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In vitro free radical scavenging and *in vivo* antioxidant potential of mulberry (*Morus indica* L.) leaves

*Bondada Andallu**, *Mahalakshmi Shankaran*, *Rajeshwari Ullagaddi*, *Shobha Iyer*

Sri Sathya Sai Institute of Higher Learning, Anantapur 515001, India

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ABSTRACT

The leaves of mulberry (*Morus indica* L.) of Moraceae, possess a number of bioactive compounds that fight against various ailments. *In vitro* free radical scavenging and *in vivo* antioxidant potential of mulberry leaves were investigated. Ethanolic extract of mulberry leaves was tested for antioxidant activity *in vitro* using butylated hydroxy toluene as a positive control. Erythrocyte membrane was used as peroxidation model system *in vitro* while elderly human volunteers who received mulberry leaf powder (5 g/day) for 60 days served as subjects for *in vivo* assessment. Mulberry leaf extract scavenged DPPH, nitric oxide and superoxide radicals in a concentration dependent manner and inhibited $FeSO_4$-induced lipid peroxidation and hydroperoxides in the erythrocyte membrane model. This was supported by significantly ($p < 0.01$) decreased lipid peroxidation in plasma (23%) and erythrocytes (49%), significantly ($p < 0.01$) elevated levels of non enzymatic antioxidants *viz.* β carotene (116%), vitamin A (69%), vitamin C (23%), vitamin E (55%) and ceruloplasmin (26%); decreased nitrite (43%) in serum and significantly elevated activity of superoxide dismutase (47%) and glutathione-S-transferase (72%) and reduced glutathione (21%, $p < 0.05$) in erythrocytes from elderly subjects treated with mulberry leaf powder. Mulberry leaves exhibited antioxidant properties postulated to be as a result of the synergistic action of free radical scavenging compounds such as carotenoids, flavonoids, moracins and others present in the leaves.

1. Introduction

Oxygen and nitrogen free radicals are generally unstable and very reactive (Evans and Halliwell, 2001). "Oxidative stress", which results from an improper balance between reactive oxygen species (ROS) and their metabolites, and antioxidant defence, is a factor in the pathogenesis of various diseases, such as cardiovascular disorders, neurological conditions, Parkinson's disease, rheumatoid arthritis, and ageing (Chen et al., 2002). Antioxidants synthesized in the body, including all antioxidant proteins and various small molecules,

* *Corresponding author at*: Sri Sathya Sai Institute of Higher Learning, Anantapur Campus, Anantapur 515001, India. Tel.: +91 08554 272567; mobile: +91 9247450990.
E-mail addresses: bandallu@gmail.com, rshobhaiyer@gmail.com (B. Andallu).
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form a defence against oxidative stress, and their levels in the body cannot be altered by simple means. On the other hand, the levels of antioxidant vitamins such as ascorbate, α-tocopherol, β-carotene and phytochemicals *viz.* polyphenols, flavonoids, can be increased easily by supplementation (Burton and Wayner, 1986). Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and micronutrients such as, selenium in glutathione, and copper and zinc in SOD as integral components of metallo-enzymes (Ray and Husain, 2002).

There has been a growing interest in replacing commercial antioxidants with natural ingredients due to the possible adverse effects of synthetic antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT), which now have restricted use in foods as they are suspected to be carcinogenic (Madhavi et al., 1995). Moreover, natural antioxidants are considered to be safe and environmentally friendly and they play a very important role in the physician's therapeutic armamentarium. Currently, the use of plants and herbs with antioxidants is gaining importance. Mulberry (*Morus* species) is one such plant with therapeutic applications. The ethanolic extract of leaves of *Morus indica* showed good hypoglycaemic and hypolipidaemic effects in alloxan-induced diabetic rats (Pradeep et al., 2010). In Thailand, beverages containing mulberry leaves (*Morus alba* L.) are being used to promote good health, especially in diabetics (Naowaboot et al., 2009). In diabetic rats, treatment with 400 mg/kg of aqueous leaf extract of *Morus rubra* significantly decreased glycosylated haemoglobin, elevated plasma insulin and C-peptide levels, altered serum lipids and enhanced the activity of erythrocyte antioxidant enzymes. Histopathological examination of pancreatic tissue revealed an increased number of islets and β-cells in the extract treated diabetic rats (Sharma et al., 2010). Antidiabetic and antioxidant effects of mulberry (*Morus indica* L.) leaves were reported by Andallu and Varadacharyulu (2003, 2007) in streptozotocin-diabetic rats.

