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Antioxidant Activity, DNA and Cellular Protective Effect of Honey from Srilanka

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ABSTRACT

Honey is a natural product produced by honey bees. Its value is increasing in food industry because of its nutritional and medicinal properties. Besides this, it also contains certain volatile chemicals, phenolic acids, flavonoids and carotenoid like substances which play a major role in antioxidant properties. The composition of honey depends on the plant species visited by the honeybees. The present study was carried out to evaluate the antioxidant activity of Srilankan honey and its role in protecting DNA and erythrocytes from free radical induced oxidative damage. Total phenolic, flavonoid and proanthocyanidin content present in honey was measured, in addition antioxidant assays like 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, reducing power, β-carotene linoleic acid assay, α-glucosidase enzyme inhibition assay, superoxide anion radical scavenging activity and ability of chelating ferrous ions were also studied.

Key words: Honey, erythrocytes, flavonoid, DNA, proanthocyanidin

INTRODUCTION

Many scientists have studied the phenolic and flavonoid content of honey to determine the correlation with floral origin (Ferreres *et al*., 1991). These compounds have major role against many human chronic disease such as neurodegenerative disorders, cancer, liver cirrhosis and cardio vascular disease (Aruoma, 1998). All these diseases are seen associated with oxidative stress. Antioxidants such as phenolics and flavonoids are very important to reduce the stress as they have the ability to scavenge Reactive Oxygen Species (ROS) free radical activities in the living cells. Many methods have been used to dictate the antioxidant activity in honey viz. determination of active oxygen species such as the superoxide anion, peroxyl and hydroxyl radicals, DPPH radical scavenging ability (Gheldof *et al*., 2002).

Erythrocytes are also found continuously exposed to both endogenous and exogenous source of ROS. They are highly prone to oxidative damage due to the presence of high concentration of hemoglobin and oxygen, potent promoters of oxidative stress (Bors *et al*., 1990). Due to these activities erythrocytes are used as a cellular model to study about biomembrane integrity in relation to oxidative damage. Strong antioxidants reduce the cellular damage caused by free radicals. Free radicals are molecules that are highly reactive due to the presence of unpaired electron on the molecule. Majority of the antioxidant available such as phenolic acid and flavonoids, show protection against ROS by neutralizing these highly reactive radicals.

Honey is not a herbal medicine, it is a plant product. Honey has been used from ancient times for various medical remedies. It is believed to be one of the most concentrated forms of sugar

available worldwide. Honey has wild therapeutic value due to which it is used in many treatments. Therapeutic value has been partly assigned to its antioxidant activity (Aljadi and Kamaruddin, 2004). Honey varies from place to place due to content and composition and with different floral sources as well as climatic and environmental conditions (Kucuk *et al*., 2007). In the present study, the protective activity of honey against DNA damage, erythrocyte hemolysis and antioxidant activity assays were studied.

MATERIALS AND METHODS

Chemicals: The λ DNA was purchased from Bangalore Genei, India. α-glucosidase and p-nitro phenyl-α-D-glucopyranoside were bought from Sigma Aldrich, USA. Butylated hydroxyanisole (BHA), Nitro Blue Tetrazolium (NBT), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, Phenazine methosulphate (PMS), trichloro acetic acid, linoleic acid, Tween 40, hydrogen peroxide and ethidium bromide were obtained from SRL, India. All other chemicals were of analytical grade. Honey sample was obtained from Trincomalee district, Srilanka. It was stored at 4°C in airtight containers until further use.

