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Original Article

Enzymatic and non-enzymatic antioxidant properties of *Indigofera longeracemosa* – An *in vitro* study

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ABSTRACT

Antioxidant compounds present in many plants can protect cells against damage caused by ROS. The present study is focused on the assessment of antioxidant potential of *Indigofera longeracemosa* by evaluating both enzymatic and non-enzymatic antioxidants. The activities of enzymatic antioxidants (Superoxide dismutase, Catalase, Glutathione-s-transferase, Glutathione peroxidase, Peroxidase, Ascorbate oxidase and Polyphenoloxidase) and non-enzymatic antioxidants (Total reduced glutathione, Vitamin E, Vitamin A and Vitamin C) were determined. From the present study, it can be concluded that the plant has the capability to scavenge the free radicals and protect against oxidative stress related disorders. In future, *Indigofera longeracemosa* may serve as a good pharmacotherapeutic agent.

Keywords: *Indigofera longeracemosa*, Enzymatic antioxidants, Non-enzymatic antioxidants, Oxidative stress, Free radicals.

INTRODUCTION

Free radicals have been implicated in causation of ailments such as liver cirrhosis, atherosclerosis, cancer, diabetes etc [1]. Free radical is any atom or a molecule, which has a single electron on its external orbit. Unstable free radicals are produced in normal metabolism when oxygen is used to burn food for energy [2]. Reactive oxygen species (ROS) such as superoxide anions, hydroxyl radical and nitric oxide inactivate enzymes and damage important cellular components causing injury through covalent binding and lipid peroxidation [3]. Mammalian cells possess elaborate defense mechanisms of metabolic enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and nonenzymic molecules like thioredoxin and thiols which play important roles in antioxidant defense systems for radical detoxification [4]. Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by other mechanisms and thus prevent diseases [5]. Although a living system possesses several natural defense mechanisms, such as enzymes and antioxidant nutrients, which arrest the chain reaction of ROS initiation and production, its continuous exposure for a long time may lead to irreversible oxidative damage [6]. Many plants often contain substantial amounts of antioxidants including vitamin C and E, carotenoids, flavonoids and tannins etc. and thus can be utilized to scavenge the excess free radicals from human body. *Indigofera longeracemosa* a tropical shrub plant belonging to the family Fabaceae has been used as a diuretic. In India, the root has also been used in tribal medicine as an antidote for all snake poison [7]. Many compounds with antimicrobial, antiulcerogenic, pharmacognostical activities have been isolated from Indigofera longeracemosa [8]. Though the chemical composition and pharmacological properties of the leaves of Indigofera longeracemosa were investigated, the antioxidant levels of this plant have not been reported previously. Therefore the main aim of this study was to evaluate the level of enzymatic and non-enzymatic antioxidants of *Indigofera longeracemosa*.

MATERIALS AND METHODS

Plant Collection: Fresh plant, I.longeracemosa was collected from Kozhijampara and authenticated from Botanical survey of India, Tamil Nadu Agricultural University, Coimbatore, India.

Plant Sample Extraction: The fresh samples were prepared by grinding one gram of *Indigofera*

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longeracemosa, 2 ml of 50% ethanol, separately, in a pre-chilled mortar and pestle and the extracts were centrifuged at 10,000 g at 4ºC for 10 minutes. The supernatants thus obtained were used within four hours for various enzymatic and non-enzymatic antioxidants assays.

Phytochemical Analysis: Preliminary phytochemical screening of the ethanolic extract of *Indigofera longeracemosa* was estimated according to the method adopted by Peach and Tracey [9].

Assay of Superoxide Dismutase (SOD): The assay of superoxide dismutase was done according to the method of Das [10]. In this method, 1.4ml aliquots of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer of pH 7.4, 0.075 ml of 20 mM L-Methionine, 0.04ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM Hydroxylamine hydrochloride and 0.1ml of 50 mM EDTA) was added to 100µl of the sample extract and incubated at 30ºC for 5 minutes. 80 µl of 50 µM riboflavin was added and the tubes were exposed for 10 min to 200 W-philips fluorescent lamps. After the exposure time, 1ml of Greiss reagent (mixture of equal volume of 1% sulphanilamide in 5% phosphoric acid) was added and the absorbance of the color formed was measured at 543 nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation under assay conditions.

Assay of Catalase (CAT): Catalase activity was assayed by the method of Sinha [11]. The enzyme extract (0.5 ml) was added to the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M H2O2, 0.4 ml H2O and incubated for different time period. The reaction was terminated by the addition of 2 ml of acid reagent (dichromate/acetic acid mixture) which was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3 by volume). To the control, the enzyme was added after the addition of acid reagent. All the tubes were heated for 10 minutes and the absorbance was read at 610 nm. Catalase activity was expressed in terms of µmoles of H2O2 consumed/min/mg protein.

