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IN VITRO STUDIES OF VARIOUS EXTRACT OF *ENSETE SUPERBUM* FOR THEIR ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY

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ABSTRACT

The present study was designed to evaluate the in-vitro antioxidant potential of various extract of Ensete superbum. The total phenolic and flavonoids content was determined in the ethanolic and aqueous extract. The plant extracts exhibited strong antioxidant and free radical scavenging activity on DPPH free radical, ABTS radical cation, scavenging of hydrogen peroxide, hydroxyl radical and superoxide radical. The antioxidant and free radical scavenging activities of the extracts were comparing to standard ascorbic acid. The extracts had good phenolic and flavonoid content. The result of present study is used to suggest a potential utility of the plant as a source of phenolic antioxidants and may provide leads in the ongoing research for natural antioxidants from Indian medicinal plants to be used in treating diseases related to free radical mediated reactions.

***Keyword**: Ensete superbum, Antioxidant, Total phenolic assay, Total flavonoid assay.*

1. INTRODUCTION

The Free radicals are responsible for chronic disorders in humans, including diabetes, cancer, atherosclerosis, arthritis, jaundice, hepatic injury, central nervous system injury, gastritis and AIDS [1, 2]. It was generated due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, which cause depletion of the antioxidants immune system, change in gene expression, and induce abnormal proteins. The most likely and practical way to fight against degenerative diseases is to improve body antioxidant status, which could be achieved by higher consumption of vegetables and fruits[3]. The antioxidants play an important role in preventing oxidative deterioration of food and indirectly eliminating radicals[4]. Oxidative atmosphere in cell is also created by the impairment in functioning of endogenous antioxidant enzymes namely superoxide dismutase, glutathione peroxidase, and catalase. These enzymes are known to be inhibited in diabetes mellitus as a result of oxidation and non-enzymatic glycosylation. Antioxidants are protective agents that inactivate reactive oxygen species, and thereby significantly delay or prevent oxidative damage[5]. Flavonoids are a group of polyphenolic compounds, which include free radical scavenging, inhibition of hydrolytic, oxidative enzymes, and antiinflammatory actions. Some evidence suggests that the biological actions of these compounds are related to their antioxidant activity. In the last two decades, there has been an explosive interest in the role of oxygen-free radicals, more generally known as "reactive oxygen species," (ROS) and of "reactive nitrogen species" (RNS) in experimental and clinical medicine.

Ensete superbum belongs to the family Musaceae, which is being widely used as Folk and ayurvedic medicine. It is widely distributed throughout Western Ghats region of India[6]. The plant has more important medicinal uses. Medicinally *Ensete superbum* is used as potent anti-fertility agent[7]. The plant was reported to possess anti-oxidant. In this study, the fraction of *Ensete superbum* were used to perform inhibitory studies on enzymes α-amylase, α- glucosidase and hypoglycemic effects of ethyl acetate fraction of *Ensete superbum* (EAFES) on high fat fed with low dose Streptozotocin induced diabetes in rats. This evaluation is required to establish potential hypoglycemic effects in type 2 diabetes of this valuable herbal preparation. The plant species (or their active constituents) identified as having high levels of *in vitro* antioxidant activity may be of value in the design of further studies to unravel novel treatment strategies for disorders associated with free radicals-induced tissue damage.

2. MATERIALS AND METHODS

2.1. Plant materials

Ensete superbum seeds were collected from Kottakal, Malappuram Dt, Kerala, India during the month of June. It was identified and authenticated by Dr. R. Prabakaran, head of the department of botany, Vivekananda College of arts and science for women, Tiruchengode, Tamilnadu, India, and the voucher specimen was deposited at the same institute for future reference.

2.2. Extraction

The dried seeds of *Ensete superbum* were washed with distilled water separately to removed unwanted foreign materials like soil and dusts. After washed, seeds were dried under shade at room temperature. It was then coarsely grounded by using mechanical device. The powdered seed material was passed through sieve no 40 and stored in an airtight container for further use. 500 grams of shade-dried powdered were extracted using Soxhlet, successively with petroleum ether, chloroform and ethanol for 72 hr. each. The extracted were concentrated to dryness in a rotavapor.

The crude extracts were prepared 1.5 kg of shade-dried powdered seeds were extracted with ethanol in a Soxhlet extractor for 72 hr. The dried powder (50gm) was extracted with water (250ml) by maceration for seven days. All the extracts were concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (40-50°C). All the extracts were stored in a refrigerator at 4°C until further use.

