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# EVALUATION OF INVITRO ANTI OXIDANT ACTIVITY ON THREE TRADITIONAL DRUGS

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**ABSTRACT**

Achyranthus aspera Linn, Moringa oleifera Lam and Scopharia dulcis Linn are used in the traditional system of medicine for the treatment of many diseases. Achyranthus aspera L (AA) belongs to the family Amaranthaceae, the whole plant and especially the roots, characterized by their uterine stimulant activity, are prescribed in the therapy of rheumatism, contusions, lumbago, osteodynia, dysuria, post-partum haematometra and dysmenorrhoea. There is no doubt that the pure Moringa oleifera L(MO) belongs to the family Moringaceae,this tree leaf is the source of incredible health benefits. It's the ultimate, natural, organic, energy and endurance health supplement. Scopharia dulcis L (SD) belongs to the family Scrophulariaceae, this plant has long held a place in herbal medicine in almost every tropical country where it grows, and it is much used by indigenous peoples. Then the antioxidant activity of the plants were evaluated by DPPH Method, Hydrogen peroxide scavenging activity, Nitric oxide scavenging activity, Antihemolytic activity, Reducing power determination methods. Three plants were estimated for their total flavonoid contents and total phenols contents. The flavonoids are a heterogeneous group of phenol compound present in the plant world. Many flavonoids, purified from medicinal plants and herbs used in the practice traditional medicine are endowed with biological effects. They include vasoprotective, anti-inflammatory, anti-allergic, anti-microbial, anti-hepatotoxic, anti-osteoporatic and anti-neoplastic. Structural aspects of antioxidant activity of flavonoid and the correlation between active oxygen scavenging and antioxidative effects of flavonoid have been studied. Flavonoids may directly scavenge some radical species by acting as chain breaking antioxidants or they may cycle other chain-breaking antioxidants such as tocopherols by donating a hydrogen atom to tocopherol radical. Transition mineral such as ferric and copper are important pro-oxidants and some flavonoids can chelate divalent metal ions, hence preventing free radical formation. And also vitamin C content present in these plants were estimated quantitatively, Vitamin C is also a good source for antioxidant activity. The plants shows significant effect on Antioxidant activity.

**KEYWORDS:** Antioxidant, Antihaemolytic, Moringaceae, Scrophulariaceae.

## 1. INTRODUCTION

Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Antioxidants (also known as free radical scavengers) function by offering easy electron target for free radicals (Padmavati P. 2004). In absorbing a free radical, antioxidants "trap" (re-energize or stabilise) the lone free radical electron and make it stable enough to be transported to an enzyme which combines two stabilized free radicals together to neutralize (Santoso U. 2004)·The oxidants / free radicals are species with very short half life, high reactivity and damaging activity towards macromolecules like proteins, DNA and lipids. These species may be either oxygen derived (ROS, reactive oxygen species) or nitrogen derived RNS, (reactive nitrogen species). The oxygen derived species include $O_2^-$ (superoxide), HO (hydroxyl), $HO_2$ (hydroperoxyl), ROO (peroxyl), RO (alkoxyl) as free radicals and $H_2O_2$ (hydrogen peroxide), HOCL (hypochlorous acid), $O_3$ (ozone) and $O_2$ (single oxygen) as non radical. Similarly, nitrogen derived oxidant species are mainly NO (nitric oxide), ONOO (peroxy nitrite, $NO_2$ nitrogen di oxide) and $N_2O_3$ (dinitrogen trioxide). This is why the body needs antioxidant compounds (Muhammad M.2005). Chemically antioxidants work by one of the following mechanisms: They donate electron, hydrogen, scavenge oxygen, scavenge free radicals.

