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Research Paper

Modulation of oxidative/nitrosative stress and mitochondrial protective effect of *Semecarpus anacardium* in diabetic rats

Aseervatham Jaya, Palanivelu Shanthi and Panchanadham Sachdanandam

University of Madras, Chennai, India

Abstract

Objectives Oxidative and nitrosative stress play an important role in the complications of diabetes mellitus. Free radicals are produced when there is an electron leak in the mitochondria and a change in the mitochondrial membrane potential. The present study was undertaken to investigate the role of *Semecarpus anacardium* in protecting the mitochondria by modulating the production of reactive oxygen species and reactive nitrogen species in diabetic rats.

Methods Diabetes was induced using streptozotocin at a dose of 50 mg/kg body weight and, starting 3 days after the induction, *Semecarpus anacardium* nut milk extract was administered for 21 days. The same duration of study was used for control, diabetes-induced and drug control groups, together with a group treated with metformin. After the experimental period, the animals were sacrificed and the levels of superoxide, hydrogen peroxide, nitrate and nitrite were estimated. Changes in mitochondrial membrane potential, intracellular reactive oxygen species and intracellular calcium were also determined. Confocal laser microscopic images were taken for mitochondria isolated from the liver and kidneys.

Key findings The results of the study revealed that *Semecarpus anacardium* was able to decrease the production of reactive oxygen and nitrogen species, and reverse the changes in mitochondrial membrane potential and the influx of calcium into the mitochondria.

Conclusions The mitochondrial protective effect may be mediated by scavenging of free radicals and complexing of metal ions by virtue of the antioxidative effect of *Semecarpus anacardium*.

Keywords confocal microscope; intracelluar calcium; mitochondrial membrane potential; reactive nitrogen species; reactive oxygen species; *Semecarpus anacardium*

Introduction

Reactive oxygen species (ROS) play an important role in a wide variety of physiological and pathological processes leading to complication of various diseases.[1] In diabetes, persistent hyperglycaemia in concert with fatty acids may cause high production of free radicals, generated in direct auto-oxidation processes of glucose.[2] Hyperglycaemia also disrupts natural antioxidant defence mechanisms.[3] Such metabolic perturbations elicit alterations in tissues that undergo insulin-independent glucose uptake, thereby provoking early tissue damage in target organs such as the ocular lens, retina, peripheral nerve and renal glomerulus.[4]

Alterations in the redox state of the respiratory chain produce ROS.[5] Electrons derived from the oxidation of substrates are funnelled through the mitochondrial complexes. Transfer of electrons through the respiratory chain generates a proton gradient, which is used for the synthesis of ATP. When the electrochemical potential difference generated by the proton gradient is high, as in the case of hyperglycaemia, the life of superoxide-generating electron transport intermediates such as ubiquinone is prolonged. Since the respiratory chain complexes are governed by the transmembrane proton gradient (ΔpH) and the membrane potential ($\Delta\Psi$mt), sufficiently high ΔpH and $\Delta\Psi$mt inhibit the proton pumps.[6] Above a threshold value even a small increase in $\Delta\Psi$mt gives rise to large stimulation of superoxide production by mitochondria.[7]

Correspondence: Sachdanandam Panchanadham, Department of Medical Biochemistry, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai 600 113, India.
E-mail: psachdanandam2000@yahoo.co.in

