Original Article

Beneficial effect of extracts of *Premna integrifolia* root on human leucocytes and erythrocytes against hydrogen peroxide induced oxidative damage

Abstract

Background: 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. *Premna integrifolia* (Verbenaceae) is an important woody, medicinal plant and has a prominent place in Ayurvedha, Siddha and Unani system of medicines. Traditionally, it has been used for various antioxidant related disorders. **Objective:** The objective of the present study was to evaluate the beneficial effect of extracts of *P. integrifolia* root on human leucocytes and erythrocytes against hydrogen peroxide (H_2O_2) induced oxidative damage. **Materials and Methods:** Chloroform:methanol (1:1) extract of *P. integrifolia* (CMEPI) and aqueous extract of *P. integrifolia* roots were used to accessed catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione (GSH) and lipid peroxidation (LPO) levels in H_2O_2 induced oxidative damage. **Results:** Results of the present study revealed that, there was an increase in the CAT, SOD, GPx and reduction of the GSH and LPO levels in H_2O_2 group compared with the control. *P. integrifolia* root extract treated groups showed the reduction of CAT, SOD, GPx and increased in the GSH and LPO levels as compared with H_2O_2 group. CMEPI was found to be more effective than aqueous. **Conclusion:** The present study suggests that, extracts of *P. integrifolia* root possess beneficial effect on human leucocytes and erythrocytes against H_2O_2 induced oxidative damage which has substantiated their use in ethnomedicine as an antioxidant. Observed effect can be attributed due to the flavonoid and phenol contents in the plant. Furthermore, *in-vitro* and *in-vivo* studies are needed to explore its effects on antioxidant system of the body for proving its clinical safety, reliability and efficacy.

Key words:
Erythrocytes, hydrogen peroxide, leucocytes, Premna integrifolia

Introduction

Premna integrifolia Linn. (Verbenaceae), commonly known as Arni or Agnimantha.[1,2] It is a large shrub or a small tree distributed on the western sea coast from Bombay to Molucca, Srilanka and the Andaman. *P. integrifolia* root extract is an active ingredient of many ayurvedic preparations such as *Arishtam*, *Avaleham*, *Kvatham*, *Ghritam* and *Tailum*.[3] Roots are used in the treatment of diabetes, chyluria, inflammations, swellings, bronchitis, dyspepsia, liver disorders, piles, constipation and fever.[1] The root of the plant mainly contains premnine, ganiarine,[4] ganikarine,[5] premnazole,[6] clerodendrin-A,[7,8] glycerine,2,5-furandione, 3-methyl-, 2-furancarboxaldehyde, 5-(hydroxymethyl)-, benzofuran, 2,3-dihydro-, 2-hydroxy-3-methylbenzaldehyde, seychellene, dodecanoic acid, 1H-cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aà,4aà,7á,7aá,7bà)]-, 2-propenoic acid, 3-(4-methoxyphenyl)-, tetramethyltricyclo [5.2.2.0(1,6)] undecan-2-ol, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, n-hexadecanoic acid, phytol, octadecanoic acid, ethyl ester, 2-phenanthrenol,

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4b,5,6,7,8,8a,9,10-octahydro-4b,8,8-trimethyl-1-(1-methylethyl)-,[9] luteolin,[10] 1β,3α,8β-trihydroxy-pimara-15-ene, 6α,11,12,16-tetrahydroxy-7-oxo-abieta-8,11,13-triene, 2α,19-dihydroxy-pimara-7,15-diene[11,12] and acteoside (Verbacoside).[13] Moreover, alkaloids, proteins, carbohydrates, amino acids, steroids, flavonoids, glycosides, tannins and phenolic compounds were found in preliminary phytochemical screening of the root extracts of *P. integrifolia*.[14] It has been reported for its potential actions such as anti-diabetic and hypoglycemic,[15,16] anti-inflammatory,[6,17] immunomodulatory[18] cardiac stimulant,[19] analgesic and antibacterial,[20] anti-arthritic,[17,21] antioxidant,[22,23] hepatoprotective and *in-vitro* cytotoxic,[22-24] anti-hyperglycemic,[25] anti-parasitic,[26] hypolipidemic and anti-obesity.[27-30]