Assay of Glutathione Peroxidase (GPX): Glutathione peroxidase was assayed according to the method of Rotruck et al., [12] with slight modifications. The reaction mixture consisting of 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H2O2, 0.2 ml of water and 0.5 ml of plant extract was incubated at 0, 30, 60, 90 seconds respectively. The reaction was terminated with 0.5 ml of 10% TCA and after centrifugation; 2 ml of the supernatant was added to 3 ml of phosphate buffer and 1ml of DTNB reagent (0.04% DTNB in 1% sodium citrate). The color developed was read at 412 nm and the enzyme activity is expressed in terms of µg of glutathione utilized/min/mg protein.

Assay of Glutathione S Transferase (Gst): Glutathione transferase activity using 2, 4 dichloronitrobenzene as substrates was assayed spectrophotometrically essentially as described by Habig et al.,[13]. The cuvettes (final volume of 3.0 ml) contained 0.1 M phosphate buffer (pH 6.5), 1 mM GSH and 1 mM of chlorodinitrobenzene and 20 µl of appropriately diluted plant extract from the different sources. Change in absorbance at 340 nm was followed against a blank containing all reactants excepting enzyme protein, Specific activity was expressed as µmol conjugate formed/min/mg protein

Assay of Peroxidase: The assay was carried out by the method of Addy and Goodman [14]. The reaction mixture consisted of 3ml of buffered pyrogallol (0.05 M pyrogallol in 0.1 M phosphate buffer (pH 7.0)) and 0.5 ml of 1% H2O2. To this added 0.1 ml plant extract and O.D. change was measured at 430 nm for every 30 seconds for 2 minutes. The peroxidase activity was calculated using an extinction coefficient of oxidized pyrogallol (4.5 litres/mol).

Assay of Ascorbate Oxidase: Assay of ascorbate oxidase activity was carried out according to the procedure of Vines and Oberbacher [15]. The sample was homogenized [1: 5 (w/v)] with phosphate buffer (0.1 M/ pH 6.5) and centrifuged at 3000 g for 15 min at 50oC. The supernatant obtained was used as source. To 3.0 ml of the substrate solution (8.8 mg ascorbic acid in 300 ml phosphate buffer, pH 5.6), 0.1 ml of the plant extract was added and the absorbance change at 265 nm was measured for every 30 seconds for a period of 5 minutes. One enzyme unit is equivalent to 0.01 O.D. changes per min.

Assay of Polyphenol Oxidase (PPO): Assay of Polyphenol oxidase activity was carried out according to the procedure of Sadasivam and Manickam [16]. To 2.0 ml of plant extract, added 3.0ml of distilled water and mixed well. 1.0ml of cathecol solution (0.4mg/ml) was added to the above solution and the reactants were quickly mixed. The enzyme activity was measured as change in absorbance/min at 490nm.

Estimation of Reduced Glutathione: The amount of reduced glutathione in the samples was estimated by the method of Boyne and Ellman [17]. 1ml of the sample extract was treated with 4.0 ml

of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl dissolved in 100ml water). After centrifugation, 2.0 ml of the protein-free supernatant was mixed with 0.2 ml of 0.4 M Na2HPO4 and 1.0 ml of DTNB reagent (40 mg DTNB in 100 ml of aqueous 1% tri sodium citrate). Absorbance was read at 412 nm within 2 minutes.

Estimation of Vitamin C: The assay mixture for vitamin C consisted of 0.1 ml of brominated sample extract, 2.9 ml of distilled water, 1 ml of 2% DNPH reagent and 1-2 drops of thiourea. After incubation at 37ºC for 3 h, the orange-red osazone crystals formed were dissolved by the addition of 7 ml of 80% sulphuric acid and absorbance was read at 540 nm after 30 minutes. Vitamin C concentration was expressed in terms of µg/mg plant tissue.

Estimation of Vitamin A: Vitamin A from the fresh and dried sample was extracted twice with 10 ml proportions of petroleum ether (40o-60oC). Pooled the extracts and washed thoroughly with water separating the layers using separating funnels. Added sodium sulphate (anhydrous) to remove the moisture. 1 ml of the ether extract was then taken and evaporated to dryness at 60oC. The dried residue was dissolved in 1 ml chloroform and used for estimation. The estimation of Vitamin A in the sample was analyzed using the method of Bayfield and Cole [18].