Medicinal plants with antioxidant activities are used in the treatment of diabetes. Plant drugs, because of the synergetic activities of the compounds and low toxicity, are preferred for the treatment of various diseases. *Semecarpus anacardium* (SA), commonly used in traditional medicine, is found in the outer Himalayas and the hotter parts of India.[8] Phytochemical analysis has revealed the presence of biflavonoids, phenolic compounds,[9] bhilawanols, sterols and glycosides,[10] and anacardic acid[11] in *Semecarpus anacardium* nuts. Drug distillation of bhilawanol gave rise to catechol and a hydrocarbon.[12] Monophenolic compounds known as semecarpol ($C_{17}H_{28}O$) have also been isolated. Vitamins, linoleic, myristic, oleic, palmitic and stearic acids are present.[13] Vijayalakshmi *et al.* have reported the presence of carbohydrates, phenols and flavonoids in the milk extract.[14] It is non-toxic up to 2000 mg/kg body weight. TLC, HPLC and HPTLC analysis of the nut and milk extract confirmed the presence of the above compounds.[15–19] The extract also possesses antioxidant, anti-inflammatory, hypoglycaemic,[20] antiarthitic and anticancer actitivies.[21] Selvam and Jachak reported anti-inflammatory activity and cyclo-oxygenase inhibitory properties.[22] In order to highlight the mitochondrial protective effect of SA, the present study was undertaken to prove that attenuation of reactive oxygen and nitrogen species drastically reduces the oxidative stress seen in diabetes.

Materials and Methods

Semecarpus anacardium (SA) nut extract contains purified nuts of *Semecarpus anacardium*, cow's milk and ghee in the ratio indicated in the Formulary of Siddha Medicine.[23] Two hundred grams of the nut was boiled three times with 500 ml of milk. Then ghee was added to it and boiled until dehydrated. The decoction was stored.[24]

Animals

Male albino rats of the Wistar strain weighing 260 ± 10 g were used in this study. The animals were housed in polypropylene cages under a controlled environment with 12 h light/dark cycles and a temperature between 27 and 37ºC, and were given a commercial diet with water *ad libitum*. All experiments involving animals were conducted according to National Institutes of Health guidelines, after obtaining approval from the Madras University Ethical Committee.

Experimental design

Male albino Wistar rats weighing 250–270 g were divided into five groups of six animals each. The details of the five groups are shown in Table 1.

Biochemical analysis

After the experimental period, the animals were killed by cervical decapitation. The liver and kidney were excised immediately and immersed in ice-cold physiological saline. Ten per cent homogenate was prepared with fresh tissue in 0.01 M Tsris-HCl buffer (pH 7.4) and used for the following assays.

Table 1 Experimental groups

Group	Description	Details
I	Control	Normal healthy controls received olive oil (0.5 ml) orally by gastric intubation for 21 days daily
II	Diabetes induced	50 mg/kg body weight streptozotocin dissolved in 0.5 ml of 0.1 M citrate buffer, pH 4.5
III	SA treated	Three days after the induction of diabetes, SA (300 mg/kg body weight dissolved in 0.5 ml olive oil) was administered daily by gastric intubation for 21 days
IV	Metformin treated	Three days after the induction of diabetes, metformin (500 mg/kg body weight dissolved in 0.5 ml physiological saline) was administered daily by gastric intubation for 21 days
V	Drug control	Animals received SA daily at a dose of 300 mg/kg body weight in olive oil (0.5 ml) orally by gastric intubation for 21 days

SA, *Semecarpus anacardium*.

Estimation of hydroxyl radicals

Hydroxyl radicals were estimated by the method of Gutteridge.[25] Samples containing 0.1 ml of the tissue homogenates and 2 ml of deoxyribose were added and incubated at 37°C for 1 h. Then 0.5 ml of thiobarbituric acid solution and 0.5 ml of trichloroacetic acid solution were mixed and heated for 15 min at 100°C. The mixture was cooled and the absorbance was read spectrophotometrically at 530 nm.

Estimation of superoxide radicals

Superoxides were estimated by the method of Nishikimi *et al.*[26] To 0.1 ml of the tissue homogenates were added 0.2 ml of 4 mM nitro-blue tetra-zolium (NBT) and 150 mM ascorbic acid and the mixture was incubated at 37°C for 10 min. Two millilitres of 0.1 M sodium hydroxide containing 24 mM sodium bicarbonate were added and the precipitate obtained after centrifugation was dissolved in 5 ml of 1,4-dioxane. The absorbance was read spectrophotometrically at 520 nm with a blank of 1,4-dioxane.