Determination of total phenolic, flavonoid and proanthocyanidin content: Phenolic concentration in the honey sample was estimated using modified Folin-Ciocalteu method (Singleton *et al*., 1999). Briefly, 1 g of honey was mixed with 5 mL of distilled water. About 0.5 mL (0.2 g mL^{-1}) of honey sample was mixed with 0.5 mL of 0.2 N Folin-Ciocalteu reagents. After 2 min, 0.5 mL (100 mg mL^{-1}) of sodium carbonate was added and was adjusted to 2 mL by adding distilled water. The contents were mixed well and allowed to stand for 2 h. Absorbance of the sample was measured at 765 nm. Gallic acid was used to calculate the standard curve (20, 40, 60, 80 and 100 mg L^{-1}) and the results obtained were expressed as milligram of gallic acid equivalents (GAEs)/100 g of honey.

The flavonoid content in the honey sample was assessed using the colorimetric assay described by Zhishen *et al*. (1999). One gram of honey was mixed with 5 mL of distilled water. One milliliter (0.2 g mL^{-1}) of this solution was mixed with 4 mL of distilled water. To this 0.3 mL of sodium nitrate and 0.3 mL of aluminium chloride was added and mixed. After 6 min, 2 mL of sodium hydroxide was added. The volume was made up to 10 mL by adding distilled water. The mixture was mixed well and the absorbance was read at 510 nm. Standard curve was plotted using Quercetin and the results obtained were expressed as milligram of Quercetin Equivalent (QE)/100 g of honey.

Proanthocyanidin content present in the sample was calculated using vanillin assay described by Sun *et al*. (1998). The 0.5 mL (0.2 g mL^{-1}) of honey solution was mixed with 3 mL of vanillin methanol solution to which 1.5 mL of hydrochloric acid was added and left at room temperature for 15 min. The absorbance was then measured at 500 nm against reagent blank. The result was expressed as milligram of Catechin Equivalent (CE)/100 g of honey.

DPPH free radical scavenging assay: The 2, 2-Diphenyl 1-picrylhydrazyl (DPPH) radical scavenging property of honey sample was determined using the method of Mensor *et al*. (2001). The 1 mL of 0.3 mM methanolic solution of DPPH was added to 2 mL of honey at varying concentrations ranging from 35, 70, 105, 140 and 175 mg mL^{-1}. The reaction mixture was kept in dark for 30 min after which the absorbance was measured at 518 nm. The control sample was prepared replacing honey with methanol. BHA was used as positive control. The percentage of radical scavenging activity was measured using the equation:

$$\text{RSA (\%)} = \frac{\text{A control-A sample}}{\text{A control}} \times 100$$

Superoxide anion radical scavenging activity: Honey sample was assessed for its superoxide radical scavenging activity using the method of Nishikimi *et al*. (1972) with slight modification. The reaction mixture contained 1 mL NBT (312 μM), 1 mL NADH (936 μM) along with different concentrations of honey (100, 200, 300, 400 and 500 mg mL^{-1}) and was made up to a total volume of 3 mL using phosphate buffer (50 mM pH 7.4). The reaction was initiated by adding PMS (120 μM) to the mixture and was incubated at room temperature for 5 min. Following this, absorbance was measured at 560 nm. Control was made without adding the test sample but with all other reagents. Gallic acid was used as standard reference. The percent of scavenging activity was calculated using the equation:

$$\text{Inhibition (\%)} = \frac{\text{A control-A sample}}{\text{A control}} \times 100$$

Ability of chelating ferrous ions: The activity of honey in ferrous ion chelation was studied by ferrous ion-ferrozine complex method (Meyer and Isaksen, 1995). The 0.8 mL of different concentrations of honey (20, 40, 60, 80 and 100 mg mL^{-1}) was mixed with 50 μL of 2 mM $FeCl_2$ and 200 μL of 5 mM ferrozine. The reaction mixture was incubated at 25°C for 10 min. Absorbance of the mixture was measured at 562 nm. BHA was used as standard. Metal chelating effect of honey was estimated using the equation:

$$\text{Activity (\%)} = \frac{\text{A control-A sample}}{\text{A control}} \times 100$$

Reducing power: The reducing property of the sample was estimated by the procedure described by Oyaizu (1986). One milliliter of honey at varying concentrations (50-250 mg mL^{-1}) was mixed with 2 mL of 0.2 M (pH 6.6) phosphate buffer and 2 mL of potassium ferricyanide (10 mg mL^{-1}). The contents were mixed well and then incubated at 50°C for 20 min. After incubation, 2 mL of trichloroacetic acid (100 mg mL^{-1}) was added to stop the reaction. After centrifugation at 3000 rpm for 10 min, 2 mL of the supernatant was diluted with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride was added. The sample was left for 10 min after which absorbance was read at 700 nm. The BHA was used as positive control.