Due to biochemical processes occurring in the body, it is normal for free radicals to be present in the body at all times. A normal healthy immune system is normally able to control the existence of free radicals and minimize their potential damage. Not all free radicals are potentially dangerous. For example, the immune system creates valuable free radicals to control and destroy virus and bacteria. Other free radicals produce vital hormones. Indeed, we need free radicals in our everyday bodily

functions. However, when the presence of free radicals increases to an abnormal level the danger begins. The danger exists in the potential for genetic material in the form of code structure to be altered in a manner that is destructive to the related Oxidation is the chemical process by which an atom, molecule or ion robs another of one or more of its electrons. Chemicals exhibiting this tendency of stealing electrons are referred to as oxidizing agents. The most familiar oxidizing agent is oxygen itself. Oxidation reactions may involve highly reactive molecules called free radicals. Free radicals can be defined as chemical species possessing an unpaired electron, which is formed by haemolytic cleavage of a covalent bond of a molecule, by the loss of a single electron from a normal molecule or by the addition of a single electron to a normal molecule. In simple words, free radicals are molecules that have lost an electron and try to replace it by reacting with other molecules (Dr. Rob Fletcher 2004).

Free radicals are considered unstable due to the existence of at least one unpaired electron. They react quickly, with other compounds, trying to capture the needed electron to gain stability (Ying, W. M 2002). Generally free radicals attack the nearest stable molecule, "stealing" its electron. When the "attacked" molecule loses its electron, it becomes a free radical itself, beginning a chain reaction. Once the process is started, it can cascade, finally resulting in the disruption of a living ce1l (Amin.I.2006). The antioxidant nutrient themselves don't become free radicals by donating an electron because they are stable in either form. Preventative antioxidants, such as catalase and superoxide dismutase, suppress the formation of free radicals. Radical-scavenging antioxidants, such as flavonoid compounds and vitamin C, serve to "mop up" excess free radicals. Vitamin E and carotenoids are very important biological antioxidants that act in both preventative and radical scavenging roles. Naturally occurring antioxidants include retinoids and tocopherols found in many animals and plants, ascorbic acid and beta-carotene, found in citrus fruit and dark green & deep orange vegetables respectively (Ying, W. M. 2002).

## 2. MATERIALS AND METHODS

### 2.1. Plant material

The leaf of Moringa oleifera Lam. And Achyranthus aspera L are available locally were collected in and around Coimbatore and Scopario dulcis L is available and collected from Palakad district in Kerala. The botanical identity has been confirmed and authenticated by the Director, Botanical survey of India, Coimbatore, No: BSI/SRC/5/23/2012-13/Tech/496. The voucher specimen has been submitted and preserved in herbarium for future reference.

### 2.2. Processing of Plant material

The plant materials were collected and shade dried at room temperature and was subjected to size reduction to get coarse powder of desired particle size. This powdered material was subjected to successive extraction. Each (1kg) powdered drugs were extracted with methanol by cold maceration method for 7 days. Then the extracts were filtered and the last traces of the solvent were evaporated under reduced pressure in a rotary evaporator to get the dry extract. The yield of the dry extracts were calculated and stored in desiccators and used for further experiments.

### 2.3. Chemical Identification test for Flavonoids

1. The methanolic extract of three plants were treated with ammonia solution. It gives red colour. It indicates the presence of Flavonoids. (Harborne J.J, 1984).
2. The methanolic extract of three plants were treated with potassium hydroxide solution. It gives canary yellow colour. This showed the presence of flavonoid. (Kokate C.K . 1998).
3. To the aqueous extract, 5 ml of dilute ammonia solution was added and followed by addition of concentrated $H_2S0_4$. A yellow colouration observed in the extract indicated the presence of flavonoid. The yellow colouration disappeared on standing.
4. A portion of the powdered plant sample was in each case heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration was observed indicating a positive test for flavonoid.

### 2.4. Estimation of total flavonoids

The flavonoid content was determined by the use of a slightly modified colorimetric method described previously by (Zhishen et al. (1999). A 0.5ml aliquot of appropriately (2mg/2ml) diluted sample solution was mixed with 2ml of distilled water and subsequently with 0.15ml of 5% NaNO2 solution. After 6 min, 0.15 ml of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, and then 2ml of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5ml, and then the mixture was thoroughly mixed and allowed to stand for another 15min. Absorbance of the mixture was determined at 510 nm versus water blank. The analysis was performed in triplicate and the results were expressed as rutin equivalent. Values are means of three independent analyses ± standard deviation (n = 3) RE – Rutin equivalent.