2.3. Total phenolic assay

The concentration of phenolic in ethanolic and aqueous extracts of *Ensete superbum* was determined using spectrophotometric method[8]. Methanolic solution of the extracts in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared**,** containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of NaHCO3. The samples were thereafter incubated in a thermostat at 45°C for 45min. The absorbance was determined using spectrophotometer at 760 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of tannic acid and the calibration line was constructed. Based on the measured absorbance, the concentration of phenolic was read (µg/ml) from the calibration line; then the content of phenolic in extracts was expressed in terms of tannic acid equivalent (TAE) (µg of TAE/g of extract).

2.4. Total flavonoid assay

The content of flavonoids examined ethanolic and aqueous extracts of *Ensete superbum* was determined using spectrophotometric method[9]. The sample contained 1ml of methanol solution of the extracts in the concentration of 1mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at 415nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of Rutin and the calibration line was constructed. Based on the measured absorbance, the concentration of flavonoid was read (µg/ml) on the calibration line; then, the content of flavonoid in extracts was expressed in terms of Rutin equivalent (RE) (µg of RE/g of extract).

2.5. *In vitro* Antioxidant activity

The *in vitro* antioxidant activity was used different extraction of Petroleum ether extraction of *Ensete superbum* (PEES), Chloroform extraction of *Ensete superbum* (CEES), Ethanolic extraction of *Ensete superbum* (EEES), Crude ethanolic extraction of *Ensete superbum* (CEEES) and Aqueous extraction of *Ensete superbum* (AEES).

2.5.1. Diphenyl picryl hydrazyl (DPPH) radical scavenging activity

The antioxidant activities of all extracts were evaluated through free radical scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. The determination was based on the method proposed by[10]. 2 ml of 0.1mM DPPH methanolic solution was added into 200 µl of sample extracts/standard of ascorbic acid (5-1000µg/ml) and 0.8 ml methanol. The mixture was thoroughly mixed and kept in the dark for 1 hr. The control was prepared by mixing 2 ml of DPPH and 1 ml methanol. The absorbance was measure at 517 nm using spectrophotometer. Samples were measured in three replicates. Percentage of DPPH scavenging activity was calculated as following formula.

% inhibition = [Absorbance control – Absorbance sample / Absorbance control] X 100

2.5.2. ABTS radical scavenging method

Free radical scavenging activity of plant samples was determined by ABTS radical cation decolorization assay[11]. $ABTS^{\cdot+}$ cation radical was produced by the reaction between 7 mM ABTS solution and 2.4 mM potassium persulfate solution (1:1), stored in the dark at room temperature for 12-16 hr. before use. $ABTS^{\cdot+}$ solution was then diluted with methanol to obtain an absorbance of 0.700 at 734 nm. After the addition of 30µl of plant extract / standard of ascorbic acid (5-1000µg/ml) and 3ml diluted $ABTS^{\cdot+}$ solution, the absorbance was measured at 30 min after the initial mixing. An appropriate solvent blank was run in each assay. All the measurements were carried out triplicate. Percent inhibition of absorbance at 734 nm was calculated using formula.

% inhibition = [Absorbance control – Absorbance sample / Absorbance control] X 100

2.5.3. Scavenging of hydrogen peroxide (H_2O_2)

The scavenging activity of extract towards Hydrogen peroxide radicals was determined by this method[12]. 2ml of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4) and 1 ml of methanolic sample [5-1000µg/ml of extract of plant / standard of ascorbic acid] was added to hydrogen peroxide solution. The absorbance of hydrogen peroxide at 230nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The experiment was repeated in triplicate. The percentage of hydrogen peroxide scavenging by the extract and standard compound was calculated using formula.

% inhibition = [Absorbance control – Absorbance sample / Absorbance control] X 100

2.5.4. Hydroxyl radical scavenging activity *p*-NDA method

Various concentration of the extract / Standard of ascorbic acid (5-1000µg/ml) in 0.5 ml of distilled DMSO were added to a solution mixture containing 0.5 ml of ferric chloride (0.1 mM), 0.5 ml of EDTA (0.1 mM), 0.5 ml of ascorbic acid (0.1 mM), 0.5 ml of hydrogen peroxide (2 mM) and 0.5 ml of *p*-NDA (0.01 mM) in phosphate buffer (pH 7.4, 20 mM) to produce a final volume of 3 ml. Absorbance was measured spectrophotometrically at 440 nm. All the measurements were carried out triplicate. Percentage inhibition was calculated[14].

