

In Vitro Antioxidant Profile of Methanol Leaf Extract of *Securidacalongepedunculata*

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Abstract: The serious problem associated with the use of plants parts for medicinal purposes is the paucity of information on the phytochemistry, pharmacology, antioxidant contents and toxicity of most of the medicinal plants. The aim of this study is to evaluate the antioxidant property of the leaves of *S. longepedunculata* with a view to elucidating its antioxidant potential. The potent secondary metabolites in the leaves of *Securidacalongepedunculata*, were extracted and the antioxidant potential, evaluated using in vitro methods. Results showed that the methanol extract scavenged 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) in a concentration dependent manner with a correlation coefficient (R^2) of 0.976, indicating antioxidant activity with effective concentration that inhibits 50 percent of the radicals (EC_{50}) of $90.02 \pm 0.2 \mu\text{g/ml}$ compared to ascorbic acid standard (EC_{50} of $98.01 \pm 0.2 \mu\text{g/ml}$). Superoxide radical scavenging activity was concentration dependent with an EC_{50} of $350.11 \pm 0.42 \mu\text{g/ml}$ compared with ascorbic acid standard with EC_{50} of $812.97 \pm 0.97 \mu\text{g/ml}$. The extract, also showed hydroxyl radical scavenging activity with an EC_{50} of $83.74 \pm 0.02 \mu\text{g/ml}$ compared to α -tocopherol standard with EC_{50} of $54.16 \pm 0.01 \mu\text{g/ml}$. There was an inverse correlation between the percentage inhibition and concentration with R^2 of -0.958. The methanol extract, also scavenged nitric oxide radical in a concentration dependent manner with 500 $\mu\text{g/ml}$ being more effective than 500 $\mu\text{g/ml}$ of ascorbic acid standard. Comparison of the anti-radical power (ARP) of DPPH (0.011), superoxide radical (0.003) and hydroxyl radical (0.012) of the extract revealed that the ARP of hydroxyl radical was most efficacious. The antioxidant vitamin contents of the extract showed that vitamin C was significantly high ($p < 0.05$), ($4.62 \pm 0.14 \text{ mg/100g}$) when compared to vitamin A ($0.902 \pm 0.05 \mu\text{g/g}$) and vitamin E ($1.474 \pm 0.01 \text{ mg/100g}$). This result suggests a moderate antioxidant activity which is healthy for a biological system to reduce the deleterious effects associated with excessive free radical generation.

Keywords: Phytochemistry, pharmacology, antioxidant contents and toxicity

I. Introduction

Antioxidants are substances that, when present in low concentrations, compared to that of an oxidizable substrate, significantly delay or prevent oxidation of the substrate (Halliwell and Gutteridge, 1989). Two classes of antioxidants are known. They include; the low molecular weight compounds such as vitamins E and C, beta-carotene, glutathione, uric acid and bilirubin, proteins such as albumin, transferrin, caeruloplasmin, ferritin, superoxide dismutase, glutathione peroxidase and catalase (Psotova et al., 2001). Typical phenolics that possess antioxidant activity have been characterized as phenolic acid and flavonoids (Kahkonen et al., 1999). The increasing awareness by consumers to issues regarding food additive safety, results in an enhanced effort in finding alternative additives and preservatives from natural and probably safer sources. Synthetic antioxidants are disadvantageous due to their side effects in vivo (Balasundram et al., 2006). In this regard, food manufacturers have been encouraged to use natural antioxidants instead of synthetic

