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Non-enzymatic and enzymatic antioxidant variations in tender and mature leaves of *Strychnos nux-vomica* L. (Family: Loganiaceae)

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Abstract

In the present investigation, *Strychnos nux-vomica*, an important plant used in traditional medicine, was evaluated for its antioxidant potential. The antioxidant potentials were examined in terms of non-enzymatic antioxidant molecules and activities of antioxidant enzymes. The non-enzymatic antioxidant molecules studied were ascorbic acid, α-tocopherol and reduced glutathione. The estimated antioxidant enzymes were superoxide dismutase, ascorbate peroxidase, catalase, peroxidase and polyphenol oxidase. The analyses were carried out in the field-collected leaf samples. It was found that plant contained a significant quantity of non-enzymatic and enzymatic antioxidants in the leaves. These findings have high significance in the pharmacological industry; however, a detailed investigation is needed to confirm this conclusion, by radical scavenging ability, which is underway in our laboratory. ***To cite this article: R. Vijayakumar et al., C. R. Biologies 332 (2009).***

Keywords: Ascorbic acid; Ascorbate peroxidase; Catalase; Peroxidase; Polyphenol oxidase; Reduced glutathione; *Strychnos nux-vomica*; Superoxide dismutase; α-tocopherol

1. Introduction

The World Health Organization (WHO) has estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. Also, modern pharmacopoeia still contains at least 25% of drugs derived from plants, and many others which are synthetic analogues built on prototype compounds isolated from plants. The Indian system of medicine, Ayurveda, Sidha and Unani uses over 2000 medicinal plants of which the Ayurvedic system of medicines uses about 700, Sidha – 600 and Unani – 700 medicinal plants in our country. Most of the plant-derived drugs were originally discovered through the study of traditional cures and folk knowledge of the indigenous people and some of these could not be substituted, despite the enormous advancement in synthetic chemistry [1].

Herbal medicine is still the mainstay of about 75% to 80% of the world population, mainly in the developing countries, with the aim of promoting primary health

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care with better cultural acceptability, human compatibility and fewer side effects. Although synthetic pharmaceuticals now dominate the drug scene, medicinal plants continue to hold a place in international health care. Awareness of the importance of natural heritage and biodiversity is also growing. India is a gold mine of treasures with traditional and practical knowledge of herbal medicines. Globally a positive trend has blossomed in favour of traditional and integrative health sciences both in research and in practice.

Cellular damage or oxidative injury arising from free radicals or reactive oxygen species (ROS) now appears the fundamental mechanism underlying a number of human diseases [2–4]. Antioxidants are radical scavengers, which protect the human body against free radicals that may cause pathological conditions such as anaemia, asthma, arthrists, inflammation, neurodegeneration, Parkinson's diseases, mongolism, ageing process and perhaps dementias [5–8]. Free radicals can be scavenged through chemoprevention utilizing natural antioxidant compounds present in foods [9] and medicinal plants [10–16]. Some medicinal plants have been shown to have chemopreventive and/or therapeutic effects on human diseases [17].

The term antioxidant refers to the activity possessed by numerous vitamins, secondary metabolites and other photochemicals to serve as protection against the damaging effects of highly reactive molecules known as the radicals [18]. The non-enzymatic system of defence against oxidative stress consists of the antioxidant molecules corresponding to glutathione, ascorbic acid, α-tocopherol, carotenoids and phenolic compounds [19]. These molecules play a key role in scavenging free radicals in plants by donating electron or hydrogen.

Key enzymes involved in the detoxification of ROS are namely SOD, CAT, peroxidase (POD), APX and other enzymes implicated in the Halliwell and Asada cycle (ascorbate-glutathione pathway) under stress condition that enhanced activity of almost all these enzymes reported [5]. SOD and CAT are required for destruction of superoxide and H_2O_2 [15]. Superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase are present in isoforms with specific subcellular localization. The enzymes of glutathione-ascorbate cycle have been implicated in mitigating the effect of reactive oxygen species [18].

Strychnos nux-vomica L. is an evergreen tree native to East India, Burma, Thailand, China, and Northern Australia. The tree belongs to the Loganiaceae family and has small flowers and orange colored fruits that are the size of an apple or orange. Inside the fruit are five seeds surrounded by a jelly-like pulp. The ash gray seeds are round and measure 1 in (2.5 cm) in diameter and are 0.25 in (0.6 cm) thick. The seeds are coated with downy hairs that give them a satiny appearance. These dried seeds are used for the treatment of nervous diseases, arthritis, and vomiting as herbal remedies in traditional Chinese medicine. 2-Strychnine and brucine are the major pharmacologically active components from *Strychnos nux-vomica*, which are central nervous stimulants, but they can also lead to lethal poisoning at high dosage [20]. Strychnine and brucine have similar pharmacological action. The curative effect of brucine amounts to 1/40 of that of strychnine, and its toxicity is 1/70 of that of strychnine. So, in the development of *Strychnos nux-vomica* and strychnine as secure, responsible curatives, it is important to study the interaction of strychnine and albumin [21].