% inhibition = [Absorbance control – Absorbance sample / Absorbance control] X 100

2.5.5. Superoxide radical scavenging activity by alkaline DMSO method

In this method, superoxide radical is generated by the addition of sodium hydroxide to air saturated DMSO. The generated superoxide remains stable in solution and reduces nitroblue tetrazolium (NBT) into formazan dye at room temperature which can be measured at 560 nm. Briefly, 0.1 ml of NBT (1 mg/ml) was added to the reaction mixture containing 1 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml water) and 0.3 ml of the extract / standard of ascorbic acid (5-1000µg/ml) in freshly distilled DMSO at various concentrations, to give a final volume of 1.4 ml. The absorbance was measured at 560 nm. Percentage inhibition was calculated[15].

% inhibition = [Absorbance control – Absorbance sample / Absorbance control] X 100

2.6. Statistical analysis

All determinations for *in vitro* study were carried out in triplicate and the values are expressed as mean ± SEM and inhibitory concentration (IC_{50}) was carried out with GraphPad Prism.

3. RESULTS AND DISCUSSION

The total phenolic content of *Ensete superbum* of crude ethanolic and aqueous extract were obtained using the regression calibration curve Y=0.0058X-0.0425, R2=0.998 and is expressed as tannic acid equivalents. Total phenol content of the ethanolic extract of *Ensete superbum* (EEES) was found to be 299µg/ml and aqueous extract of *Ensete superbum* (AEES) was found to be 275µg/ml respectively (Figure 1). Total phenolic content was high in ethanolic extract compared to the aqueous extract. It shows the ethanolic extract of *Ensete superbum* posse's high antioxidant ability.

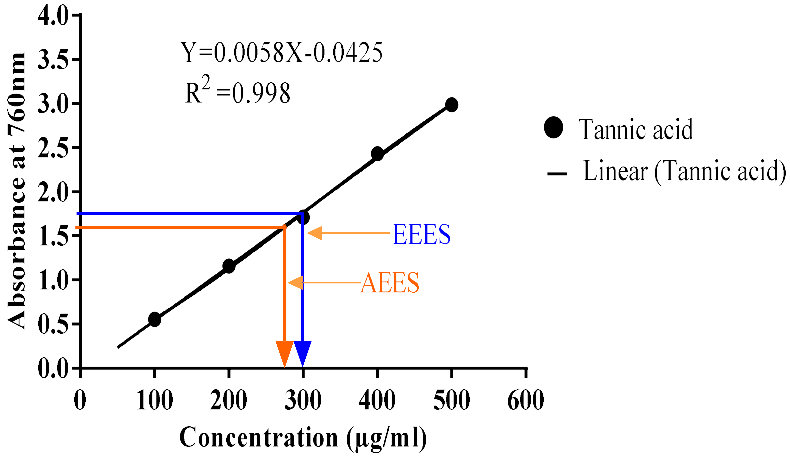

Figure 1: Total Phenolic content

The total flavonoids content was obtained using the regression calibration curve Y=0.0012X-0.042 with Rutin equivalent. The Total flavonoid content of the ethanolic extract was found to be 304µg/ml and aqueous extract was found to be 222µg/ml respectively (Figure 2). Total flavonoid content was high in ethanolic extract compared to the aqueous extract.

