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**Original Research Paper**

# EVALUATION OF *IN VITRO* ANTIOXIDANT ACTIVITY OF ROOT BARK OF *CHROMOLAENA ODORATA*-AN IMPORTANT ANTIDIABETIC MEDICINAL PLANT

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## ABSTRACT

Three different extracts of *Chromolaena odorata* root bark were tested for their total phenolic contents, total antioxidant activity, DPPH, ferric reducing activities ($Fe^{3+}$- $Fe^{2+}$) and $H_2O_2$ scavenging activity. Total phenolic contents (TPC) were high in CoEAE when compared with CoMeE and CoAqE. In redox potential assay methods CoMeE showed maximum total antioxidant activity, DPPH activity ($IC_{50}$) and ferric reducing activity when compared with CoEAE and CoAqE. Hydrogen peroxide decomposing capacity was higher in CoMeE ($IC_{50}$) when compared with CoEAE and CoAqE. The phytochemical screening revealed the presence of saponins, tannins, triterpenoids, and phenols are common in both extracts, alkaloids and carbohydrates were present only in CoMeE. The scientific experimental investigations of the present study revealed that potential antioxidants (may be phenolic, flavonoids, tannins, saponins and triterpenoids) present in CoMeE and CoEAE can be used against the free radical associated degenerative diseases. But proper toxicity evaluation is needed to proceed further for clinical therapeutic use.

**Keywords:** *Chromolaena odorata,* Polyphenolics, DPPH, Hydrogen peroxide.

## INTRODUCTION

Reactive Oxygen Species (ROS) are known to be produce in all normal aerobic cells as a byproduct of metabolisms and are accelerated by external factors like smoking, chlorine in drinking water, pollutants like pesticides, food additives, drugs and antibiotics.[1] Most of the investigations were focused on oxygen centered radicals like hydroxyl radicals and superoxide's which were most reactive and hydrogen peroxide ($H_2O_2$) which was highly cytotoxic agents. Due to the presence of unpaired electrons they can potentially causes oxidative damage starting from outer membrane (lipid peroxidation), intracellular organelles and components such as enzymes, proteins and ultimately DNA strands breakdown leads to permanent tissue damage.[2] Exposure of organisms to free radicals from internal and external sources arises biological complex array of endogenous radical scavenging proteins, enzymes, numerous antioxidant compounds like vitamin-E, C and uric acid prevent the accumulation of pro-oxidant molecules that induce oxidative stress.[3] The above mentioned biological and non-biological antioxidants can neutralize the ROS by oxidizing themselves. Imbalance between the pro-oxidants (increase) produced and inadequate anti-oxidants in the biological system can divert the normal physiological condition to abnormal pathological condition. Generation of ROS associated with

many pathological problems like cancer, diabetes, cardiovascular diseases, atherosclerosis, coronary heart diseases, neurodegenerative disorders, alzimer's disease, parkinson's disease and with acceleration of age were reported.[4] These are the major life threatening diseases in the present world. To overcome the common causative agents (ROS) in all degenerative diseases, synthetic antioxidants like BHT, BHA, propylgallate and tetrabutylqunone were supplemented with food[5] were reported to possess adverse side effects. Hepatotoxicity and carcinogenesis were also reported in laboratory animals.[5] The uncontrolled ROS and adverse side effects of synthetic antioxidants directed the use of medicinal herbs supplements that own effective antioxidant property at the same time little or no side effects. Polyphenolic compounds and other considerable major antioxidants in majority of dietary and medicinal plants can remove toxic radicals by different ways.[6-8] The therapeutic use of medicinal plants with high concentrations of natural antioxidants can control the morbidity and mortality in radical associated chronic diseases.[9]

*Chromolaena odorata* belongs to the family Asteraceae, native of North America and introduced to tropical Asia. *Chromolaena odorata* possess anti-inflammatory activity[10], anti-oxidant properties.[11] No reports were available up to our knowledge on invitro antioxidant activity of the present tested plant. The present study was planned to evaluate the antioxidant capacity of *Chromolaena odorata* root bark in invitro.

## MATERIALS AND METHODS

### Collection of Plant Material

Root bark was collected from *Chromolaena odorata* plants in herbal garden and surroundings of Dravidian university campus, Kuppam, Andhra Pradesh India. Plant material was dried in shade and powdered. The voucher specimen (SVUBH/2010/1165) was deposited in the herbarium of Department of Botany, S.V. University, Tirupathi, Chittoor District, Andhra Pradesh, India.

