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Research Article

ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANT PROPERTIES OF *Tylophora pauciflora* Wight and Arn. – AN *IN VITRO* STUDY

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ABSTRACT

Objectives: Many oxidative stress related diseases are as a result of accumulation of free radicals in the body. A lot of researches are going on worldwide directed towards finding natural antioxidants of plants origins. In this study, we assessed enzymatic and non-enzymatic antioxidant properties of *Tylophora pauciflora.*

Method: The enzymatic antioxidants (Superoxide dismutase, Catalase, Glutathione-s-transferase, Glutathione peroxidase, Peroxidase, Ascorbate oxidase and Polyphenoloxidase) and non-enzymatic antioxidants (Total reduced glutathione and Vitamin C) activities were determined.

Results: The present study revealed that *Tylophora pauciflora* has an excellent source of enzymatic and non-enzymatic antioxidants.

Conclusion: The present study, results has capability to scavenge the free radicals and protect against oxidative stress causing diseases. In future *Tylophora pauciflora* may serve as a good pharmacotherapeutic agent.

Keywords: Tylophora pauciflora, enzymatic antioxidants, Non-enzymatic antioxidants, Oxidative stress, Free radicals

INTRODUCTION

Free radicals are more with one or more unpaired electrons [1, 2]. These highly reactive molecules attack the nearest stable molecule to obtain an electron. Subsequently, the targeted molecule becomes a free radical itself and initiates a cascade of events that can ultimately lead to cellular damage [3]. However, at physiological levels, free radicals also help preserve hemostatic by acting as signal transducers [4]. Free radicals are the new "buzz word" in pathophysiology today. They have a special affinity for lipids, proteins and nucleic acids (DNA). Most molecules have all their electrons in pairs and are therefore not free radicals. Molecules are held together by pairs of electrons forming stable bonds, but breaking a bond forms highly reactive free radicals [5].

Plants have been an important source of medicine and has helped human in the maintenance of health for thousands of years [6]. According to the World Health Organization estimates that up to 80 percent of people still rely mainly on traditional remedies such as herbs for their health needs due to better cultural acceptability, fewer side effects and better compatibility with the human body [7]. Its civilization is very ancient and the country as a whole has long been known for its rich resources of medical plants [8].

Tylophora pauciflora Wight and Arn is a synonym of *Vincetoxicum pauciflorum* (Wight & Arn.) Kuntze distributed throughout South India, especially in the Southern Western Ghats [9]. Generally *Tylophora* genus plant has been traditionally used for the treatment of allergic and inflammatory disorders like bronchial asthma, rhinitis, and whooping cough, antitumor, anticancer, immune-modulatory, anti-arthritis, anti-asthmatic, smooth muscle relaxant and anti-lupus activity.

The main objective of this study is, to evaluate the level of enzymatic and non enzymatic antioxidants of Tylophora *pauciflora.*

MATERIALS AND METHODS

PLANT COLLECTION

The Fresh whole plant of *Tylophora pauciflora* used for the investigation was obtained from Tirunelveli district, Tamilnadu, India. The plant was identified by Dr. Kalidass, Botanical survey of India, TNAU Campus, Coimbatore. The plant sample was collected and deposited in the Herbarium of the Botany Department, Bharathiar University, Coimbatore. The voucher number is 06155.

PLANT SAMPLE EXTRACTION

The fresh samples were prepared by grinding one gram of *Tylophora pauciflora* in 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.

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 H_2O_2, 0.4 ml H_2O 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 H_2O_2 consumed/min/mg protein.

ASSAY OF GLUTATHIONE PEROXIDASE (GP_X)

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 H_2O_2, 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 spectrophotometric ally 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% H_2O_2. 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 50°C. 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 and 3.0ml of distilled water added and mixed together. 1.0ml of cathecol solution (0.4mg/ml) 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 (TRG)

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 Na_2HPO_4 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. GSH concentration was expressed as nmol/mg protein.

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.

STATISTICAL ANALYSIS

The results obtained were expressed as mean ±SD.