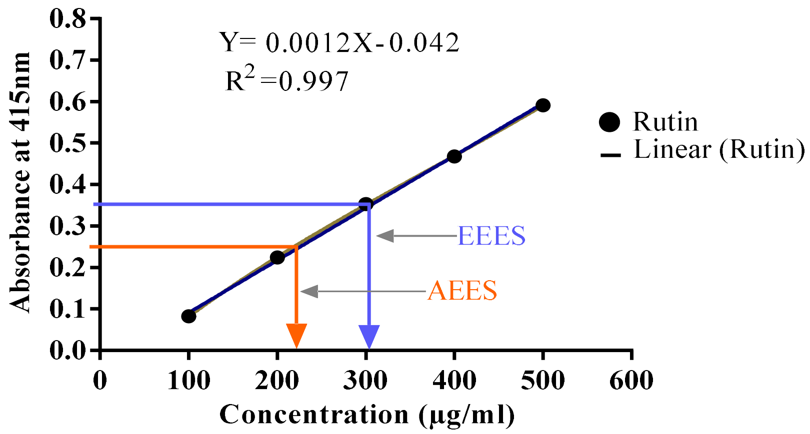

Figure 2: Total Flavonoid Content

In the last two decades, there has been an explosive interest in the role of oxygen free radicals, more generally known as "reactive oxygen species" (ROS) and of "reactive nitrogen species" (RNS) in experimental and clinical medicine[16]. Beneficial effects of ROS involve physiological roles in cellular responses, as for example in defense against infectious agents and in the function of a number of cellular signaling systems. One further beneficial example of ROS at low concentrations is the induction of a mitogenic response. In contrast, at high concentrations, ROS can be important mediators of damage to cell structures, including lipids membranes, proteins and nucleic acids termed as oxidative stress[17]. Reactive oxygen species and reactive nitrogen species such as superoxide anions, hydroxyl radical and nitric oxide inactivate enzymes and damage intracellular components causing injury through covalent binding and lipid peroxidation. Antioxidants are compounds that hinder the oxidative processes and thereby delay or prevent oxidative stress[18].

Table - 1: *In vitro* antioxidant activity of *Ensete superbum*

Extract	IC_{50} values ± SE (µg/ml) by methods*				
	DPPH	ABTS	H_2O_2	*p*-NDA	Alkaline DMSO
Pet Ether	378.4±3.66	241.2±2.64	268.0±0.91	292.5±4.11	316.2±3.36
Chloroform	318.8±2.33	237.0±2.80	261.2±1.66	189.6±1.44	315.8±3.93
Successive Ethanol	217.1±1.95	167.3±2.10	149.2±1.28	150.8±1.50	158.0±1.14
Crude Ethanol	203.6±2.24	165.1±2.71	161.1±2.15	145.5±1.48	155.8±2.15
Crude Aqueous	234.5±2.84	234.3±2.18	252.6±2.06	192.4±1.30	167.1±0.43
Standard	191.6±2.02	138.3±2.47	150.7±1.80	141.6±3.16	141.9±1.26

***Average of three determinations; Data are expressed as mean ± SEM**

The antioxidant activity of DPPH method, the ethanolic extract of *Ensete superbum* showed good antioxidant activity. The IC_{50} values were found in PEES 378.4±3.66, CEES 318.8±2.33, EEES 217.1±1.95, CEEES 203.6±2.24, AEES 234.5±2.84 and standard ascorbic acid is 191.6±2.02 respectively (Table 1 and Figure 3). The DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants[19]. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[20]. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. The experimental data of the extracts revealed that the extracts are likely to have the effects of scavenging free radicals. From the result, we observe that a dose dependent relationship in the DPPH radical scavenging activity.

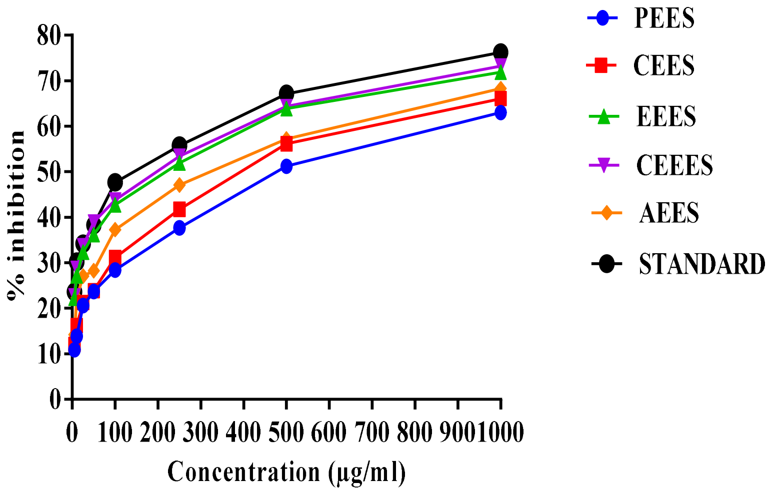

Figure 3: DPPH radical scavenging activity

In the ABTS method, the extracts showed the potent radical scavenging activity in concentration dependent manner in all the extracts. The IC_{50} values were found in PEES 241.2±2.64, CEES 237.0±2.80, EEES 167.3±2.10, CEEES 165.1±2.71, AEES 234.3±2.18 and standard ascorbic acid is 138.3±2.47 respectively (Table 1 and Figure 4). ABTS radical scavenging activity is relatively, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS for the estimation of antioxidant activity[21]. Here, the extracts radical scavenging activity showed a direct role of its phenolic compounds in free radical scavenging.