### 2.5. Estimation of Total Phenols

The total phenol content was determined according to the method described by (Siddhuraju and Becker, 2003). Ten micro litre aliquots of the extracts (2mg/2ml) were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-Ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The analysis was performed in triplicate and the results were

expressed as tannic acid equivalents. Values are means of three independent analyses ± standard deviation (n = 3) TAE – Tannic acid equivalent.

**2.6. Estimation of total lipid content**

Estimation of total lipid content was determined by (Chung et al., 1980,) method. About 10g of the samples was used to extract lipids with 150 ml of petroleum ether for 16 hr, at a solvent condensation rate of 2–3 drops/sec according to AACC Approved Method 30-25 with minor modifications of sample size and extraction time. The obtained extract was concentrated and evaporated at room temperature to dryness. The weight of extract gives the total lipid content which was expressed as mg/g dry matter.

**2.7. Vitamin C Determination**

This method determines the vitamin C concentration in a solution by a redox titration using iodine. Vitamin C, more properly called ascorbic acid, is an essential antioxidant needed by the human body (Henry Tauber 2011). As the iodine is added during the titration, the ascorbic acid is oxidised to dehydroascorbic acid, while the iodine is reduced to iodide ions. **ascorbic acid + I2 → 2 I− + dehydroascorbic acid** Due to this reaction, the iodine formed is immediately reduced to iodide as long as there is any ascorbic acid present. Once all the ascorbic acid has been oxidised, the excess iodine is free to react with the starch indicator, forming the blue-black starch-iodine complex. This is the end point of the titration. Standard solution of Vitamin C tablet is dissolved in 200ml of distilled water. The freshly collected drugs are cleaned and cut into small pieces and grinded well with addition of little water, and collect 25 ml of fresh juice. Decanted the liquid into volumetric flask, strain the ground drug rinsing the pulp with 10ml water and collect all filtrate into flask. Make up to 100ml with distilled water. Pipette out 20 ml of the liquid sample into 250ml conical flask and add about 150ml distilled water and1ml starch indicator solution. Titrate the solution with 0.005mol iodine solution. End point is the permanent trace of dark blue-black colour due to starch iodine complex (Pharmacopoeia of India 1997). Repeat the titration to obtain concordant value. The results are given in the table no.1.

**3. ANTIOXIDANT ACTIVITY**

**3.1. DPPH radical scavenging activity**

The free radical scavenging activity was measured by (Kumaran A 2007) method, the decrease in absorbance of methanolic solution of DPPH. A stock solution of DPPH (33mg$L^{-1}$) was prepared in methanol and 5ml of this stock solution was added to 1ml of the plant extract solutions at different concentrations (25,50,75,100,150,200,250, 2500ug/$ml^{-1}$). After30min, absorbance was measured at 517nm and compared with the standard ascorbic acid (10-50ug$ml^{-1}$) pH 7.4.Percentage of DPPH scavenging activity of the plant extracts and the standard was calculated. The percentage extract of inhibition was calculated by the formula [(Ao-A1)/Ao] x100, when Ao is the absorbance of the control & A1 is the absorbance of the extract/standard.

**3.2. Reducing power determination**

The reducing power of the extracts was determined by (Oyaizu.M.1986) method with different concentrations of extracts/standard (50-250mgm/ml) in methanol were mixed with phosphate buffer (PH 6.6) and incubated with (2.5ml) of potassium ferricyanide solution (1%w/v) at 50$^{o}$C for 20 min. Then 2.5 ml of trichloroacetic acid was added to the mixture and which was then centrifuged for 10 min. The supernatant (2.5ml) was mixed with distilled water (2.5ml) and ferric chloride (0.5ml) and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicates increased reducing power.

**3.3. Scavenging of hydrogen peroxide**

The ability of three extracts to scavenge hydrogen peroxide was determined (Ruch.R.J.1984) by a solution of hydrogen peroxide (2mol/l) was prepared in phosphate buffer (PH 7.4). Hydrogen peroxide concentration was determined by spectrometric ally absorbance at 230nm.Extracts were prepared at the concentration of 50-250mgm/ml and added to the hydrogen peroxide solution (0.6ml). Blank solution contains phosphate buffer without hydrogen peroxide. For each concentration a separate blank sample was used for background subtraction. The % of inhibition activity was calculated from the formula $[(A_0 – A_1)/A_0]$ X 100.Where $A_0$ is the absorbance of the control and $A_1$is the absorbance of extract/standard.

