CELL BIOCHEMISTRY AND FUNCTION
Cell Biochem Funct 2009; **27**: 358–363.
Published online 22 June 2009 in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/cbf.1581

Effect of *Terminalia chebula* aqueous extract on oxidative stress and antioxidant status in the liver and kidney of young and aged rats

Ramalingam Mahesh*, Shanmugham Bhuvana and Vava Mohaideen Hazeena Begum

Department of Siddha Medicine, Faculty of Science, Tamil University, Thanjavur, Tamilnadu, India

We evaluated the preventive effects of *Terminalia chebula* (*T. chebula*) aqueous extract on oxidative and antioxidative status in liver and kidney of aged rats compared to young albino rats. The concentrations of malondialdehyde (MDA), lipofuscin (LF), protein carbonyls (PCO), activities of xantione oxidase (XO), manganese-superoxide dismutase (MnSOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), and glucose-6-phosphate dehydrogenase (G6PDH), levels of glutathione (GSH), vitamin C and vitamin E were used as biomarkers. In the liver and kidney of aged animals, enhanced oxidative stress was accompanied by compromised antioxidant defences. Administration of aqueous extract of *T. cheubla* effectively modulated oxidative stress and enhanced antioxidant status in the liver and kidney of aged rats. The results of the present study demonstrate that aqueous extract of *T. cheubla* inhibits the development of age-induced damages by protecting against oxidative stress.

KEY WORDS — antioxidants; liver and kidney; oxidative stress; plant extract; *Terminalia chebula*

INTRODUCTION

Aging is characterized by a progressive decline of cellular functions. Reactive oxygen species (ROS) are involved in the aging process and result mainly from non-enzymatic processes in the liver.[1] It is widely accepted that disorganized free radical reactions linked to oxygen metabolism or 'oxidative stress' play an important role not only in normal aging but also in many age-related degenerative processes.[2–4] Aging has been shown to result in increased superoxide anion, hydrogen peroxide, and hydroxyl radical resulting in oxidative protein damage in the liver.[5] Liver aging is associated with morphological changes such as a decrease in size attributable to decreased hepatic blood flow. Mitochondria appear to be the major source of the oxidative lesions that accumulate with age and these lesions have been proposed as the major cause of cellular aging and death.[6] Renal changes that occur with aging are: decreased renal weight, thickening of the intrarenal vascular intima, sclerogenous changes of the glomeruli, and infiltration of chronic inflammatory cells and fibrosis in the stroma. Altered renal tubular function, including impaired handling of water, sodium, acid, and glucose, is also frequently present in old age.[7] The aging kidney is constantly exposed to the effects of a variety of potential toxic processes and impairment in the ability to concentrate urine and to conserve sodium and water. Aging is associated with the development of glomerulosclerosis and interstitial fibrosis.[8]

Resistance to oxidative stress is critical for all biological membranes because of their essential role in cellular physiology and maintenance of homeostasis. Age related variations in the antioxidant defences of the organism have been suggested to be the cause of increased susceptibility to drugs and diseases in advanced age.[9–11] An imbalance between the formation and removal of reactive oxygen species (ROS) and the development of oxidative stress has been widely purported to play a important role in drug toxicity, ischemic damage, neoplastic transformation and metastasis, and cardiovascular, neurodegenerative, and age-associated diseases, as well as in differentiation, development, and aging.[9,12] We have used this model to document the anti-aging efficacies of several dietary agents and medicinal plants. Recently, medicinal plants rich in antioxidant phytochemicals have received growing attention as potential preventive agents. These are recognized to exert their preventive effects by scavenging ROS and detoxifying potent genotoxic oxidants.

Terminalia chebula (Combretaceae) (Chebulic myrobalan in English) is a native plant in India and used in Indian system of medicines such as Ayurveda and Siddha. It is a well-known ayurvedic rasayana which possesses an adaptogenic property.[13] *T. chebula* is one of the ingredients in popular ayurvedic formulation of Triphala. The important active principle constituents of *T. chebula* are chebulagic, chebulinic acid, corilagin,[14] beta-sitosterol, gallic acid, terchebulin, caffeic acids, carbohydrates, etc.[15] It is highly