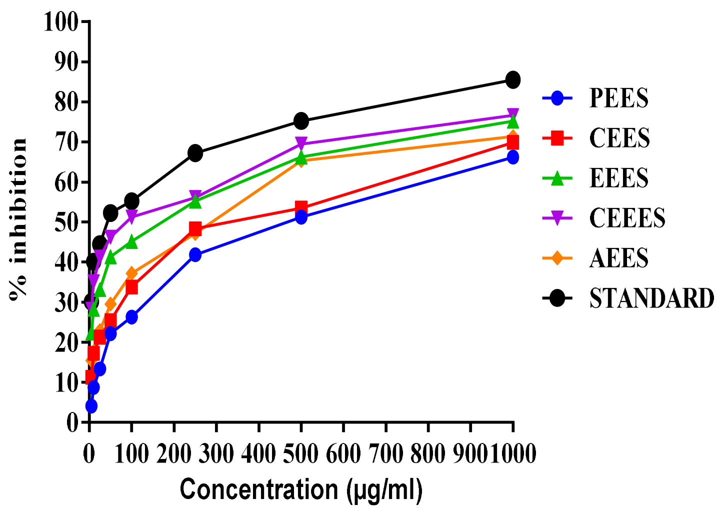

Figure 4: ABTS radical scavenging activity

Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive species. The superoxide radical is known to be produced *in vivo* and can result in the formation of hydrogen peroxide via dismutation reaction. Moreover, the conversion of superoxide and hydrogen peroxide into more reactive species. Hydrogen peroxide itself is not very reactive, but sometimes is toxic to cell because it may give rise to hydroxyl radical in the cells[22]. Therefore, removing of hydrogen peroxide is very important for antioxidant defense in cell system. The ethanolic extracts EEES showed near IC_{50} value 149.2±1.28 compared to standard ascorbic acid IC_{50} value of 150.7±1.80 and the extracts IC_{50} values were found in PEES 268.0±0.91, CEES 261.2±1.66, CEEES 161.1±2.15 and AEES 252.6±2.06 respectively (Table 1 and Figure 5).

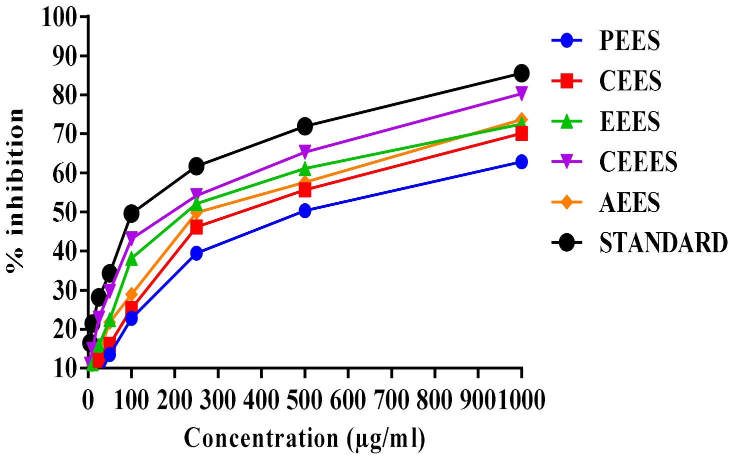

Figure 5: Scavenging of hydrogen peroxide

In the p-NDA method, among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules[23]. In p-NDA method, the hydroxyl radical is generated through Fenton reaction. In this reaction, iron-EDTA complex reacts with hydrogen peroxide in presence of ascorbic acid to generate hydroxyl radical which can bleach p-NDA specifically. The extracts show potent scavenging activity by inhibition of bleaching of p-NDA. The crude ethanolic extracts showed near IC_{50} value 145.5±1.48 compare to standard ascorbic acid IC_{50} value of 141.6±3.16. The extracts of in PEES 292.5±4.11, CEES 189.6±1.44, EEES 150.8±1.50 and AEES 192.4±1.30 and standard ascorbic acid is respectively (Table 1 and Figure 7). The scavenging activity may be due to the presence of various phytochemical including polyphenols and flavonoids in the extracts.

