Asparagus Racemosus Supplementation Ameliorate Age-related Oxidative Damage in Skeletal Muscle Lysosome of Aged Rats

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ABSTRACT: Aging is the accumulation of diverse deleterious changes in the cells and tissues leading to increased risk of diseases. Oxidative stress is considered as a major risk factor and contributes to age-related increase in oxidative damage during aging. Age associated changes occur in many cellular organelles. Changes in the lysosomes of senescent tissues and organisms are common and have been used as biomarkers of aging. Cellular aging represents slowly developing functional decline of lysosomes compartment and secondary to oxidant-induced damage and lipofuscin accumulation. Lipofuscin consider as a hallmark of aging and their deposition ultimately increases the oxidative damage. In the present study, we have evaluated the salubrious role of asparagus racemosus root extract (ARRE) on accumulation of oxidative damage products such Malondialdehyde (MDA), Protein carbonyls (PCO), lysosomal marker enzymes acid phosphatase and Cathepsin D activity, aging marker lipofuscin and membrane bound H⁺ATPase activity in skeletal muscle lysosome of aged rats. Our results, thus, revealed that ARRE has ameliorating effect on the accumulation of age-related oxidative damages and restored the enzyme activity and decreased the lipofuscin content in skeletal muscle lysosomes. This ameliorating activity of ARRE mainly attributed to the presence of enriched therapeutic phytochemical constituents, which act synergistically to alleviate the indices of oxidative stress, is associated with aging.

KEY WORDS: Asparagus racemosus root extract, Lysosome, Aging, Lipofuscin, Malondialdehyde, Protein carbonyl.

Introduction

Aging is characterized by slow, progressive, structural and functional changes that take place at cellular, tissue and organ level. These changes resulting in gradual functional decline, decreased adaptability and ability to face stress and increased probability of age-associated diseases including cardio vascular disease, cancer, diabetic, Alzheimer’s etc. Age associated changes occur in many cellular organelles including lysosomes and mitochondria. Changes in the lysosomes of senescent tissues and organisms are common and have been used as biomarkers of aging. Lysosomes of skeletal muscle show the most remarkable age-related changes because they are non-proliferative and play a major role in cellular aging and death (Cuervo and Dice, 2000). Cellular aging represents slowly developing functional decline of lysosomes compartment and secondary to oxidant-induced damage and lipofuscin accumulation (Terman et al., 2006).

Lysosomes are membrane bound vesicles involved in intracellular digestion. They contain a variety of hydrolytic enzymes that are optimally active at an acidic pH (Holtzman, 1989; Kornfeld and Mellman, 1989; Futai et al., 1998). These hydrolases require an acidic environment for activity and become inactivated at a neutral pH. The intralysosomal environment is maintained at (pH) 4.5 by membrane integrated H⁺ATPase. (Dell’Angelica, 2000). Lysosomal enzymes degrade not only cytosolic biomolecule like protein, carbohydrate, lipid and nucleic acid but also whole organelles including mitochondria, endoplasmic reticulum, ribosome, peroxisome and proteosomes (Cuervo, 2004; Levine and Klipnsky, 2004). The lysosomal compartment has multiple functions and it may be indicator of adaptive abilities. It is known that lysosomes are especially sensitive to oxidative stress (Li et al., 1998). It was investigated that hydroxyl radicals destabilize lysosomal membranes and thereby cause leakage of lysosomal enzymes to the cytosol with ensuing cellular degeneration or even death (Hellquist et al., 1997). Normally damaged macromolecules and organelles are efficiently degraded in the lysosome, resulting in the successful recycling (Brunk and Terman, 2002b).

As age advances, lysosomal degradation capacity decreases that contribute to increased accumulation of incompletely degraded intralysosomal waste material as lipofuscin (age pigment) which starts to accumulate in postmitotic cells from early life and then gradually increases with age advancing (Terman et al., 2006). Lipofuscin consider as a hallmark of aging and their deposition ultimately decreases cellular adaptability and promotes the development of age-related pathologies, including neuro degenerative diseases, heart failure and macular degeneration (Terman and Brunk, 2004).
The enhancement of lipofuscin formation by oxidative stress and its attenuation by the use of antioxidant and iron chelators suggest a potential anti-aging strategy (Terman et al., 2006). The substance centrophенoxine is claimed to promote the removal of lipofuscin from cells, which is consistent with the antioxidant properties (Nagy et al., 1994). Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, low toxicity and economic viability (Auddy et al., 2003).

