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Antioxidant activity, repair and tolerance of oxidative DNA damage in different cultivars of Sugarcane (*Saccharum officinarum*) leaves

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Abstract

High intake of natural antioxidants has been associated with lower incidence of chronic diseases such as cancer and heart diseases. Thus, there is need to find out the crops rich in antioxidants and phenolic compounds. The aqueous extracts of leaves of thirteen varieties of sugarcane were studied for their antioxidant activity and protective effect on DNA damage. 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and phosphomolybdenum reduction assays were used to determine the antioxidant activities in the leaves. Different varieties of sugarcane showed the higher antioxidant properties (IC_{50} values ranged between 18.75±1.2 to 27.67±0.45 µg/ml) and demonstrated their ability to protect against DNA damage induced by hydroxyl radical generated in the Fenton's reaction. The high antioxidant activity and protection against DNA damage of sugarcane may be partly due to their phenolic and flavonoid contents. In here, we suggest that the hot water extracts of sugarcane leaves could provide health and functional food effects due to their antioxidant properties.

Keywords: Sugarcane, DNA damage, antioxidant activity, phenolics, hydroxyl radical.
Abbreviations: DPPH-2,2-diphenyl-1-picrylhydrazyl radical, GAE-Gallic acid equivalent, IC_{50}- Inhibitory concentration, Imax-maximum inhibition.

Introduction

The human body is continuously attacked by both the exogenous and endogenous reactive oxygen species (ROS), causing oxidative damage to the cells. Among different ROS, the hydroxyl radical (^{o}OH) is usually highly reactive and short-lived and is known to cause damage to cellular components including DNA (Chatgilialoglu and Neill, 2001). Our body possesses a natural defense system of complex antioxidant mechanisms to neutralize reactive oxygen species. Damaged DNA can be repaired enzymatically and regain its normal functions (Friedberg et al., 1995). However, misrepair of DNA damage could result in mutations such as base substitution and deletion which leads to carcinogenesis (Dreher and Junod, 1996). In the last several decades, there has been a great interest in identifying free radical scavengers or antioxidants that can inhibit or retard the oxidative DNA damage. These antioxidants supplied by diets, include vitamin C, vitamin E, carotenoids (β-carotene, α-carotene, β-cryptoxanthin, lutein, zeaxanthin and lycopene) to several polyphenolic compounds including flavonoids (catechins, flavonols, flavones and isoflavonoids) which can impede carcinogenesis by scavenging free radicals or interfere with the binding of carcinogens to DNA (Craig, 1997; Stoner and Mukhtar, 1995). Accordingly, the plants rich in antioxidants, have received the greater attention and have been studied extensively, since they can reduce the risks for cardiovascular disease or several types of cancers (Kromhout et al., 1995; Hertog et al., 1993). Sugarcane is an important cash crop of Pakistan. It is mainly grown for sugar and sugary production. It is an important source of income and employment for the farming community of the country. It also forms essential item for industries like sugar, chip board, paper, barrages, confectionery, used in chemicals, plastics, paints, synthetics, fiber, insecticides and detergents (Aslam and Khan, 2001). Sugarcane, popularly known as noble cane, due to its high sucrose content and low fiber content is one of the most important industrial crops of the world. The sugarcane juice contains flavonoids such as apigenin, luteolin and tricin derivatives and among phenolics, hydroxycinnamic, caffeic and sinapic acid, representing a total content of around 160 mg/L (Joaquim et al., 2006) whereas, sugarcane leaves contains luteolin-8-*C*-(rhamnosylglucoside) as major compound with radical scavenging activity (Fabiana et al., 2008). It is a principal raw material for the sugar industry as 70% of the world's sugar comes from sugarcane. Besides sugar production, large number of population in the tropics and subtropics relishes its juice and consume raw cane. In the Pakistan, chewing raw sugarcane is recommended for sound and healthy body. Both the roots and stems of sugarcane are used in medicine to treat skin and urinary tract infections, as well as for bronchitis, heart conditions, loss of milk production, cough, anemia, constipation as well as general debility. It is also used to treat jaundice and lowering blood pressure (Mira et al., 2011). The DPPH radical has been widely used to test the ability of compounds as free-radical scavengers or hydrogen donors and to evaluate the

