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IN VITRO EVALUATION OF ANTIOXIDANT PROPERTIES OF SUGARCANE EXTRACTS RICH IN DIETARY NUCLEOTIDES

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ABSTRACT
Food not only provides essential nutrients required for life, but also bioactive compounds useful to maintain good health and disease prevention. It's a fairly new approach in nutracueticals that involves finding plant based natural sources, that are rich in nucleosides-nucleotides along with potent antioxidant properties. High intake of natural antioxidants has been associated with lower incidence of chronic diseases such as cancer and heart diseases. Thus, this study was conducted to evaluate a multi-bio-functionality of the aqueous extracts of sugarcane (SE1, SE2 and SE3; rich uridine and uridine rich derivatives) for their antioxidant activity and high phenolic content. The assays involved different levels of antioxidant action such as DPPH (2, 2-diphenyl-1-picrylhydrazyl), ABTS (2, 2 -azinobis (3-ethylben- zothiazoline 6-sulfonate)), FRAP (ferric reducing antioxidant potential), and ORAC (oxygen radical absorption capacity). It is interesting to note, that these extracts displayed antioxidant activity higher than 50% even at a low concentration. Results of the DPPH, ABTS, FRAP, and ORAC assays indicate that all the three extracts examined showed significant antioxidant activities. Among these, SE3 extract seems to be the more effective antioxidant. The ORAC values are used as standard measures for comparing the antioxidant activity ranging from 602±3.7-1236±4.4 (µg TE/g). The high antioxidant activity of sugarcane extracts may be partly due to their total phenolic and flavonoid contents. In conclusion, this study reveals the ability of sugarcane extracts to scavenge free radicals, reduce iron complex and inhibit lipid peroxidation. It may explain possible mechanisms by which sugarcane extracts exhibits their beneficial effects in relation to its reported health benefits.

KEY WORDS: Antioxidant assays, Dietary nucleotides, Phenolic content and Sugarcane extracts.

INTRODUCTION
Antioxidants are the compounds, which scavenge the free radicals and other Reactive Oxygen Species (ROS) which causes oxidative damage implicating in the etiology of cancer, cardiovascular diseases and other degenerative disorders. However natural antioxidant mechanism can be inefficient and hence dietary intake of antioxidant compounds is necessary (Rakesh *et al.,* 2011). Also the nutritional research has focused on the antioxidant potential of various foods. In recent times, natural foods have gained considerable interest among nutritionists, food manufacturers and consumers due to their perceived safety, potential therapeutic value, and long shelf life (Siro *et al.,* 2008). Dietary intake of various substances like vitamins, minerals, herbs or other plant products including amino acids, antioxidants and nucleosides-nucleotides are essential for certain deficiencies and disease associated pathologies. The Nutraceutical approach is a new way to find a plant based natural sources that are rich in nucleosides-nucleotides and also have antioxidant properties. Human body requires dietary nucleic acids/nucleotides to meet its physiological requirements in certain conditions particularly, during rapid growth, limited food supply and metabolic stress (Gill, 2002; Abtahi *et al*., 2011; Sonkawade *et al.,* 2012). Synthetic supplements rich in antioxidants or nucleotides are available in the market. There are no reports available on plant-based compounds, which are rich in nucleic acids and antioxidants. Sugarcane is the world's largest commercial crop cultivated extensively for its sucrose content and ethanol production. Sugarcane products contain various phenolic compounds and its extracts displayed a wide range of biological activities including antioxidant, anti-inflammatory, anti-thrombosis, immune-stimulation and anti-stress effects (Shreeramulu *et al.,* 2013 and Vinutha *et al.,* 2014). In continuation of our previous studies, the present study was conducted using three different sugarcane extracts rich in dietary nucleotides (uridine and its derivatives). There has been an upsurge of interest in exploring the potent antioxidant activity of these sugarcane extracts by using different approaches and models such as its use in reducing power, superoxide anion radical scavenging; it is also widely used for its electron or radical scavenging activities against DPPH (2,2- diphenyl-1-picrylhydrazyl), ABTS (2,2 - azinobis (3-ethylben- zothiazoline 6-sulfonate)), FRAP (ferric reducing antioxidant potential), and ORAC (oxygen radical absorption capacity) assays (Bertrand *et al.,* (2006); Dudonne *et al.,* 2013; Saiqa *et al.,* 2014; Shreeramulu *et al.,* 2013 and Vinutha *et al.* (2014). Further, the total phenol

