

Anti-Oxidant activity of various leaf extracts of mulberry species in rotenone induced oxidative stress model of rat

Sheeba Saji Samuel^{*1}, Abhishek Desai¹, R Raghuram¹ and Venkatesh Kumar R²

¹Division of Pharmacology, CSIR-Central Drug Research Institute, Jankipuram Extn, Lucknow-226031.

²Department of Applied Animal Sciences, BabasahebBhimraoAmbedkar University, Lucknow-226025.

*Corresponding author: E-Mail: shebs9179@gmail.com, Mob: 91-9839705280.

ABSTRACT

The oxidative stress is an important cause of a number of diseases in humans, which can be prevented or reduced by timely intervention of anti-oxidants. Rotenone induced oxidative stress model of rat has been used to assess the anti-oxidant profile of nine freshly prepared aqueous extracts of mulberry leaf. The results suggest that pre-treatment with four varieties S-146, AR-14, BR-2 and S-1 have shown highly significant anti-oxidant activity by attenuating both malondialdehyde (50.49%, 36.14%, 41.36%, 37.13%) and superoxide dismutase (54.01%, 40.18%, 34.82%, 29.74%) levels in the brain of experimental animals. The remaining extracts were relatively less active. The most significant ones AR-14 and S-146 are being tried in focal cerebral ischemia model of rat to assess their neuroprotective (anti-oxidant) activity. The potent mulberry extracts can be used as a dietary supplement to suppress the oxidative stress.

KEY WORDS: Anti-oxidant, MDA, SOD, Rotenone, Mulberry.

1. INTRODUCTION

Oxidative stress is produced as a result of imbalance between highly reactive oxygen species (ROS) produced in the cell and the ability of endogenous anti-oxidant system to scavenge them. The cellular free radicals (hydroxyl (OH·), superoxide (O₂^{-·}) and nitric monoxide (NO·)) can interact with macromolecules, membrane lipids, proteins, enzymes and DNA, causing cell damage. This oxidative damage can result in the development of a wide range of ailments such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, chronic inflammation, cardiovascular diseases, stroke, aging and other degenerative diseases in humans (Kathy, 2003; Maritim, 2003). In normal physiological conditions, the free radicals are immediately consumed by the body's naturally produced antioxidants like superoxide dismutase, catalase and peroxidase enzymes. However, when the level of free radicals becomes quite high in the body, the endogenous antioxidant system is unable to neutralize the excess of free radicals produced. At the same time, antioxidants, such as glutathione, vitamin E, vitamin C, also help in regulating the ROS generated.

Diet is a major source of antioxidants, and medicinal herbs are rich source of antioxidants. Therefore, consumption of fruits and vegetables lower the risk of several diseases, caused by oxidative stress (Willett, 2002). Their antioxidant activity is owing to the presence of phytochemicals, such as polyphenols, carotenoids and vitamin E and C. The substantial evidence is available supporting the role of dietary antioxidants and polyphenolic compounds from botanical sources like green tea extract, *Ginkgo biloba* extract and red wine/resveratrol to combat oxidative stress (Bridi, 2001). These antioxidants act as a major defense against free radical-mediated toxicity. Hence, antioxidants as supplements are now being looked upon as persuasive therapeutics against oxidative stress and related damage. Based on the growing interest in mulberry due to its nutritional value, and biological activities, it was decided to study the *in vivo* anti-oxidant activity of mulberry leaf extracts.

Mulberry belongs to the family *moraceae*, is a perennial herb with highly branching shoot systems. It is one of the herbs which are used in medicine since ancient times due to its chemical novelty and pharmacological functions. The plant is reported to contain the phytoconstituents as tannins, phytosterols, saponins, triterpenes, flavanoids, morusinic acid, anthocyanins, anthroquinones, glycosides and oleanolic acid as the main active constituents (Nomura, 1983). The medicinal uses of the plant reported so far include analgesic, antiasthmatic, antirheumatic, antitussive, astringent, diaphoretic, diuretic, emollient and expectorant, hypotensive and as brain tonic (Fukai, 1985). It was reported that higher amount of quercetin in the leaves of mulberry is responsible for reduction of oxidation process (Enkhmaa, 2005; Chen and Li, 2007). Hypoglycemic activity of dried leaves of mulberry has been also reported (Lemus, 1999). The ethanolic as well as the aqueous extract of mulberry leaves contains oxyresveratrol and 5,7-dihydroxycoumarin 7-methyl ether which scavenge superoxide and have antioxidant potential (Oh, 2002). The plant leaf extract has also been used in various studies to prove its neuroprotective effect as well (Tong, 2006).

