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EVALUATING THE ANTIOXIDANT ACTIVITIES IN THE LEAF EXTRACT OF A MEDICINAL PLANT, *ABUTILON INDICUM* (LINN.) SWEET

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ABSTRACT
The plant kingdom is the treasury of pharmaceutically important components. The secondary metabolites from plants can play an important role in disease healing, used to restore health and also given the lead structures for the development of synthetic molecules. The plant species *Abutilon indicum* (Linn.) belongs to family Malvaceae, commonly known as Khangi in India, Country Mallow in English and Atibala in Sanskrit. Various parts of this plant are traditionally used to treat inflammation, piles, and gonorrhea and as an immune stimulant, anthelminthic, anti diabetic, nervine tonic etc. The present work is focused on protein profiling and also analyzing the antioxidant activity including superoxide dismutase (SOD), Catalase (CAT) and Peroxidase (PX) (Guaiacol units) from the leaves of *A. indicum*. The report has shown that total 12.5±3.6mg of protein was found per gm of fresh leaves. The data from native gel analysis has clearly shown the antioxidant activity of *A. indicum* leaves.

KEYWORDS: Antioxidant; *Abutilon indicum*; Reactive Oxygen Species; Native gel.

INTRODUCTION

Oxygen plays a vital role in the progress of aerobic life processes. More than 5% of inhaled oxygen is converted to superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) by univalent reduction of O_2. Those free radicals popularly known as reactive oxygen species (ROS) are always threatened to cells [1]. However the highly powerful antioxidant system of the cell will be effective in prevention of those free radical formations by scavenging or promotion of their decomposition without any untoward effect. But when the balance between ROS production and antioxidant defenses is lost, the free-radical-mediated 'oxidative stress' results oxidative damage of the cellular macromolecules (lipids, proteins, and nucleic acids), leading finally to various pathological conditions. It had shown that oxidative stress is among the major causative factors for cardiovascular dysfunction, gastroduodenal pathogenesis, neurodegenerative diseases and metabolic dysfunction of almost all the vital organs, cancer and premature aging [2, 3].

At present, there is an increasing interest in pharmacological evaluation of various plants in ayurvedic system of medicine, and the scientific community is looking forward in the usage of plant species as elements of complementary and alternative medicine, in term of control the oxidative stress mediated disorders. Moreover plant derived natural products such as steroids and terpenoids have got attention in recent years [4, 5]. Hence the growing application of plant derived natural resources toward the pharmaceutical aspect, make aspiration that it will be in tune to occupy 40% of the synthetic pharmaceutical market by 2012 [6].

In ancient scriptures of Ayurvedic, four different plants viz., bala, atibala, mahabala and nagabala are together called as Bala catustaya. Out of which bala and atibala are commonly used as traditional medicines. Atibala is a stronger diuretic, whereas bala is a more effective heart tonic. The plant atibala grows throughout India [7] and in Sri Lanka. The botanical name of atibala is *Abutilon indicum (Linn.)* sweet belongs to family Malvaceae. The perennial shrub grows upto 1.25- 2 meters in height. The various parts of this plant are in use in the traditional system of Indian medicine. The report had shown that plant is used in treatment of inflammation, piles, gonorrhea and as an immune stimulant [8]. The leaves are in use as astringent [9], root and bark are used as aphrodisiac, anti diabetic, nervine tonic, and diuretic, where as seeds are used as aphrodisiac and in urinary disorders [10]. Moreover *Abutilon indicum* has analgesic [11], hypoglycemic [12], and hepato protective activity [13]. The present investigation was attempted to reveal the antioxidant activity of *Abutilon indicum* (Linn.) sweet leaves which is in use as an ethno medicine in India and several other parts of the world.

MATERIALS AND METHODS

Plant Material

Fresh leaves of the plant *A. indicum* were collected from herbal garden at Botany Department, Utkal University, Vani Vihar, Bhubaneswar, Orissa, India (20º18'05.44"N, 85º50'30.76"E), and confirmed the species from authentic sources. The collected leaves were dried in shade, crushed to coarse powder and used for further studies. A voucher of the specimen was deposited at the same centre.

Chemicals

Chemicals and reagents were purchased from Sigma Chemical Co. Agarose was obtained from Bio-Rad. Protein marker was purchased from New England Biolab. All other chemicals and solvents used in this study were of the reagent grade.