content and various flavonoids present in these extracts were also determined. However, very little information is available regarding peroxyl radicals, particularly against azo-initiator , 2 -azobis (2-amidinopropane) dihydrochloride (AAPH) peroxyl radicals against sugarcane antioxidant activity. These techniques have shown different results among plants tested across different laboratories (Dudonne *et al.,* 2013). In this investigation, water is used as an extraction solvent to extract the hydrophilic antioxidants. Indeed, for use in food and nutraceuticals, aqueous plant extracts are nutritionally more relevant and would have obvious advantages in relation to certification and safety (Moller *et al.,* 1999). Therefore, analysis of these compounds is important to provide safe, effective and practical methods for increasing longevity while ensuring good quality of life. It is also important for improving and assuring food quality.

MATERIALS AND METHODS

Sugarcane samples

Samples were collected from local market and they were sterilized and spray dried for longetivity and extended studies. An analytical high performance liquid chromatography method was used for the analysis of uridine, uridine 5’-monophosphate and 2', 3', 5'-tri-O-acetyl uridine in samples of fresh, condensed and concentrated powdered sugarcane juice (SE1, SE2 and SE3) respectively.

Preparation of sugarcane extracts

5 g of the ground sugarcane samples were prepared in 20 ml distilled water. Samples were centrifuged at 10,000 g for 15 min at 10 ºC, the supernatant was collected and filtered (Dalvi *et al.,* 2014). Extraction was performed in triplicate and all samples were freshly prepared for each experiment.

EVALUATION OF ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT

Following experimentswere performed on each sugarcane extract in aqueous solution.

Folin-Ciocalteu Assay

Soluble phenolic compounds (PC) in sample extracts were determined using the Folin-Ciocalteu reagent as per the method described by Sreeramulu *et al.* (2013). A sample aliquot of 100 µL was added to 900 µL of water, 1 mL of Folin-Ciocalteu reagent (1:2, v/v) and 2 mL of 10% sodium carbonate were added sequentially, mixed thoroughly and incubated for one hour at room temperature and the absorbance was measured at 765 nm in visible spectrophotometer. Gallic acid used as standard and the total phenolic content expressed as milligrams of Gallic acid equivalent (GAE) per gram sample.

Determination of Flavonoid content

Aluminium Chloride spectrophotometric method was used for the determination of total flavonoid content according to Abbas *et al*., (2013) with slight modification. 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 M Sodium acetate and 2.8 ml of water. The same volume of distilled water in the blank substituted the volume of 10 % aluminium chloride. 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.

Oxygen Radical Absorbance Capacity

The ORAC assay was carried out according to the method of Dudonne *et al*., (2013). Sodium fluorescein stock solution (4.19×10−3 mM) was prepared in 75 mM potassium phosphate buffer (pH 7.4) and stored at -4°C. Sodium fluorescein working solution was made daily by further diluting the stock solution in 75 mM potassium phosphate buffer. Trolox standard solution was prepared 20 µl each of sample; potassium phosphate buffer and Trolox standard solution were added in 96-well microtiter plate. Sodium fluorescein working solution (160 µl) was then added into the wells. AAPH solution was prepared last by diluting 0.110 g of AAPH in 5.0 ml of 75 mM potassium phosphate buffer (pH 7.4). AAPH solution (20µl) was added last into the well via an automatic dispenser. Thus, the total volume for each well was 200 µl, and the fluorescence intensity was measured every 2 min for 2 h by Tecan Infinite® PRO reader with i-control™ software.

DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to Sreeramulu *et al.* (2013). This method is based on the ability of the antioxidant to scavenge the DPPH cation radicals. 100 µl of sample extract or standard was added to 2.9 ml of DPPH reagent (0.1 mM in methanol) and vortexed vigorously. The reaction tubes were incubated in dark for 30 min, at room temperature and the discoloration of DPPH was measured against a reagent blank at 517 nm. Percentage inhibition of the discoloration of DPPH by the sample extract was expressed as Trolox and Ascorbic acid equivalents. The percent of DPPH discoloration of the samples was calculated according to the formula:

Antiradical activity (%)= $(A_{control}-A_{sample})/A_{control} X100$

ABTS – radical scavenging assay

In this assay, the radical scavenging activities of each extract were determined by using ferrylmyoglobin/ABTS protocol by Dudonne *et al.* (2006). The stock solutions of 500 mM ABTS diammonium salt, 400 mM myoglobin, 740 mM potassium fericyanide, and 450 mM H_2O_2 were prepared in phosphate-buffered saline (PBS) (pH 7.4). Metmyoglobin (MbIII) was prepared by mixing equal amounts of myoglobin and potassium ferricyanide solutions. The reaction mixture (total volume 2ml) contained the following substances (final concentrations in the reaction mixture): ABTS (150 mM), MbIII (2.5 mM), 16.8 ml of the sample, and 978 ml PBS. The reaction was initiated by adding 75 mM H_2O_2 (330 ml) and the lag time in seconds, before absorbance of $ABTS^+$at 734 nm began to increase was recorded. The calibration curve was plotted at various concentrations of the standard antioxidants (L-ascorbic acid or Trolox). All determinations were assayed in triplicate and mean values were calculated and the results were expressed in TE µg/ml.

Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power (FRAP) was determined according to Sreeramulu *et al.* (2013). This method is based on the ability of the sample to reduce $Fe^{+3}$to F^{+2}ions. In the presence of TPTZ, the F^{+2}-TPTZ complex exhibits blue color, which has absorption maxima at 593 nm. Briefly, 3.0 ml of working FRAP reagent was added to a suitable volume of the sample extract was taken to suit in to standard range. After incubation for 6 min at room temperature the absorbance was measured at 593 nm against Troloxas standard and the results were expressed in TE µg/ml.

Superoxide Radical Scavenging Assay

The superoxide radical scavenging assay of sugarcane extracts were determined according to the methodology described by Vinutha *et al.* (2014). Briefly, an aliquot (10-50 µL) of samples and standard antioxidant Trolox mixed with 1 mL of Nitro blue tetrazolium, NBT (156 µM) and 1 ml of NADH (468 µM). Reaction was initiated by addition of 100 µL of Phenazine methosulphate (60 µM) and the reaction mixture was incubated at room temperature for 5 min. The absorbance was read at 560 nm against blank and percentage of inhibition was calculated.

Reducing power Assay

The reducing power was determined according to the method reported earlier Vinutha *et al.* (2014). Different concentrations of sugarcane extract (0.5- 2.5 mg/mL) or standard antioxidant Trolox, (10-50 µg/mL) mixed with an equal volume of 0.2 M Phosphate buffer, pH 6.6 and 1 % potassium ferricyanide. The mixture incubated at 50 ^{0}C for 20 min. An equal volume of 10 % trichloro acetic acid was added to the mixture and centrifuged at 3000 g for 10 min. The upper layer of the solution mixed with distilled water and 0.1% $FeCl_3$ at a ratio of 1:1:2 (v/v/v) and the absorbance measured at 700 nm. The increased absorbance of the reaction mixture indicated increased reducing power.

Statistical analysis

All the experiments were carried out in triplicates (n = 3) and the results expressed as mean ± standard deviation (SD) using Microsoft Excel.

RESULTS

Total phenolic content

The present results, TPC value of sugarcane extracts ranged between 1682±2.1-3837±3.5 µg TE/g (Table 1). High amount of TPC were found in SE3 (3837±3.5 µg TE/g) followed by SE2 (2560±4.1 µg TE/g) and SE1 (1682±2.1µg TE/g). The total amount of phenolic compound was calculated in microgram of Gallic acid equivalents (GAE)/g.

Total flavonoid content

The total flavonoid contents ranged between 1307±5.7-2781±6.1 µg QAE/g (Table 1). SE3 showed the high content of flavonoids followed by SE2 (1982±3.4µg QAE/g) and SE1 (1307±5.7µg QAE/g). The total amount of phenolic compound was calculated in microgram of Quercetin equivalents (QAE)/g.

ORAC assay

The ORAC values are used as standard measures for comparing the antioxidant activity of food materials (Table 1). Our results show the values of sugarcane extracts ranging from 602±3.7-1236±4.4 µg TE/g are similar to or higher than the values observed for some fruits and vegetables.