Estimation of hydrogen peroxide

Hydrogen peroxide (H_2O_2) was estimated by the method of Wolff.[27] To 0.1 ml of the tissue homogenates, 0.05 ml of FOX reagent was added and the mixture incubated for 30 min at 37°C. After 30 min of incubation, the solutions were read spectrometrically at 560 nm.

Estimation of NO_x metabolites

Nitrate and nitrite in serum and urine were determined by the method of Kerber *et al.*[28] To 200 μl of urine sample, 400 μl of sulphosalicylic acid was added to precipitate the protein. The suspension was centrifuged at 3000 rpm for 5 min. Two 200-μl aliquots were pipetted out into different tubes. To one set of tubes, 300 μg of cadmium pieces were added and incubated with vortexing for 20 min. To all the tubes, 150 μl of Griess reagent was added and the colour developed was read at 540 nm by spectrophotometry.

Estimation of reactive oxygen species

Mitochondria were isolated by the method of Johnson and Lardy[29] and the purity was assessed by the assay of succinate dehydrogenase.

Generation of ROS was evaluated by using dichlorofluorescein diacetate ($DCFH_2$-DA) as a probe, according to the method of LeBel *et al.* as modified by Kim *et al.*[30] DCF formation was measured using excitation at 488 nm and emission at 525 nm for 30 min, using a Shimadzu-1601 fluorescence spectrometer.

Confocal laser scanning microscopic images were obtained using a confocal laser scanning microscope (Molecular Devices, Sunnyvale, CA USA) after loading the isolated mitochondria with $DCFH_2$-DA. The images were obtained after excitation at 488 nm and emission at 525 nm.

Measurement of mitochondrial membrane potential

The measurement of changes of mitochondrial membrane potential (MMP) was conducted according to the method of Palmeira *et al.*,[31] using rhodamine-123 (Rh-123) as the fluorescent probe, read in a spectrofluorimeter at excitation and emission wavelengths of 490 and 515 nm.

Estimation of intracellular calcium

Intracellular Ca^{2+} ion concentration was determined by the dual wavelength method described by Grynkiewicz *et al.*[32] using a calcium-sensitive fluorescent probe, Fura-2AM. Concentration of free Ca^{2+} ion was determined using the dual wavelength method with excitation at 340 and 380 nm and emission at 510 nm wavelength.

Statistical analysis

The values are expressed as mean ± SD for six rats in each group. Statistically significant differences between the groups were calculated using one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls for multiple comparisons using the Statistical Package for Social Sciences (SPSS) computer package. Values of $P < 0.05$ were considered to be significant.

Results

Effect of *Semecarpus anacardium* on the production of reactive oxygen and nitrogen species and hydroxyl radical levels

The effects of SA on the production of superoxide radical and hydrogen peroxide and hydroxyl radicals in the liver and kidney are shown in Table 2. Increased levels of superoxide and hydrogen peroxide ($P < 0.05$) were seen in the untreated diabetic Group II animals which significantly decreased ($P < 0.05$) on administration of SA (Group III). When Group III and Group IV animals were compared, SA was more effective in preventing the production of superoxide radicals and hydrogen peroxide than metformin. Non-significant values were obtained when Group I and Group V animals were compared.

Increased production of hydroxyl radicals was seen in Group II animals and this decreased in the drug-treated groups ($P< 0.05$) animals. The levels of NO_x metabolites in serum and urine are also given in Table 2. The levels of these metabolites in serum and urine of diabetic animals increased 1.36-fold and 1.4-fold in Group II animals. This was significantly decreased ($P< 0.05$) on drug administration.

Effect on the total reactive oxygen species production

Figure 1 shows the total ROS produced, as detected by $DCFH_2$-DA. This compound diffuses through the cell membrane and is enzymatically hydrolysed by intracellular esterase to dichlorofluorescein (DCF), which is highly fluorescent in the presence of ROS. In diabetic animals, due to the shift towards the pro-oxidant state, ROS are produced in increased amounts, and because of this higher levels of DCF fluorescence are seen in Group II animals. The decrease in the DCF fluorescence in drug-treated animals shows the ROS-scavenging effect of the drug. The fluorescence in metformin-treated animals was significantly ($P < 0.05$) increased when compared to SA treated animals.