* Correspondence to: Dr R. Mahesh, Oriental Medicine Research Institute, College of Oriental Medicine, Dongguk University, 707, Seokjangdong, Gyeongju, Gyeongbuk 780-714, Republic of Korea. H/P: +82-10-8727-0427, South Korea. E-mail: melanimahesh@gmail.com

Received 18 January 2009
Revised 12 May 2009
Accepted 14 May 2009

nutritious and could be an important source of dietary supplement in vitamin C, energy, protein, amino acids and mineral nutrients.[16] In our previous studies, *T. chebula* has proven *in vitro* free radical scavenging[17] and *in vivo* properties.[18–21] In the present study we investigated the effect of *T. chebula* aqueous extract on mitochondrial antioxidant defense and macromolecular damage in the liver and kidney of aged rats compared with young rats.

MATERIALS AND METHODS

Preparation of T. chebula aqueous extract

The fruits of *T. chebula* ripen from November to March and fall soon after ripening. The fully ripe fruits were collected from the ground as soon as they have fallen and shade dried. The dried fruit skins were hammered in to small pieces and heated in 800 ml distilled water for 24 h in water bath at 40°C. This process was repeated twice. The final yield of the aqueous extract used for this study was 47.6%.

Animals

Young (3–4 months; 120–150 g) and aged (22–24 months; 380–410 g) male albino Wistar rats were selected for this experiments. The rats were housed in polypropylene cages on a 12L: 12D cycle and fed *ad libitum* on commercial laboratory food pellets and water. All animal experiments were conducted as per the instructions of Institutional Animal Ethics Committee.

Experimental design

The animals were divided into four groups of six; Group I: control young rats received sterile water only. Group II: young rats were treated orally with *T. chebula* aqueous extract at a dose of 200 mg/kg body weight in 1.5 ml sterile water orally for 4 weeks. Group III: control aged rats received sterile water only. Group IV: aged rats were treated orally with *T. chebula* aqueous extract as a dose of 200 mg/kg body weight in 1.5 ml sterile water orally for 4 weeks.

Isolation of mitochondria and post-mitochondrial fractions

After 4 weeks of the experimental period, animals were anaesthetized with Thiopentone sodium (50 mg/kg). Liver and kidney tissues were excised immediately and immersed in physiological saline. The mitochondria were isolated with fresh tissues by the method of Johnson and Lardy.[22] A 10% (w/v) homogenate was prepared in 0.25 M sucrose solution and centrifuged at $600 \times g$ for 10 min. The supernatant fraction was decanted and centrifuged at $15\,000 \times g$ for 5 min. The resultant supernatants were stored as post-mitochondrial fractions and the resultant mitochondrial pellet was then washed and resuspended in 0.25 M sucrose. The purity of mitochondria was assessed by the assay of specific marker enzyme, succinate dehydrogenase,[23] Mitochondrial protein was estimated by the method of Lowry *et al*.[24]

Biochemical analysis

Lipid peroxidation was assessed biochemically by determining the level of malondialdehyde (MDA).[25] The lipofuscin was determined by the method of Tappel *et al*.[26] The protein carbonyl (PCO) content was analyzed using 2,4-dinitrophenylhydrazine (DNPH) as described by Levine *et al*.[27] The activity of xanthione oxidase was assayed by the method of Stripe and Della Corte.[28] Manganese-superoxide dismutase (Mn-SOD) activity was measured by the method of Kakker *et al*. [29] using NADH-PMS-NBT. Catalase (CAT) activity was measured according to the method of Beers and Sizer.[30] Glutathione peroxidase (GPx) was estimated by Rotruck *et al*.[31] glutathione reductase (GR) activity by the procedure of Stall *et al*. [32] glutathione-s-transferase (GST) according to Habig *et al*.[33] and glucose-6-phosphate dehydrogenase (G6PDH) by Korenberg *et al*.[34] Reduced glutathione (GSH) was measured as described by Ellman[35] using 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent. Ascorbic acid (vitamin C) and α-tocopherol (vitamin E) contents were assayed according to Omaye *et al*.[36] and Desai,[37] respectively.

Statistical analysis

The values are expressed as mean $\pm$ standard deviation (SD). The results were computed statistically (SPSS software package) using one-way analysis of variance. Tukey-Kramer multiple comparisons test *post hoc* testing was performed for intergroup comparisons using the least significance (LSD) test. A p-value < 0.05 was considered significant.