compounds to maintain the nutritional values of their products. For instance, commercial antioxidants such as butylatedhydroxytoluene (BHT) and butylatedhydroxyanisole (BHA) can be replaced by plant extracts particularly polyphenols (Balasundram et al., 2006). Antioxidants usually prevent food oxidation, normally initiated by free radicals formed during the food's exposure to environmental factors such as air, light and temperature (Hrasnet al., 2000). Reactive oxygen species (ROS), which consist of free radicals such as hydroxyl radical (OH[•]), superoxide radical (O₂^{•-}), Nitric oxide (NO[•]), peroxy (RO₂[•]), lipid peroxy (LOO[•]) radicals and non-free radical species such as hydrogen peroxide (H₂O₂), singlet oxygen (O₂⁻¹), ozone (O₃) and lipid peroxide (LOOH), are different forms of activated oxygen (Halliwell et al., 1999; Yildrinet al., 2000; Gulcinet al., 2002). ROS are produced by all aerobic organisms and can easily react with proteins, lipids, lipoproteins and DNA, leading to an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily repair the resulting damage (Halliwell, 2007). During times of environmental stress, systemic ROS levels can increase dramatically. Cumulatively, ROS could lead to many pathophysiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity (Kourounakiset al., 1999; Gulcin and Oktay, 2002). Generation of free radicals during metabolism, givesrise to oxidative stresswhich could play a role in heart diseases, neurodegenerative diseases, cancer and in aging process (Astley, 2003). Antioxidants oppose these effects, thus lowering the risk of diseases (Atoui, 2005). Securidacalongepedunculata Linn belonging to the family of polygalaceae, is a spiny semi-deciduous shrub that grows to about 12m tall (Owoyeleet al., 2006). Eaten as vegetable, *S. longepedunculata* was reported to have analgesic, anti-inflammatory and hypoglycemic potentials (Ajiboyeet al., 2006), and is highly efficacious in treating malaria and rheumatism (Gill, 1992). It is also used in bacterial chemotherapy (Akinniyiet al., 1996; Mohammed et al., 2012). The plant is also used in the treatment of cough, headache, constipation, wound, sore throat and gout (Ojewale, 2008). Its anti-snake venom potential has also been reported (Wannaget al., 2005). Some secondary metabolite contents such as alkaloids and flavonoids and related compounds such as methyl salicylate has been reported (Iddagoda and Thamara, 2003; Abdullahi and Lawal, (2010).

II. Materials and Methods

Collection of plant sample and Extraction of Securidacalongepedunculata Constituents

The plant was harvested in a natural habitat at Nsukka, Enugu State Nigeria and identified by Alfred Ozioko of the Bioresources Diversity and Conservative Programme (BDCP) Nsukka. After shade drying, one hundred and fourteen (114g) of the leaves were weighed after pulverizing, soaked with 80% methanol for 24hrs and filtered with Whatman filter paper no 1. The filtrate was concentrated using rotary evaporator, and the solid extract stored in freezer at 4 °C till used.

Chemicals

All chemicals used in this study were of analytical grade and products of Sigma Aldrich, (USA), British Drug House (BDH) (England), Burgoyne, (India), Harpkin and Williams, (England), Qualikems (India), Fluka (Germany), May and Baker, (England)

Quantitative 1,1-diphenyl-2-picrylhydrazyl(DPPH) Radical Scavenging Assay

The scavenging activity on DPPH free radical by the extract was tested using the method of Gyamfi, et al., (1999) with the following modifications. One milliliter (1ml) of the extract at different concentrations (1.25-160µg/ml) was added followed by addition of 0.5 ml of 0.076 mM DPPH. The solution was made up to 2 ml by 80% methanol and the solution was

allowed to stand at room temperature in the dark for 30 min. The negative control was 0.076 mM DPPH[·] in methanol. L-ascorbic acid was used as the positive control. Thereafter, the absorbance of the assay mixtures was read using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Huston Texas) at 517 nm using DPPH[·] radical scavenging activity was calculated using the equation;

$$\% \text{ scavenging activity} = 100 - \left(\frac{\text{Abs of Sample}}{\text{Abs of Control}} \times 100 \right)$$

Abs= absorbance

The effective concentration at 50% inhibition (EC₅₀ value was calculated using a plot of % inhibition against different concentrations of the extract and it represented the concentration of the sample leading to 50% reduction in the initial DPPH[·] concentration. Triplicate values were taken.