Antioxidants that are mainly supplied as dietary consumptions can impede carcinogenesis by scavenging oxygen radicals or interfering with the binding of carcinogens to deoxyribonucleic acid. This includes vitamin C and E, carotenoids (cryptoxanthin, lutein, zeaxanthin, lycopene) and several polyphenolic compounds including flavonoids (catechins, flavonols, flavones, isoflavonoids).[31,32] The antioxidative potentials of phenolic compounds can be attributed to their strong capability to transfer electrons to reactive oxygen species or free radicals, chelating metal ions by activating antioxidant enzymes and inhibitory oxidases.[33] In addition, free radicals and reactive oxygen species are constantly generated *in-vivo* and cause oxidative damage to biomolecules, a process held to check only by the existence of multiple antioxidants or repair systems as well as the replacement of damaged lipids or proteins.[34] As plants could represent a source of natural compounds with antioxidant activities, many studies have been conducted for the antioxidant activities of many plant extracts and their constituents,[35-38] due to their low toxicity and great medicinal value. Therefore, by keeping this view in mind the author has evaluated beneficial effect of extracts of *P. integrifolia* root on human leucocytes and erythrocytes against H_2O_2 induced oxidative damage.

Materials and Methods

Chemicals

Hydrogen peroxide (H_2O_2, 3%) (Universal), pyrogallol (high-media), butylated hydroxyl anisole, nitro blue tetrazolium, thiobarbituric acid (TBA), trichloroacetic acid (TCA), ethylene diamine tetra-acetic acid, ferrous chloride, ascorbic acid, etc. were obtained from Sigma-Aldrich, USA & SD Fine-Chem Ltd., Mumbai. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) activity were determined using commercial available enzyme kits (Ransel, RANDOX/RS-504 and Ransod, RANDOX/SD-125, RANDOX Laboratories, Crumlin, UK). All other chemicals and reagents used for experimental work were of AR grade.

Plant material and preparation of extracts

Fresh, well-developed *P. integrifolia* plants and their roots were collected from the region of North Karnataka, India in the month of September 2008 and it was authenticated by a taxonomist, Department of Botany, Basaveshwar Science College, B.V.V.S. Campus, Bagalkot, Karnataka, India. Voucher specimen (No. B.Sc/Bot/13/08) was deposited in the same. Roots were dried in the shade and powdered. The root powder was extracted with petroleum ether (40-60°C) and subsequently with 1:1 ratio of chloroform-methanol (55-60°C) for 24 h by using soxhlet apparatus. The residue was used for the preparation of aqueous extract; the powder was heated at 20-22°C in distilled water. After filtration through Whatmann filter paper no. 40, the extract was evaporated to dry by slow heating and continuous stirring on water bath. After the residue extractions, the excess solvents were completely removed by using a rotatory flash evaporator to get concentrated, then completely dried in freeze drier and preserved in an airtight container under refrigeration. Percentage yield of chloroform-methanol and aqueous extract were found to be 1.29% and 5.43% based on the initial plant material used for the extraction. The extracts used for the present study were formulated using 1% tween 80 with distilled.

Isolation of leucocytes

Human polymorphonuclear leukocytes were isolated from the freshly sampled venous blood of healthy volunteers using dextran (Macrodex: 6% dextran in 0.9% NaCl solution) and heparin tubes (25,000 IU/ml).[39,40]

Isolation of erythrocytes

Fresh venous blood was collected from the healthy volunteers 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, containing 140 mM NaCl) 3 times and erythrocytes were hemolyzed with ice-cold distilled water.[41]

Preparation of incubations with *P. integrifolia* root extracts

Leucocyte and erythrocytes hemolysate obtained from healthy volunteers were divided into control, H_2O_2 and *P. integrifolia* extracts group.[42,43] All the incubation mixtures were prepared are as per below procedures.

For leucocytes

- Group I: Control, 50 µl of leucocytes hemolysate and 1050 µl of PBS
- Group II: H_2O_2, 50 µl of leucocytes hemolysate, 50 µl of 10 mM H_2O_2 and 1050 µl of PBS
- Group III: Chloroform:methanol extract, 50 µl of leucocytes hemolysate, 50 µl of 10 mM H_2O_2, 1050 µl of PBS and 500 µl of chloroform:methanol extract
- Group IV: Aqueous extract, 50 µl of leucocytes hemolysate, 50 µl of 10 mM H_2O_2, 1050 µl of PBS and 500 µl of aqueous extract.