antioxidative activity of plant extracts and foods (Porto et al., 2000; Seow et al. 2012). In view of the potential role of sugarcane as a dietary source of flavonoids as well as its possible use as functional food, this study was aimed to evaluate the antioxidant activity of thirteen different varieties of sugarcane, their phenolic and flavonoid contents and to examine their protective effect on hydroxyl radical mediated DNA damage. To our knowledge this is first report on the antioxidant and DNA protecting ability of these sugar cane cultivars. The study is particularly important in view of the fact that most the studies were carried out on sugarcane juice.

Results

DPPH radical scavenging activity

The scavenging activity of extracts against DPPH$^{\bullet}$ was concentration-dependent (Fig 1). Significant differences in scavenging percentage between extracts were observed and the results clearly indicate that all the extracts exhibited high antioxidant activity. Among different genotypes HSF-240, CPF-7-400, HSF-242, NSG-60 significantly displayed higher percentage of DPPH radical scavenging activities (Fig 1 a), whereas US-133, US-778, CPHF-35, US-623, CPF-234 also showed good antioxidant properties (Fig 1 b). It is interesting to note, that the aqueous extracts of these genotypes displayed antioxidant activity higher than 50% even at a low concentration of 25 µg/ml. The extract concentration that cause 50% scavenging of DPPH$^{\bullet}$ (IC_{50} value) and maximum inhibition percentage (I_{max}) are shown in Figure 2. The IC_{50} is inversely proportional to the scavenging percentage of the extract. The IC_{50} values of different genotypes of sugarcane varied between 18.75±1.2 to 27.67±0.45 µg/ml showing the antioxidant activity of sugarcane. Among different genotypes, HSF-240 showed the highest antioxidant activity (IC_{50}, 18.75±1.2 µg/ml) whereas, CP-43-33 showed relatively less antioxidant activity (IC_{50}, 27.67±0.45 µg/ml). NSG-60, US-133, US-778, CPHF-35 showed similar IC_{50} and I_{max} values (Fig 2). However, HSF-240 demonstrated the lowest IC_{50} values whereas, CP-43-33 demonstrated the highest Imax value at a concentration of 300 µg/ml (Fig 2).

Total antioxidant activity

The total antioxidant activity of the extract (equivalent to ascorbic acid) ranged between 55-75.26 µg/ml. HSF-240 showed the highest antioxidant activity (75.26 µg/ml) whereas, NSG-60 showed the least antioxidant activity (55 µg/ml). CPHS-35, CPF-247, SPF-234, US-114 and SPF-238 demonstrated the similar antioxidant activities (Fig 3).

Total phenolic content

Mean values for the total phenolic content of thirteen genotypes of sugarcane extracts are shown in Figure 4. The amount of total phenolics was found to be different in different genotypes and varied between 33.78 to 65.7 mg GAE/g of dry weight. The highest amount of phenolic contents was detected in HSF-240, US-778, CPHF-35, NSG-60, and the lowest in CP-43-33. The high antioxidant activity of HSF-240 (IC_{50}, 18.75 µg/ml) is due to the presence of high phenolic contents i.e. 65.7 mg GAE/g of extract as compared to the other genotypes of sugarcane. CP-43-33 showed the lowest phenolic content i.e. 33.78 mg GAE/g, concluded that CP-43-33 had less antioxidant activity (IC_{50}, 27.67 µg/ml). The