RESULTS

Estimation of Enzymatic Antioxidants

The level of enzymatic antioxidants such as SOD, CAT, GPx, GST, Ascorbate Oxidase, Peroxidase, and Polyphenol oxidase values showed in table 1. SOD and CAT in *Tylophora pauciflora* were found to be 29.78±0.57units/mg protein and 39.87±0.51µmole of H_2O_2 consumed/min/mg proteins respectively. In this study the polyphenol oxidase level was found to be 2.19±0.127 µmoles/g tissue in fresh sample of *Tylophora pauciflora.* Ascorbate oxidase activity was found to be 27.23±0.57 unit/g sample in *Tylophora pauciflora*. The activity of glutathione peroxidase and Glutathione S transferase in *Tylophora pauciflora* was found to be 261.53±2.54and 293.11±1.57 respectively. In our study the polyphenol oxidase level was found to be 2.19±0.127 µmoles/g tissue in fresh sample of *Tylophora pauciflora*

Table1: Levels of enzymatic antioxidants present in fresh sample of T. pauciflora

S.NO	Parameters	Values
1	Superoxide Dismutase	29.78±0.57
2	Catalase	39.87±0.51
3	Glutathione Peroxidase	261.53±2.54
4	Glutathione S transferase	293.11±1.57
5	Ascorbate Oxidase	27.23±0.57
6	Peroxidase	381.57±2.19
7	Polyphenol Oxidase	2.19±0.127

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 level of non-enzymatic antioxidants such as Total reduced glutathione and Vitamin C showed in table 2. The activity of total reduced glutathione and vitamin C was found to be 61.21±1.89 and 251.56±2.45 respectively.

Table 2: Levels of non-enzymatic antioxidants present in fresh sample of *T. pauciflora*

S.NO	Parameters	Values
1	Total reduced glutathione	61.21±1.89
2	Vitamin C	251.56±2.45

Values are expressed as Mean±SD (n=3)

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

DISCUSSION

Oxidative damage has been suggested to occur as a consequence of reactive oxygen species (ROS) produced as a byproduct of ETC in mitochondria. A number of studies have been suggested that ROS can affect critical events associated with many disorders [18]. It gets special attention due to many factors such as drought, cold, heat, herbicides and heavy metals, because they harm the cell by raising the oxidative level through loss of cellular structure and function [19], hence demands the detoxification agents like enzymes such as SOD, catalase and peroxidase [20] and non enzymatic antioxidants such as flavones, anthocyanin, carotenoids and ascorbic acid [21].The formation of ROS is prevented by an antioxidant system: low molecular mass antioxidants (ascorbic acid, glutathione, and

tocopherols), enzymes regenerating the reduced forms of antioxidants, and ROS-interacting enzymes such as SOD, peroxidases and catalases [22]. The SOD enzyme destroys the superoxide radical; however, as a result of that it creates hydrogen peroxide, which also has high toxic properties [23]. It has been reported as one of the most important antioxidant defense enzyme that scavenge superoxide anion by converting to hydrogen peroxide thus diminish the toxic effect caused by this radical [24].

Catalase is a tetrahedrical protein, constituted by four heme groups which catalyze the dismutation of hydrogen peroxide in water and oxygen [25] Phenol oxidases are copper proteins catalyse the aerobic oxidation of certain phenolic compounds to quinones. Polyphenol oxidase is one of the major enzymes that have a role in the biosynthesis of lignin and defense against water stress by scavenges H_2O_2 in chloroplasts [26, 27].

Ascorbate oxidase is a member of the multicopper oxidase family. It catalyzes the oxidation of ascorbic acid to dihydroascorbic acid. Recently, the enzyme has been used for clinical and food analyses of L-ascorbic acid [28]. The role of this enzyme is to regulate the levels of oxidized and reduced glutathione and NADPH [19]. The definitive biological function of ascorbate oxidase is not clear, although it has been reported that the enzyme may participate in a redox system involving ascorbic acid [29].

Glutathione S-transferases (GSTs), a family of cytosolic multifunctional enzymes. It catalyzes the conjugation of glutathione with a variety of reactive electrophilic compounds, thereby neutralizing their active electrophilic sites and subsequently making the parent compound more water soluble. Glutathione peroxidases are substantially more efficient on a molar basis than other enzymes [30, 31]. Glutathione peroxidase acts as a radical scavenger, membrane stabilizer [32] and precursor of heavy metal binding peptides [33].

Glutathione, a major non-protein thiol in living organisms, plays a central role in coordinating the body's antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption. Reduced thiols have long been reported to be essential for recycling of antioxidants like vitamin E and vitamin C [34]. The concentration of different non-enzymatic antioxidants in fresh sample of *Tylophora pauciflora* was also assessed.

Vitamin C is regard as the first line natural antioxidant defense in plasma and a powerful inhibitor of LPO [35]. Vitamin C is a water soluble antioxidant. It acts as a free radical scavenger. It scavenges peroxyradicals [36]. Vitamin C protects non-smokers against the harmful effects of ROS from passive smoking [37]. It has been found in the chloroplast, cytosol, vacuole and extracellular compartments of the plant cells and shown to function as a reluctant for many free radicals [38].

CONCLUSION

Based on all these finding it is suggested that this plant is a potential source of natural antioxidant 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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