RESULTS

Table 1 details the levels of liver and kidney mitochondrial MDA, and post-mitochondrial LF, PCO and activity of XO in control and *T. chebula* treated young and aged rats. The levels of MDA, LF, PCO, and activity of XO were significantly increased in liver and kidney of aged control rats compared to young control rats. The increase was 36.64, 18.35, 18.00, and 29.41% for MDA, LF, PCO, and XO in liver; and 31.34, 54.35, 15.19 and 15.63% for MDA, LF, PCO, and XO in kidney of aged control rats. Reduced levels of MDA, LF, PCO, and activity of XO were observed in *T. chebula* supplemented aged rats, with the decrease being 35.88, 28.36% for MDA, 16.91, 47.17% for LF, 16.33, 14.35% for PCO, and 25.21, 13.54% for XO in liver and kidney of aged rats, respectively. *T. chebula* supplementation to young rats significantly decreased the levels of LPO (14.46%), PCO (9.28%) and the activity of XO (11.90%) in liver mitochondria and did not bring noticeable alteration in the kidneys of young rats.

The activities of liver and kidney mitochondrial MnSOD, CAT, GPx, GR, GST, and post-mitochondrial G6PDH in

DOI: 10.1002/cbf

Table 1. Effect of *T. chebula* aqueous extract on mitochondrial MDA and post-mitochondrial LF, PCO and XO in young and aged rats

	Young		Aged	
	Control	Treated	Control	Treated
Liver				
MDA (nmol MDA formed/mg protein)	1.66 ± 0.11	1.42 ± 0.13 [a§]	2.62 ± 0.16 [a‡]	1.68 ± 0.14 [b‡]
LF (% relative fluorescence/gm tissue)	227 ± 18	206 ± 14	278 ± 17 [a‡]	231 ± 18 [b‡]
PCO (nmol of DNPH incorporated/mg protein)	4.42 ± 0.24	4.01 ± 0.21 [a§]	5.39 ± 0.29 [a‡]	4.51 ± 0.26 [b‡]
XO (μg of uric acid formed/min/mg protein)	0.84 ± 0.03	0.74 ± 0.06 [a§]	1.19 ± 0.07 [a‡]	0.89 ± 0.05 [b‡]
Kidney				
MDA (nmol MDA formed/mg protein)	1.84 ± 0.10	1.77 ± 0.11	2.68 ± 0.12 [a‡]	1.92 ± 0.09 [b‡]
LF (% relative fluorescence/gm tissue)	210 ± 11	192 ± 13	460 ± 12 [a‡]	243 ± 17 [b‡]
PCO (nmol of DNPH incorporated/mg protein)	4.02 ± 0.20	3.79 ± 0.24	4.74 ± 0.22 [a‡]	4.06 ± 0.27 [b‡]
XO (μg of uric acid formed/min/mg protein)	0.81 ± 0.05	0.72 ± 0.06	0.96 ± 0.08 [a†]	0.83 ± 0.06 [b†]

Each value is expressed as mean ± SD for six rats in each group. Superscript letters represent $p < 0.05$ (Tukey-Kramer Multiple comparisons Test).
[a]As compared with Young control,
[b]As compared with Aged control.
[§]$p < 0.05$; [†]$p < 0.01$; [‡]$p < 0.001$.

control and *T. chebula* treated young and aged rats are presented in Table 2. The activities of MnSOD, GR, GST and post-mitochondrial G6PDH were found to be significantly lower (37.37, 37.25, 34.69, and 49.54% in liver and 38.51, 45.75, 30.30 and 38.33% in kidney, respectively) while the activities of CAT and GPx were higher (19.76, 19.10% in liver and 15.85, 14.10% in kidney, respectively) in aged rats. Supplementation with *T. chebula* increased mitochondrial MnSOD, GR, GST, and post-mitochondrial G6PDH activities in liver and kidney mitochondria of aged rats (32.59, 35.53, 35.35, and 47.77% in liver and 35.83, 46.24, 31.85, and 39.34% in kidney, respectively). *T. chebula* supplementation also normalized the activities of CAT and GPx in mitochondria of aged rats in a significant manner (20.63, 16.21% in liver and 13.21, 12.77% in kidney, respectively) and increased the activities of the enzymes GST (4.85% in liver and 6.38% in kidney) and G6PDH (8.19% in liver only).