Flavonoids and phenolic compounds widely distributed in plants have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic etc., (Miller, 1996). They were also suggested as a potential iron chelator (Boyer et al., 1988; Havsteen, 1983; Borsari et al., 2001). Plant extracts increasingly used as phytotherapeutics are still a large source of natural antioxidants. Natural antioxidants strengthen the endogenous antioxidant defense from ROS ravage and restore the optimal balance by neutralizing the reactive species. They are gaining immense importance by virtue of their critical role in healthy old age and disease prevention.

In Indian system of medicine Asparagus racemosus Willd root (Liliaceae) (Eng: Wild asparagus, Tamil: Thanner Vittan Kizhangu,) is an important medicinal plant. Traditionally it is used as health tonic (Pandey and Chunekar, 1998) and common Indian home remedy used as a rejuvenator, promoter of strength, breast milk and semen (Dash, 1991). Roots of the plant have been used in the Indian traditional system of medicine for the treatment of various ailments in human being (Kirtikar and Basu, 1975; Nadkarni, 1976; Goyal et al., 2003). Asparagus racemosus is a well known ayurvedic rasayana which prevent aging, increase longevity, impart immunity, improve mental function and add vigor and add vitality to the body and also used in nervous disorders, dyspepsia, tumors, inflammation, hyperdipsia, neuropathy and hepatopathy (Sharma, 2001). Asparagus racemosus has also been reported to have potent adaptogenic activity (Rege et al., 1999) and antioxidant property (Kamat et al., 2000).

The aim of this study was to evaluate the salubrious role of asparagus racemosus root extract (ARRE) on accumulation of oxidative damage products such malondialdehyde (MDA), protein carbonyls, lysosomal marker enzymes such acid phosphatase and Cathepsin D activity, aging marker lipofuscin and membrane bound H' ATPase activity in skeletal muscle lysosome of aged rats.

Material and Methods

Plant Material

The roots of the Asparagus racemosus were collected from the kolli hills, Tamil Nadu, South India. The collected roots were identified and authenticated by a botanist Prof. Dr. M. Jegadeesan, Department of Environmental and Herbal science, Tamil University, Thanjavur, Tamil Nadu. A voucher specimen (Specimen no: 29) has been deposited at the Herbarium of our department. The roots were cut into small pieces and shade dried at room temperature for 15 days and powdered finely then used for extraction.

Preparation of plant extract

A required quantity of the powder (5g) was suspended in a measured amount of distilled water (600ml). The suspension was boiled until the quantity was reduced to 100ml. The resultant decoction was cooled and used in the present study. The concentration of resultant decoction was 50 mg/mL. For experiments 500mg/kg body weight of asparagus racemosus root extract (ARRE) was used. This effective dose of ARRE was selected based on the dose dependent studies carried out in aged rats (Velavan et al., 2006).

Animal

Male albino rats of wistar strain approximately 3-4 months old rats weighing approximately 140-160g (young) and 24-26 months old rats weighing approximately 380-410g (aged) were used in this study. They were healthy animals from Sri Venkateswara Enterprises, Bangalore, India. The animals were housed in spacious polypropylene cages bedded with rice husk. The animal room was well-ventilated and maintained under standard experimental conditions (Temperature 27 ± 2 ºC and 12 hours light/dark cycle) throughout the experimental period. All the animals were fed with standard pellet diet (Gold Mohur, Mumbai, India) and water ad libitum. They were acclimatized to the environment for 1 week prior to experimental use. The study protocol was carried out as per the rules and regulation of the institutional animal’s ethics committee (IAEC).