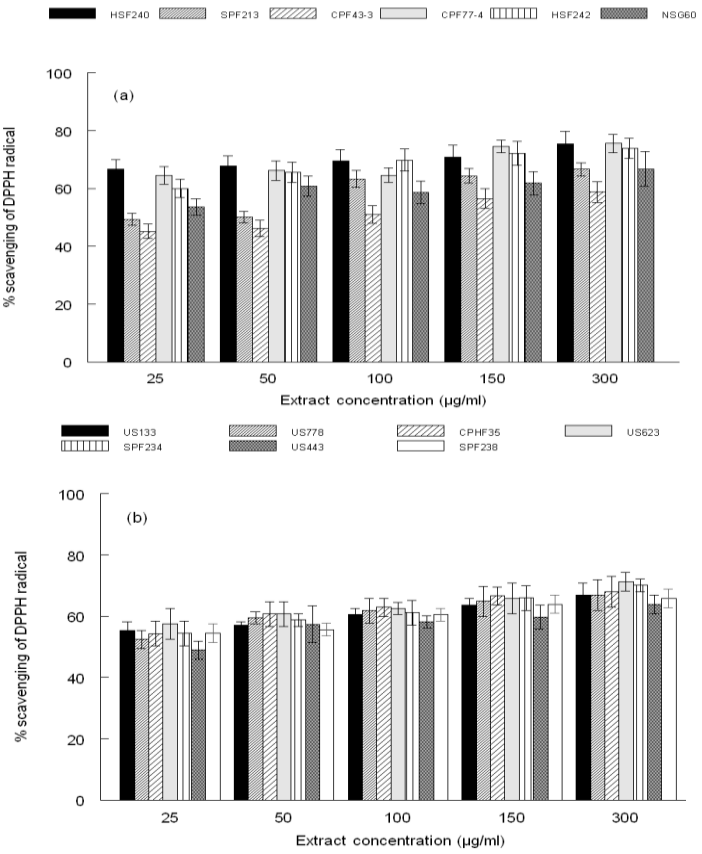

Fig 1. Antioxidant activity of aqueous extract of different cultivars of sugarcane leaves. (a,b) DPPH radical scavenging activity of different genotypes of sugarcane. Values are means ± SD (n=3).

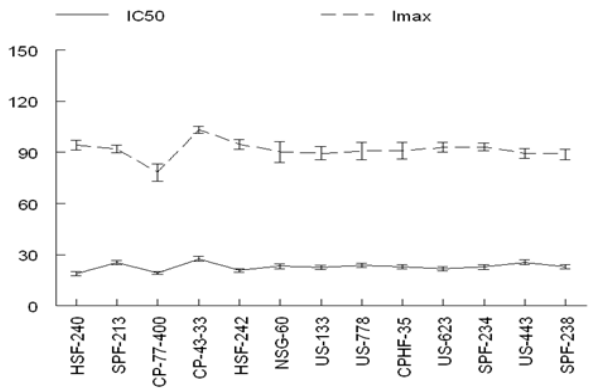

Fig 2. Calculated IC_{50} values (µg/ml) and Imax (extract concentration that causes maximum percentage of scavenging) values for different sugarcane cultivars on DPPH radical scavenging.

results of total phenolic content, showed the variation among different genotypes. However, this variation was non-significant ($P > 0.05$).

Flavonoid contents

The flavonoid contents ranged between 7.3-63.1mg/g (Fig 5). HSF-240 and SPF-213 showed the least content of flavonoids. Whereas, CP-77-400, HSF-242, NSG-60, US-778 and US-114 showed the higher amount of

flavonoids. The results of flavonoid content, showed the variation among different genotypes. However, this variation was non-significant (P > 0.05).

Protection against DNA damage

In order to study the protective effects of sugarcane aqueous extract on hydroxyl radical-mediated DNA strand breaks, thirteen genotypes of sugarcane were used in the site specific DNA damage assay. Incubation of sugarcane DNA with Fe(II) and H_2O_2 for 40 minutes resulted in production of hydroxyl ions, whereby indicating that both single-strand and double-strand DNA breaks can be induced by $FeSO4/H_2O_2$ at the indicated concentrations and incubation time (Fig 6). The gel pattern of DNA exposed to $FeSO_4$ + H_2O_2, in the presence (Lane 2-14) and the absence of extracts (Lane 1) of sugarcane is shown in Figure 6. The control showed the absence of specific band in treated DNA (shredding) which indicates DNA damage. Different genotypes of sugarcane extracts at a concentration of 100 µg/ml significantly reduced the DNA damage (Fig 6).