**3.4. Assay of nitric oxide scavenging activity**

The nitric oxide scavenging activity of the samples was measured according to the method of (Sreejayan and Rao 1997). 3ml of Sodium nitropruside in 0.2 M in phosphate buffered saline (pH 7.4) with different concentrations of methanolic extracts and incubate at room temperature for 150 min. The same reaction mixture without the methanol extract but the equivalent amount of methanol served as the control. After the incubation period, 0.5 ml of Griess reagent (1% sulphanilamide, and 0.1% N-napthyl ethylene diamine dihydrochloride in 2% $H_3PO_4$) was added. The absorbance of the chromophore was measured at 546nm. Quercetin and the same mixture of the reaction without the sample extracts were employed as positive and negative control. Percentage radical scavenging activity was calculated as follows: % NO radical scavenging activity = (control OD - sample OD / control OD) x 100.The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC 50) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

**3.5. Antihemolytic activity**

The antihemolytic activity was determined by (Naim M, 1976) method.The erythrocytes from wistar albino rat

blood were separated by centrifugation and washed with 0.2M phosphate buffer (pH 7.4). The erythrocytes were then diluted with phosphate buffered saline to give 4% suspension 200 - 1000µg of extracts in saline buffer was added to 2 ml of the erythrocyte suspension and the volume was made up to 5 ml with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 ml of H2O2 solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H2O2 in reaction mixture was adjusted to bring about 90% haemolysis of blood cells after 120 min. After incubation the reaction mixture was centrifuged at 1500 rpm for 10 min and the extent of haemolysis was determined by measuring the absorbance at 540 nm corresponding to haemoglobin liberation. Quercetin and the same mixture of the reaction without the sample extracts were employed as positive and negative control. The analysis was performed in triplicates and results were expressed in terms of percentage activity and IC50.

### 3.6. HPTLC analysis

Pre coated silicagel 60 F 254 (10x10 cm) (Merck) were used. Samples were applied with a 100µl sample syringe (Hamilton, Bonaduz, Switzerland) using a Linomat V system (Camag, Muttenz, Switzerland). Methanolic extract of three samples of 10µl were applied as 6 mm bands with a 10mm distance between the bands. Plates were developed in a pre saturated vertical twin trough glass chamber (Camag) for 20 min using Toluene: Ethyl acetate: Formic acid (6:6:1), by volume as a mobile phase. After development, the plates were dried and the components were visualized by UV irradiation at 254,366 nm. Antiradical activity of each component of the extract was estimated on intensity of disappearance and after dipping the plates into DPPH solution and observing the plates at 517 nm. A violet/purple background of plate and was identified by densitometric scanning at 517 nm as negative peak (Camag TLC scanner 3 under software control of Win Cats version 1.3.2). Each determination was carried out in triplicate. In order to calibrate the method, stock solutions of the reference compounds were prepared in methanol (0.5 mg/ml) and various amounts of these solutions were analyzed by HPTLC exactly as described above and calibration curves were prepared by plotting height of negative peak versus concentration. The method was applied for measuring the free radical-scavenging activity of phenol compounds of plants extract separated by normal phase TLC. The ID50 values for the major phenol substances of the extracts i. e., the dose of the compounds required to scavenge 50% of DPPH were calculated.

The post chromatographic derivatization of plates in HPTLC – DPPH method given in this study can be successfully used for the qualitative analysis of free radical scavengers in complex mixtures. This study has established that all the detected compounds within the extract were capable of scavenging DPPH radicals. From the estimated ID50 values.