Extraction, Estimation and Analysis of Total Leaf

Protein

Total leaf protein was extracted by the polyvinyl polypyrrolidone (PVP) precipitation method. 0.5 g of fresh leaf tissue was homogenized for 1 min in 50 mM sodium phosphate buffer containing 10% (w/v) insoluble PVP using a pre-chilled mortar and pestle and incubated overnight at 4ºC. The homogenized sample was centrifuged at 48,000 x g for 20 min at 4ºC. The supernatant was collected and kept under -20ºC for protein estimation and enzyme assay. The protein estimation was done by the method of Lowry [14]. Protein in the unknown sample was estimated at wavelength 750 nm using bovine serum albumin as standard and expressed the volume in gm per fresh weight basis.

Total leaf protein concentration was analyzed on SDS-PAGE electrophoresis. After estimation of protein, the supernatant sample was diluted with 50 mM sodium phosphate buffer to make the final concentration 25, 50 and 100 μg mL $^{-1}$ of protein. All the diluted samples were mixed with equal volumes of solubilizing buffer [62.5 mM Tris-HCl, pH 6.8, 20% (w/v) glycerol, 2% (w/v) SDS, 5% (w/v) 2-mercaptoethanol and 0.01% bromophenol blue] and heated for 4 min at 95º C, cooled on ice before loading. A 10% separating gel and 4% stacking gel were made according to Laemmli [15]. The electrophoresis running buffer was prepared with 25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3. The gels were stained by silver staining method until the background was clear [16].

Enzyme Assay

Super oxide dismutase

The activity of SOD was assayed according to Misra and Fridovich [16]. About 200 mg fresh tissues were homogenized in 5 ml of 100 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and 2% (w/v) polyvinyl pyrrolidone (PVP). The extract was filtered through muslin cloth and centrifuged at 22,000 g for 10 min at 4ºC. The supernatant was dialyzed in cellophane membrane tubing against the cold extraction buffer for 4 h with 3 - 4 changes of the buffer and then used for the assay. The assay mixture in a total volume of 3 ml containing 50 mM sodium carbonate-bicarbonate buffer (pH 9.8), 0.1 mM EDTA, 0.6 mM epinephrine and enzyme. Epinephrine was the last component to be added. The adrenochrome formation in the next 3 min at the interval of 15sec. was recorded at 475 nm in a UV-Vis spectrophotometer. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions. SOD activity was expressed as nKat/ min/ mg of protein.

Catalase

The activity of catalase was assayed according to Beers and Sizer [17]. Fresh 200 mg leaf sample was homogenized in 5 ml of 50 mM Tris-/NaOH buffer (pH 8.0) [0.5 mM EDTA, 2% (w/v) PVP and 0.5% (w/v) Triton X-100, pH-8]. The homogenate was centrifuged at 22,000 x g for 10 min at 4 º C and after dialysis supernatant was used for enzyme assay. Assay mixture in a total volume of 1.5 ml contained 1000 μl of 100 mM KH_2PO_4 buffer (pH 7.0), 400 μl of 200 mM H_2O_2 and 100 μl of enzyme. The decomposition of H_2O_2 was determined by reading the absorbency at 240 nm. Decrease of absorbance was recorded at every 15 sec up to 3 min. Catalase activity was expressed as nKat/ min/ mg of protein.

Peroxidase

PX (Guaiacol units) was assayed by following the method described by Kar and Feierabend [18] with some modification. Fresh leaf sample (200 mg) was homogenized in 5 ml of cold 50 mM Na-phosphate buffer (pH- 7.0). The homogenate was centrifuged at 22 000 x g for 10 min and the dialyzed enzyme extracts were used for the assay. The assay mixture was composed of 1.5 ml of 0.1 M sodium phosphate buffer, 0.5 ml of 30 mM guaiacol, 0.5 ml of 30 mM hydrogen peroxide, 0.5 ml of enzyme extract, at pH 6.8. Peroxidase activity was calculated by using the extinction co-efficient of 26.6/mM/cm for tetraguaiacol at 470 nm in every 15 second interval up to 3 min. The total concentration of PX activity was expressed as nKat/min/mg.

Enzyme Activity on native gels

For the analysis of SOD and CAT activity, the extracted enzyme samples were loaded onto 13% non-denaturing polyacrylamide gels and run at 120 V for 12 h at 4ºC. The Catalase gel was stained by Vitoria *et al.* (2001) method [19]. Gel was incubated in 0.003% H_2O_2 for 10 min and developed in a solution of 1% (w/v) $FeCl_3$ and 1% $K_3Fe(CN)_6$ (w/v) solution for 10 min. The SOD gel was stained with Coomassie Brilliant Blue R-250. One unit of native PAGE protein molecular weight marker was applied to gel to serve as a positive control of SOD and CAT activity.