Table 3 shows the levels of liver and kidney mitochondrial GSH, VIT-C. and VIT-E in control and *T. chebula* treated young and aged rats. The levels of GSH, VIT-C, and VIT-E were lower in liver and kidney mitochondria of aged control rats (18.01, 26.39, and 30.70% in liver and 24.22, 28.75, and 25.36% in kidney, respectively). Supplementation with *T. chebula* increased the levels of GSH, VIT-C, and VIT-E in liver and kidney mitochondria of aged rats, the increase being 18.09, 24.85% for GSH, 26.71, 28.30% for VIT-C, and 27.25%, 24.27% for VIT-E in liver and kidney of aged rats, respectively. In young rats, *T. chebula* only increased GSH levels in liver (10.04%) and kidney (4.83%) mitochondria.

Table 2. Effect of *T. chebula* aqueous extract on enzymatic antioxidants in young and aged rats

	Young		Aged	
	Control	Treated	Control	Treated
Liver				
MnSOD (50% reduction of NBT/min/mg protein)	3.37 ± 0.20	3.55 ± 0.18	2.11 ± 0.24 [a‡]	3.13 ± 0.23 [b‡]
CAT (μmol H_2O_2 consumed/min/mg protein)	4.59 ± 0.31	4.51 ± 0.22	5.72 ± 0.28 [a‡]	4.54 ± 0.25 [b‡]
GPx (μmole GSH utilized/min/mg protein)	6.44 ± 0.24	6.53 ± 0.20	7.96 ± 0.26 [a‡]	6.67 ± 0.23 [b‡]
GR (nmol NADPH oxidized/min/mg protein)	5.61 ± 0.28	5.73 ± 0.31	3.52 ± 0.26 [a‡]	5.46 ± 0.25 [b‡]
GST (μmoles of CDNB-GSH conjugated/min/mg protein)	0.98 ± 0.02	1.03 ± 0.03 [a§]	0.64 ± 0.02 [a‡]	0.99 ± 0.04 [b‡]
G6PDH (Units/min/mg protein)	3.25 ± 0.18	3.54 ± 0.14 [a§]	1.64 ± 0.16 [a‡]	3.14 ± 0.19 [b‡]
Kidney				
MnSOD (50% reduction of NBT/min/mg protein)	3.35 ± 0.18	3.42 ± 0.21	2.06 ± 0.16 [a‡]	3.21 ± 0.23 [b‡]
CAT (μmol H_2O_2 consumed/min/mg protein)	4.14 ± 0.20	4.20 ± 0.15	4.92 ± 0.22 [a‡]	4.27 ± 0.22 [b‡]
GPx (μmole GSH utilized/min/mg protein)	5.18 ± 0.27	5.07 ± 0.31	6.03 ± 0.25 [a‡]	5.26 ± 0.29 [b‡]
GR (nmol NADPH oxidized/min/mg protein)	4.35 ± 0.24	4.48 ± 0.24	2.36 ± 0.26 [a‡]	4.39 ± 0.22 [b‡]
GST (μmoles of CDNB-GSH conjugated/min/mg protein)	1.32 ± 0.06	1.41 ± 0.03 [a†]	0.92 ± 0.04 [a‡]	1.35 ± 0.03 [b‡]
G6PDH (Units/min/mg protein)	1.80 ± 0.08	1.88 ± 0.06	1.11 ± 0.08 [a‡]	1.83 ± 0.05 [b‡]

Each value is expressed as mean ± SD for six rats in each group. Superscript letters represent $p < 0.05$ (Tukey-Kramer Multiple comparisons Test).
[a]As compared with Young control,
[b]As compared with Aged control.
[§]$p < 0.05$; [†]$p < 0.01$; [‡]$p < 0.001$.

DOI: 10.1002/cbf

Table 3. Effect of *T. chebula* aqueous extract on mitochondrial non-enzymatic antioxidants in young and aged rats