## 4. RESULTS AND DISCUSSION

At present it is globally accepted that herbal based drugs have many advantages over the synthetic drugs. However, one of the major problems in utilization of herbal drugs is correct diagnosis of the medicinal plants that are used either in the traditional systems or modern systems of preparation of the drugs (.Tarwadi K.2007). In this present study various tests have been conducted qualitatively to find out the presence or absence of bioactive compounds such as flavonoids terpenoids, tannins, Phenolic substances, and vitamin C are detected in three plants, which could made the plant useful for treating different ailments as having a potential of providing useful drugs of human use. The quantitative estimation of protein, Lipid, flavonoid and vitamin C are giving good profile for these drugs. It gives an idea about the drug to go for herbal formulations.

### 4.1. QUANTITATIVE PHYTOCHEMICAL ESTIMATION

**Table No. 1. Quantitative estimation of Protein, Lipid, Phenol, Flavonoid and Vitamin C**

| Sample | Total protein(mg/g dry matter | Total Lipid (mg/g Dry matter | Total Phenols(mg TAE/g extract) | Flavonoid content(mg RE/g) | Vitamin C (mg/g) |
|---|---|---|---|---|---|
| AA | 7.4± 0.17 | 18.00± 0.14 | 23.57± 0.48 | 1.06± 0.100 | 3.305± 0.23 |
| MO | 15.7 ± 0.20 | 12.73 ± 0.08 | 61.56± 2.50 | 3.62 ± 0.020 | 0.826± 0.52 |
| SD | 17.23 ± 0.29 | 14.00 ± 0.45 | 70.16 ± 12.52 | 1.95 ± 0.030 | 3.205± 0.41 |

**Values represents the mean ± SD number of readings in each group = 3**

### 4.2. Antioxidant activity

The flavonoids are a heterogeneous group of phenol compound present in the plant world. The functions of flavonoids in plants include pigmentation, protection against UV light and microorganisms, defense against grazing animals or a regulatory function for enzymes and signal substances for nitrogen fixing bacteria.(Emmy Hainida Khairul Ikram, et. al. 2009). The flavonoids are abundant in the human diet. They are principally found in fruits, vegetables and popular drinks, such as red wine, tea, coffee and beer. Many flavonoids, purified from medicinal plants are herbs used in the practice traditional medicine, are endowed with biological effects. Flavonoids may directly scavenge some radical species by acting as chain breaking antioxidants or they may cycle other chain-breaking antioxidants such as tocopherols by donating a hydrogen atom to tocopherol radical (Morton, JF 1992). Transition mineral such as

ferric and copper are important pro oxidants and some flavonoids can chelate divalent metal ions, hence preventing free radical formation.

These three plants are having antioxidant activity. It was evaluated by five methods. In the Hydrogen peroxide scavenging activity method the IC50 values are calculated and compared with the standard Ascorbic acid , it shows 140 µg of AA, 166 µg of MO, and 122 µg of SD is equivalent to that of 12.5 µg of ascorbic acid. By the antihemolytic activity method the IC50 values are calculated and compared with the standard Quercetine. The result shows 211. 29µg of AA, 209.8 µg of MO and 225.59 µg of SD is equivalent to 57.06 µg of Quercetine. Flavonoids are phenolic compounds, present in several plants, which inhibit lipid per oxidation and lipoxygenases in vitro and in presence of free metal ion ($Fe^{3+}$).By the reducing power activity method the IC50 values are calculated and compared with the standard Epicatechine. The result shows 114 µg of AA, 125 µg of MO, and 111 µg of SD is equivalent to 12.5 µg of Epicatechine activity.

Nitric oxide is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes. Excess concentration of nitric oxide is implicated in the cytotoxic effects observed in various disorders like AIDS, cancer, Alzheimer's and arthritis (Tarwadi K 2009). Oxygen reacts with the excess NO to generate nitrite and peroxy nitrite anions, which act as free radicals. From results of Nitric oxide method, it proved that the aqueous leaf extract of AA has effective anti oxidant activity. These extract compete with oxygen to react with NO and thus inhibit the generation of the nitrite and peroxy nitrite anions. In the Nitric oxide scavenging activity method the IC50 values are calculated and compared with the standard Quercetine IC50 value. It shows 294.16 µg of AA, 280.39 µg MO, and 297.04 µg of SD is equivalent to 25 µg of Quercetine.