For erythrocytes

- Group I: Control, 750 µl of erythrocyte hemolysate, 1000 µl of PBS and 250 µl of distilled water
- Group II: H_2O_2, 750 µl of erythrocyte hemolysate, 50 µl of 10 mM H_2O_2, 950 µl of PBS and 250 µl of distilled water
- Group III: Chloroform:methanol extract, 750 µl of erythrocyte hemolysate, 50 µl of 10 mM H_2O_2, 950 µl of PBS, 250 µl of distilled water and 250 µl of infusions of chloroform:methanol extract
- Group IV: Aqueous extract, 750 µl of erythrocyte hemolysate, 50 µl of 10 mM H_2O_2, 950 µl of PBS, 250 µl of distilled water and 250 µl of infusions of aqueous extract.

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, GSH and LPO levels were determined.

Assays of Antioxidant Enzyme Activity

Assay of CAT

The reaction mixture consisted of 1 ml of 50 mM PBS (pH-7) and 2 ml of diluted leucocytes or erythrocytes homogenates as per above mentioned groups. The mixture was incubated at 25°C for 3 min after which 1 ml of 30 mM H_2O_2 was added. The decomposition of H_2O_2 was followed directly by a decrease in absorbance at 240 nm at 25°C in a UV-Visible spectrophotometer (SHIMADZU EUROPA GmbH, Germany, UV-1601).[44] The results were expressed for the leucocytes as U/mg protein and erythrocytes as U/g hemoglobin (Hb).

Assay of SOD

SOD activity was determined using the RANDOX Ransod enzyme kit. This method employs xanthine and xanthine oxidase generated superoxide radicals, which reacts 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.[42] The results were expressed for the leucocytes as U/mg protein and erythrocytes as U/g Hb.

Assay of GPx

GPx activity was determined using the RANDOX-Ransel enzyme kit. In this method, GPx catalyses the oxidation of GSH by H_2O_2. In the presence of GSH reductase and reduced nicotinamide adenine dinucleotide phosphate, the oxidized glutathione 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.[42] The results were expressed for the leucocytes as U/mg protein and erythrocytes as U/g Hb.

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 color was used to measure–SH groups by spectrophotometrically at 412 nm.[45,46] A volume of 1 ml of erythrocyte and leucocyte homogenates were taken. Following this, i.e., 4 ml of 5% TCA was added in centrifuge tubes. This mixture was centrifuged at 1000 rpm for 15 min. 2 ml of 50 mM PBS (pH-8) and 250 µl of 5 µM DTNB 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.[42] The results were expressed for the leucocytes as U/mg protein and erythrocytes as U/g Hb.

Determination of LPO

LPO was measured by TBA method.[46,47] Test solutions of 0.5 ml were added to 4.0 ml of N/12H_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 3000 rpm and the supernatant was discarded. 2.5 ml N/12H_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.[42] The results were expressed for the leucocytes as nmol/mg protein and erythrocytes as nmol/g Hb.

Determination of Hb

Hb was determined by Drabkin method in erythrocyte hemolysate.[48] Drabkin's reagent contains potassium cyanide and potassium ferricyanide. Hb reacts with ferricyanide to form methemoglobin, which is converted to stable cyanmethemoglobin (HiCN) by the cyanide. The intensity of the color is proportional to Hb concentration and is compared with a known cyanomethemoglobin standard at 540 nm.[49]

Determination of total protein

Total protein in leucocyte hemolysate was determined by Lowry method with some modifications using bovine serum albumin as standard.[50] The Lowry reaction consists of the Biuret reaction followed by the reduction under alkaline conditions of the Folin-Ciocalteu reagent. Copper ions facilitate the reduction process. The sensitivity of the test depends upon the composition of the protein. The product of the reaction, i.e., heteropolymolybdenum blue, is intense blue with the absorption at 550 nm.

Statistical analysis

All experimental data were statistically analyzed and expressed as mean ± standard deviation using one-way analysis of variance.