Chemicals

Ethylene diamine tetra acetic acid (EDTA), Trichloro acetic acid (TCA), Thio barbituric acid (TBA), Tris-HCL and Guanidine hydrochloride were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used were of analytical grade with high purity and were obtained from Glaxo Laboratories, Mumbai, India, and Sisco Research Laboratories, Mumbai, India.
Grouping of animals

Body weights of the animals were recorded and they were divided into 4 groups of 6 animals each as follows.

Group 1 : Control young rats
Group 2 : Young rats administered ARRE (500mg/Kg b.wt/day) orally for four weeks
Group 3 : Control aged rats
Group 4 : Aged rats administered ARRE (500mg/Kg b.wt/day) orally for four weeks.

After the completion of experimental regimen, the rats were fasted over night. The skeletal muscle was excised immediately and immersed in physiological saline. A known weight of them was used for homogenate preparation. Lysosomes (pellet) were isolated from the homogenate and used for various biochemical analyses.

Isolation of Lysosomes

Skeletal muscle lysosomes were isolated by the method of Jozwik et al. (2002) with minor modification. A 10% tissue homogenate was prepared in 0.1M phosphate buffer (pH 7.0). The homogenate was centrifuged at 600g for 10 min at 4°C. The pellet (nuclear fraction) was discarded and post-nucleat supernatant was recentrifuged at 9000g for 15 min at 4°C. The pellet (mitochondrial fraction) was removed and Post-mitochondrial supernatant was recentrifuged at 16000g for 30 min at 4°C to obtain the lysosomal enriched pellet. Lysosomal pellet was diluted in a 0.1M phosphate buffer (pH 6.0) with Triton X-100 and finally frozen at -20°C. The clear supernatant was subjected to determination of the activities of lysosomal enzyme. Lysosomal protein was estimated by the method of Lowry et al. (1951).

Biochemical analysis and enzyme assay

Determination of MDA and PCO

Protein carbonyl content was determined by the reliable method based on the reaction of carbonyl groups with 2,4-dinitro-phenylhydrazine to form 2,4-dinitrophenylhydrazone as suggested by Levine et al. (1994). The protein carbonyl content was expressed as nmol of DNPH incorporated/mg protein in tissues. Malondialdehyde (MDA) was estimated by the thiobarbituric acid assay method of Beuge and Aust (1978). The results were expressed as nmols MDA formed/mg protein in tissues.

Determination of Acid phosphatase and Cathepsin-D

Acid phosphatase activity was measured by the method of Annon (1963). The Acid phosphatase activity was expressed as mmol of phenol liberated /min/mg protein. Cathepsin-D was measured by the method of Takahashi and Tang (1981). Cathepsin D is expressed mmol of tyrosine liberated/h/mg protein.

Determination of Lipofuscin and H^+ATPase activity

Lipofuscin concentration was measured by the method of Tappel et al., (1973). Lipofuscin content was expressed as fluorescence arbitrary units/gm tissue. H^+ATPase assay was assayed according to the protocol Gupta et al., (1991)and Manzoor et al., (1999) with some modifications. The H^+ATPase activity was assessed by the measurement of the produced inorganic phosphate by Fiske and Subba Row (1925) method and results were expressed as specific activity (nmoles of inorganic phosphorus liberated/ hour/mg protein).

Statistical analysis

All the values were expressed as means ± SD of six rats from each group and statistically evaluated by one-way analysis of variance (ANOVA). The means were tested for significance by Tukey’s test for multiple comparisons (Harvey, 1998). A value of $p<0.001$ and $p<0.001$ were considered as significant.

Results

The Group 3 aged rats showed a significant increase in MDA, PCO and lipofuscin in skeletal muscle lysosome as compared to Group 1 control young rats (Table 1). ARRE treated Group 4 aged rats showed a significant decrease in MDA, PCO and lipofuscin in skeletal muscle lysosome as compared to Group 3. The Group 3 aged rats showed a significant increases in cathepsin D, acid phosphatase and significant decrease of H^+ATPase activity in skeletal muscle lysosome as compared to Group 1 control young rats (Table 2). ARRE treated Group 4 aged rats showed a significant decrease in cathepsin D, acid phosphatase activity and a significant increase H^+ATPase activity in skeletal muscle lysosome as compared to Group 3. In young rats Group 2 ARRE administration showed a lowered MDA, lipofuscin, PCO content and non-significant changes (NS) in all other parameters as compared to Group 1 control young rats.
Table 1. Effect of *Asparagus racemosus* on MDA, PCO and lipofuscin in skeletal muscle lysosome of control and experimental rats.