Table 2 Effect of *Semecarpus anacardium* on the levels of superoxide, hydrogen peroxide, hydroxyl radicals and NO_x

	Group I Control	Group II STZ	Group III STZ + SA	Group IV STZ + metformin	Group V SA
Superoxide radical					
Liver	22.64 ± 1.9	38.94 ± 4.26^{a*}	25.51 ± 2.93^{b*}	33.14 ± 3.13^{b*c*}	24.96 ± 1.94^{dNS}
Kidney	15.46 ± 1.72	29.18 ± 3.0^{a*}	18.35 ± 2.03^{b*}	24.62 ± 2.31^{b*c*}	15.13 ± 1.69^{dNS}
Hydrogen peroxide					
Liver	236.58 ± 27	301.43 ± 30^{a*}	257.72 ± 28^{b*}	284.13 ± 26^{b*c*}	235.86 ± 22^{dNS}
Kidney	196.28 ± 21	262.35 ± 23.1^{a*}	203.46 ± 22.8^{b*}	229.53 ± 24^{b*c*}	195.11 ± 17.8^{dNS}
Hydroxyl radical					
Liver	7.4 ± 0.71	13.54 ± 1.1^{a*}	7.9 ± 0.7^{b*}	10.8 ± 1.03^{b*c*}	7.36 ± 0.78^{dNS}
Kidney	5.9 ± 0.5	10.8 ± 1.0^{a*}	6.3 ± 0.6^{b*}	8.4 ± 0.8^{b*c*}	5.8 ± 0.5^{dNS}
NO_x					
Serum	8.32 ± 0.91	15.38 ± 1.5^{a*}	9.84 ± 1.0^{b*}	12.2 ± 1.03^{b*c*}	8.16 ± 0.78^{dNS}
Urine	2.38 ± 0.30	4.52 ± 0.40^{a*}	2.48 ± 0.22^{b*}	3.11 ± 0.38^{b*c*}	2.26 ± 0.24^{dNS}

Units: superoxide radical, μmol of NBT/min/mg protein; hydrogen peroxide, μmol/min/mg protein; hydroxyl radical, nmol/min/mg protein; NO_x, μmol/dl in serum, μmol/24 h in urine. SA, *Semecarpus anacardium*; STZ, streptozotocin. [a]Group II vs Group I, [b]Group III and IV vs Group II, [c]Group IV vs Group III, [d]Group V vs Group I. *Statistical significance at $P < 0.05$, NS, non-significant.

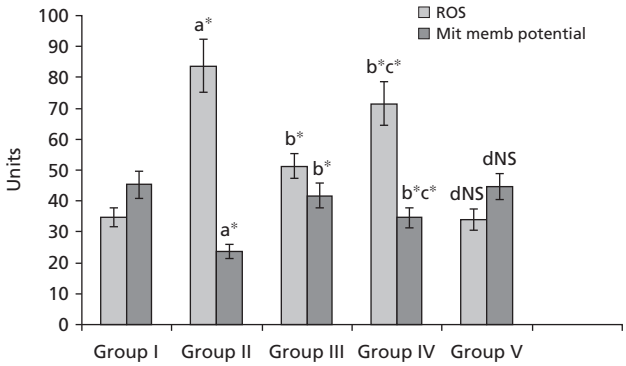

Figure 1 Effect of *Semecarpus anacardium* on the production of total reactive oxygen species and mitochondrial membrane potential in liver mitochondria of control and experimental animals. Units: reactive oxygen species, dichlorofluorescein fluorescence/min/mg protein; mitochondrial membrane potential, rhodamine-123 fluorescence/min/mg protein. Values are expressed as mean ± SD for six animals. [a]Group II vs Group I, [b]Group III and IV vs Group II, [c]Group IV vs Group III, [d]Group V vs Group I. [*]Statistical significance at $P < 0.05$, NS, non-significant, ROS, reactive oxygen species. Mit memb potential, mitochondrial membrane potential

Figures 2a and 2b show the confocal laser microscopic images of isolated liver and kidney mitochondria of normal and experimental animals after loading the cells with $DCFH_2$-DA. In the absence of ROS, normal mitochondria give a green colour (Group I), whereas in the presence of ROS they emit an orange-red colour (Group II). The free radical scavenging effect of SA is evident from the overlay of both the emission and excitation images (Group III). Administration of SA did not produce any toxic side effects to the animals, as is evident from the images obtained for Group V animals.