There are many works reported regarding the alkaloid metabolism and medicinal properties of this plant. To the best of our knowledge, no information on the contents of non-enzymatic and enzymatic antioxidants *Strychnos nux-vomica* is available. The purpose of this study was to provide additional information on the non-enzymatic (AA, toc, GSH and total phenols) and enzymatic (SOD, APX, POX, CAT and PPO) antioxidant constituents of *Strychnos nux-vomica* leaves.

2. Materials and methods

2.1. Sample collection

The fresh leaves of *Strychnos nux-vomica* were collected from the sacred groves of Ernakulam, Kerala, India. Leaves were washed and used for analyzing non-enzymatic and enzymatic antioxidant potentials.

2.2. Non-enzymatic antioxidant estimations

2.2.1. Ascorbic acid (AA) content

AA content was assayed as described by Omaye et al. [22]. The extract was prepared by grinding 1 g of fresh material with 5 ml of 10% TCA, centrifuged at 3500 rpm for 20 min, reextracted twice and the supernatant made up to 10 ml and used for assay. To 0.5 ml of extract, 1 ml of DTC reagent (2,4-dinitrophenyl hydrazine-thiourea-$CuSO_4$ reagent) was added, incubated at 37 °C for 3 h and 0.75 ml of ice-cold 65% H_2SO_4 was added, and allowed to stand at 30 °C for 30 min; the resulting colour was read at 520 nm in spectrophotometer (U-2001-Hitachi). The AA content was determined using a standard curve prepared with AA and the results were expressed in mg g^{-1} dry weight (DW).

2.2.2. Reduced glutathione (GSH)

The (GSH) content was assayed as described by Griffith and Meister [23]. 200 mg fresh material was ground with 2 ml of 2% metaphosphoric acid and centrifuged at 17 000 rpm for 10 min. Adding 0.6 ml 10% sodium citrate neutralized the supernatant. 1 ml of assay mixture was prepared by adding 100 µl extract, 100 µl distilled water, 100 µl 5,5-dithio-bis-(2-nitrobenzoic acid) and 700 µl NADPH. The mixture was stabilized at 25 °C for 3–4 min. Then 10 µl of glutathione reductase was added, read the absorbance at 412 nm in spectrophotometer and the GSH contents were expressed in $\mu g\, g^{-1}$ fresh weight (FW).

2.2.3. α-Tocopherol (α-toc) content

α-Toc content was assayed as described by Backer et al. [24]. 500 mg of fresh tissue was homogenized with 10 ml of a mixture of petroleum ether and ethanol (2:1.6 v/v) and the extract was centrifuged at 10 000 rpm for 20 min and the supernatant was used for estimation of α-toc. To one ml of extract, 0.2 ml of 2% 2,2-dipyridyl in ethanol was added and mixed thoroughly and kept in dark for 5 min. The resulting red colour was diluted with 4 ml of distilled water and mixed well. The resulting colour in the aqueous layer was measured at 520 nm. The α-toc content was calculated using a standard graph made with known amount of α-toc.

2.3. Antioxidant enzyme extractions and assays

2.3.1. Ascorbate peroxidase (APX, EC 1.11.1.1)

The activity of APX was determined by the method of Asada and Takahashi [25]. The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 200 µl of enzyme extract. The absorbance was read as the decrease at 290 nm against the blank; a correction was made for the low, non-enzymatic oxidation of ascorbic acid by H_2O_2 (extinction coefficient 2.9 $mM^{-1}\, cm^{-1}$). The enzyme activity was expressed in units mg^{-1} protein (U = change in 0.1 absorbance $min^{-1}\, mg^{-1}$ protein).

