ORIGINAL ARTICLE

Protective effect of *Amorphophallus campanulatus* tuber extracts against H_2O_2 induced oxidative damage in human erythrocytes and leucocytes

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Background: *Amorphophallus companulatus* (Araceae) is a tuberous medicinal plant commonly used in Ayuvedic medicines as well as tribal medicines of India. **Aim:** Aim of the present study was to investigate the protective effect of *A. companulatus* tuber extracts against H_2O_2 induced oxidative damage in human erythrocytes and leucocytes. **Setting and Design**: The experiment was set and design as per available method in the literatures. Three measurements were performed under each set of extracts. **Materials and Methods:** The extracts of tuber of *A. companulatus* such as methanol, ethanol, acetone (70%) and hydro-alcohol (1:1) used to assess catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione (GSH) and lipid peroxidation (LPO) levels of human erythrocytes and leucocytes. **Statistical Analysis**: All experimental data were statistically analysed and expressed as means ± SD by using the one-way analysis of variance. **Results:** Results of present studies revealed that, increased in the CAT, SOD, GPx and reduction of GSH and LPO levels in H_2O_2 group compared with control group. The extracts of tuber of *A. companulatus* treated groups showed effective reduction of CAT, SOD, GPx and increased the GSH and LPO levels as compared with H_2O_2 group on human erythrocytes and leucocytes. The methanol extract was found more effective than others. **Conclusion:** The present findings suggest that, the extracts of tuber of *A. companulatus* possess protective effect against H_2O_2 induced oxidative damage. Furthermore, these tuber extracts may appeared to be beneficial in preventing H_2O_2 oxidative human red blood cell (RBC) damage in human and can improve RBC membrane permanence. The tubers of *A. companulatus* are the potential source of natural antioxidants for the treatment and prevention of disease in which LPO takes place.

Key words: *Amorphophallus campanulatus* tuber, erythrocytes, H_2O_2, leucocytes, phosphate buffer solution

INTRODUCTION

Oxidative damage as a result of an increase in the free radical load and/or decrease in the efficiency of the antioxidant systems has been implicated in many human diseases.[1,2] According to generally accepted mechanisms, major deleterious effects are caused by hydroxyl radical (OH) generated from H_2O_2 and by the superoxide (O_2^-) species generated in the presence of redox active transition metals.[3,4] Many endogenous and exogenous defence mechanisms are available in living organisms to limit the levels of reactive oxygen species (ROS) and the damage caused by them.[5] These include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and many non-enzymatic antioxidant compounds such as polyphenols, tocopherols, ascorbic acid, uric acid, glutathione (GSH) and other thiol protein groups to protect the functional and structural integrity of biological molecules such as proteins, lipids and nucleic acids.[6,7] It has been reported that the unbalanced ROS production and antioxidant cell defences have been associated in the physiological and pathological conditions such as aging, cancer, rheumatoid arthritis, atherosclerosis and neurodegenerative diseases.[8-12] However, there is an increasing interest in the protective biochemical function of naturally occurring antioxidants in biological systems and on the mechanism of their action.[13] As plants could represent a source of natural compounds with antioxidant activities, many studies have been conducted searching for the antioxidant activities of many plant extracts and their constituents.[14-17] Hence, in line with above studies, here, we have evaluated the protective effect of *Amorphophallus companulatus* tuber extracts against H_2O_2 induced oxidative damage in human erythrocytes and leucocytes.

Access this article online	
Quick Response Code:	Website: www.greenpharmacy.info
	DOI: 10.4103/0973-8258.116387

A. campanulatus (Araceae) a tuberous, stout indigenous herb commonly known as elephant foot yam, suran, grown as vegetable.[18,19] *A. campanulatus* is basically a