β-carotene linoleic acid assay: The β-carotene linoleic acid assay was performed according to the method described by Miller (1971). The β-carotene solution was made by dissolving 2 mg of β-carotene in 10 mL of $CHCl_3$. The $CHCl_3$ was removed under vacuum following which 2 mL of the solution was pipetted out into 100 mL round bottom flask. To this, linoleic acid (40 mg), Tween 40 (400 mg) was added and made upto 100 mL with distilled water. The contents were mixed well and shaken vigorously. About 4.8 mL of this emulsion was added to each test tube containing 200 μL of different concentration of honey (50-250 mg mL^{-1}). Absorbance was measured at 470 nm immediately after adding emulsion to the each tube (t = 0). The tubes were then incubated in water bath at 50°C for 2 h and absorbance was noted again (t = 120). A solution with 100 μL of solvent and 4.8 mL of emulsion was used as negative control. BHA was used for comparison. The percentage of inhibition of β-carotene oxidation was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{A_{S(120)} - A_{C(120)}}{A_{C(0)} - A_{C(120)}} \times 100$$

where, $A_{S(120)}$ is absorbance of the sample at t = 120 min, $A_{C(0)}$ is the absorbance of control at t = 0 min and $A_{C(120)}$ is the absorbance of control at t = 120 min.

Prevention of λ DNA damage by honey: The prevention of λ DNA damage by honey was performed according to the method of Ghanta *et al*. (2007). The reaction mixture includes: The λ DNA (0.5 μg), with (10 mg) and without honey were mixed with 25 mM H_2O_2, 1 mM $FeSO_4$. $7H_2O$ in tris buffer (10 mM, pH 7.4) making the reaction volume to 20 μL. The samples were incubated for 2 h at 37°C. The λ DNA mixed with only honey was also tested. Samples were run on 1% agarose gel prepared in Tris acetate EDTA buffer (pH 8.5) at 50 V at room temperature and was then visualized in gel documentation.

Inhibition of rat erythrocyte hemolysis: All the animal experiments were performed with the approval from institutional animal ethical committee. Male wistar rats of body weight ranging from 200-250 g were chosen for the study. The animals were provided with standard diet and conditions. Animals were sacrificed under anesthesia and the blood was collected by heart puncture in heparinized tubes. Erythrocyte isolation was done according to the method of Yuan *et al*. (2005) and Yang *et al*. (2006). Blood samples collected in heparin tubes were centrifuged at 1500 g for 5 min at 4°C. Erythrocytes were separated out from buffy coat and plasma was washed thrice with 10 volumes of 20 mM PBS. Each time it was centrifuged at 1500 g for 5 min and supernatant and buffy coat formed was carefully removed. Erythrocytes thus obtained were stored at 4°C and was used within 6 h for further studies.

In vitro rat erythrocyte hemolysis inhibition assay was performed according to the method described by Tedesco *et al*. (2000). The H_2O_2 (100 μL of 200 μM in PBS pH 7.4) was used as free radical initiator. The 200 μL of 10% erythrocyte suspension in PBS was mixed with 50 μL of honey made at different concentrations (5-25 mg mL^{-1} honey in PBS pH 7.4). The mixture was then incubated at 37°C for 30 min, after which it was centrifuged at 2000 g for 10 min. The 200 μL of resulting supernatant was added to 800 μL of PBS and absorbance was measured at 410 nm. The inhibitory effect of honey was compared with a standard antioxidant BHA. Similarly, complete hemolysis was studied by treating erythrocytes with hydrogen peroxide and without sample.