Hydroxyl Radical (OH[·]) Scavenging Assay

The 2-deoxy ribose assay was determined using the method of Halliwell et al., (1987). Each reaction mixture contained the following final concentrations of reagents in a final volume of 1ml: 2-deoxyribose (2.5 μM), Potassium phosphate buffer (20 mM pH 7.4), FeCl₃ (100 μM), EDTA (104 μM), H₂O₂ (1mM), and L-ascorbic acid (100 μM). Solutions of FeCl₃ and L-ascorbic acid were prepared immediately before use in distilled water. The mixtures were incubated for 1hr at 37⁰C, followed by addition of 1ml of 1% (w/v) thiobarbituric acid (TBA) in 0.05M NaOH and 1ml of 2.8% trichloroacetic acid (TCA). The resulting mixtures were heated for 15 minutes at 100⁰C. After cooling on ice, absorbance was read at 532 nm. Inhibition of 2-deoxyribose degradation expressed in percentage, was calculated using the following equation:

$$\% \text{ inhibition} = \frac{A_o - A_s}{A_o} \times 100$$

A_o= absorbance of the control

A_s = absorbance of the test sample

The IC₅₀ value was estimated using a plot of % inhibition against different concentrations of the extract which represented the concentration of the sample leading to 50% inhibition of degradation of 2-deoxy ribose. Triplicate values were taken.

Superoxide Radical Scavenging Assay

Superoxide radical scavenging assay was performed according to the methods of Beauchamp and Fridovich, (1971) and Martinez et al. (2001). This was based on the capacity of the extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). Briefly, each 3.0 ml reaction mixture contained 0.05 M Phosphate-buffered saline (PBS) (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, NBT (75 μM) and 1.0 ml of test sample solution (10-250 μg/ml). The entire reaction assembly was enclosed in a box lined with aluminum foil. The test tubes were kept in front of a fluorescent light and absorbance was read at 560 nm after 20min. The percentage inhibition of superoxide generation was obtained by comparing the absorbance of the control and those of the reaction mixture containing test sample as per the equation:

$$\% \text{ inhibition} = \frac{A_o - A_s}{A_o} \times 100$$

Where A_o= absorbance of the control

A_s= absorbance of the test sample

Triplicate values were taken.

In Vitro Nitric Oxide Radical (NO[•]) Scavenging Assay

Nitric oxide radical (NO[•]) generated from sodium nitroprusside (SNP) was measured according to the method of Marcocci et al. (1994). Four different test tubes were set up. Test tube one (T₁) contained 8 ml of sodium nitroprusside (SNP) mixture (5 mM SNP in phosphate buffered saline (PBS) pH 7.3).

Test tube two (T₂) contained 4 ml of extract (500µg/ml) + 4 ml SNP in PBS. Test tube three (T₃) contained 4 ml extract (250µg/ml) + 4 ml SNP in PBS and Test tube four (T₄) contained 4 ml ascorbic acid standard (500µg/ml) + 4 ml SNP in PBS. The mixtures were incubated at 25°C for 180 minutes in front of a fluorescent tube. The NO radical generated in each test tube photochemically interacted with oxygen to produce the nitrite ion (NO₂⁻) which was assayed using a spectrophotometer at 30 min intervals. The standard was sodium nitrite and the standard curve was generated thus:

Different concentrations of sodium nitrite (7.78µg/ml-100µg/ml) were put in ten test tubes.

This was followed by the addition of 1.0 ml of Griess Reagent (1% Sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) to each of the ten test tubes. The absorbance of the chromophore (purple azo dye) formed during the diazotization of nitrite ions with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was measured at 546nm. The nitrite generated in the presence of the plant extract was extrapolated from the standard curve plotted between absorbance and their corresponding concentrations (nitrite ions generated from sodium nitrite).

Determination of Total Phenolic Contents

The total phenolics were determined using the method of Veligoluet al., (1998). With slight modifications. 100µl of the extract (1mg/ml) dissolved in ethanol, was mixed with 750µl of Folin-Ciocalteu's Reagent (previously diluted 10-fold with distilled water) and allowed to stand for 5 minutes. Seven hundred and fifty micro liter (750µl) of Na₂CO₃ (60 g/l) solution was then added to the mixture. The absorbance was read using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Houston Texas) at 725nm. Results were expressed as Gallic acid equivalents. Triplicate values were taken.