There is very little reported evidence of the antioxidant potential of mulberry leaves, however, oxidative stress during ageing is yet to be studied, therefore it is worthwhile to investigate the *in vitro* free radical scavenging and *in vivo* antioxidant potential of mulberry (*Morus indica* L.) leaves in elderly human beings.

2. Methods and materials

All chemicals and solvents were of analytical grade, obtained from SRL and Merck, Mumbai, India. β-carotene, BHT and 1,1-diphenyl, 2-picryl hydrazyl (DPPH) were obtained from Sigma–Aldrich Chemicals, USA.

2.1. Plant material and extraction

Young, tender mulberry (*Morus indica* L.) leaves plucked from healthy plants in bulk from the Regional Sericultural Research Station (RSRS), Raptadu, Anantapur District were identified by the director of RSRS, Dr. Suryanarayana, and a voucher specimen (RSRS Farm, CUR&BA 45358(SKU) stored in the Dept. of Botany, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, India. The leaves were thoroughly washed under running tap water, shade dried for three days, powdered and packed in polythene covers to be used for both *in vitro* and *in vivo* experiments. For *in vivo* experiments, the leaf powder was prepared freshly at weekly intervals.

2.2. Assay of non-enzymatic antioxidants in the leaves

Total phenolics (Caboni et al., 1997), β-carotene (Raghuramulu et al., 1983), and vitamin C (Roe and Kuther, 1943) were estimated in the fresh leaves.

2.3. In vitro antioxidant assays

Dried leaf powder was weighed and extracted in a Soxhlet extractor using hexane for 6 h for the removal of fatty matter. The defatted material was extracted in a Soxhlet extractor with 95% ethanol for 72 h, the extract was concentrated in a vacuum evaporator and the residue was stored in a desiccator after noting the yield for subsequent experiments. The scavenging activities of various concentrations (50–250 μg/mL) of the extract *viz.* DPPH (Sreejayan and Rao, 1996), nitric oxide (NO; Sreejayan and Rao, 1997), superoxide (Robak and Gryglewski, 1988) radicals, and reducing power (Yen and Duh, 1993) were estimated. The influence of the extract on $FeSO_4$-induced lipid peroxidation (malondialdehyde) and diene conjugates (hydroperoxides) (Buege and Aust, 1978) in erythrocyte membrane (Dodge et al., 1963) model was determined.

2.4. Evaluation of in vivo antioxidant effects

2.4.1. Selection of participants, treatment and analyses

Both males and females within 60–75 years of age in the ratio of 1:1, with no specific complications were selected from a home for the elderly on the basis of a specific questionnaire. Of the selected participants, 30 served as controls and 30 as experimentals. The experimental group received dried mulberry leaf powder (5 g/day) in two equal doses for a period of 60 days. Ethical approval for the study was obtained from an institutional ethical committee, Sri Sathya Sai Institute of Higher Learning, Prashanti Nilayam, Andhra Pradesh, India and the investigation was carried out following ethical guidelines for conducting experiments on human subjects. Informed written consent was obtained from all the participants and were monitored by a local physician throughout the period of study. At the initial and final stages of the experimental period, blood was drawn for the assay of various parameters. Lipid peroxidation in plasma (Buege and Aust, 1978) and erythrocytes (Stocks and Dormandy, 1971), ceruloplasmin (Raghuramulu et al., 1983), vitamin A and β-carotene (Henry et al., 1995), vitamin C (Roe, 1961) and vitamin E (Desai, 1984) in serum were estimated.

2.5. Preparation of erythrocyte haemolysate

2.5.1. Isolation of erythrocytes

Erythrocytes were separated from whole blood containing ethylenediaminetetraacetic acid (EDTA) (Beutler et al., 1963). In the barrel of a 5-mL syringe, USP-grade cotton (boiled for 5 min with each of five changes of distilled water, equilibrated with 0.9% sodium chloride, physiological saline) was packed