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<th>Parameters</th>
<th>Young rats</th>
<th>Aged rats</th>
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<td></td>
<td>Group 1</td>
<td>Group 2</td>
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<td></td>
<td>(Control)</td>
<td>(ARRE treated)</td>
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<tr>
<td>MDA (U*)</td>
<td>1.29 ± 0.14</td>
<td>1.18 ± 0.11</td>
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<tr>
<td>PCO (U**)</td>
<td>3.56 ± 0.32</td>
<td>3.08 ± 0.26</td>
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<td>Lipofuscin (U***</td>
<td>5.37 ± 0.45</td>
<td>5.21 ± 0.44</td>
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Values are expressed as mean ± SD for six rats in each group.
U* nmole of MDA formed/mg protein.
U** nmole of DNPH incorporated/mg protein.
U*** fluorescence arbitrary units/gm tissue.
<sup>a</sup> As compared with young control rats (p< 0.001)
<sup>b</sup> As compared with aged control rats (p< 0.001)
<sup>c</sup> As compared with young control rats (p< 0.01)

Table 2. Effect of *Asparagus racemosus* on cathepsin D, acid phosphatase and H⁺ATPase activities in skeletal muscle lysosome of control and experimental rats.

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<td></td>
<td>(Control)</td>
<td>(ARRE treated)</td>
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<tr>
<td>Cathepsin D (U*)</td>
<td>3.56 ± 0.32</td>
<td>3.28 ± 0.26</td>
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<tr>
<td>Acid phosphatase (U**)</td>
<td>7.86 ± 0.63</td>
<td>8.61 ± 0.73</td>
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<td>H⁺ ATPase (U***</td>
<td>3.72 ± 0.31</td>
<td>3.78 ± 0.32</td>
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Values are expressed as mean ± SD for six rats in each group.
U* μmole of tyrosine liberated/hour/mg protein.
U** μmole of phenol liberated /min/mg protein
U*** μmole of inorganic phosphorus liberated/ hour/mg protein
<sup>a</sup> As compared with young control rats (p< 0.001)
<sup>b</sup> As compared with aged control rats (p< 0.001)
<sup>c</sup> As compared with aged control rats (p< 0.01)
Discussion

Aging affects all types of muscle cells, yet the degree of age-related changes is the highest in skeletal muscle. Consequences of aging changes in muscle are declining physiological function and loss of muscle strength, typically associated with reduced physiological activity and this may contribute to a reduction in the life span (Carmeli et al., 2002). Certain properties of muscle may render it especially susceptible to free radical injury (Chan and Decker, 1994). The skeletal muscle tissue is expected to suffer the maximum damage because it has been shown that post mitotic tissues accumulate damage faster than mitotically active tissues (Kowald and Kirkwood, 2000). Thus, maintenance of lysosomal function may be important to maintain overall skeletal muscle function.