α-glucosidase enzyme inhibition: The reaction mixture includes 50 μL of p-nitro phenyl-α-D-glucopyranoside and different concentrations of honey (2, 4, 6, 8 and 10 mg mL in phosphate buffer). The initiation of the reaction was by adding 100 μL of α-glucosidase enzyme and the volume was made up to 2 mL by adding 0.1 M phosphate buffer. The absorbance was read at 405 nm and the enzyme reaction was compared without adding the sample.

Statistical analysis: The experiments were conducted in triplicate and the data obtained was represented as Mean±SD. Duncan's new multiple range tests was used to find the difference of means.

RESULTS

Total phenolic, flavanoid and proanthocyanidin content: Polyphenols are a major group that influences the functional properties of honey, specifically antioxidant properties. The total phenolic

content present in Srilankan honey was found to be 129 mg GAE/100 g of honey. The flavonoid and proanthocyanidin content was calculated to be 98 mg QE/100 g of honey and 67 mg CE/100 g of honey, respectively.

DPPH radical scavenging assay: The DPPH is a stable nitrogen based free radical which is extensively studied to evaluate the radical scavenging activity of various compounds. The DPPH assay showed a color reduction in the presence of honey, which indicates the antioxidant molecules present in the sample was capable enough in quenching DPPH free radicals converting them to colorless (2, 2-diphenyl 1-hydrazine) product resulting in a decrease in absorbance. Presence of high DPPH scavenging activity corresponds to high levels of antioxidant potential of the sample. Srilankan honey at varying concentrations (35-175 mg mL^{-1}) was assessed, of which it showed maximum scavenging activity of 69.5% at a concentration of 175 mg mL^{-1}. Scavenging activity was found increasing as concentration increases (Fig. 1).

Superoxide anion radical scavenging assay: Superoxide radical is highly toxic, which can indirectly initiate lipid peroxidation by forming highly reactive species like hydroxyl radical. Superoxide radicals were generated in PMS-NADH system by oxidation of NADH and it was assayed by assessing NBT reduction forming blue formazan (Kanatt *et al*., 2007). Honey exhibited a dose response inhibition of superoxide anion radicals. The percentage inhibition of superoxide generation was found to be increasing with increase in concentration and maximum activity (60%) was seen at a concentration of 500 mg mL^{-1} (Fig. 2).