	Young		Aged	
	Control	Treated	Control	Treated
Liver				
GSH (μg/mg protein)	10.66 ± 0.54	11.85 ± 0.50 [a†]	8.74 ± 0.46 [a‡]	10.67 ± 0.53 [b‡]
VIT-C (μg/mg protein)	9.17 ± 0.49	9.23 ± 0.44	6.75 ± 0.46 [a‡]	9.21 ± 0.52 [b‡]
VIT-E (μg/mg protein)	4.43 ± 0.27	4.51 ± 0.22	3.07 ± 0.26 [a‡]	4.22 ± 0.24 [b‡]
Kidney				
GSH (μg/mg protein)	9.66 ± 0.33	10.15 ± 0.24 [a§]	7.32 ± 0.26 [a‡]	9.74 ± 0.31 [b‡]
VIT-C (μg/mg protein)	8.07 ± 0.38	8.14 ± 0.31	5.75 ± 0.30 [a‡]	8.02 ± 0.35 [b‡]
VIT-E (μg/mg protein)	4.18 ± 0.32	4.26 ± 0.34	3.12 ± 0.30 [a‡]	4.12 ± 0.28 [b‡]

Each value is expressed as mean ± SD for six rats in each group. Superscript letters represent $p < 0.05$ (Tukey-Kramer Multiple comparisons Test).
[a]As compared with Young control,
[b]As compared with Aged control.
[§]$p < 0.05$; [†]$p < 0.01$ [‡]$p < 0.001$.

T. chebula did not cause any significant changes in VIT-C and VIT-E in young rats.

DISCUSSION

Liver has one of the highest antioxidant enzyme activities in the body and is involved in major detoxification functions. With aging, tissues are subjected to numerous influences such as metabolic state and nutritional status and show a number of characteristics consistent with oxidative injury.[9,12]

In the rat, aging is associated with enhanced ROS generation and oxidative stress through lipid peroxidation resulting in an elevation of lipofuscin content.[38] In the present study, the increased levels in mitochondrial MDA and post-mitochondrial lipofuscin observed in liver and kidney of aged rats support the concept of a strong oxidative stress and enhanced ROS generation in aging. This agrees with Liu and Mori[39] and Dogru-Abbasoglu *et al*. [40] who argued that increased lipid peroxidation is not an inevitable consequence of aging. Supplementation with *T. chebula* decreased liver and kidney mitochondrial MDA and post-mitochondrial lipofuscin concentrations in aged rats. In young rats *T. chebula* decreased malondialdehyde in liver mitochondria but did not bring noticeable alterations in kidney malondialdehyde or liver and kidney lipofuscin content. The decrease in the malondialdehyde and lipofuscin levels could be due to the chelating property of *T. chebula* and inhibition of lipid peroxidation.[17]

Aldehydes, such as 4-hydroxy-2-nonenal or malondialdehyde produced during lipid peroxidation can be incorporated into proteins by reaction with either the ε-amino moiety of lysine or the sulfhydryl group of cysteine residues to form carbonyl derivatives.[41] In the present study, an increased level of protein carbonyls was observed in liver and kidney post-mitochondrial fraction of aged control rats. Previous studies demonstrated that normal aging is associated with an increase in oxidatively modified amino acids, these being used as markers to oxidative protein damage.[42,43] Supplementation with *T. chebula* reduced the level of protein carbonyl in liver and kidney of aged rats. In young rats, *T. chebula* decreased the levels of protein carbonyls in liver mitochondria but not in the kidney. The decreased level of lipid peroxidation in supplementation with *T. chebula* may reduce the levels of oxidation to protein in aging. Administration of free radical scavengers, such as flavonoids and polyphenolic acids prevents oxidation of lipids and proteins.[44]

Xanthine oxidase has been implicated in oxidative injury to tissues. In the present study, an increased activity of post-mitochondrial xanthine oxidase was observed in aged rats suggesting the presence of superoxide radicals, and/or Fenton-type reactions.[45] *T. chebula* reduced the activity of xanthine oxidase in both the liver and kidney of aged rats while in young rats only the liver showed a decrease in mitochondrial xanthine oxidase. Cos *et al*.[46] reported that flavonoids inhibit xanthine oxidase activity.

Cellular defence mechanisms against superoxide include a series of linked enzyme reactions which remove superoxide and repair radical induced damage. In our study, liver and kidney mitochondrial MnSOD activity declined with aging. The lower activity of MnSOD could be a consequence of an excess of ROS generation.[47] *T. chebula* supplementation increased the level of MnSOD to near that seen in younger animals, but had no effect on MnSOD activity in young rats. This suggests that the phytochemicals present in *T. chebula* scavenge superoxide radicals and other free radicals only in the aged rats.