TABLE 1. Total phenolic and flavonoid content of sugarcane juice and their antioxidant activities measured by ORAC assay

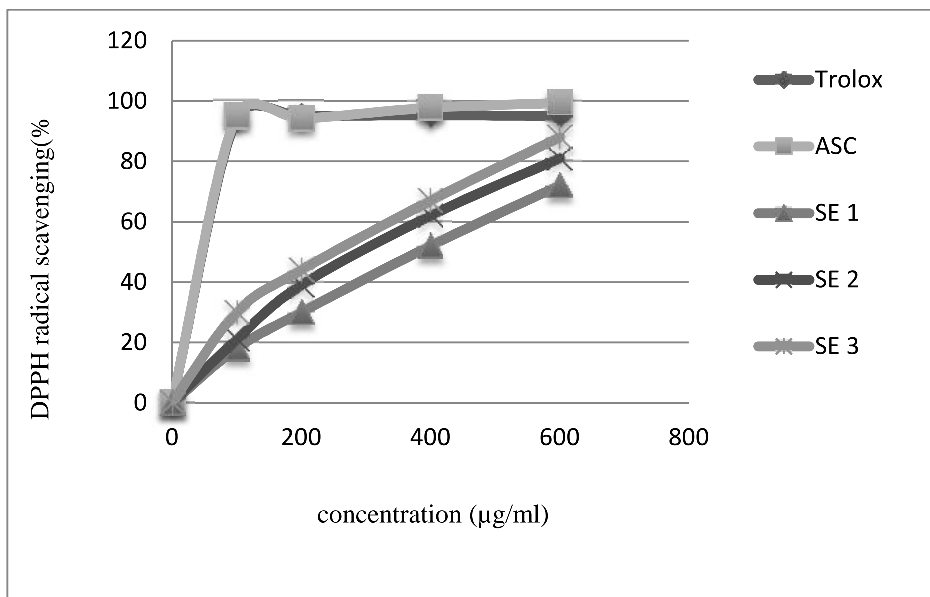
Sugarcane extracts	Total phenolic content (µg GAE/g)[a]	Total flavonoid content (µg QAE/g)[b]	ORAC value (µg TE/g)[c]
SE1	1682±2.1	1307±5.7	602±3.7
SE2	2560±4.1	1982±3.4	870±5.3
SE3	3837±3.5	2781±6.1	1236±4.4

Data are expressed as mean ± standard deviation.
[a]Data are expressed as µg Gallic acid equivalents (GAE) / g
[b]Data are expressed as µg of Quercitin acid equivalent (QAE) / g
[c]Data are expressed as µg of Trolox equivalent (TE) / g.

DPPH radical scavenging activity

The scavenging activity of extracts against DPPH was concentration-dependent (Fig. 1) Concentration response curves of two standard antioxidants; Trolox and ascorbic acid were obtained (100-600 µg/ml). Significant differences in scavenging percentage between extracts were observed and the results clearly indicate that all the extracts exhibited high antioxidant activity. Among different sugarcane extracts, SE3 displayed significantly higher percentage of DPPH radical scavenging activities, whereas SE2 and SE1 also showed good antioxidant properties (Fig. 1). It is interesting to note, that the aqueous extracts of these extracts displayed antioxidant activity higher than 50% even at a low concentration. The extract concentrations that cause 50% scavenging of DPPH (IC_{50} value) are plotted in Figure 1. The IC_{50} is inversely proportional to the scavenging percentage of the extract. The IC_{50} values of sugarcane extracts varied between 248±1.2 -380±0.45 µg/ml showing the antioxidant activity. Among different extracts, SE3 was the most active sample showing the highest antioxidant activity (IC_{50}, 248 ±1.2 µg/ml) whereas, SE2 and SE3 showed relatively less antioxidant activity (IC_{50}, 294±0.82 and 380±0.45 µg/ml) respectively. The high content of phenolic compounds in extracts (Table 1) may explain the good activity of them against the DPPH radical.