Lysosomes, ubiquitous in all animal cells as an acidic compartment with limiting membranes are able to degrade unneeded intra- and extracellular materials to biological monomers and contain various types of proteinases expect for metalloproteinases (Terman et al., 2006). An increased volume and fragility of lysosomes are common findings for senescent organisms. Compared to other intracellular membranes, lysosomal membranes are very sensitive to free radical damage and an age related increase in oxidation of lipids or proteins within the lysosomal membrane may be the cause of the increased fragility, and reduced fusion of lysosomes with autophagic vacuoles. Lysosomes of senescent organisms are filled with the aging pigment as lipofuscin (Cuervo and Dice, 2000). Lipofuscin accumulates in the lysosomal vacuole of a variety of post mitotic cell types including skeletal muscle, during aging in man and animals (Marzabadi and Jones, 1992). Lipofuscin consider as a hallmark of aging, which is primarily composed of oxidatively modified protein and lipid degradation residues. It also contains some carbohydrate and traces of metals including copper, manganese, aluminum, zinc, calcium and high content of iron (Terman et al., 2006). The increased amount of iron within lipofuscin granules, promoting generation of ROS by stimulating Fenton-like reactions. Cellular injury increases due to a rise in ROS generation results in lysosomal membrane destabilization (Essner and Novikoff, 1960; Brunk and Ericsson, 1972), resulting in hydrolytic enzymes leak into the cytosol and trigger apoptotic pathway (Bidere et al., 2003; Brunk and Neuzil, 2001; Cirman et al., 2004; Johansson et al., 2003; Zhao and Antunes, 2003). Thus, these remarkable organelles are primarily meant for the digestion of a variety of biological material and secondarily cause aging and death of animal cells.

The study of lipid peroxidation is attracting much attention in recent years due to its role in disease processes. It has been implicated in the pathogenesis of a number of diseases including aging (Devasagayam et al., 2003). It is now generally accepted that lipid peroxidation and its products (MDA) play an important role in liver, kidney, skeletal muscle and brain toxicity (Poli et al., 1987; Cojocel et al., 1984; Usyal et al., 1989). MDA is the major reactive aldehyde resulting from the peroxidation of biological membrane PUFA and used as an indicator of tissue damage and oxidative stress (Halliwell, 1991; Ohkawa et al., 1979; Vaca et al., 1988). The occurrence of lipid peroxidation in biological membranes results in impaired membrane function, decreased fluidity, inactivitation of membrane bound receptors and enzymes (Halliwell and Gutteridge, 1989).

Oxidative modification alters the function of proteins and is thought to play an important role in the decline of cellular function during aging. Free radicals produced during oxidative stress can damage the peptide backbone, resulting in the generation of protein carbonyls (Levine and Stadtman, 2001). Lipid peroxidation may also bring about protein damage by inactivation of membrane bound enzymes either through direct attack by free radicals or through chemical modification by its end products, malondialdehyde and 4-hydroxynonenal (Halliwell and Gutteridge, 1999).

The action of reactive oxygen species on lipids and protein increases with age promote lipid peroxidation and protein oxidation, which are an important key factors in the damage caused to the lysosomal membrane and inactivation of membrane bound ATPase. The level of these oxidized molecules can be quantitated by measurement of MDA and PCO content, which has been shown to increase in muscle during aging (Reznick and Packer, 1994; Navarro, 2004). Determination of MDA and PCO content used as a marker for membrane damage (Sohal, 2002; Spiteller, 2001) In the present study, we also observed the increased content of MDA and PCO in skeletal muscle of aged untreated rats as compared to young rats. The increased content of MDA and PCO indicates the membrane damage in the lysosome. Supplementation of ARRE decreased the content of MDA and PCO in aged rats as compared to young control rats indicate the antioxidative activity of ARRE. Our earlier reports reveals that the quantitative analysis of ARRE indicated the presence of flavonoids (36.7±3.9 mg/100ml), polyphenols (88.2±9.3 mg/100ml) and vitamin-C (42.4±5.1 mg/100ml) (Velavan et al., 2007). It is well known that the flavonoid and polyphenols are natural antioxidant but also have been reported to significantly decreased MDA and PCO content (Russo et al., 2000; Ferguson, 2001; Erlejman et al., 2004 ). The reduction in lipid peroxide levels may be due to the electron and H+ donating capacity of flavonoids present in ARRE, which seem to contribute to the termination of lipid peroxidation chain reaction based on their reducing power.

An acidic internal pH is an intrinsic property of lysosomes. The pH of lysosome is maintaining by vacuolar H+ - ATPases (i.e., acidification of the compartment) and
provides an optimal lysosomal enzyme function. The plasma membrane H^+ ATPases is an important new target of therapeutic intervention (Haque et al., 2005). One of the most important targets of reactive oxygen species (ROS) is the membrane lipid, which undergo peroxidation. Lipid peroxidation is a key factors in the damage caused to the lysosomal membrane and disturb the H^+ ATPases which lead to change in the intralysosomal pH (Carsten, 2004). Impaired lysosomal acidification will be inactivation of lysosomal hydrolases with subsequent intralysosomal storage of undigested material and promotion of lipofuscin formation (Ivy et al., 1984). Lipofuscin is considered to be the end product of peroxidation, fragmentation and polymerization of lipids and proteins (Marzabadi and Jones, 1992).