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Received: 16-12-2012; **Accepted:** 06-04-2013

crop of south East Asian origin. In India, it is commonly known as Suran or Jimmikand. It grows in wild form in Philippines, Malaysia, Indonesia and other South East Asian countries. This tuber is consumed by many people as a food and widely used in many Ayurvedic preparations.[20] The tubers are rich in nutrients. It is a healthy low-fat food containing a good source of protein as well as starch.[21] The tuber of *A. campanulatus* is used for tumour, rheumatoid arthritis, carminative and liver tonic. It is also used in piles and given as the restorative in dyspepsia debility, anti-inflammatory, anti-haemorrhoidal, haemostatic, expectorant and anthelmintic.[22-24] *A. campanulatus* has been reported for its potential actions such as hepatoprotective,[25-28] antioxidant,[27-35] antitumor,[35] analgesic,[36-38] *in-vitro* cytotoxic, antibacterial, antifungal,[39-43] anthelmintics,[44,45] anti-inflammatory,[38,46] curative effect,[47] gastro protective,[48,49] antidiarrhoeal,[50] immunomodulatory,[51] central nervous system activities.[52-54] From plant mainly isolated quercetin,[25] 3,5-diacetyltambulin,[40] amblyone,[41] salviasperanol.[42] Moreover, total polyphenols, flavonoids and tannin contents from the present study extracts were also estimated.[29]

MATERIALS AND METHODS

Chemicals

GPx and SOD activity were determined using commercial available enzyme kits such as Ransel, RANDOX/RS-504 and Ransod, RANDOX/SD-125, RANDOX Laboratories, U.K. Hydrogen peroxide, 3% (Universal Laboratories Pvt. Ltd.), pyrogallol (High media), butylated hydroxyl anisole, potassium ferricyanide, nitro blue tetrazolium, thiobarbituric acid (TBA), trichloroacetic acid ethylene diamine tetra acetic acid, ammonium thiocyanate, potassium persulfate, ferrous chloride, ascorbic acid were obtained from Sigma Chemicals, USA and SD. Fine-Chem Ltd., Mumbai. All other chemicals and reagents used for experimental work were of analytical grade.

Plant Material and Preparation of Extracts

Tubers of *A. campanulatus* were collected from the region of Sagar district of Madhya Pradesh, India and it was authenticated by a botanist, Department of Botany, Safia Science Collage, Bhopal, Madhya Pradesh, India and voucher specimen (192/BOTANY/Safia/19) was deposited in the same. The 700 g of air dried powdered of tubers were extracted with methanol, ethanol, acetone (70%) and hydro-alcohol (1:1) respectively. After the extraction, excess solvent was completely removed by using a rotatory flash evaporator to get concentrated, then completely dried in freeze drier and preserved in airtight container under refrigeration. The extracts were used for the estimation of total polyphenols, flavonoids and tannin contents.

Isolation of Erythrocytes

Fresh blood samples from healthy volunteers (12-15 ml) were collected and centrifuged at 3000 rpm for 15 min and plasma and buffy coats were removed. Red cells were washed with phosphate buffer solution (PBS) (pH 7.00, containing 140 mM NaCl) 3 times and erythrocytes were haemolyzed with ice-cold distilled water.[55]

Isolation of Leucocytes

Human polymorphonuclear leukocytes were isolated from freshly sampled venous blood (12-15 ml) of healthy volunteers by using dextran (Macrodex: 6% dextran in 0.9% NaCl solution) and heparin tubes (25,000 IU/ml).[56,57]

Preparation of Incubations with *A. Campanulatus* Extracts

Erythrocytes and leucocyte haemolysate obtained from healthy donors were divided into three groups:Control group, H_2O_2 group (oxidative stress H_2O_2 and oxidative stress group) and *A. campanulatus* extracts group.[13] All the incubation mixtures were prepared as per below procedures.

For erythrocytes

Group I: Control contains erythrocyte haemolysate 750 µl, PBS 1000 µl and distilled water 250 µl.

Group II: H_2O_2, erythrocyte haemolysate 750 µl, H_2O_2 (10 mM) 50 µl, PBS 950 µl and distilled water 250 µl.

Group III: Methanol extract, erythrocyte haemolysate 750 µl, H_2O_2 (10 mM) 50 µl, infusions of methanol extract 250 µl and PBS 950 µl.

Group IV: Ethanol extract, erythrocyte haemolysate 750 µl, H_2O_2 (10 mM) 50 µl, infusions of ethanol extract 250 µl and PBS 950 µl.