Effect on mitochondrial membarane potential

The effect of SA on the MMP in the liver of experimental animals is shown in Figure 1. In diabetic animals MMP is altered due to increased production of free radicals. Drug administration repolarised the mitochondrial membrane and reversed the MMP back to near-normal levels.

Effect on intracellular Ca^{2+}

The effect of SA on intracellular Ca^{2+} in liver mitochondria for control and experimental animals is shown in Figure 3. The increase in the intracellular Ca^{2+} content seen in Group II animals was significantly ($P < 0.05$) reversed on drug administration (Group III and Group IV). SA was more efficient in preventing the entry of Ca^{2+} into the mitochondria than metformin. Non-significant values were obtained for all the parameters when Group I and Group V animals were compared, proving the non-toxic nature of the drug.

Discussion

Reactive oxygen species comprise a wide variety of molecules, including singlet oxygen, superoxide anion, hydrogen peroxide, lipid peroxides, the thiylperoxyl radical, the ferryl radical and the hydroxyl radical. Over-production of superoxide during diabetes activates protein kinase C, causes accumulation of advanced glycation end-products and increases the flux of glucose through the aldose reductase pathway.[33] The oxidant H_2O_2 is a byproduct of normal cellular respiration and is also formed from the superoxide anion by the action of superoxide dismutase. H_2O_2 has been reported to damage pancreatic β cells[34] and inhibit insulin signalling. Hydroxyl radicals (·OH) are the most fearsomely reactive of the group of ROS and reactive nitrogen species