Discussion

Antioxidants are substances that neutralize free radicals and their negative effects. They act at different stages (prevention, interception and repair) and by different mechanisms: reducing agents by donating hydrogen, quenching singlet oxygen, acting as chelators and trapping free radicals (Devasagayam et al., 2004). Research studies report that the fruits, vegetables, grains and other plants are an important source of polyphenols, bioactive compounds that have been found to provide a very strong antioxidant and free radical scavenging activity. Free radicals involved in the process of lipid peroxidation are considered to play a major role in numerous chronic pathologies such as neurodegerative disorders, cancer and cardiovascular diseases (Halliwell, 1992). DPPH$^{\bullet}$ is considered to be a model of a stable lipophilic radical. A chain reaction of lipophilic radicals is initiated by lipid autoxidation. Antioxidants react with DPPH$^{\bullet}$, reducing the number of DPPH free radicals to the number of their available hydroxyl groups. Therefore the absorption at 517 nm is proportional to the amount of residual DPPH$^{\bullet}$. It is visually noticeable as a discoloration of DPPH$^{\bullet}$ from purple to yellow. When the DPPH radical scavenging activity was analyzed the strongest activity was observed for HSF-240 and CP-77-400. Whereas, the least antioxidant activity was shown by CP-43-33 on the basis of their IC_{50} values. The results obtained in this study showed the high antioxidant activity of sugarcance cultivars and suggest their use in diseases arising from free radicals. The antioxidant activity of sugarcane is also supported by the literature (Joaquim et al. 2006; Fabiana et al., 2008; Kadam et al., 2007). The antioxidant activity of aqueous extract sugarcane leaves is supported by the studies of Pei-Ying Yu (2009) who reports that three varieties of sugarcane displayed significant total antioxidant activity, reducing activity and liposome peroxidation inhibition studies. Phenolics are the most wide spread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelators. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers (Rafael et al., 2008). Several studies have shown that the higher antioxidant

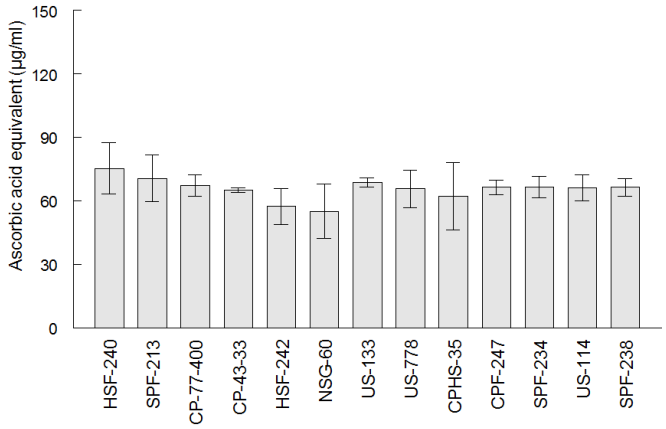

Fig 3. Total antioxidant activity measured by phosphomolybdenum reduction method. Values are mean ± SD (n=3).