Therefore, a search for novel natural resource with anti-oxidant activity is of particular interest. The study is aimed to evaluate the anti-oxidant property of aqueous mulberry leaf extracts using rotenone induced oxidative stress model in rats.

2. MATERIALS AND METHODS

Animals: Adult male Sprague Dawley rats, 200±10g, were used in the experiments. The animals were procured from the National Laboratory Animal Centre, CSIR-Central Drug Research Institute, Lucknow. All animal experiments

were done strictly in compliance with the guidelines for care and use of animals after necessary approval of the Institutional Animal Ethical Committee. Rats were allowed food and water *ad libitum* throughout the experiment.

Preparation of aqueous leaf extracts: Fresh mulberry leaves of both (*Morusalba* and *Morusindica*) nine varieties-V-1, S-1, S-13, S-146, AR-12, AR-14, S-1635, BR-2 and TR-10, were obtained from mulberry plantation in Babasaheb Bhimrao Ambedkar University, Lucknow. The leaves were cleaned, dried and grinded into a fine powder. The resulting powder was then passed through an 80-mesh sieve and the powder was kept in a sealed aluminum foil at 4 °C, till further use.

The aqueous extract of powder was prepared following the standard method (Katsube 2006). Briefly, 2 g of mulberry leaves powder was soaked in 200 ml of boiling water for 20 minutes. The mixture was cooled at room temperature before being filtered through Whatman no.1 filter paper and then lyophilized. The freeze-dried solid extract was transferred in plastic tubes and stored at -20 °C to protect from light. The extract was re-dissolved in double distilled water in desired concentration prior to use in all the experiments.

Rotenone induced oxidative stress model: Rotenone, a phyto-toxin obtained from roots of *Derris sp* belonging to *Leguminosae* family is a broad-spectrum pesticide and is generally used to induce oxidative stress. Rotenone interferes with the electron transport chain in mitochondria thus affecting the ATP synthesis. It causes neurotoxicity by inhibiting the oxidation of NADH to NAD, which blocks the oxidation of substrates such as glutamate, α -ketoglutarate and pyruvate, thereby generating ROS and other free radicals leading to various adverse effects in cellular physiology (Uversky, 2004).

Treatment schedule: Eleven experimental groups comprising of 8-10 male rats were used in the study. Group I was taken as sham. Animals of Group II were subjected to oxidative stress by rotenone treatment alone. Rotenone was dissolved in DMSO and administered orally at a dose of 75 mg/kg to induce oxidative stress model in rats. The group III to XI animals received pretreatment with nine varieties of mulberry leaf extracts (100mg/kg, p.o.) Each one hour prior to challenge with same dose of rotenone. Animals were sacrificed after one hour post rotenone challenge and brain tissue was gently removed in chilled ice-cold conditions for further studies.

Estimation of oxidative stress markers: Malondialdehyde (MDA) and Superoxide Dismutase (SOD) were monitored in the brain homogenates of experimental animals as *in vivo* biomarker for oxidative stress. The procedure of estimating both the biomarker is detailed below.

Malondialdehyde estimation in brain: Biological specimens contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydro-peroxides and aldehydes, which gets elevated as a result of oxidative stress. Since, brain tissue is rich in lipid content hence vulnerable to lipid peroxidation. TBARS return to normal level over a period of time, depending upon the presence of anti-oxidants. In practice, TBARS are expressed in terms of malondialdehyde (MDA) equivalents. As an important biomarker of lipid peroxidation, The MDA level was estimated using the standard protocol (Okhawa 1979) as an important biomarker of lipid peroxidation. It is determined based on its reaction with two molecules of TBA with one molecule of MDA and measured in acidic solution at 532 to 535 nm. The MDA concentration (nmol/mg protein) in the samples was extrapolated from the standard curve obtained by plotting the optical density of the standard MDA concentrations.

Superoxide Dismutase estimation in brain: SOD was estimated using the standard method (Fridovich and McCord, 1969). The SOD activity is based on the inhibition of auto-oxidation of epinephrine. One unit is equal to the amount of the enzyme required to inhibit the auto-oxidation of epinephrine by 50%.

Statistical analysis: Data was analyzed by one-way analysis of variance (ANOVA). Newman – Keuls multiple comparison test was performed for comparison among different groups. The P value * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ were considered statistically significant.

3. RESULTS

The anti-oxidant activity of nine mulberry extracts has been monitored in terms of attenuation in the MDA and SOD levels in the brain homogenates of rats challenged with rotenone. This was done to select potent anti-oxidant extracts for the bio-evaluation in the disease models exhibiting profound oxidative stress. The results have been summarized as percentage decrease in MDA and SOD contents with each test group (Table 1).