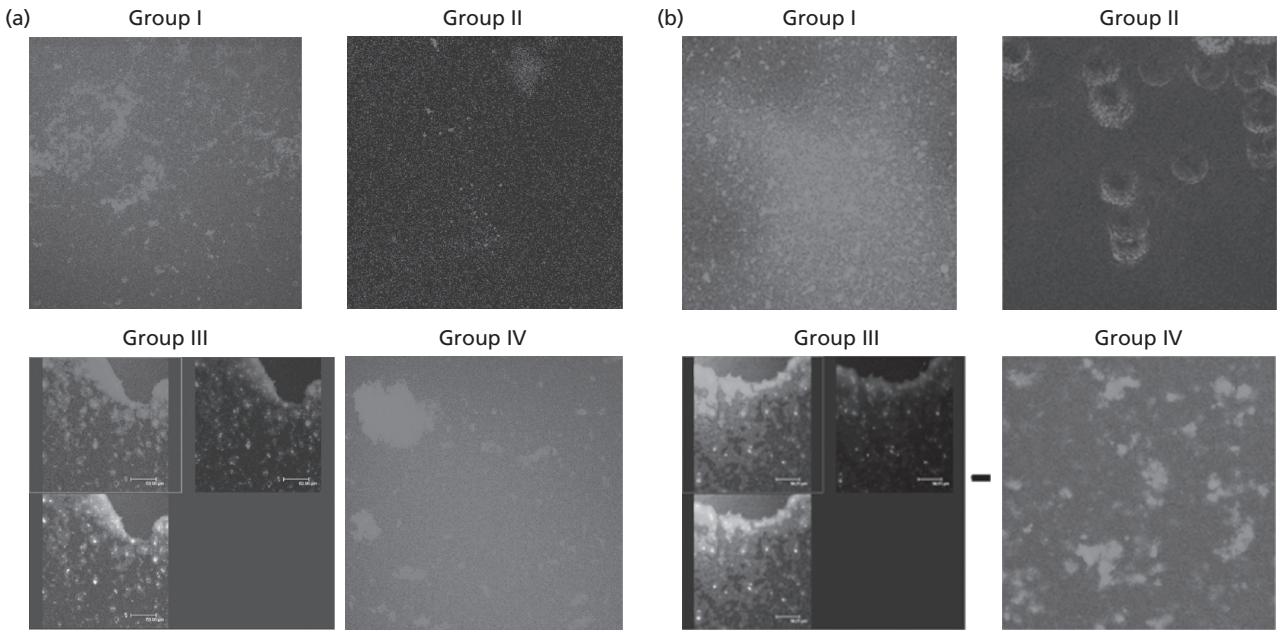

Figure 2 Confocal images of isolated mitochondria in normal and *Semecarpus anacardium* treated animals. (a), liver mitochondria; (b), kidney mitochondria

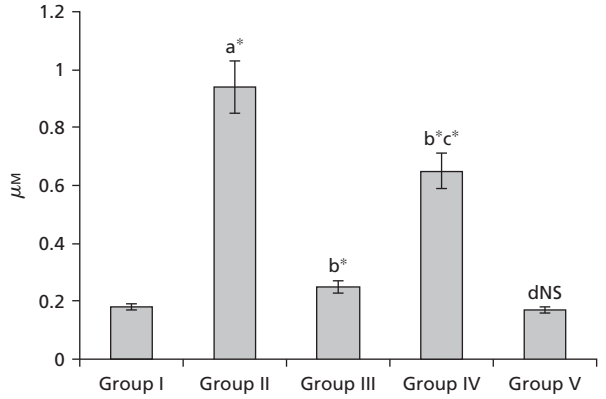

Figure 3 Effect of *Semecarpus anacardium* on the intracellular calcium levels in the liver mitochondria of control and experimental animals. Values are expressed as mean ± SD for six animals. [a]Group II vs Group I, [b]Group III and IV vs Group II, [c]Group IV vs Group III, [d]Group V vs Group I. *Statistical significance at $P < 0.05$, NS, non-significant

(RNS). Hydroxyl radicals produced by streptozotocin (STZ) are the main mediators of cell damage.[35] The decrease in hydroxyl levels in the drug-treated groups may be due to the presence of flavonoids in SA. Flavonoids, because of their antioxidant activity and transitional metal chelation, interfere with the conversion of hydrogen peroxide to hydroxyl radicals.[36] The flavonoids present in SA scavenge superoxide and inhibit xanthine oxidase as a result of the presence of hydroxyl groups on the flavone skeleton.[37]

Nitric oxide (NO) acts as a pro-oxidant at high concentrations and is a precursor of oxidizing and nitrating species. Due to its short half-life, NO is unstable and only can be estimated from its stable breakdown products, nitrite and nitrate (collectively NO_x). In diabetes, NO metabolites can be detected within 5 days, which confirms that augmented synthesis is both renal and systemic.[38] Under elevated superoxide levels in diabetes, more NO can react with superoxide, forming peroxynitrite, which exerts further deleterious effects. In addition, peroxynitrite is a strong oxidant and is more stable than NO or superoxide, which leads to diabetic complications. Nitric oxide has adverse effects on the β cell, where it inhibits insulin secretion, disrupts electron transport, promotes apoptosis and increases lipid peroxidation.[39] Nitric oxide molecules are directly scavenged by flavonoids.[40] Due to the presence of conjugated ring structures and hydroxyl groups, most phenolic compounds have the potential to function as antioxidants by scavenging the superoxide anion, singlet oxygen and lipid peroxy radicals, thereby stabilising the free radicals involved in the oxidative process through hydrogenation or complexing with oxidising species. SA has also been reported to scavenge free radicals and nitric acid.[41] Flavonoids present in SA may be responsible for the scavenging of ROS and RNS. ROS are generally considered to be cytotoxic, and inhibit insulin gene transcription.[42] The lowered levels of ROS seen in SA-treated groups may be attributed to the presence of polyphenols.[43]