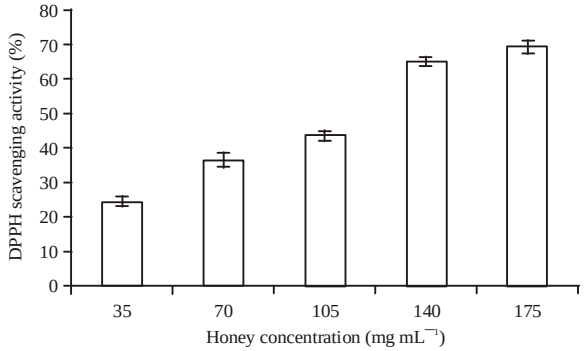

Fig. 1: DPPH radical scavenging effect of Srilankan honey sample

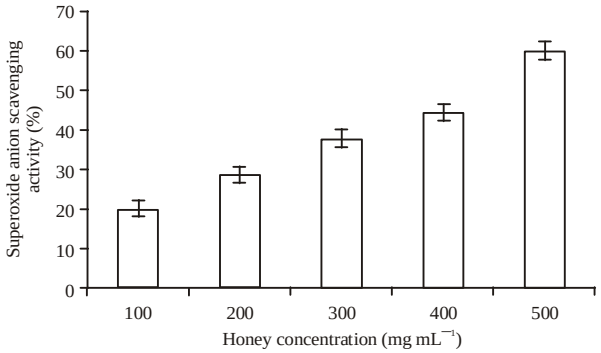

Fig. 2: Superoxide anion radical scavenging activity of Srilankan honey sample

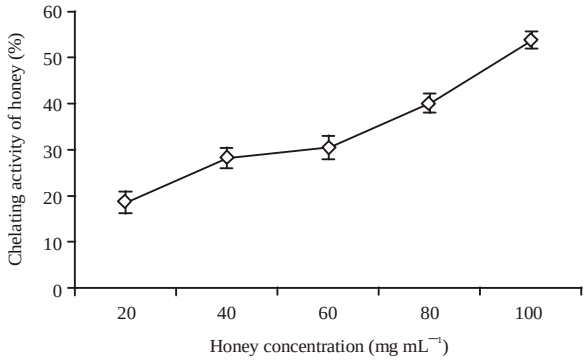

Fig. 3: Ability of chelating ferrous ions by Srilankan honey

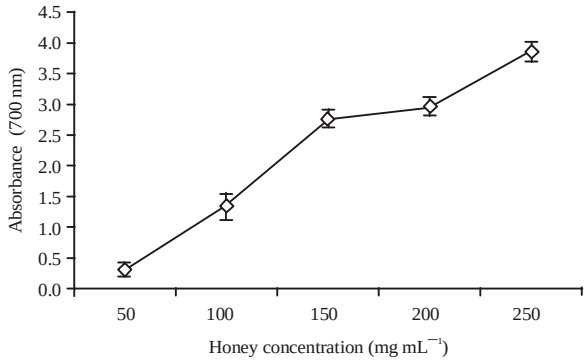

Fig. 4: Reducing power of Srilankan honey

Metal chelating property: Metal chelating assay measures the ability of antioxidants to compete with ferrozine in chelating ferrous ions and preventing formation of complex (Elmastas *et al*., 2006). Antioxidants form insoluble metal complex with ferrous ion and thereby, prevents the interaction between metal and lipid (Hsu *et al*., 2003). Chelating activity of the sample was assessed by measuring the rate of colour reduction. Chelating capacity increased with increase in concentration. At highest calculated concentration, honey sample exhibited 54.2% activity indicating the presence of ample amount of antioxidants in the sample (Fig. 3).

Reducing power: The reducing power of honey is due to the presence of reductants in the solution which exhibits antioxidant activity through breaking the free radical chain by donating a hydrogen atom (Xing *et al*., 2005). The Fe^{3+}/ferricyanide complex get reduced to ferrous form signifying the antioxidant capacity of the sample. The reducing power of honey increases as concentration increases (Fig. 4).

β-carotene bleaching assay: The β-carotene has got antioxidant properties that helps in neutralizing free radicals, reactive oxygen molecules damaging lipids in cell membranes. The β-carotene bleaching assay is basically used to measure the activity of a compound to inhibit the oxidation of β-carotene. The free radical formed by linoleic acid oxidizes β-carotene resulting in its degradation. On addition of honey, oxidation of β-carotene was minimized due to the action of antioxidants present in the sample. The percentage of oxidation inhibition was found increasing in dose dependent manner (Fig. 5), 63.9% at a maximum concentration of 250 mg mL^{-1}.