Determination of Flavonoids

One gram (1g) of the extract was taken and macerated with 20ml of ethyl acetate for 10 min. It was centrifuged for 5 minutes at 1000 rpm. Five milliliter (5ml) of the supernatant were each transferred into three test tubes into which 5ml of 1M ammonium hydroxide were poured and shaken vigorously for 2 to 5 minutes. The upper layers were discarded, and the absorbance of the lower layer was read using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Houston Texas) at 470nm. The blank was 1N ammonia solution. Flavonoid calculation was done using standard curve. The standard curve was prepared in the same way the test sample was prepared. The absorbance of the standard was plotted against the concentration of the standard flavonoid. The flavonoid was quantified using the following:

$$\frac{\text{Abs of Sample}}{\text{Slope (Standard)}} \times \text{dilution factor}$$

Triplicate values were taken.

Determination of Tannin

One gram (1g) of the extract was macerated with 20ml of methanol for 10min. It was centrifuged for 5 min at 1000 rpm. The supernatant (2ml) were poured into three test tubes. Methanol, (3ml) were each poured into the test tubes followed by 0.3ml of 0.1M ferric chloride in 0.1M HCl and then mixed thoroughly. To the mixture, was added 0.3ml of 0.0008M potassium ferricyanate, and mixed. The absorbance of the solutions in each of the tubes were read against the blank using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Huston Texas) at 750nm after 5 min, but not exceeding 30 min. The concentration of tannin was extrapolated from the standard curve (prepared as described earlier). Triplicate values were taken.

Statistical Analysis

Data were reported as means \pm SEM, where appropriate. One-way analysis of variance (ANOVA) and correlation analysis were used to analyze the experimental data and Duncan multiple test range was used to compare the group means obtained after each treatment with control measurements. Differences were considered significant when $P \leq 0.05$.

III. Results and Discussion

Effect of Methanol Extract of Securidacalongepedunculata (MESL) on DPPH Radical Scavenging Activity

The quantitative DPPH analysis revealed DPPH radical scavenging activity of the plant part. The percentage inhibition was concentration dependent with an effective concentration at 50% (EC_{50}) of $90.02 \pm 0.02 \mu\text{g/ml}$ compared to ascorbic acid standard with EC_{50} of $98.01 \pm 0.02 \mu\text{g/ml}$. (Table 1) The percentage inhibition against concentration showed a statistically strong significant positive correlation ($P \leq 0.01$), correlation coefficient (R^2) of 0.976. It, also, confirmed the ability of the extract to donate hydrogen atom to the stable free radical. The addition of the extract showed a progressive decrease in absorbance. DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerize, like most other free radicals (Sagar and Singh, 2011). On mixing DPPH solution with a substance that can donate a hydrogen atom, it gives rise to the reduced form (Sagar and Singh, 2011). The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine (Rajesh and Natvar, 2011). Besides, cysteine, glutathione, ascorbic acid, tocopherol, poly-hydroxyl aromatic compounds reduce DPPH by their hydrogen donating ability (Blois, 1958). The extract has ascorbic acid (vitamin C) and protein (cysteine might be one of amino acids that make up the sequence of the concerned protein found in the extract). These and other related compounds found in the extract might be responsible for the quenching of the DPPH radical.

Table 1 Inhibition of DPPH radical by MESL

Concentration ($\mu\text{g/ml}$)	Percentage Inhibition (%) ⁿ
9	83.17 \pm 0.00
19	84.62 \pm 0.01
30	85.58 \pm 0.00
42	90.22 \pm 0.00
54	91.03 \pm 0.01
68	93.59 \pm 0.00
83	94.23 \pm 0.00

EC₅₀ of extract (µg/ml) is 90.02±0.02 compared to the standard
EC₅₀ (µg/ml) of ascorbic acid standard (98.01±0.02)
n= 3 (readings were determined at triplicates)