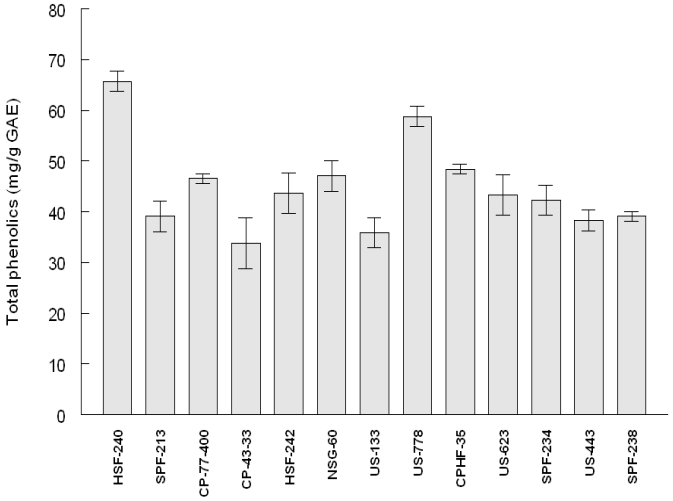

Fig 4. Total phenolic content among different varieties of sugarcane. Values are mean ± SD (n=3). The varieties are non-significantly different (P > 0.05) by DMR test from each other.

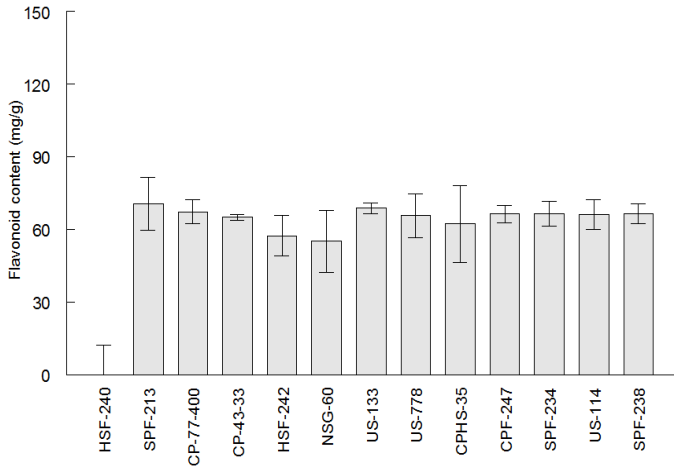

Fig 5. Total flavonoid content among different varieties of sugarcane. Values are mean ± SD. The values are non-significantly different (P > 0.05) from each other by DMR test from each other.

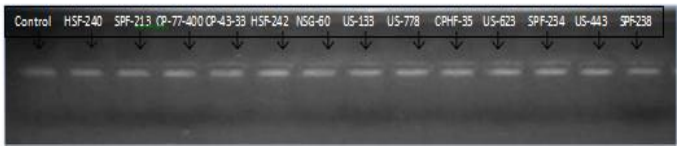

Fig 6. Effect of different varieties of sugarcane on DNA damage induced with Fe + H_2O_2 (Control). The Lanes (2-14), $FeSO_4$ and H_2O_2 in the presence of aqueous extracts of different cultivars of sugarcane.