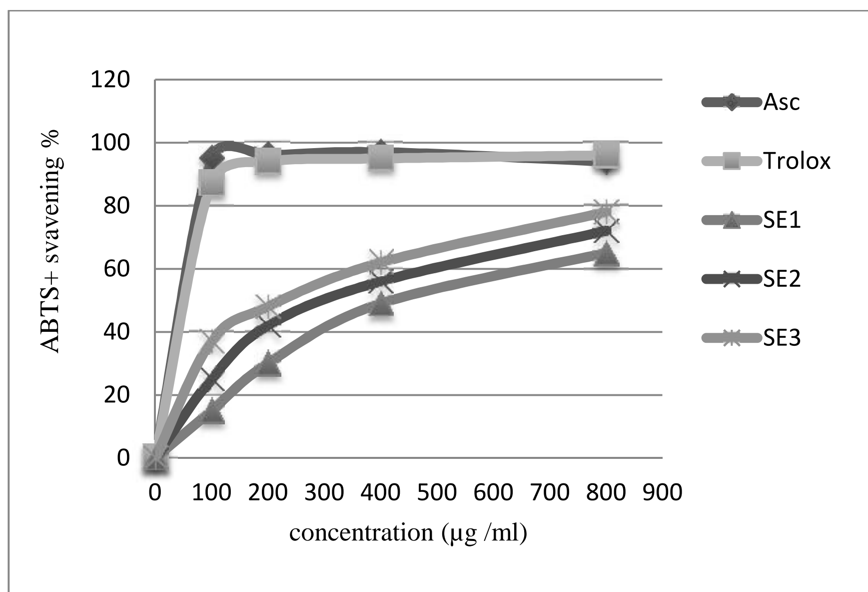
FIGURE 1



ABTS - radical scavenging assay

In these studies concentration response curves of two standard antioxidants, Trolox and ascorbic acid were obtained. Aqueous solutions of sugarcane extracts exhibited moderate to high free radical scavenging capacity (Fig. 2) SE1 and SE2 comparatively has high IC_{50} values of 300±3.4 and 410±6.2 µg/ml respectively, thus corresponding to weak activities, whereas SE3 showed greater activities. SE3 showed the strongest inhibition of ABTS oxidation (IC_{50}) 220±5.7 µg/ml).

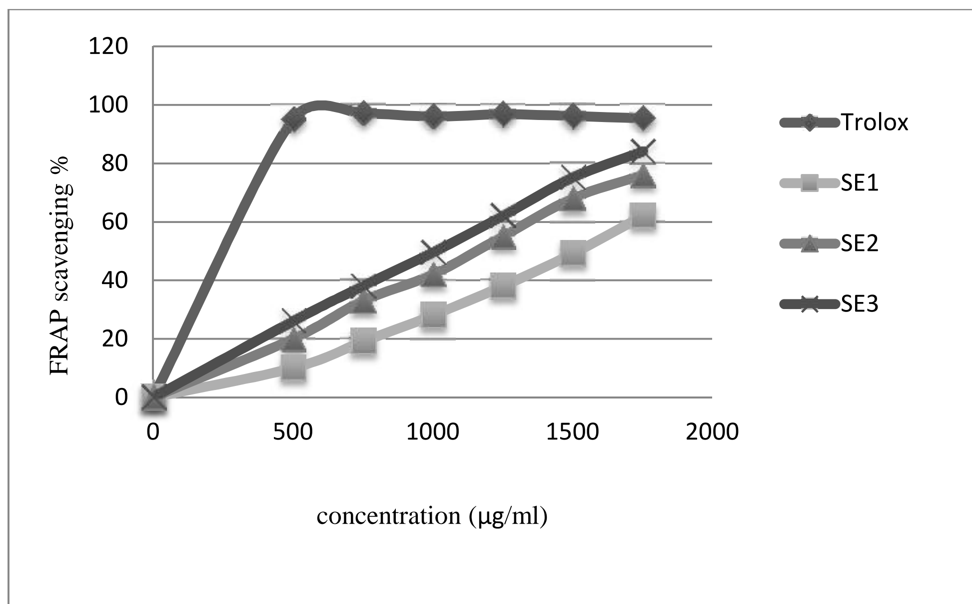
FIGURE 2



Ferric reducing antioxidant power (FRAP) assay

In FRAP radical scavenging activity (Trolox equivalents) showed marked variation among the three sugarcane extracts studied (Fig. 3) The IC_{50} values of sugarcane extracts varied between 1030±1.3-1520 ±2.8 µg/ml showing the antioxidant activity with the highest activity being found in the SE3 1030±1.3followed by the SE2 1140±4.8 µg/ml.