RESULTS AND DISCUSSION

The previous studies had shown the antioxidant activity from different parts of the plant rather than the leaf of *A. indicum.* The present studies have shown the total protein profiling as well as analyzing the antioxidant activities of the herbal plant leaves. The experimental results have demonstrated that the total protein of 12.5±3.6mg was found for per gm of fresh leaves the molecular weight of the most abundant protein of the leaves was approximately 50.8 kDa (Fig. 1).

FIGURE 1

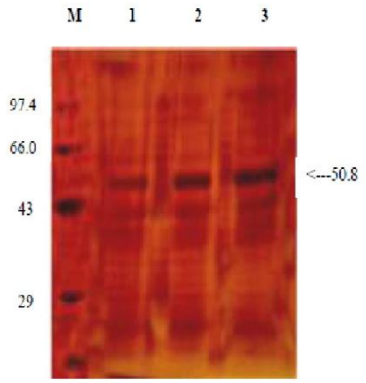

Fig 1: SDS-PAGE analysis of leaf proteins extracted from *Abutilon indicum*
(M- Low weight molecular marker in KDa. 1, 2 and 3 represent the different concentrations of total protein 25, 50 and100µg/ well respectively. The highest protein obtained is 50kDa.)

Superoxide radicals are generated during the metabolism of aerobic organisms, either as an end product of enzymatic reactions or as accidental side products of cellular redox reactions. SOD is an essential component of the antioxidative defense system in plants and animals [20],

and it dismutates two superoxide radicals (O_2^{*+}) to H_2O_2 and O_2. Superoxide radical scavenging activity is generally based on the anion radical which is associated with PMS-NADH system.

The present data have shown that the high activity of SOD (2.84±0.69 Kat/min/mg) in leaf extract of *A. indicum* and also observed a clear 36 kDa SOD protein band in native gel (Fig 2). The elevation in absorbance was identified and understood that the elevation is due to adrenochrome formation (Fig 4). It was reported that high PX activities can disturb the SOD activity. However, SOD activity had been increased under salinity, water stress, γ-radiation, and UV-radiation, Cd, Pb, Al and Cu toxicity [21]. In contrast to this, in the present study, the SOD activity of the collected plant leaf was not affected by the above mentioned factors. Transgenic plants over-expressing SOD, show increased tolerance towards oxidative damage caused due to harsh environmental conditions and among antioxidant enzymes the activity levels of SOD are of more relevance in maintenance of the overall defense system of plants subjected to oxidative stress.

FIGURE 2 & 4

Fig 2: Native gel analysis of SOD from *Abutilon indicum*
M- Molecular Marker of SOD. 1, 2 and 3 represent the different concentration of SOD concentration in 25, 50 and100µg/ well respectively.

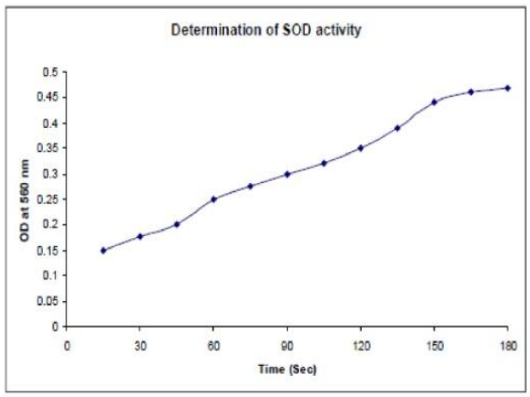

Fig 4: Superoxide dismutase activity of leaf extract from *Abutilon indicum*.

Natural antioxidants that are present in herbs are responsible for inhibiting or preventing the deleterious consequences of the oxidative stress. Herbs contain free radical scavengers like polyphenols, flavonoids and phenolic compounds. CAT is widespread in nature, having been found in all aerobic organisms studied to date. CAT is universally present as an oxido-reductase that decomposes H_2O_2 to water and molecular oxygen and it is one of the key enzymes involved in removal of toxic peroxides [23]. CAT assay was studied for 3 min with the absorbance at 240 nm against blank. In every 15 sec the absorbance was recorded and it was found that the activity was gradually decreased (Fig. 5). It was found that the leaves of *A. indicum* contained 0.22 ±0.44 Kat/min/mg of protein. The molecular weight of CAT in A. indicum was measured by native gel analysis to be 28 kDa (Fig. 3). More over in staining, it was clearly noted that both the catalase marker and the leaf extract of *A. indicum* were in a straight line (Fig 3) which indicate that the leaves of *A. indicum* have CAT activity.