activity associated with medicinal plants is attributed to the total phenolic compounds (Cheung et al., 2003). It is considered that phenolic compounds contribute to the overall antioxidant activity of sugarcane. Therefore, the content of total phenolics in sugarcane extracts was determined. Phenolics are important mainly because of their function to scavenge the free radicals in the human body and to help maintaining healthy body by scavenging or removing the reactive oxygen species (ROS). The results revealed that different cultivars of sugarcane are rich in phenolics (Fabiana et al., 2008). The potential beneficial effects of the high antioxidant activity and protections of cells from free radical attack seem clear (Halliwell, 1997). In the leaves of sugarcane the major phenolics are luteolin 8-*C*-rhamnosylglucoside, diosmetin-8-glucoside, vitexin, orientin, tricin-7-O-neohesperidoside and tricin derivatives (Fabiana et al. 2008) which are responsible for the antioxidant activity of sugarcane. In the phosphomolybdenum assay, which is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity), the sugarcane extracts demonstrated electron-donating capacity showing their ability to act as chain terminators, transforming relative free radical species into more stable non-reactive products (Dorman et al., 2003). Hydroxyl radicals generated by the Fenton reaction are known to cause oxidatively induced breaks in DNA strands to yield its fragmented forms. The free radical scavenging activity of sugarcane was studied on genomic DNA. The treatment of super coiled DNA with Fenton's reagent directed the alteration of DNA to open circular form. The addition of extracts to the reaction mixture substantially decreased the DNA strand scission and retained the super coiled form, thus increased the native form of DNA. However, HSF-240 containing higher amount of phenolics and antioxidant activity relatively showed less protection against DNA damage compared to the other cultivars (CP-77-400, CP-43-33 and HSF-242 etc). The high DNA damage protecting ability of CP-77-400 and CP-43-33 is due to the presence of flavonoids despite of the fact that these extracts contained lesser amount of phenolics. Based on the previous data, it is possible that the powerful antioxidant activity of sugarcane aqueous extracts is given by the presence of substances with hydroxyls. In this context, flavonoids possess an ideal structure for the scavenging of free radicals since they present a number of hydroxyls acting as hydrogen donators acting as an important antioxidant agent (Cao et al., 1997). Compounds with antioxidant activity may exhibit pro-oxidant behavior under certain conditions. Pro-oxidant activity can accelerate damage to molecules such as DNA, carbohydrates, or proteins (Auroma et al., 1997). In fact, polyphenols are an important group of pharmacologically active compounds. They are considered to be the most active antioxidant derivatives in plants (Edenharder et al., 2003; Park et al., 2004). However, it has been shown that the phenolic content does not necessary follow the antioxidant activity. Antioxidant activity is generally the result of the combined activity of a wide range of compounds, including phenolics, peptides, organic acids and other components (Gallardo et al., 2006). This is first report to date on the protective effect of sugarcane leaves on DNA damage. Kadam et al. (2008) reported that sugarcane juice has protective effect against radiation induced DNA damage. DNA strand scission is induced by hydroxyl radicals. Hydroxyl radical is the most reactive radical found in biological systems. It can abstract hydrogen atoms from biological molecules, including thiols, leading to the formation of sulphur radicals capable to combine with oxygen to generate oxysulfur radicals, which damage biological molecules (Jihed et al., 2011). Sugarcane extracts are able to exhibit the protection on DNA damage caused by hydroxyl radical, which might be due to their chelating activity on iron or hydroxyl radical scavenging or both (Sabir et al., 2012).

Material and methods

Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH Sigma, 90.0%), Folin-Ciocalteu reagent (2 N), 1,1,1-tris (hydroxymethyl) ethane (Tris), Low melting agarose and gallic acid were purchased from Sigma and Aldrich Chemicals (St, Louis, MO, USA). Ethanol (95%), iron sulphate, phosphate buffer, hydrogen peroxide, sodium acetate and sodium carbonate were purchased from Bio-Chemical (Lahore). All the chemicals and reagents were of analytical grade.

Sugarcane samples

Studies were conducted on thirteen genotypes of sugarcane which were collected from different growing areas of Pakistan. Genotypes selected for this experiment were HSF-240, SPF-213, CP-7-400, HSF-242, CP-43-33, NSG-60, US-133, US-778, CPHF-35, US-623, CPF-234, US-443 and SPF-238. All the genotypes were grown under glass house conditions under normal soil conditions in Faculty of agriculture, University of the Poonch Rawalakot, Pakistan.

Preparation of sugarcane extracts

The air dried leaves of sugarcane at full maturity (5 g) were ground and soaked in boiling water (250 ml) for 15 mins, allowed to cooled and filtered using whatman filter paper. The obtained residue was further extracted twice and finally the whole extract was concentrated. The extract weight and percentage yield were found to be 0.8-1% respectively. The serial dilution of the extract was made to obtain the desired concentration of plant for experiment.

Antioxidant activity by DPPH radical scavenging

The antioxidant activities of the sugarcane extracts were measured using the stable DPPH radical according to the method of Hatano et al. (1998). Briefly 0.25 mM solution of DPPH radical (0.5 ml) was added to the sample solution in ethanol (1 ml) at different concentrations (25-300 µg/ml) of aqueous extract of sugarcane. The mixture was shaken vigorously and left to stand for 30 minutes in the dark, and the absorbance was measured at 517 nm. The capacity to scavenge the DPPH radical was calculated using the following equation: (%) scavenging = $[(Ao - A_1)/Ao)] \times 100$, Where, Ao is the absorbance of the control reaction and A_1 is

the absorbance of the sample itself. The IC_{50} values (Extract concentration that cause 50% scavenging) were determined from the graph of scavenging effect percentage against the extract concentration. All determinations were carried out in triplicate.