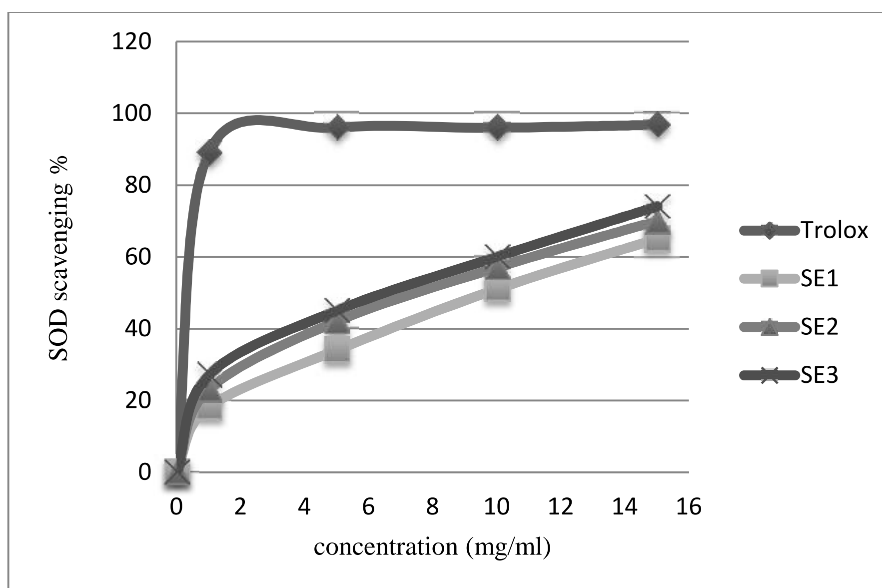
FIGURE 3



Superoxide Radical scavenging Assay

The increased superoxide radical scavenging ability of SE3 is studied as given in Fig. 4. All the three sugarcane extracts are able to scavenge superoxide radical at different concentration dependent manner with IC_{50} SE1, SE2 and SE3 9.9±4.9 mg/mL, 7.4±7.3 mg/mL, 6.5±5.4 mg/mL respectively. SE3 was found to be more potent superoxide radical quencher.

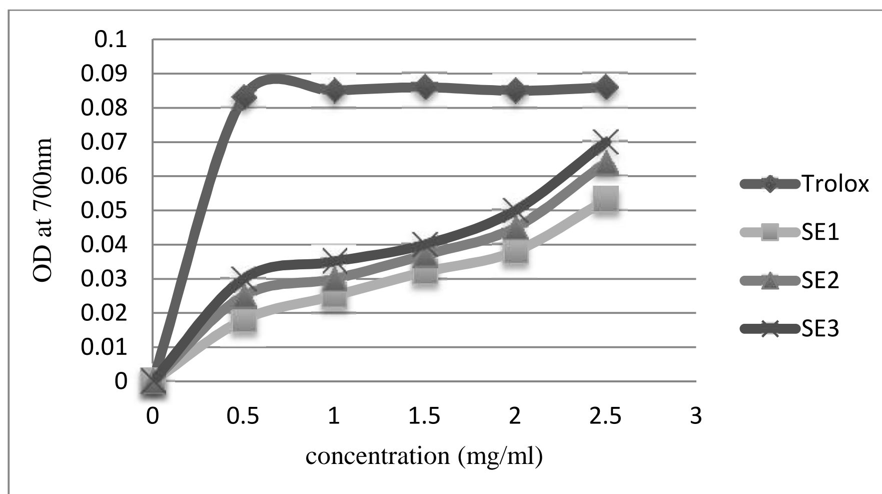
FIGURE 4



Reducing power Assay

The reducing activity of sugarcane extracts is based on reduction of Fe^{3+}/ferricyanide complex to Fe^{2+}ferrous complex monitored by measuring the formation of perl's Prussian blue at 700nm. Reducing potential of all sugarcane extracts indicated increased reducing power ability in dose dependent manner. SE3 sample showed highest reducing power potential is shown in Fig. 5.