FIGURE 3 & 5

Fig 3: Native gel analysis of CAT from *Abutilon indicum*
(M- Molecular Marker for catalase. 1, 2 and 3 represent the different concentration of Catalase concentration in 25, 50 and100µg respectively.)

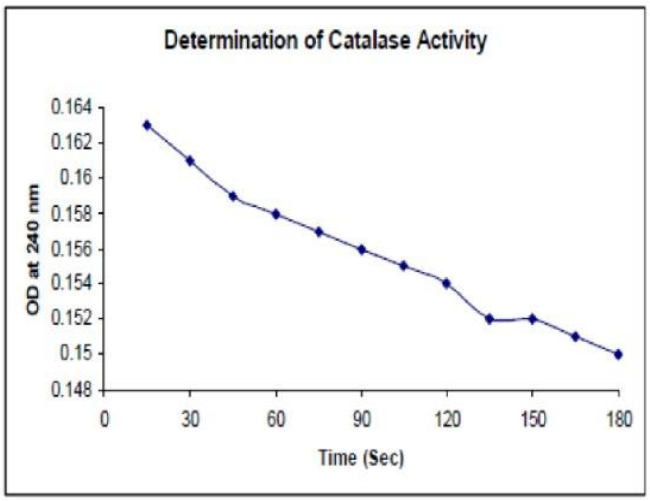

Fig 5: Catalase activities of leaf extract from *Abutilon indicum*.

PX is a family of wide-spread enzymes [22] and is widely accepted as 'stress enzymes' [23], which perform distinct tasks. On the one side they act as preventive antioxidants to detoxify damaging lipid peroxides [24] or other organic hydroperoxides from blood and organic substrates. On the other hand PX functions as starters for oxidative reactions, thereby generating a source for reactive oxygen species like HOCl or OCI-. Such reactions are induced by activated neutrophils in the course of their antibacterial and antifungal actions [24]. PX can also modify low density lipoprotein (LDL) cholesterol in the presence of H_2O_2 or lipid hydroperoxide (LOOH) [25]. Vice versa, a special type of PX, the glutathione peroxidases (GPX), is very important as antioxidant defense enzymes, removing peroxides and H_2O_2 by coupling its reduction to H_2O or LOH with oxidation of reduced glutathione (GSH).

The assay of PX (Guaiacol units) in the leaf sample of *A. indicum* indicated that initially the concentration of PX was increase but later due to substrate saturation, the optical density of sample was constant (Fig 6). Moreover the low activities of PX (0.066±0.004 Kat/min/mg) also make sure that the presence of PX is not making any effect on the SOD activity in *A. indicum*.

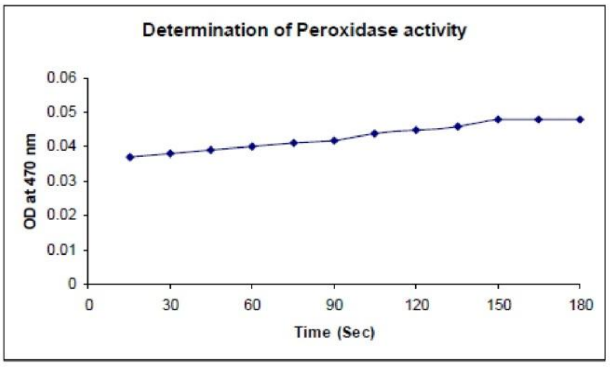

Fig 6: Peroxidase activity of leaf extract from *Abutilon indicum*.

CONCLUSION

Reactive oxygen species (ROS) generated by oxidative stress in large quantities is one of the most important reasons for cancer, ageing and for various disorders. To certain extent, to overcome the damage, every living being will have its own antioxidant protective mechanism. When the stress is more, it may require supplementing the system with antioxidants as a concentrate. Various forms of antioxidants are in use for therapy and still there is a search to find few more sources for anti oxidants. The present study is focused on the plant *A. indicum* and results indicate that the plant has the enzymatic components associated with SOD, CAT, PX make possible to defense against ROS. The antioxidant activity in *Abutilon* species may be due to the higher phenolic content. Hence the result reveals on the strong antioxidant properties of *A. indicum* and it will be use as a potential source for pharmaceutical application in term of antioxidant drug and other aspect.

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