DPPH is relatively stable nitrogen centred free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine (Udupa, K.N.1995). The solution therefore loses colour stoichometrically depending on the number of electrons taken up. Substances capable of donating electrons/hydrogen atoms are able to convert DPPH (Purple) into their no radical form 1, 1-diphenyl-2- picrylhydrazine (Yellow), a reaction which can be followed spectrophotometrically. Free radical scavenging activity of the methanol leaf extract of SD, AA and MO is concentration dependent, as the concentration of the test compounds increases, the radical scavenging activity increases. In the DPPH scavenging activity method the IC50 values are calculated and it gives a good result of 384 µg of AA, 364 µg of MO, and 326 µg of SD is equivalent to 24 µg of Ascorbic acid.

The DPPH scavenging data suggests that the extracts of three plants are capable of scavenging free radicals at physiological pH; thus, they should be able to prevent the initiation and propagation of free radical-mediated chain reactions by stabilizing reactive species via electron or hydrogen donation before such deleterious reactions can occur. The post chromatographic derivatization of plates in HPTLC - DPPH method given in this study can be successfully used for the qualitative analysis of free radical scavengers in complex mixtures. From the above evaluation methods it shows the antioxidant activity in an order of SD> MO>AA when compared with different standards.

**FIGURES**

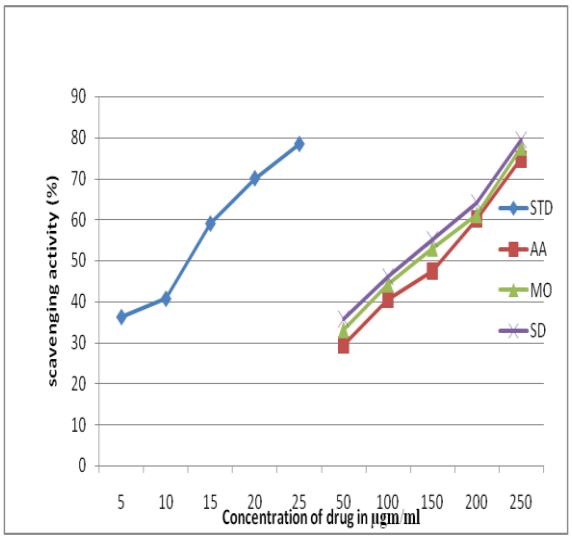



**Fig.1.Hydrogen peroxide method**

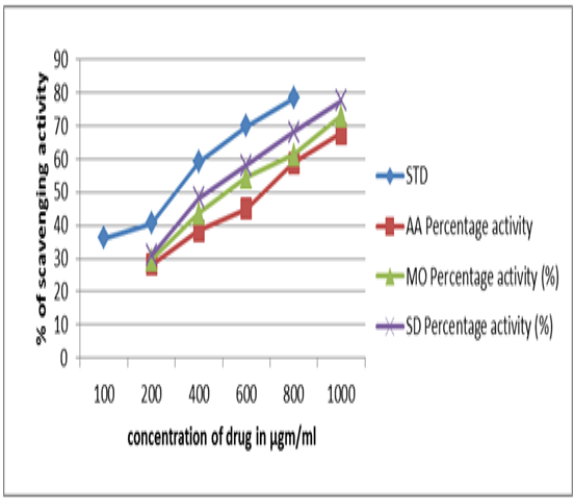



**Fig.2 Antihemolytic activity**

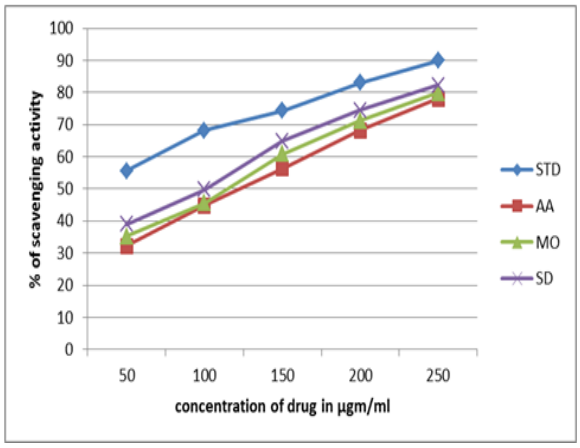



**Fig.3.Reducing power determination**

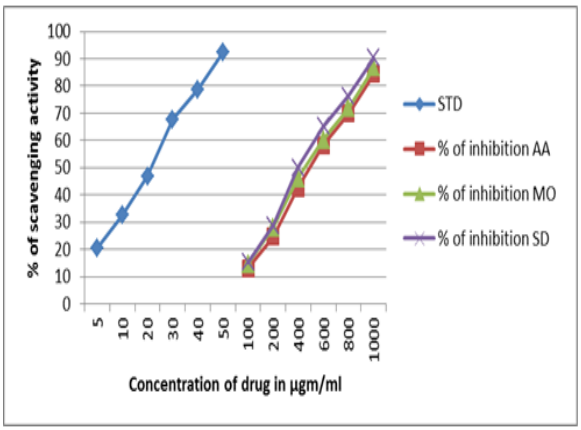



**Fig.5. DPPH Radical scavenging activity**

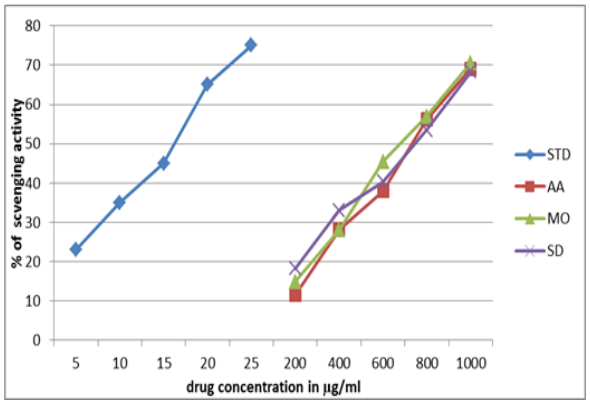
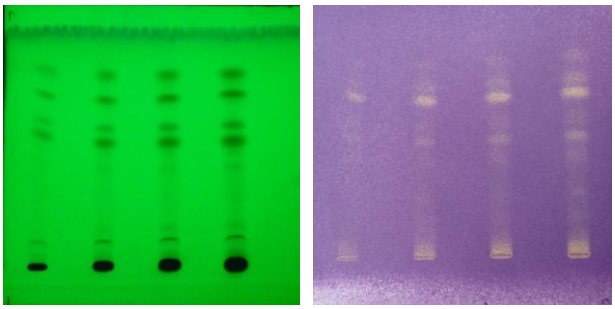