FIGURE 5



DISCUSSION

Nucleotide and nucleoside are of a rich diet source and have many associated health benefits, such as effect of nucleotides on the immune system (Sanchez-Pozo, 2002, Lee *et al*., 2007 and Abtahi *et al*., 2011). However, there are many additional benefits to dietary nucleic acids that have not been widely publicized. Supplementary nucleotides can promote the neutralization of internal toxins. It can aid in the detoxification process in two ways, first of all, they stimulate liver action, keeping the liver healthy and promoting its natural action in neutralizing toxins. Secondly, the uracil or its derivatives can act as a powerful antioxidant, removing free radicals and protecting the body from oxidative damage (Akhatova *et al.,* 2011). Synthesized dietary sources have many side effects. Researchers have proved that nucleotides rich natural sources like plant parts, mushroom, yeasts, chlorella, sugarcane components can be beneficial for various sicknesses (Tibbets, 2002 and Weinberg *et al.,* 2011). In continuation with the previous studies, the sugarcane extracts are good sources of uridine and its derivatives (Sonkawade *et al.*, 2012). It's surprising and interesting that sugarcane components have various beneficial physiological functions. These functions are very useful and make these extracts effective as ingredients in functional foods, health foods, and functional animal feed (Walter, 2012).

In the present investigation, the bioactivity studies of sugarcane extracts were investigated. The results include, total phenolic content, total flavonoids content, DPPH radical scavenging activity, ABTS, FRAP, SOD, reducing power and ORAC assay. The antioxidant activity of phenolic compounds is mainly due to their redox properties and chemical structure, which allow them to act as reducing agent, hydrogen donators and singlet quenchers, and chelating transitional metals, inhibiting lipoxygenase and scavenging free radical. Folin- Ciocalteu reagent was used to determine total polyphenols in samples (Gulcin, 2012). The total phenolic and flavonoid contents in sugarcane extract were found to be directly proportional to their antioxidant effects as shown in Table 1. The phenols and flavonoids are potent scavengers of peroxyl radicals and hence exhibit inhibition of peroxyl radical induced PE oxidation in ORAC assay (Josheph *et al*., 2006). The ORAC values are used as standard measures for comparing the antioxidant activity of food materials. Our results show that values of sugarcane extracts ranging from 602±3.7-1236±4.4 (µg TE/g) are similar to or higher than the values observed for some fruits and vegetables (Dudonne *et al*., 2009).

Several studies have shown that the higher antioxidant activity associated with sugarcane plant is attributed to the total phenolic compounds (Jian *et al.,* 2014; Kadam *et al*., 2007; Mauricio *et al*., 2006 and Owino *et al*., 2014). The higher phenolic concentration corresponds to greater antioxidant capacity. This hypothesis is in accordance with the data reported by many researchers who have proved high correlations between phenolic content and antioxidant activities in different plants such as herbs, fruits (Sreeramulu *et al.* 2013). The antioxidant capacity of the sugarcane extract is also studied by the free radical scavenging methods DPPH, ABTS and FRAP and was proven to be high. Generally, high content of antioxidants obtained by the FRAP method corresponds with the high content of antioxidants determined by the $ABTS^{+}$ method (Grabek-Lejko *et al.,* 2013). Superoxide radical ($O_2^{\cdot -}$) called as hyperoxide are ROS generated during normal physiological process mainly in mitochondria during cellular respiration. Although superoxide radicals are weak oxidants but they give rise to powerful and dangerous hydroxyl radicals as well as singlet oxygen, which induce oxidative damage in biomolecules. Reducing power is one mechanism of action of antioxidants and may serve a significant indicator of potential antioxidant activity. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Vinutha *et al.,* 2014).

The results obtained in this study showed the high antioxidant activity of sugarcane extracts (Figs. 1-5) and

suggest they can be used against diseases arising from free radicals. The highest activity was found in SE3. Finally, the results in this study indicate that the examined sugarcane extracts contain certain amounts of polyphenols and flavonoids, proving them to be perfect sources of antioxidants. It is difficult to compare the results obtained in this work with those available in the literature because researchers use different methods to estimate antioxidant capacity, using different standards, incubation time, concentrations, etc. It was, however, confirmed that food found to display a high total antioxidant capacity using one antioxidant assay will most likely also be found to display a high antioxidant capacity using another assay (Carlsen *et al.,* 2010).

CONCLUSION

Sugarcane extracts rich in uridine and its derivatives have the ability to quench oxidative radicals and thereby reducing oxidized stress. They contain highest amount of phenolic compounds exhibited through effective radical scavenging activity and reducing power. As the plant extracts were quite safe and the use of synthetic antioxidant was limited because of their toxicity, therefore these sugarcane extracts can be exploited as antioxidant additives. Altogether, the current results support the notion that natural phenolics and antioxidants present in sugarcane extract could be a useful alternative therapy for relative oxidative stress

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