Group V: Acetone extract, erythrocyte haemolysate 750 µl, H_2O_2 (10 mM) 50 µl, infusions of acetone extract 250 µl and PBS 950 µl.

Group VI: Hydro-alcohol extract, erythrocyte haemolysate 750 µl, H_2O_2 (10 mM) 50 µl, infusions of acetone extract 250 µl and PBS 950 µl.

For leucocytes

Group I: Control contains leucocytes hemolysate 50 µl and PBS 1050 µl.

Group II: H_2O_2, leucocytes haemolysate 50 µl, H_2O_2 (10 mM) 50 µl and PBS 1050 µl.

Group III: Methanol extract, leucocytes haemolysate 50 µl, H_2O_2 (10 mM) 50 µl, methanol extract 500 µl and PBS 1050 µl.

Group IV: Ethanol extract, leucocytes haemolysate 50 µl, H_2O_2 (10 mM) 50 µl, ethanol extract 500 µl and PBS 1050 µl.

Group V: Acetone extract, leucocytes haemolysate 50 µl, H_2O_2 (10 mM) 50 µl, acetone extract 500 µl and PBS 1050 µl.

Group VI: Hydro-alcohol extract, leucocytes haemolysate 50 µl, H_2O_2 (10 mM) 50 µl, hydro-alcohol extract 500 µl and PBS 1050 µl.

All the above experimental groups were incubated in a shaking water-bath (60 rpm) for an hour at 37°C. Following the incubation, CAT, SOD, GPx, LPO GSH levels were determined. Three measurements were performed under each set of *A. campanulatus* extracts.

Assays of Antioxidant Enzyme Activity

Assay of CAT

The reaction mixture consisted of 1 ml PBS (50 mM, pH 7.00) and 2 ml diluted erythrocytes or leucocytes homogenate. The mixture is incubated at 25°C for 3 min and the reaction by the addition of 1 ml of 30 mM H_2O_2. The decomposition of H_2O_2 was followed directly by the decrease in absorbance at 240 nm at 25°C in a spectrophotometer (Shimadzu UV-1601).[58] The results were expressed for erythrocytes as AU/g hemoglobin (Hb) and for the leucocytes as U/mg protein.

Assay of SOD

SOD activity was determined using the RANDOX Ransod enzyme kit. This method employs xanthine and xanthine oxidase (XOD) generated superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitropheno)-5-phenyltetrazolium-chloride to form the red formazon dye. The SOD activity was measured by the degree of inhibition of this reaction.[13] The results were expressed for erythrocytes as U/g Hb and for leucocytes as U/mg protein.

Assay of GPx

GPx activity was determined using the RANDOX-Ransel enzyme kit. In this method, GPx catalyses the oxidation of GSH by hydrogen peroxide. In the presence of GSH reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH), the oxidised glutathione (GSSG) was immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$ (oxide form). The decrease in absorbances at 340 nm was measured.[13] The results were expressed for erythrocytes as U/g Hb and for leucocytes as U/mg protein.

Determination of GSH Contents

GSH was determined by using 5, 5'-di-thio-bis-2-nitro benzoic acid (DTNB). In this method, molecule of DTNB was reduced to 2-nitro-5-mercapto benzoic acid (NMBA) by GSH. NMBA was deep yellow and this colour was used to measure-SH groups by spectrophotometrically at 412 nm.[59] Erythrocyte and leucocyte haemogenate samples (1 ml) were taken. Following this, i.e., 4 ml of 5% trichloroacetic acid (TCA) was added in centrifuge tubes. This mixture was centrifuged at 1000 rpm for 15 min. PBS (50 mM, pH 8.00) 2 ml and 5 µM DTNB 250 µl were mixed with each of 200 µl erythrocyte and leucocyte supernatants. This absorbance of the mixture was measured against blank tube (added 200 µl distilled water instead of supernatant) at 412 nm.[13] The results were expressed for erythrocytes as µg/g Hb and for leucocytes as µg/mg protein.