Effect on MDA levels: Rotenone induced the ROS generation led to brain oxidative stress by lipid peroxidation as revealed by significant increase 138% in MDA content. The results indicate that one hour pre-treatment with mulberry leaf extracts prior to rotenone challenge in general caused significant reduction in MDA levels from 13.30 to 50.49%. The maximum reducing effect was observed with extract S-146 which attenuated the MDA levels by 50.49% followed by 41.36% by BR-2. Further the decrease of 36.14% and 37.13% in MDA was also observed with extract AR-14 and S-1 respectively. Whereas, the reduction in MDA by the other extracts V-1, S-13, AR-12, S-1635, TR-10 varied considerably and was less pronounced (Fig.1).

Effect on SOD levels: Superoxide serves an important role in signaling processes, cell division and lipid peroxidation. However, when overproduced, the free radical can even initiate lipid peroxidation, protein oxidation, and DNA damage, leading to cell dysfunction and death by apoptosis or necrosis. A reduction in superoxide level

offers a defense against these cellular damages. As the SOD levels which got elevated due to the increased oxidative stress induced by rotenone were significantly reduced by pre-treatment with mulberry leaf extracts. There was a significant reduction (54.01 %) in the brain of rats treated with S-146, further AR-14 treated rats showed a reduction by 40.18%. There was also significant 34.82 % and 29.74 % reduction in SOD content by BR-2 and S-1 respectively. The other mulberry varieties V-1, S-13, AR-12, S-1635, TR-10 however, had also significant effect but it was far less compared to S-146 and AR-14 (Fig.2).

DISCUSSION

Table.1. A comparative effect of pre-treatment with mulberry leaf extracts (100 mg/kg p.o.) one hour prior to challenge with rotenone (75 mg/kg p.o.) on MDA and SOD levels in rat brain.(n=8-10 each).

Extract	% Change in brain tissue	
	MDA	SOD
Rotenone	138.30↑	189.62↑
V-1	16.36↓	13.89↓
S-1	37.13↓	29.74↓
S-13	22.12↓	15.54↓
S-146	50.49↓	54.01↓
AR-12	22.54↓	21.38↓
AR-14	36.14↓	40.18↓
S-1635	29.22↓	8.64↓
BR-2	41.36↓	34.82↓
TR-10	13.30↓	20.78↓

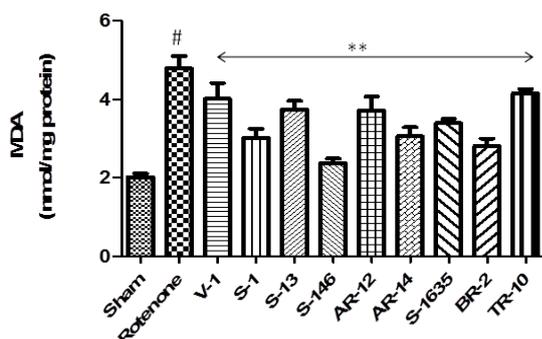


Figure.1. Effect of pretreatment with mulberry leaf extracts (100 mg/kg p. o.) one hour prior to rotenone challenge on MDA levels in brain homogenate of rats.

Significance

P<0.001 vs sham, *** P<0.001 vs rotenone

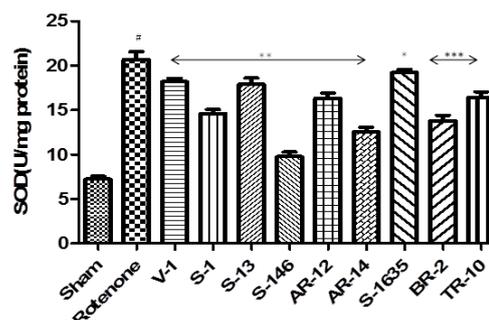


Figure.2. Effect of pretreatment with mulberry leaf extracts (100 mg/kg p.o.) one hour prior to rotenone challenge on SOD levels in brain homogenate of rats.