Increased electron generation by electron donors (NADH and $FADH_2$) during hyperglycaemia increases the flux of electrons though the electron transport chain (ETC), which in turn increases the ATP/ADP ratio and leads to hyperpolarisation of the MMP. The high electrochemical potential difference generated by the proton gradient leads to partial inhibition of the electron transport in complex III, resulting in an accumulation of electrons to coenzyme Q. In turn this drives partial reduction of O_2, to generate the free radical anion, superoxide.[44] This accelerated reduction of co-enzyme Q and generation of ROS is believed to be the fundamental source of mitochondrial dysfunction. The MMP of diabetic animals decreased due to increased ROS formation. Administration of SA to diabetic animals may have caused uncoupling of oxidative phosphorylation, like metformin, due to the presence of anacardic acid in the drug, which has an uncoupling effect.[45]

In the presence of Ca^{2+}, oxidative alterations of protein thiols in the mitochondrial inner membrane promote non-specific inner membrane permeabilisation, referred to as mitochondrial permeability transition (MPT). MPT is characterised by a progressive permeabilisation of the inner mitochondrial membrane, which gradually becomes permeable to protons, ions and even small proteins, leading to complete collapse of the membrane potential. An increase in $[Ca^{2+}]i$, could stimulate either ADP phosphorylation or gluconeogenesis or both. Lack of oxygen could lower the membrane potential and Ca^{2+} uptake could raise the pH of the mitochondrial matrix. Higher $[Ca^{2+}]i$ decreases the membrane potential, causes slight alkalinisation of the matrix and increases the probability of opening the membrane permeability pore, leading to cell death. Pore opening is inhibited by antioxidants[46] through chelation of Ca^{2+}.[47] The poly phenols present in SA, due to their antioxidant and metal chelating properties, may be responsible for decreasing intracellular Ca^{2+}.

Flavonols inhibit lipid peroxidation by chelating metal ions, which initiates a chain reaction. Flavonoids can chelate metal ions due to the presence of hydroxyl groups attached to the ring structure. Three important structural features are essential for antioxidant activity: (1) the 3,4-dihydroxy catechol configuration on the B ring, which gives most stable phenoxy radicals after the donation of hydrogen atom, (2) the 2,3-double bond in conjunction with the 4-carbonyl group on the C ring, which allows a delocation of an electron from phenoxy radicals on the B ring to the C ring, and (3) the 3-hydroxy group in combination with the 2,3-double bond, which increases the resonance stabilisation for electron delocalisation across the molecule.[48]

The antioxidant activity of SA is due to the presence of biflavonoids such as semecarpuflavonone, jeediflavonone, galluflavonone, nalluflavonone, semecarpetin and anacardiflavonone,[49] biflavonoids A, 3,8-binaringenin and 3,8-biliquiritigenin and biflavonones such as A1, A2 and tertrahydrobustaflavone. Flavonones present in SA contain a catechol moiety in the B ring, which could be responsible for its radical-scavenging activity. In galluflavonone, a catechol moiety is present in the secondary flavonone ring. The 4-oxo group present in all the biflavonones in SA contributes to the free radical quenching activity. The 5-OH

and 7-OH groups present in jeediflavonone also confer scavenging potential. Semecarpuflavonone, semecarpetin and galluflavonone have a 7-OH group that may contribute to the free radical chelating activity of SA.[50] The hypoglycaemic effect of the components is responsible for the reduction in blood glucose levels.[51] These components in SA may have a synergetic effect against the deleterious effects of the free radicals, either by scavenging them or by preventing the initiation or propagation of a chain reaction.

Conclusions

Oxidative stress, the result of imbalance between the production of free radicals and its destruction, plays an important role in complications of diabetes. Drugs that have the ability to modulate membrane potential or transmembrane gradient can decrease mitochondrial ROS production. Administration of SA helps in scavenging free radicals and alleviates the complications of diabetes. From the above results it can be inferred that SA, due to its antioxidant property and hypoglycaemic effect, is able to scavenge the ROS/RNS produced and protect mitochondria against oxidative stress.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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