Hydrogen peroxide, a precursor of more potent radical species, is scavenged at higher concentrations by CAT and at lower concentrations by GPx. In our study, the liver and kidney mitochondrial CAT and GPx activities were increased in aged control rats indicates that CAT and GPx are more responsive to the increased hydrogen peroxide concentration in this tissue, emphasizing its role in the control of cellular lipid peroxide concentration.[48] This is important in the maintenance of cellular differentiation. The age-associated increase in antioxidant enzymes may be an attempt by the organism to counter balance the decrease in the reducing power of the tissues mediated by GSH.

DOI: 10.1002/cbf

However, in senescent animals, the total antioxidant capacity of cells should not be sufficient to scavenge the ROS generated.[49] Supplementation with *T. chebula* showed that the CAT and GPx activities in liver and kidney mitochondria were decreased to that seen in younger animals suggesting that *T. chebula* acts through controlling cellular lipid peroxide concentration and other free radicals as reported *in vitro*.[17]

With a aging reduction in protein synthesis occurs due to decreased ATP production. This also may be the reason for the reduction in the activities of free-radical protective antioxidant enzymes. In the present study, the antioxidant enzymes such as mitochondrial GR, GST and post-mitochondrial G6PDH activities were decreased in liver and kidney of aged animals. Decreases in these antioxidant enzymes activities has been reported with advancing age.[50] These free-radical protective enzymes were correlated with increases in free radical induced cellular damage as measured by several assays, including sensitivity to lipid peroxidation, protein oxidation, and oxidative stress.[50] *T. chebula* enhanced the activities of GR, GST, and G6PDH in liver and kidney of aged rats indicating that *T. chebula* may protect these enzymes from further peroxidative damage by increasing the overall protein synthesis and GSH reproduction by flavonoids and polyphenols.[51]

In the present study, the level of mitochondrial GSH was decreased in liver and kidney of aged control rats. Thus, the increased generation of ROS and lipid peroxides during oxidative stress observed in aged animals could be related to decline in GSH levels has been reported.[52,53] *T. chebula* restored the liver and kidney mitochondrial GSH level to that seen in young rats. T. chebula supplementation to young rats increases the level of liver and kidney mitochondrial GSH as compared with young control rats. It promotes the scavenging ability against free radicals induced oxidative stress in young rats. Flavonoids increase the expression of γ-glutamylcysteine synthetase and showed concomitant increase in the intracellular glutathione concentrations.[54]

In addition to GSH, vitamins C and E are interrelated by recycling processes.[55] Recycling of tocopheroxyl radicals to tocopherol is achieved by reaction with ascorbic acid. Dehydroascorbic acid is formed in reaction with reduced GSH. McCay *et al*.[56] have shown the presence of a liable glutathione dependent factor, which cycles the tocopheroxyl radicals to tochopherol. If recycling of tocopheroxyl radicals to tocopherol is a major mechanism for maintenance of tissue tocopherol levels, deficiency of ascorbic acid is expected to result in depletion of tissue tocopherol. In the present study, vitamins C and E levels were decreased in liver and kidney mitochondria of aged control rats suggesting the recycling of tocopheroxyl radicals to tocopherol may have been hindered, resulting in elevated lipid peroxidation reactions.[56] Supplementation with *T. chebula* increased the liver and kidney mitochondrial vitamin C and E in aged rats. The possible mechanisms are (i) increase in ascorbic acid absorption, (ii) stabilization of ascorbic acid, (iii) reduction of dehydroascorbate to ascorbic acid,[57] (iv) metabolic sparing of ascorbic acid, and (v) influenced in the ascorbic acid biosynthesis by flavonoids.[58] Phenolics and some flavonoids, owing to their intermediate redox potential and physiochemical characteristics, can possibly act an interface between ascorbate and tocopherol.[59] This shows the efficacy of *T. chebula* in enhancing liver and kidney functions.

Overall, the study concluded that supplementation of *T. chebula* on liver and kidney in aged rats reduces oxidative stress in aged rats by alleviating lipid peroxidation through scavenging of free radicals and increasing the activities of antioxidants. The antioxidant activities of *T. chebula* might be due to the presence of phytochemicals such as flavonoids, polyphenols, etc. Use of *T. chebula* may offer therapeutic benefit, by assisting the liver and kidney in the management of oxidant/antioxidant imbalance.

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DOI: 10.1002/cbf

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DOI: 10.1002/cbf