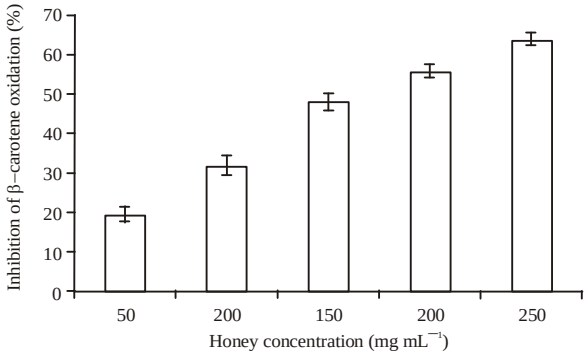

Fig. 5: Oxidation inhibition of β-carotene by Srilankan honey

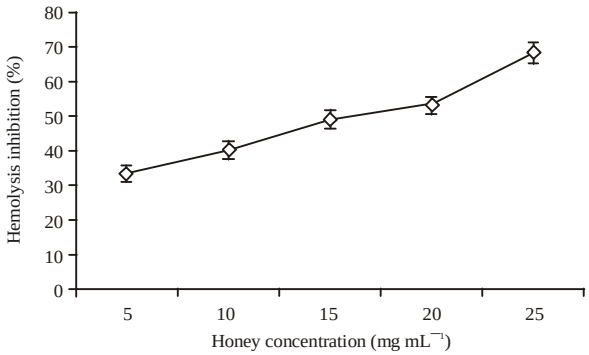

Fig. 6: Erythrocyte hemolysis inhibition by using Srilankan honey

Rat erythrocyte hemolysis inhibition: The H_2O_2 results in damaging erythrocytes leading to the release of hemoglobin into the medium and the color produced was measured to study the extend of erythrocyte damage. Protective action of honey was compared with a known antioxidant, BHA. The inhibitory action of honey against erythrocyte hemolysis was studied in a dose dependent manner. The IC_{50} value for honey was found to be 15.5 mg mL^{-1} (Fig. 6). The results proved the inhibitory action of honey towards hemolysis of rat erythrocyte.

Inhibition of α-glucosidase: The α glucosidase is seen associated with type 2 diabetes as it alters normal glucose metabolism resulting in high levels of glucose. Several studies have proved that by inhibiting the enzyme action, glucose levels can be brought back to normal limits (Puls *et al*., 1977; Shim *et al*., 2003). In the study, action of honey in inhibiting the enzyme action was assessed and the results showed that more the sample concentration, more the percentage of inhibition of enzyme activity (Fig. 7).

Prevention of λ DNA damage by honey: Honey sample was tested for its role in preventing DNA damage caused by free radicals like H_2O_2 and $FeSO_4$. Figure 8 showed electrophoresis result of λ DNA. DNA with $FeSO_4$ and H_2O_2 showed a decreased band intensity at end of 2nd h (lane 2) indicating damage. In the presence of honey, λ DNA damage were not observed showing its protective activity (lane 3). λ DNA along with honey was studied and the result showed the DNA was intact in the presence of honey sample (lane 4). Thus, proves the role of honey in preventing DNA from oxidative damage.

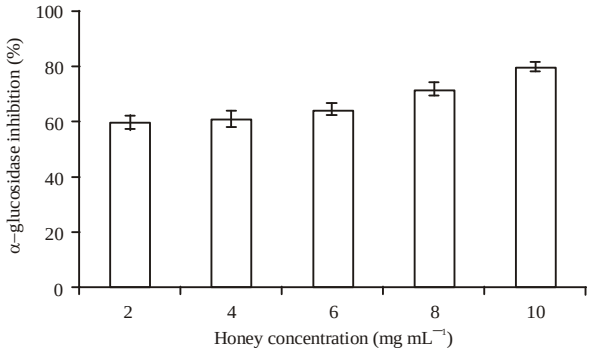

Fig. 7: Inhibition of α-glucosidase on action of Srilankan honey. Values are Mean±SD (n = 3)