Effect of MESL on Hydroxyl Radical (OH·) Scavenging Activity

The extract showed scavenging activity against hydroxyl radical (OH·) with an inverse relationship between the extract concentrations and % inhibition. The percentage inhibition against concentration showed a statistically significant ($P \leq 0.05$) negative correlation with correlation coefficient (R^2) of -0.958. The EC₅₀ of the extract was 83.74±0.02 (µg/ml) as compared to 54.16±0.01 (µg/ml) of α-tocopherol standard (Table 2). Hydroxyl radical is a reactive oxygen species and it is a form of activated oxygen (Halliwell and Gutteridge, 1999). Its anti-radical power is 0.012 compared to 0.003 of superoxide. This suggested a stronger scavenging activity of the extract on hydroxyl radical compared to the extract's ability to scavenge superoxide radical. Hydroxyl radicals were generated using ascorbic acid-iron EDTA (Jayabalan, et al; 2007). Hydroxyl radical is an extremely reactive species formed in biological systems and has been implicated as highly damaging in free radical pathology, capable of damaging almost every molecule found in living cells (Hochstein and Attallah, 1988). This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity (Kappus, 1991). Hydroxyl radical is considered to be one of the quick initiators of lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids (Kappus, 1991). Polyphenolic compounds are known to have antioxidant activity and the activity of the extract in scavenging hydroxyl radical could be due to these polyphenolic compounds. This activity was believed to be mainly due to their redox properties which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Ali et al, 2010).

Table 2 Inhibition of OH radical by MESL

Concentrations (µg/ml) Percentage Inhibition (%) ⁿ

5	90.24±0.00
10	85.37±0.00
25	65.00±0.01
50	63.41±0.01
125	34.15±0.02
EC ₅₀ (µg/ml)	83.74±0.02

EC₅₀ (µg/ml) of the extract is 83.74±0.02 as compared to the standard

EC₅₀ (µg/ml) of α-tocopherol (standard) = 54.16±0.01

n= 3 (triplicates)

Effect of the Extract(MESL) on Superoxide Radical Scavenging Activity

There was a moderate superoxide radical scavenging. The percentage inhibition against concentration showed a statistically significant ($P \leq 0.01$) positive correlation with correlation coefficient (R^2) of 0.993. The EC₅₀ of the extract was 350.11±0.42(µg/ml) while that of ascorbic acid standard was 812.97 ± 0.97 (µg/ml) (Table 3). In cellular oxidation reactions, superoxide radicals are normally formed and their effects can be magnified because they produce other kinds of cell-damaging free-radicals and oxidizing agents. The extract inhibited the photochemical reduction of nitro blue tetrazolium (NBT) by superoxide in a

concentration dependent manner as shown in Table 3. The decreases in absorbance at 560nm with antioxidants thus indicated the consumption of superoxide anion in the reaction mixture (Palashet al, 2009). The generation of superoxide radicals was reported to be inhibited by tannins (Chung et al, 1998). Tannins were contained in the extract. This further confirmed the antioxidant property of the extract, thereby making it an agent that could ameliorate the deleterious effect of free radicals (reactive oxygen species) which are sometimes generated in vivo (Nelson and Cox, 2005). The principal chain-breaking antioxidants, in vivo, are superoxide dismutase, which acts in the aqueous phase to trap superoxide free radicals ($O_2^{\cdot-}$), urate and vitamin E, which act in the lipid phase to trap ROO radicals (Murray et al, 2006). The extract might, therefore, have a boosting effect on in vivo antioxidants.

Table 3 Superoxide radical scavenging activity of the extract

Concentrations ($\mu\text{g/ml}$)	Percentage inhibition (%) ⁿ
10	16.61 \pm 0.07
25	20.09 \pm 0.11
50	23.26 \pm 0.06
125	26.27 \pm 0.13
250	41.46 \pm 0.13

EC₅₀ ($\mu\text{g/ml}$) of extract is 350.11 \pm 0.42 compared to the standard
EC₅₀ ($\mu\text{g/ml}$) of Ascorbic acid (Standard) = 812.97 \pm 0.97
n = 3 (triplicates)

Effect of MESL on Nitric Oxide Radical Scavenging Activity

The extract, also showed nitric oxide radical scavenging activity (Fig.1). Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions (Rajesh and Natvar, 2011). Securidacalongepedunculata had higher scavenging activity at 500 $\mu\text{g/ml}$. The fluctuating nature observed at different intervals, might be as a result of steady state complex formation which tend to proceed to form nitrite ion or dissociate to form nitric oxide. This is healthy in a system where nitric oxide serves like two- edged sword. It could be beneficial or deleterious. This equilibrium point can be drawn to favour nitric oxide's (NO^{\cdot}) role in immune system integrity or to nitrite (NO_2^-) which indicated reduced production of nitrite ions. Thus, there is need for a moderate scavenging activity for nitric oxide. If this were not so, nitric oxide's role in immunity would be compromised. These different assays (DPPH, hydroxyl, superoxide and nitric oxide radicals) are needed to confirm the scavenging activity of a given extract since the antioxidant activity measured depends substantially on the test system used (Janaszewska and Bartosz, 2002). Therefore it is recommended that conclusion should be based on at least two different systems (Moon and Shinamoto, 2009).