Determination of LPO

LPO was measured by TBA method.[60,61] This method evaluates oxidative stress by measuring MDA, the last product of lipid breakdown caused by oxidative stress. All experimental groups of erythrocyte and leucocyte homogenate samples were used. Test solutions (samples and standards) of 0.5 ml were added to 4.0 ml of N/12 H_2SO_4 followed by the addition of 0.5 ml of 10% phosphotungustic acid and allowed to stand at room temperature for 5 min. and then centrifuged for 10 min at 3,000 rpm and supernatant was discarded. 2.5 ml N/12 H_2SO_4 and 0.2% TBA was added to these tubes and allowed to stand at boiling water bath for 60 min. After being cooled with tap water, 3 ml of the mixture of n-butanol and HCl (15:1, v/v) was added, and the mixture was shaken vigorously and absorbance of the organic layer (upper layer) was measured at 532 nm.[13] The results were expressed for erythrocytes as nmol/g Hb and for leucocytes as nmol/mg protein.

Determination of Hb Concentration

Hb concentration was determined by Drabkin method in erythrocyte hemolysate.[62]

Determination of Total Protein Concentration

Total protein concentration in leucocyte haemolysate was evaluated by using bovine serum albumin as standard.[63]

Statistical Analysis

All experimental data were statistically analysed and expressed as means ± SD by using one-way analysis of variance.

RESULTS AND DISCUSSION

Erythrocytes and leucocytes are critical targets for natural products and plants as well as many other drugs. Moreover, human erythrocytes and leucocytes are excellent subjects for studies of the biological effects of free radicals, since they are both structurally simple and easily obtained. Indeed, they have been used as a model for the investigation of free-radical induced oxidant stress because of several reasons, they are continually exposed to high oxygen tensions, they are unable to replace damaged components, the membrane lipids are composed partly of polyunsaturated fatty acid side chains, which are vulnerable to per oxidation and they have antioxidant enzyme systems.[64]

In-vitro oxidative haemolysis of human red blood cells (RBCs) was used as a model to study the free

radical induced damage of biological membranes and the inhibitory effect of natural antioxidants. Potential sources of antioxidant compounds have been searched in many types of plant materials such as fruits, leaves, seeds etc.[65] ROS such as hydrogen peroxide, organo peroxide, super oxide anion and OH are generated in biological systems by aerobic metabolism and also by exogenous sources such as drugs, ultra violet light, ionizing radiation and pollution systems.[66] According to generally accepted mechanisms, major deleterious effects are caused by OH generated from H_2O_2 and by the superoxide species generated in the presence of redox active transition metals.[3,4] H_2O_2 is the primary product of the reduction of O_2 by numerous oxidases such as XOD, uricase, D-amino acid oxidase, and α-hydroxy acid oxidase localized in peroxisones.[67] H_2O_2 is decomposed to H_2O and O_2 by a reaction catalysed by redox active metal complexes of which CAT and peroxidase are the most OH is responsible for DNA damage.[68]

A. campanulatus is a natural product that is high in fibre, rich in potassium, calcium (50 mg/g), phosphorus (34 mg/g), vitamin A (260 IU/g) and vitamin B_6 as well as with trace minerals such as selenium, zinc and copper.[21] Therefore, by keeping this view in mind, the protective effect of extracts of *A. campanulatus* tuber against H_2O_2 induced oxidative damage in human erythrocyte and leucocytes were established by this study. The results of our present study were in line with the above findings, as we have observed. Figures 1 and 2 showed that increased in the CAT, SOD, GPx and reduction of GSH and LPO levels in H_2O_2 group compared with control group. The extracts of *A. companulatus* tuber treated groups showed effective reduction of CAT, SOD, GPx and increased the GSH and LPO levels as compared with H_2O_2 group on human erythrocytes and leucocytes. The methanol extract showed more effective activity than others. However, all extracts of *A. companulatus* tuber were found protective on GSH levels of erythrocytes and leucocytes, which is consistent with its flavonoid and total phenol contents.[69] GSH is released by erythrocytes in response to oxidative stress, presumably to protect the essential thiol groups on the membrane surface. The increased use of the GSH and export of GSH catabolic products indicate an increased need for GSH in banked erythrocytes, probably to combat storage-mediated oxidative stress.[70] All extracts of *A. companulatus* tuber were found protective on LPO levels of erythrocytes and leucocytes against H_2O_2-induced oxidative damage.