2.3.2. Superoxide dismutase (SOD, EC 1.15.1.1)

The activity of SOD was assayed as described by Beauchamp and Fridovich [26]. The reaction mixture contained 1.17×10^{-6} M riboflavin, 0.1 M methionine, 2×10^{-5} M KCN and 5.6×10^{-5} M nitroblue tetrazolium (NBT) salt dissolved in 3 ml of 0.05 M sodium phosphate buffer (pH 7.8). Three ml of the reaction medium was added to 1 ml of enzyme extract. The mixtures were illuminated in glass test tubes by two sets of Phillips 40 W fluorescent tubes in a single row. Illumination was started to initiate the reaction at 30 °C for 1 h. Identical solutions that were kept under dark served as blanks. The absorbance was read at 560 nm in the spectrophotometer against the blank. SOD activity is expressed in U mg^{-1} protein (U = Change in 0.1 absorbance $hour^{-1}\, mg^{-1}$ protein).

2.3.3. Catalase (CAT, 1.11.1.6)

The activity of CAT was measured according the method of Chandlee and Scandalios [27] with small modification. The assay mixture contained 2.6 ml of 50 mM potassium phosphate buffer (pH 7.0), 0.4 ml of 15 mM H_2O_2 and 0.04 ml of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in units mg^{-1} protein (U = 1 mM of H_2O_2 reduction $min^{-1}\, mg^{-1}$ protein).

2.3.4. Polyphenol oxidase (PPO, EC 1.10.3.1)

The assay of PPO was carried out by the method of Kumar and Khan [28]. Assay mixture for PPO contained 2 ml of 0.1 M phosphate buffer (pH 6.0), 1 ml of 0.1 M catechol and 0.5 ml of enzyme extract. This was incubated for 5 min at 25 °C, after which the reaction was stopped by adding 1 ml of 2.5 N H_2SO_4. The absorbancy of the purpurogallin formed was read at 495 nm. To the blank 2.5 N H_2SO_4 was added of the zero time of the same assay mixture. PPO activity is expressed in U mg^{-1} protein (U = Change in 0.1 absorbance $min^{-1}\, mg^{-1}$ protein).

2.3.5. Peroxidase (POX, EC 1.11.1.7)

POX was assayed by the method of Kumar and Khan [28]. Assay mixture of POX contained 2 ml of 0.1 M phosphate buffer (pH 6.8), 1 ml of 0.01 M pyrogallol, 1 ml of 0.005 M H_2O_2 and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25 °C after which the reaction was terminated by adding 1 ml of 2.5 N H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 N H_2SO_4 at zero time. The activity was expressed in U mg^{-1} protein. One U is defined as the change in the absorbance by 0.1 $min^{-1}\, mg^{-1}$ protein. The enzyme protein was estimated by the method of Bradford [29] for all the enzymes.

2.4. Statistics

Each parameter was analysed with at least seven replicates and a standard deviation (SD) was calculated and data are expressed in mean ± SD of seven replicates.

3. Results and discussion

India is a veritable emporium of medicinal plants. It is one of the richest countries in the world as regards genetic resources of medicinal plants. Even though the landmass of India occupies only two per cent of the globe, it occupies 11% of the total known world flora and is one of the world's top 12 mega diversity nations. Two of the '18 hot spots' in the world are in India. More over the agro climatical conditions are conducive for introducing and domesticating new exotic plant varieties. It is necessary that indigenous plants are to be identified in terms of their total antioxidant capacities, to maintain a constant supply of medicinal plants. Through this study, we have explored the non-enzymatic and enzymatic antioxidant variations in tender and mature leaves of *Strychnos nux-vomica* L. of the family Loganiaceae.

In indigenous/traditional systems of medicine, the drugs are primarily dispensed as water decoction or ethanolic extract. Fresh plant parts, juice or crude powder are a rarity rather than a rule [12,30]. Thus medicinal plant parts should be authentic and standardized with scientific validation of individual constituents. It has been estimated that just 10–15% of the world's higher order plants have been investigated for bioactive compounds [31]. The production of secondary metabolites by plants has become an active field of study because of its potential as a source of valuable pharmaceutical compounds [13–15,32]. The medicinal plant examined in this study has long been used in Indian traditional medicine, Ayurveda as well as many other traditional drug practices of the rest of the world.

Free radicals can be formed via enzymatic reaction from non-autooxidizable substances such as halo alkanes, phenols, nitrocompounds and aromatic amines [33,34]. Free radicals are also formed when cell constituents are exposed to ionizing radiation [5]. The presence of oxygen in the cellular environment represents a constant oxidative threat to cellular structure and process [19]. Generation of ROS have greater toxicity potential on biomolecules and membranes [15,16]. Reactive oxygen species are an entire class of highly reactive molecules derived form the metabolism of oxygen [2–7]. Moreover, ROS can cause extensive damage to cells and tissues, during infections and various degenerative disorders, such as cardiovascular disease, aging, and neuro-degenerative diseases like Alzheimer's disease, mutations and cancer [35]. The major defense systems of any organism against ROS injury are based mainly on antioxidant molecules and activities of antioxidant enzymes.