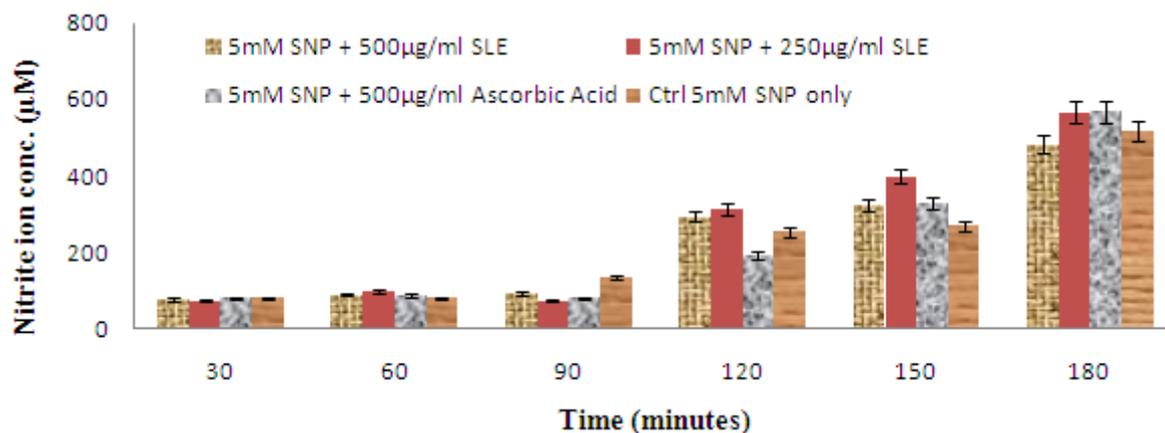


Fig.1 nitric oxide radical scavenging activity of MESL

Comparison of the Anti Radical Power of the Extract against DPPH· Superoxide Radical and Hydroxyl Radical Scavenging Activity

Anti Radical Power (ARP) is the inverse of the effective concentration of extract that can scavenge 50 % of the oxidant (EC_{50}). It shows the strength of the extract in quenching the oxidants (NO , OH , O_2 among others). The higher the ARP the more powerful the extract was in quenching the radical with respect to the method used. In this study, the ARP against Hydroxyl radical was the highest, showing that the extract had the ability to quench hydroxyl radical more than the other radicals compared (Fig. 2).

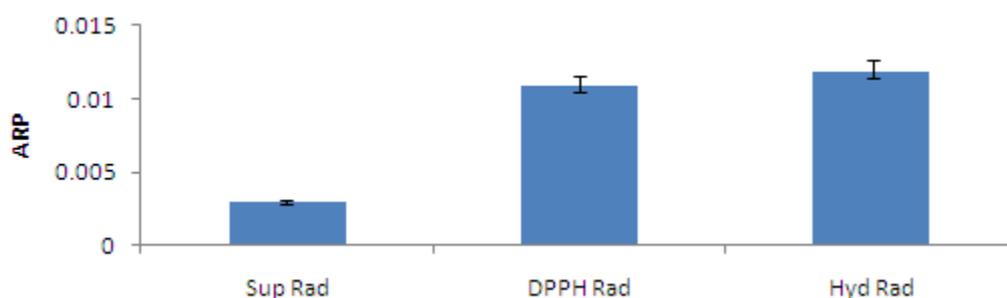


Fig.2 Different anti radical power (ARP) of the extract against the different free radicals used.

IV. Conclusion

The antioxidant property of *Securidacalongepedunculata* points to its relevance in ameliorating different deleterious effects associated with reactive oxygen species. However, there is a need to study extensively the *in vivo* antioxidant effect so as to ascertain whether what happened *in vitro* would be duplicated *in vivo* notwithstanding the moderate antioxidant effect is healthy as it would allow the system to use its free radical arsenal as well as moderate the adverse effects of over generation of free radicals.

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