(n=8-10 each). Significance # P<0.001 vs sham, *** P<0.001, **P<0.01, *P<0.05 vs rotenone

It is now well recognized that anti-oxidants can be therapeutically used to counter cellular damage caused by oxidative stress as well as these can also successfully scavenge free radicals to prevent further damage. The free radicals are chemical species produced in the body which contains one or more unpaired electrons and therefore are highly unstable and cause damage to other molecules by extracting electrons from them to stabilize themselves. The reactive oxygen species (ROS) are formed 'in vivo' such as superoxide anion radical (O_2^-) and hydrogen peroxide, which produces hydroxyl radicals. The latter is highly reactive and mainly responsible for oxidative stress. The defense against the radicals is provided by a number of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). SOD converts O_2^- to H_2O_2 and GPx and CAT convert H_2O_2 to H_2O . The enzymatic anti-oxidants along with other non-enzymatic anti-oxidants such as ascorbic acid, vitamin-E and glutathione tend also reduce levels of ROS. But when there is an imbalance due to overproduction of free radicals induced by exposure to external oxidant substances or a decrease in anti-oxidant defenses, the oxidative stress results causing damage to lipid, protein and DNA which eventually leads to cell death either via apoptosis or necrosis. Oxidative stress is associated with number of diseases including diabetes, inflammation, arthritis, dyslipidemia, cardiovascular diseases including cerebral ischemia (Mehta, 2008, Raghubir, 2010).

Antioxidants play an important role in protecting damage caused by oxidative stress. Plants having phenolic compounds are reported to possess antioxidant properties. The present study was designed to investigate the antioxidant properties of the mulberry leaf extract which contains phenolics, flavonoids, glycosides, saponins, vitamin, and minerals. The available literature indicates that different parts of the plant have been used to treat a variety of diseases. The plant has found useful in the treatment of a number of diseases as reported earlier (Katsube,

2006; Kang, 2006) Moreover, it has also been reported to possess several pharmacological activities such as antioxidative, antiinflammatory (Kim, 1998) and antihyperlipidemic (Kim, 2001) as well.

The pharmacological activities may be due to the presence of polyphenolic compounds in the plant mainly the anthocyanins. The anthocyanins are considered to be very good antioxidants and their high activity can be attributed to their peculiar structure, having the oxonium ion in the C ring (van Acker, 1996). Recent study has shown that anthocyanins and the other phenolics possess high antioxidant activity (Linghong, 2012) which may be well compared with the activity of the established antioxidants α -tocopherol (Kähkönen, 2003).

Our study with mulberry leaf extracts had displayed anti-oxidant property against rotenone induced oxidative stress. It is interesting to find that some of the extracts exhibited highly significant anti-oxidant activity as evidenced by reduced level of MDA and SOD in the brain tissue of rats challenged with oxidative marker, rotenone. Our results indicated that aqueous mulberry leaf extract exhibited strong antioxidant property this is confirmatory with the results obtained by Naowaboot, 2009.

The broad range of antioxidant activity of these extracts indicates the potential of the mulberry plant as a rich source of pharmaceuticals with potential to reduce oxidative stress. Therefore, these two varieties AR-14, S-146 of mulberry leaf extract seem to possess potent antioxidant activity in *in vivo* rotenone-induced oxidative model in rat. Hence, this plant could serve as an effective free radical scavenger which may be further used to reduce the oxidative stress induced by certain diseases. We have found the significant neuroprotective activity of one of the extracts, MLE-AR-14 in focal cerebral ischemia model of rats (Samuel, 2016). Further, the neuroprotective efficacy of the extract S-146 is being investigated in focal cerebral ischemia model in rats because the cerebral ischemia is known to cause severe oxidative stress (Mehta, 2008, Raghbir, 2010).

4. CONCLUSION

Thus the study has provided a good lead about the use of selected extracts for overall reducing oxidative stress associated with number of diseases. The extracts with potent anti-ischemic activity can be used as neuroprotectants. Hence the mulberry leaves can be safely used as food supplement and even can be therapeutically exploited.

5. ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Director, CDRI, Lucknow for providing the laboratory facility and BBAU, Lucknow for providing mulberry leaf samples from the mulberry plantation for the successful completion of the study.