DNA Extraction

DNA was extracted from leaves of sugarcane with new modified PEG method (Li and Midmore, 1999). DNA of all sample were stored at -80 ^{o}C and the presence of DNA was confirmed by gel electrophoresis (1.5%).

Site-specific hydroxyl radical-mediated DNA strand breaks

The site specific hydroxyl radical-mediated DNA strand breaks were measured by the procedure described by Yeung et al. (2002) with some minor modifications. Briefly, 0.5 µg DNA was incubated with 1 µl of 1 mM $FeSO_4$, 1 µl of 10% H_2O_2, 3 µl of 100 µg/ml of aqueous extracts of sugarcane, and the final volume was made up to 15 µl with 50 mM phosphate buffer (pH 7.0). The mixture was incubated in water bath at 37 ^{0}C for 30 mins. After the incubation, the sample was immediately loaded in a 1.5% agarose gel along with 3 µl ethidim bromide, containing 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA, and electrophoresed in a horizontal slab apparatus in Tris/boric/EDTA gel buffer. The gel was then photographed under UV light.

Total Antioxidant assay

The assay was based on the reduction of molybdenum, Mo (VI)-Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH (Prieto et al., 1999). The extract (0.1 mg/ml) was mixed with 3 ml of the reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM amonium molybdate). The tubes were incubated at 95 ^{0}C for 90 mins. The mixture was cooled to room temperature and the absorbance of the solution was measured at 695 nm.

Determination of Phenolics content

The total phenolics content as gallic acid equivalent was determined by the method of Singleton et al. (1999). The aqueous extract (0.5 ml) of sugarcane leaves was added to 2.5 ml, 10% Folin-Ciocalteau's reagent (v/v) and 2 ml of 7.5% sodium carbonate. The reaction mixture was incubated at 45 ^{0}C for 40 minutes and the absorbance was measured at 765 nm in the spectrophotometer. Gallic acid was used as a standard phenol. The mean of three readings was used and the total phenol content was expressed as milligrams of gallic acid equivalents/g extract.

Determination of Flavonoid content

Flavonoids were expressed as quercetin equivalents. Quercetin was used to make the calibration curve [0.04, 0.02, 0.0025 and 0.00125 mg/ml in 80% ethanol (v/v)]. The standard solutions or extracts (0.5 ml) were mixed with 1.5 ml of 95% ethanol (v/v), 0.1 ml of 10% aluminium chloride (w/v), 0.1 ml of 1 mol/l sodium acetate and 2.8 ml of water. The volume of 10% aluminium chloride was substituted by the same volume of distilled water in the blank. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The mean of three readings was used and the total flavonoid content was expressed as milligrams of quercetin equivalents/g of extract (Kosalec et al., 2004).

Statistical analysis

The results were expressed as means ± standard deviation. The data was analyzed by one way AOVA and different group means were compared by Duncan's multiple range (DMR) test where necessary. $P < 0.05$ was considered significant in all cases. The software Package Statistica was used for analysis of data.

Conclusions

In conclusion the results of the present study revealed that the different cultivars of sugar cane are rich source of antioxidants and has effectively protected the DNA damage. Among different cultivars HSF-40 showed the highest antioxidant and the phenolic content. Whereas, cultivars CP-43-33 containing less phenolic content showed higher protection against DNA damage. The investigation results that these cultivars can be used not only as accessible source of natural antioxidants but also as an ingredient of the functional food and control of degenerative diseases. In general, the variation among phytochemical contents and antioxidant activity indicates that the genetic variability used can be used to develop cultivars with enhanced health benefits.

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