grant KB. Epidemic dropsy in Poona 1969 (clinical features and 1 year follow up). *J Assoc Physicians India* 19: 641–646, 1971.
48. Wagner TC and Scott MD. Single extraction method for the spectrophotometric quantification of oxidized and reduced pyridine nucleotides in erythrocytes. *Anal Biochem* 222: 417–426, 1994.

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2. Challagundla K. Babu, Kausar M. Ansari, Sanjay Mehrotra, Satyananda Patel, Madhu Dikshit, Mukul Das. 2010. Activation of inflammatory response and apoptosis of polymorphonuclear leukocytes in patients with argemone oil poisoning. *Chemico-Biological Interactions* **183**:1, 154-164. [CrossRef]
3. E VRUBLOVA, J VOSTALOVA, R VECERA, B KLEJDUS, D STEJSKAL, P KOSINA, A ZDARILOVA, A SVOBODOVA, V LICHNOVSKY, P ANZENBACHER. 2008. The toxicity and pharmacokinetics of dihydrosanguinarine in rat: A pilot study. *Food and Chemical Toxicology* **46**:7, 2546-2553. [CrossRef]
4. C BABU, K ANSARI, S MEHROTRA, R KHANNA, S KHANNA, M DAS. 2008. Alterations in redox potential of glutathione/glutathione disulfide and cysteine/cysteine disulfide couples in plasma of dropsy patients with argemone oil poisoning#. *Food and Chemical Toxicology* **46**:7, 2409-2414. [CrossRef]