In the present study, decline in the activity of H^+ ATPases and increased content of lipofuscin were observed in skeletal muscle of aged untreated rats when compared to young rats. Reversion of H^+ ATPases activity and decreased levels of lipofuscin in *asparagus racemosus* treated animals may be due to the stabilizing property of the ARRE on lysosomal membrane which could have been imparted by the flavonoids. The drug may modify the lysosomal membrane in such a way that it is capable of fusing with the plasma membrane and thereby preventing the discharge of acid hydrolases or by inhibiting the release of lysosomal enzymes. (Carevic, 1988). The stabilizing property of the ARRE on lysosomal membrane to maintain the intralysosomal pH. Acidic nature of lysosomal compartment promote the activity of hydrolytic enzymes that degraded the lipofuscin. The membrane stabilizing property of ARRE may be due to the presence of flavonoids, polyphenol and vitamin-C. Numerous reports have been appeared on the inhibition of release of acid hydrolases by flavonoids. (Havsteen, 1983). Several studies have also shown that flavonoids interact with cell membranes, improving their fluidity, thereby protecting them from lipid peroxidation (Saija et al., 1995).

Lysosome contains a battery of hydrolytic enzymes such as acid phosphatase, cathepsin, beta glucuronidase etc. If the lysosomal membrane is damaged or destabilized then these marker enzymes are released. Hence, the assay of these enzymes can be used as an index of lysosomal membrane damage. The release of lysosomal enzymes is related to necrosis of death of the cell or pathological stress full conditions. Lysosomal damage is well established as a biomarker of stress in a wide range of vertebrates and invertebrates. (Bindu et al., 2005). The most powerful hydrolytic enzymes are the cathepsins. Cathepsin D (cat D) is a lysosomal aspartic protease that is widely distributed in tissue cells and has been shown to be involved in aging and certain pathological condition. (Shibata et al., 2001). Acid phosphatase is localized in cellular lysosomes. Any type of stress burden results the significant fluctuations of cathepsin and acid phosphatase activity (Jozwik et al., 2002). The digestive enzymes of cellular compounds are confined to the lysosomes in the best interest of the cell. Escape of these enzymes into cytosol will destroy the functional macromolecules of the cell and result in many complications. The occurrence of several diseases (e.g. arthritis, aging, muscle diseases etc..) has been mainly attributed to the release of lysosomal enzymes (Holtzman, 1989).

In the present study, the significant elevation of acid phosphatase and Cathepsin D activity were observed in skeletal muscle of aged untreated rats as compared to young rats. Results indicate that aged untreated rats exposed to severe oxidative stress, has to lysosomal membrane destabilization. This may be due to aged rats increase accumulation of lipofuscin that causes lipid peroxidation of lysosomal membrane through Fentone like reaction and thereby release of acid phosphatase and Cathepsin D into cytosol. Administration of ARRE to aged rats restored the activity of acid phosphatase and Cathepsin D in skeletal muscle indicate that the stabilization of lysosomal membrane and prevent the membrane peroxidation. Present finding is in agreement with the Samarth et al. (2001) studies.

The results of the present study indicate that the oxidative damage ameliorating activity of ARRE in aged rats may probably relate to a counteraction of free radicals by its antioxidant nature of ARRE, to a strengthening of skeletal muscle lysosomal membrane by its membrane stabilizing action through its ability to decrease the levels of lipofuscin, lipid peroxidation and protein carbonyl which is used as a marker for oxidative damage. This ameliorating activity of ARRE is mainly attributed to the presence of enriched therapeutic phytochemical constituents, which act synergistically to alleviate the indices of oxidative stress associated with aging.

References