REFERENCES

- Bridi R, Crossetti FP, Steffen VM, Henriques AT, The antioxidant activity of standardized extract of Ginkgo biloba (EGb 761) in rats, *Phytother Res*, 15(5), 2001, 449-51.
- Chen J, Li X, Hypolipidemic effect of flavonoids from mulberry leaves in triton WR-1339 induced hyperlipidemic mice, *Asia Pac. J. Clin. Nutr*, 16, 2007, 290-294.
- Enkmaa BK, Shiwaku T, Katsube K, Kitajima E, Anuurad, Yamasaki M, Mulberry (*Morus alba* L.) leaves and their major flavonol quercetin 3-(6-malonylglucoside) attenuate atherosclerotic lesion development in LDL receptor-deficient mice, *J. Nutr*, 135, 2005, 729-734.
- Fridovich I, McCord JM, Superoxide dismutase, An enzymic function for erythrocyte hemocuprein, *J Biol Chem*, 244(22), 1969, 6049-6055.
- Fukai T, Hano Y, Hirakura K, Nomura T, Uzawa J, Fukushima K, Structures of two natural hypotensive Diels-alder type adducts, mulberrofurans F and G, from the cultivated Mulberry tree, *Chem Pharm Bull*, 33, 1985, 3195-3204.
- Kähkönen MP, Heinonen M, Antioxidant activity of anthocyanins and their aglycons, *J Agr Food Chem*, 51, 2003, 628-633.
- Kang TH, Hur JY, Kim HB, Ryu JH, Kim SY, Neuroprotective effects of the cyanidin-3-O-[beta]-d-glucopyranoside isolated from mulberry fruit against cerebral ischemia, *Neurosci Lett*, 391, 2006, 122-126.
- Kathy K, Griendling, Garret A, FitzGerald, *Clinical Cardiology, New Frontiers Oxidative Stress and Cardiovascular Injury, Circulation*, 108, 2003, 1912-1916.
- Katsube K, Imawaka TN, Kawano Y, Yamazaki Y, Shiwaku K, Yamane Y, Antioxidant flavonol glycosides in mulberry (*Morus alba* L.) leaves isolated based on LDL antioxidant activity, *Food Chem*, 97, 2006, 25-31.
- Kim HB, Kim SY, Ryu KS, Lee WC, Moon JY, Effect of methanol extract from mulberry fruit on the lipid metabolism and liver function in cholesterol-induced hyperlipidemia rats, *Korean Journal of Sericultural Science*, 43, 2001, 104-108.

Kim SY, Park KJ, Lee WC, Antiinflammatory and antioxidative effects of Morus spp. fruit extract, Korean Journal of Medicinal Crop Science, 6, 1998, 204–209.

Lemus I, Garcia R, Delvillar E, Knop G, Hypoglycemic activity of four plants used in Chilean popular medicine, Phytother Res, 13, 1999, 91–4.

Linghong L, Xiangyang W, Maomao Z, Weiguo Z, Fang L, Ye Z, Liuqing Y, Chemical composition, nutritional value, and antioxidant activities of eight mulberry cultivars from China, Pharmacogn Mag, 8(31), 2012, 215–224.

Maritim AC, Sanders RA, Watkins III JB, Diabetes, oxidative stress, and antioxidants: A review, Journal of Biochemical and Molecular Toxicology, 17(1), 2003, 24–38.

Mehta SL, Manhas N, Raghbir R, Molecular targets in cerebral ischemia for developing novel therapeutics, Brain Research Review, 54, 2007, 34–66.

Naowaboot J, Pannangpetch P, Kukongviriyapan V, Kongyingyoes B, Kukongviriyapan U, Antihyperglycemic, antioxidant and antiglycation activities of mulberry leaf extract in streptozotocin-induced chronic diabetic rats, Plant Foods for Human Nutrition, 64(2), 2009, 116–121.

Nomura T, Fukai T, Hano Y, Nemato K, Terada S, Kuramochi T, Constituents of cultivated Mulberry tree. Planta Medica, 47, 1983, 151–156.

Oh H, Ko EK, Jun JY, Oh MH, Park SU, Kang KH, Hepatoprotective and free radical scavenging activities of prenylflavonoid coumarin and stilbene from Morus alba, Planta Med, 68, 2002, 932–934.

Ohkawa H, Ohishi N, Yagi K, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem, 95(2), 1979, 351–358.

Raghbir R, Nakka VP, Mehta SL, Endoplasmic reticular stress in brain damage, Methods Enzymology, 489(15), 2010, 261–277.

Samuel SS, Desai A, Raghbir R and Kumar RV, Neuroprotective profile of mulberry leaf extract in focal cerebral ischemia model in rats, Indian Journal of Research in Pharmacy and Biotechnology, 4(3), 2016, 2320 – 347.

Tong HK, Hye RO, Sun MJ, Jong HR, Mee WP, Yong KP, Sun Y K, Enhancement of Neuroprotection of Mulberry Leaves (*Morus alba* L.) prepared by the Anaerobic Treatment against Ischemic Damage, Biol. Pharm. Bull, 29(2), 2006, 270–274.

Uversky VN, Neurotoxicant induced animal models of Parkinson's disease: understanding the role of rotenone, maneb and paraquat in neurodegeneration, Cell Tissue Res, 318, 2004, 225–241.

van Acker SABE, van den Berg DJ, Tromp MNJL, Griffioen DH, van Bennekom WP, van der Vijgh WJF, Structural aspects of antioxidant activity of flavonoids, Free Radical Biology and Medicine, 20, 1996, 331–342.

Willett WC, Balancing life-style and genomics research for disease prevention, Science, 296, 2002, 695–698.