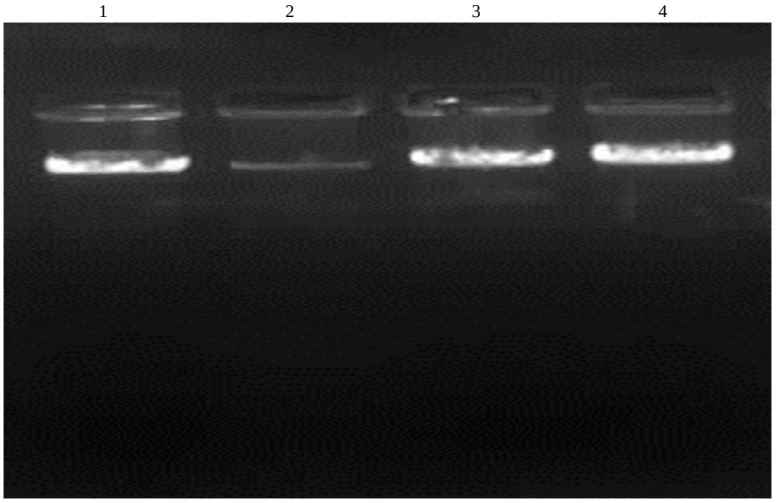

Fig. 8: Agarose gel electrophoresis image of λ DNA damage inhibition by Srilankan honey after 2 h incubation of reaction mixture. Lane 1: 0.5 μg λ DNA alone, Lane 2: 0.5 μg λ DNA+1 mM FeSO4.7H_2O+25 mM H_2O_2, Lane 3: 0.5 μg λ DNA+1 mM FeSO4.7H2O+25 mM H_2O_2+10 mg of honey and Lane 4: 0.5 μg λ DNA+10 mg of honey

DISCUSSION

Honey is a well known natural antioxidant produced by honey bees. Honey contains phytochemical components like phenolic acid, flavonoids, vitamins, enzymes and some amount of mineral content particularly copper and iron (Erlund 2004; Meda *et al*., 2005), which are found beneficial to humans in one way or the other. Most of the chronic diseases like cancer, coronary and neurological degeneration happen as an after effect of oxidative damage to the cells. Use of honey in therapeutics can be attributed to its antioxidant capacity in fighting against free radicals causing oxidative damage. Several studies have proven that, presence of polyphenols like phenolic acid, flavonoids and proanthocyanidin have a beneficial effect in scavenging harmful reactive oxygen species (Sakihama *et al*., 2002). Antioxidant activity of polyphenols is derived from the presence of (-OH) in aromatic ring, which mediates the redox reaction and there by, leads to scavenging of free radicals (Raina *et al*., 2003). Free radical scavenging activity of the sample was assessed by observing decrease in absorbance as concentration increases. The decreased absorbance

corresponds to increased scavenging activity by the sample. Metal ion chelating capacity is significant in deciding the antioxidant status of the compound because it reduces the concentration of ferrous ions catalyzing lipid peroxidation (Mohan *et al*., 2012). The presence of reductants in the solution serves as a significant indicator of its antioxidant capacity as they help in breaking the free radical chain by donating a hydrogen atom. In β-carotene linoleic acid assay, linoleic acid forms hydroperoxides as free radicals, which oxidizes β-carotene resulting in increased lipid peroxidation by reactive oxygen species (Jayasri *et al*., 2008). From the result obtained, it was shown that administering honey resulted in a reduction in β-carotene oxidation. This was because the antioxidants present in the sample interacted with free radicals, preventing them from acting on β-carotene. The hydroxyl radicals attack hydrogen atoms of λ DNA resulting in nick formation leading to single strand or double strand breaks in DNA (Balasubramanian *et al*., 1998). The results obtained from the study showed protective role of honey in preventing DNA damage. The preventive potential may be due to the free radical scavenging activity of polyphenols present in honey sample. Erythrocytes are highly susceptible to peroxidation due to the presence of high membrane concentration of poly unsaturated fatty acids and oxygen transport associated with hemoglobin molecules. Hence, it was chosen for the study. On addition of honey, the induction of hemolysis initiated by H_2O_2 was inhibited and the condition was ameliorated. Hence, the antioxidant action of honey on free radical induced erythrocyte hemolysis inhibition was proved. Based on all these results, we conclude that honey sample obtained from Srilanka exhibited satisfactory antioxidant properties and that it helps in preventing DNA and erythrocytes from oxidative damage.

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