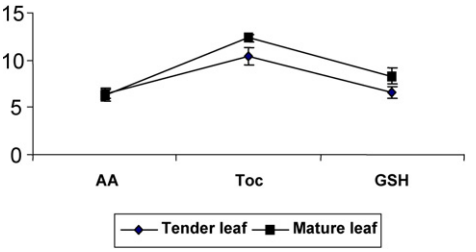

Fig. 1. Variations in the non-enzymatic antioxidant contents (AA, α-toc and GSH) in tender and mature leaves of *Strychnos nux-vomica*. Values are given as mean ± SD of seven experiments in each group. (AA – ascorbic acid; α-toc – α-tocopherol; GSH – reduced gluthione.)

3.1. Non-enzymatic antioxidant estimations

3.1.1. Ascorbic acid (AA) content

The AA content showed a less variation in tender and mature leaves of *Strychnos nux-vomica* (Fig. 1). The mature leaves showed high levels of AA content (6.23 mg g^{-1} FW) when compared to the tender (6.17 mg g^{-1} FW). AA is a very important reducing substrate for H_2O_2 detoxification in photosynthetic organisms [36]. AA participates in the removal of H_2O_2 as a substrate of APX.

3.1.2. α-toc content

The α-toc content varied in tender and mature leaves of *Strychnos nux-vomica* (Fig. 1). Here also, the mature leaves showed high levels of α-toc content when compared to the tender. The highest content was recorded in mature leaves samples and it was 12.38 mg g^{-1} FW. The lowest content was in tender samples and it was 10.41 mg g^{-1} FW. α-Toc was consumed predominantly as a radical scavenging antioxidant against lipid peroxidation [37].

3.1.3. GSH content

The GSH content varied in tender and mature leaves of *Strychnos nux-vomica* (Fig. 1). The highest content was recorded in mature leaf samples and it was 8.31 μg g^{-1} FW. The lowest content was in tender leaves and it was 6.54 μg g^{-1} FW. GSH is another most important non-enzymatic antioxidant molecule, which functions as an effective ROS detoxifier [38].

3.2. Antioxidant enzymes (Fig. 2)

The activities of antioxidant enzymes varied in tender and mature leaves of *Strychnos nux-vomica*. The highest activity of APX enzyme was recorded in mature leaves samples (19.85 units mg^{-1} protein). The highest

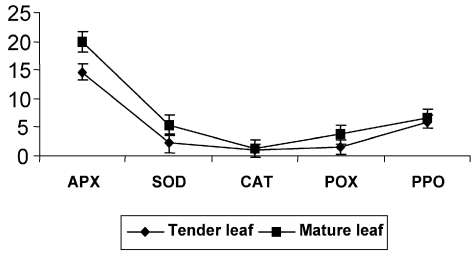

Fig. 2. Variations in the antioxidant enzymes (APX, SOD, CAT, POX and PPO) in tender and mature leaves of *Strychnos nux-vomica*. Values are given as mean ± SD of seven experiments in each group. (APX – ascorbic acid; SOD – superoxide dismutase; CAT – catalase; POX – peroxidase; PPO – polyphenol oxidase.)

activity of SOD enzyme (5.34 units mg^{-1} protein) was observed in mature leaves.

SOD is a major scavenger of superoxide anion radical ($O_2^{\bullet -}$) that catalyses the dismutations of $O_2^{\bullet -}$ with great efficiency resulting in the production of H_2O_2 and O_2 [39]. The higher activity of antioxidant enzyme CAT (1.21 units mg^{-1} protein) was recorded in mature leaves.

The enzymes PPO and POX also showed variation in its activity among mature and tender leaves. PPO is believed to be ubiquitous in the plant kingdom and it is primarily associated with enzymatic browning and off-flavour generation [40]. These enzymes are modulated in various diseases by free radical attack [35]. Thus, maintaining the balance between the rate of radical generation and the rate of radical scavenging is an essential part of biological homeostasis [36].

Here from this study, it can be concluded that, the mature leaves of *Strychnos nux-vomica* are good source of non-enzymatic and enzymatic antioxidant components. Our results points out the significance of *Strychnos nux-vomica* as an important medicinal plant, which have good antioxidant potentials throughout its tender as well as mature leaves. Further studies are required to isolate individual active principles and antioxidant activity of individual extracts of different parts through radical scavenging assay; their pharmacological validation in terms of modern medicine will be of great medicinal importance in future and that is underway in our laboratory.

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