Estimation of Vitamin E: The tissue were homogenized in a blender. Weighed accurately 2.5 g of the homogenized tissue into a conical flask. Added 50ml of 0.1 N sulphuric acid slowly without shaking. Stoppered and allowed to stand overnight. The next day the content of the flask were shaken vigorously and filtered through Whatman No.1 filter paper, discarding the initial 10-15 ml of the filtrate. Aliquots of the filtrate were used for the estimation. The estimation of Vitamin E in the sample was analyzed using the Emmerie-Engel method, as described by Rosenberg [19].

Statistical Analysis: The results obtained were expressed as mean ±SD.

RESULTS

Phytochemical screening: The phytochemical constituents of the sample was presented in the Table 1.

Table 1: Phytochemical Screening of *Indigofera longeracemosa*

Phytochemical compounds	Ethanolic extract
Alkaloids	+
Saponins	+
Terpenoid	+
Tannins & Phenolic compounds	+
Flavonoids	+
Carbohydrates	+
Aminoacids & Proteins	-
Steroids	+

'+'Present '_' Absent

Estimation of Enzymatic Antioxidants: The levels of enzymatic antioxidants such as SOD, CAT, GPx, GST, Ascorbate Oxidase, Peroxidase, and Polyphenol oxidase were represented in table 2. SOD and CAT in *Indigofera longeracemosa*, were found to be 18.58±0.30units/mg protein and 29.37±0.81µmole of H_2O_2 consumed/min/mg proteins respectively. In this study the polyphenol oxidase level was found to be 2.23±0.17µmoles/g tissue in fresh sample of *Indigofera longeracemosa*. Ascorbate oxidase activity was found to be 29.23±0.93unit/g sample in *Indigofera longeracemosa*. The activity of glutathione peroxidase and Glutathione S transferase in *Indigofera longeracemosa* was found to be 221.83±1.54 and 193.51±0.57 respectively.

Table2: Levels of enzymatic antioxidants present in fresh sample of *I. longeracemosa*.

S.NO	Parameters	Values
1.	Superoxide Dismutase	18.58±0.30
2.	Catalase	29.37±0.81
3.	Glutathione Peroxidase	221.83±1.54
4.	Glutathione S Transferase	193.51±0.57
5.	Ascorbate oxidase	29.23±0.93
6.	Peroxidase	281.57±1.29
7.	Poly phenol oxidase	2.23±0.17

Values are expressed as Mean±SD (n=3)

Units: SOD: Units/mg protein, Catalase: µmole of H_2O_2 consumed/min/mg protein, GPx: µg of glutathione oxidized/min/mg protein; GST: µmoles of CDNB-GSH conjugate formed/min/mg protein, Peroxidase: µmoles/g sample; Ascorbate oxidase: unit/g sample, Polyphenol oxidase: µmoles/g tissue

Estimation of Non-Enzymatic Antioxidants: The levels of non-enzymatic antioxidants such as Total reduced glutathione and Vitamin C were represented in table 3. The activity of total reduced glutathione and vitamin C was found to be 61.21±1.89 and 51.56±1.45 respectively.

Table 3: Levels of non-enzymatic antioxidants present in fresh sample of *I. longeracemosa*.

S.NO	Parameters	Values
1.	Total reduced Glutathione	61.21 ± 1.89
2.	Vitamin C	51.56± 1.45
3.	Vitamin A	66.98± 0.37
4.	Vitamin E	18.12± 0.86

Values are expressed as Mean±SD (n=3)

Units: Total reduced glutathione: nM/mg plant tissue, Vitamin C: µg/mg plant tissue , Vitamin A: µg/mg plant tissue, Vitamin E: µg/mg plant tissue

DISCUSSION

Reactive oxygen species are produced naturally in cells as byproducts of the metabolism of oxygen as well as in response to various environmental stresses including UV radiation, pollutants, and heat exposure. Additionally, ROS levels can be altered by disease and injury, including cancer, neurodegenerative disease, cardiovascular disease, ischemia, stroke and aging. Reactive oxygen species also play an important role in cell signaling, a process called redox signaling. The regulation of ROS within cells is important for maintaining a proper homeostasis. Enzymatic and nonenzymatic antioxidants normally counteract damaging effects of intracellular ROS by either repairing the oxidative damage or directly scavenging oxygen radicals. The three most important specialized antioxidant enzymes are the superoxide dismutase (SOD) that converts O_2 into H_2O_2 which is detoxified into H_2O and O_2 by either catalase (CAT) or peroxidases [20]. Superoxide dismutase (SOD) scavenges harmful superoxides (O_2-) within cells protecting them from harmful oxidation of lipids, proteins and nucleic acids. Its altered expression levels have been linked to Down's syndrome, ALS and various cancers. Within a cell, the superoxide dismutases (SODs) constitute the first line of defence against ROS [21]. Catalase catalyzes the reduction of H_2O_2 to water and it can also remove organic hydroperoxides. Nervous system in body is sensitive to free radical damage due to rich content of easily oxidizible fatty acids and relatively especially low content of antioxidants including catalase. CAT, associated with other enzymatic antioxidants (peroxidases, super-oxide dismutase) is capable of removing, neutralizing, or scavenging oxy-intermediates [22]. GPx is inactivated by a variety of physiological substances, including nitric oxide and carbonyl compounds in vitro and in cell culture. Decreased GPx activity has also been reported in tissues where oxidative stress occurs in several pathological animal models. The accumulation of increased levels of peroxide resulting from inactivation of GPx may act as a second messenger and regulate expression of anti-apoptotic genes and the GPx itself to protect against cell damage [23]. Glutathione peroxidase (GPx), an enzyme that is uniquely positioned in the ROS degradation pathway to protect cells from excessive levels of hydrogen peroxide (H_2O_2) and intracellular lipid peroxides [24]. It is believed that the glutathione peroxidase enzyme, protects the erythrocyte against peroxides that are generated intracellularly or exogenously [25]. Glutathione peroxidases are substantially more efficient on a molar basis than other enzymes. Glutathione peroxidase (GPx), by