Results and Discussion

Recently, there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing free radical-induced tissue injury.[51] Besides, well-known and traditionally used natural antioxidants from teas, wines, fruits, vegetables and spices, some natural antioxidants (e.g., rosemary and sage) are already exploited commercially either as antioxidant additives or as nutritional supplements.[52] Human leucocytes and erythrocytes are excellent subjects for studies of biological effects of free radicals, since they are both structurally simple and easily obtained. They are critical targets for natural products and plants as well as many other drugs. Indeed, they have been used as a model for the investigation of free-radical induced oxidative stress due to several reasons such as, 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.[53] Antioxidants exert their action either scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms.[54] Results of this study was in line with the above findings, as the author has observed that, there was an increased in the CAT, SOD, GPx and reduction of the GSH and LPO levels in H_2O_2 group compared with the control. Extracts of *P. integrifolia* root treated groups showed the reduction of CAT, SOD, GPx and increased the GSH and LPO levels as compared with H_2O_2 group as shown in Figures 1 and 2. Chloroform:methanol (1:1) extract of *P. integrifolia* root was found to be more effective than aqueous. The extracts used under the study exhibited antioxidant activity against H_2O_2 induced oxidative damage due to the presence of flavonoids and phenolic compounds which have been reported to possess free radical scavenging activities. It has been mentioned that antioxidant activity of plants might be due to their phenolic compounds.[55] Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic oxidative enzymes and anti-inflammatory action.[56] Both extracts were found protective on GSH and LPO levels, which is consistent with its flavonoid and total phenolic contents.[57] GSH is released by erythrocytes in response to oxidative stress, presumably to protect the essential thiol groups on the membrane surface.[58] Hence, this beneficial effect of extracts of *P. integrifolia* root on human leucocytes and erythrocytes against H_2O_2 induced oxidative damage is the index to reduce injurious effects or to preserve the normal physiological mechanisms that have been distributed by an oxidative stress inducing agents. Therefore, the significant number of modern drugs are thus based on or derived from the medicinal plants.

Conclusion

Therefore, it can be concluded that the extracts of *P. integrifolia* root possessed beneficial effects on human leucocytes and erythrocytes against H_2O_2 induced oxidative damage which is substantiated their use in ethnomedicine as an antioxidant. *P. integrifolia* roots are the potential source of natural antioxidants which would be helpful in treating and preventing many free radical mediated diseases. Observed effect can be attributed due to the flavonoid and phenol contents in the plant. Furthermore, *in-vitro* and *in-vivo* studies are needed to explore its effects on antioxidant system of the body for proving its clinical safety, reliability and efficacy.

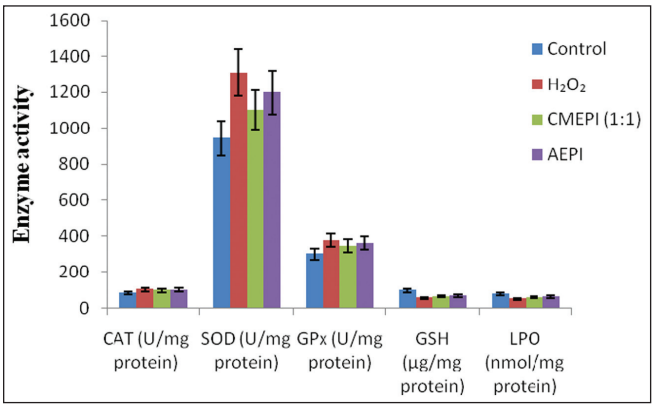

Figure 1: Effect of extracts of *Premna integrifolia* on human leucocytes. All values are replicate of three observations and expressed as mean ± standard deviation. CAT – Catalase; SOD – Superoxide dismutase; GPx – Glutathione peroxidase; GSH – Glutathione; LPO – Lipid peroxidation

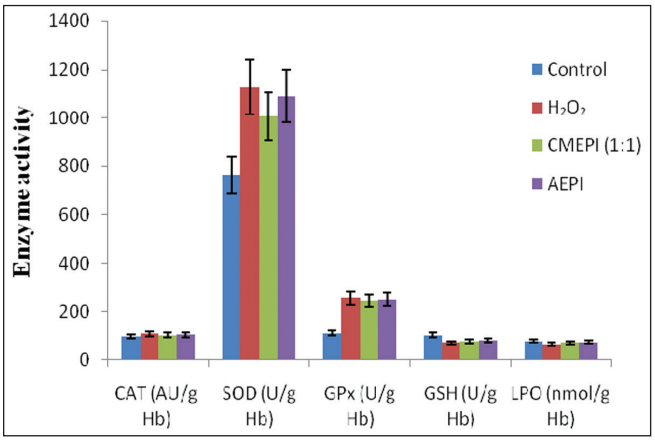

Figure 2: Effect of extracts of *Premna integrifolia* on human erythrocytes. All values are replicate of three observations and expressed as mean ± standard deviation. CAT – Catalase; SOD – Superoxide dismutase; GPx – Glutathione peroxidase; GSH – Glutathione; LPO – Lipid peroxidation

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