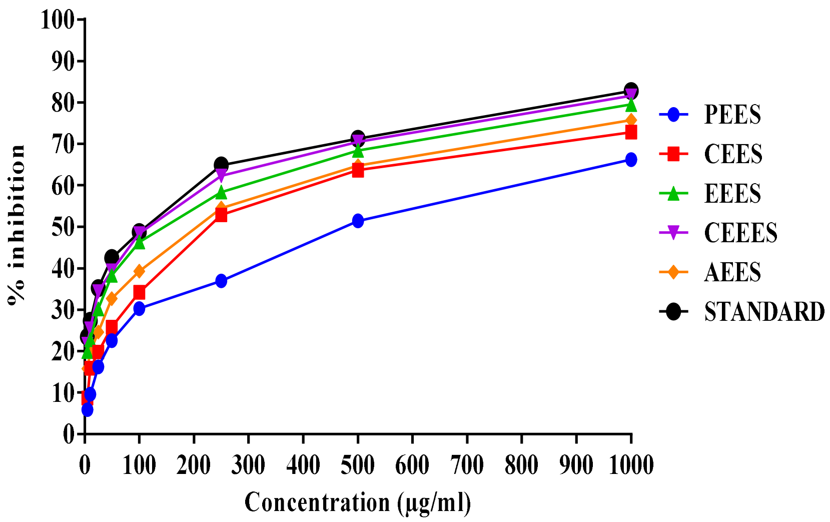

Figure 7: Hydroxyl radical scavenging activity *p*-NDA method

The alkaline DMSO method, the extracts are moderately inhibited the superoxide radical generation. The IC_{50} values were found in PEES 316.2±3.36, CEES 315.8±3.93, EEES 158.0±1.14, CEEES 155.8±2.15, AEES 167.1±0.43 and standard ascorbic acid is 141.9±1.26 respectively (Table 1 and Figure 8). Therefore, the phenolic compounds of extracts may be involved in scavenging hydrogen peroxide. The extracts are found to be an efficient scavenger of superoxide radical generated in alkaline DMSO system. The result clearly indicates that the plant extracts have a noticeable effect as scavenging superoxide radical.

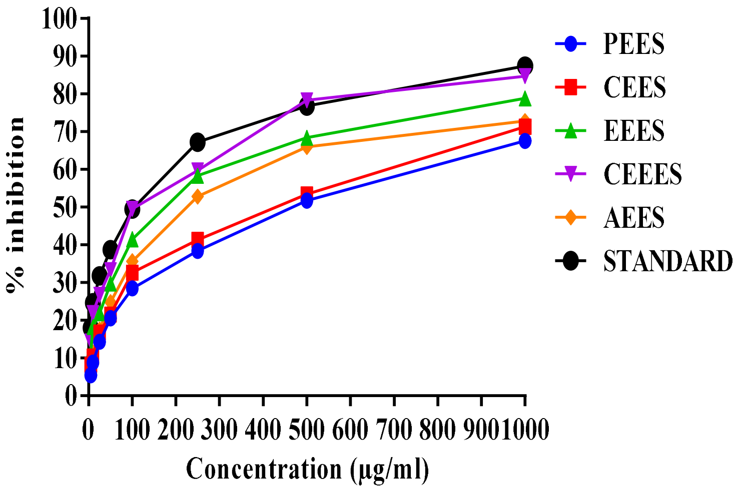

Figure 8: Superoxide radical scavenging activity by alkaline DMSO method

4. CONCLUSION

The results from various free radicals scavenging system reveal that extracts of *Ensete superbum* have significant antioxidant activity. IC_{50} values obtained were comparable with that of the standard of ascorbic acid and crude ethanolic extract of *Ensete superbum* showed relatively more antioxidant activity than other extracts. Since free radicals are different chemical entities, it is essential to test the extracts against many free radicals prove their antioxidant activity. However, the difference in the activity of extracts may be due to the different chemical entities of the free radicals. In this study, significant linear relationship was found between the antioxidant activity and total phenol and flavonoid contents, indicating these compounds could be major contribution to antioxidant activity. Further phytochemical compounds and ensure the medicinal properties of the plant *in vivo* studies.

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