virtue of its ability to catabolize both H_2O_2 and lipid peroxides, is uniquely positioned to protect tissues from ROS. Glutathione *S*-transferases (GSTs) are evolutionarily conserved enzymes that are important in the detoxification of many xenobiotic compounds. These enzymes catalyze the conjugation of glutathione to electrophilic substrates, producing compounds that are generally less reactive and more soluble. This facilitates their removal from the cell via membrane-based glutathione conjugate pumps. The broad substrate specificity of GSTs allows them to protect cells against a range of toxic chemicals [26]. Also, when constitutive GST activity is inhibited, accumulation of products of lipid peroxidation occurs, resulting in increased cellular apoptosis [27]. However, GST activity can sometimes be deleterious to the cell. For example, dihaloalkanes are bioactivated by conjugation with glutathione, generating more genotoxic metabolites [28]. Polyphenol oxidases (PPOs) catalyze the O_2-dependent oxidation of mono- and *o*-diphenols to *o*diquinones, highly reactive intermediates. A defensive role for PPO has frequently been suggested due to the conspicuous appearance of PPO reaction products upon wounding, pathogen infection, or insect infestation, and due to the inducibility of PPO in response to various abiotic and biotic injuries or signaling molecules [29]. Ascorbate oxidase (AO) is a cell wall-localized enzyme that uses oxygen to catalyse the oxidation of ascorbate (AA) to the unstable radical monodehydroascorbate (MDHA) which rapidly disproportionates to yield dehydroascorbate (DHA) and AA, and thus contributes to the regulation of the AA redox state [30]. Recently, the enzyme has been used for clinical and food analyses of L-ascorbic acid. Several recent studies documented the importance of intracellular GSH, via glutathione peroxidase and the GSH redox cycle, in protecting cells from oxidative stress caused by oxygen-derived species [31,32]. Depletion of GSH results in oxidative stress and increased cytotoxicity, whereas elevation of intracellular GSH levels is recognized as an adaptive response to oxidative stress [33].

Vitamin C is the major water-soluble antioxidant present within the cell and extracellular fluids. Vitamin C readily scavenges free radicals and may thereby prevent oxidative damage of important biological macro molecule [34]. This vitamin is able to provide protection against phagocyte-derived oxidants by reducing the adhesion of phagocytes to endothelium, attenuating respiratory burst, and preventing subsequent lipid peroxidation [35]. Vitamin E appears to play a critical role in protecting the cell membrane from free radical reactions and peroxidation of polyunsaturated fatty acids (PUFA) [36]. Vitamin E is one of the few nutrients for which supplementation with higher than recommended has been shown to enhance immune response and good flavor, prevents microbial deterioration and resistance to diseases [37]. Many studies have suggested that high intake of Vitamin E may slow down the development and progression of atherosclerosis. Some clinical trials also reported the beneficial effects of Vitamin E supplementation in the secondary prevention of cardiovascular events [38]. Vitamin A (retinol) is essential for a diversity of physiological processes, including vision, embryonic development, skin differentiation, spermatogenesis, and immune system function [39]. Vitamin A and retinoid, either topically or orally administered, were able to induce complete remission in a high proportion of patients with basal cell and advanced squamous cell carcinoma [40].

CONCLUSION

Plant based products have been in use for medicinal, therapeutic and other purposes right from the dawn of history. Based on all these finding it is suggested that, *Indigofera longeracemosa* can be considered as a potential source of natural antioxidants that could have great importance as therapeutic agents in preventing or slowing the oxidative stress related degenerative diseases such as cancer and various other human ailments.

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