Erythrocytes and leucocytes are rich in non-conjugated polyunsaturated fatty acids with reactive methylene groups that are susceptible to hydrogen atom obstruction. MDA is an end product of erythrocyte membrane LPO. MDA

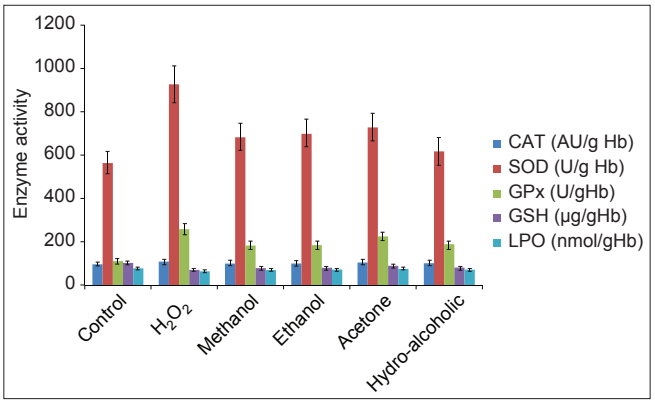

Figure 1: Effect of extracts of *A. campanulatus* tuber on human erythrocytes. (All values are replicate of three observations and expressed as mean ± S.D. GSH – Glutathione; LPO – Lipid peroxidation; CAT – Catalase; SOD – Superoxide dismutase; GPx – Glutathione peroxidase)

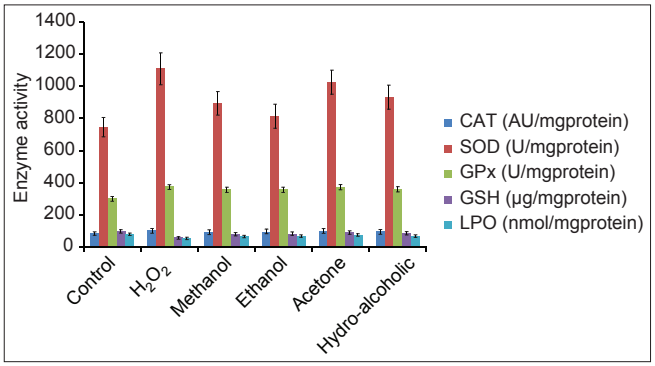

Figure 2: Effect of extracts of *A. campanulatus* tuber on human leucocytes. (All values are replicate of three observations and expressed as mean ± S.D. GSH – Glutathione; LPO – Lipid peroxidation; CAT – Catalase; SOD – Superoxide dismutase; GPx – Glutathione peroxidase)

a highly reactive bi-functional molecule has been shown to cross-link erythrocyte and leucocytes phospholipids and proteins to impair a variety of the membrane related functions and ultimately leading to diminished erythrocytes and leucocytes survival.[13]

CONCLUSION

The present findings suggest that, extracts of *A. companulatus* tuber possess protective effect against H_2O_2 induced oxidative damage. Furthermore, these tuber extracts may appeared to be beneficial in preventing H_2O_2 oxidative RBC damage in human and can improve RBC membrane permanence. The tubers of *A. companulatus* are a potential source of natural antioxidants for the treatment and prevention of disease in which LPO takes place. Thus, it is suggested that assessment of active constituents and clinical evaluation of *A. companulatus* tuber would give a positive lead in the successful treatment of against H_2O_2 induced oxidative damage diseases. However, further series of studies are required to prove its clinical reliability, safety and efficacy.

ACKNOWLEDGMENTS

Authors are thankful to the Principal, Adina Institute of Pharmaceutical Sciences, Sagar for providing the necessary facilities during this research work. Authors also wish to thanks Pinnacle Biomedical Research Institute, Bhopal.

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How to cite this article: Bais SS, Mali PY. Protective effect of *Amorphophallus campanulatus* tuber extracts against H_2O_2 induced oxidative damage in human erythrocytes and leucocytes. Int J Green Pharm 2013;7:111-6.

Source of Support: Nil, **Conflict of Interest:** None declared.