**Fig.4. Nitric oxide scavenging activity**



**256nm** **After spraying DPPH**

**Fig: 6. DPPH METHOD BY USING HPTLC**

## 5. CONCLUSION

The HPTLC method for separation and identification of antiradical activity with the DPPH radical using post chromatographic derivatization allows an analyst to estimate the antioxidant contribution of each component within a total extract. Isolation and identification of active compounds within plant extracts is a difficult, long, and expensive process. Plate derivatization techniques can be used as a cheap, fast, and efficient alternative. The HPTLC-DPPH method given in this study can be successfully used for the qualitative analysis of free radical scavengers in complex mixtures. From the densitogram and combined activity profile figures, sufficient data are available to identify known compounds or the chemical class of unknown components which possess radical – scavenging activity. The observed chromatographic behaviour provides important information about conditions required for the isolation of new analysts with such important activity. In this paper The antioxidant activity was measured as free radical scavenging activity method, Nitric oxide scavenging, Antihemolytic activity method, DPPH method, Reducing power determination method, Hydrogen peroxide method. All the methods show good response due to the presence of phenolic compounds and flavonoids in three species. Among all the three extracts tested, showed highest antioxidant activity with IC 50 value in the order of SD>AA >MO when compared to the standards. The result reveals that all the extracts have the scavenging character in accordance with the standards. The further work has been developed for the isolation of particular phenolic compound for this activity, and also can be used for the new formulation development.It is well known that Phenols, Flavonoids and vitamin C have beneficial effect on health.

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