### Preparation of Extracts

The powdered plant material was defatted with petroleum ether (60-70$^0$c) then sequentially extracted with increasing polarity solvents first with Ethyl acetate and then the filtrate was concentrated in a rotary vacuum evaporator (CoEAE; yield: 2.9%w/w) under reduced pressure and the residue was extracted with methanol (CoMeE; yield: 10%w/w), concentrated in a rotary vacuum evaporator. The same procedure was repeated with water (CoAqE; yield: 8%w/w).

### Determination of Total Phenolic Contents

Total phenolic contents of CoMeE and CoEAE were measured by Folin-ciocalteu method. The amount of phenolics in the selected medicinal plant extracts was determined with Folin-Ciocalteu reagent using the method.[12] To 0.5 ml of each sample (triplicate), 2.0 ml of Folin-Ciocalteu reagent (10%) was mixed and then 4.0 ml of $Na_2CO_3$ (7.5% W/V) was added and the resulting mixture incubated at room temperature for 30 min. Standard curve was prepared using 25, 50, 75 and 100 µg/ml methanolic galic acid solutions. Results were expressed as milligrams galic acid equivalent per gram extract (mg GAE/g extract).

### Determination of Total Antioxidant Activity

Total antioxidant activity of CoMeE and CoEAE were quantitatively measured[13] and was expressed in ascorbic acid equivalents. An aliquot of 0.3 ml of extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, 4mM ammonium molybdate). The reaction mixture was incubated after capping the test tubes at 95$^0$C for 90 min. then the samples were removed and cooled to room temperature. The absorbance was measured at 695 nm against blank (reagent solution-3 ml). Results were expressed in ascorbic acid equivalents by using standard ascorbic acid graph.

### Determination of DPPH Free Radical Scavenging Activity

DPPH radical scavenging activity was measured by using modified method of Khtoon *et al.*, 2013.[14] 2 ml of each extract and control at different concentrations (12.5, 25, 50, 75 and 100

µg/ml) were added to 3ml of freshly prepared DPPH solution (0.004%) in methanol. The reaction mixture was incubated for 30 min in dark and absorbance was measured at 515 nm using spectrophotometer. All the concentrations were run in triplicate. The degree of decolorization of DPPH from purple to yellow colour indicated the scavenging efficiency of extract. The free radical scavenging activity of extracts and positive control (ascorbic acid) was measured in terms of hydrogen donating or radical scavenging ability using DPPH stable radical.

Percentage inhibitions of DPPH free radical scavenging activity was calculated using the fallowing equation.

$$\% \text{ Reduction scavenging activity} = \frac{\text{Control absorbance - Test absorbance}}{\text{Control absorbance}} \times 100$$

Calculation of $IC_{50}$ value using graphical method.

### Determination of Hydrogen Peroxide Radical Scavenging Assay

The ability of extracts to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.*, 1998.[15] A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4) and concentrations were determined spectrophotometrically at 230 nm. Various concentrations of extracts (25 – 100 µg/ml) in methanol were added to a hydrogen peroxide solution (0.6 ml, 40 mM) and the absorbance of hydrogen peroxide at 230 nm was determined after 19 min against a blank solution in phosphate buffer without hydrogen peroxide. Tests were carried out in triplicate. Ascorbic acid was used as a standard. The percentages of scavenging of hydrogen peroxide of extracts were calculated using the following equation:

$H_2O_2$ scavenging activity (%) = $[(A_0 - A_1) / A_0] \times 100$.

### Determination of FRAP (Ferric Reducing/Antioxidant Power) Assay

The FRAP assay was performed as described by Benzie and Strain.[16] Briefly, the working FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in 10:1:1 ratio prior to use and heated to 37°C in an hot water bath for 10 min. 1mL various concentrations of each extract (25, 50, 75, 100 µg/ml) were allowed to react with 3.0 ml of the FRAP reagent. The reaction mixture was kept in dark at room temperature for 30 min. The absorbance of the colored product (ferrous tripyridyltriazine complex) was then recorded at 593 nm using UV-visible spectrophotometer. A higher absorbance reading indicated a higher reducing power. Tests were carried out in triplicate. Ascorbic acid was used as a standard.

### Phytochemical Screening

Phytochemical screening were carried out for alkaloids, flavonoids, tritrpenes, tannins, Saponins, phenols, carbohydrates and proteins in ethyl acetate and methanolic extract as per the standard methods (17, 18).

### Statistical Analysis

All the values for the obtained results (triplicate) were expressed in mean±SD by using MS excel software of Windows7.

## RESULTS

### Total Phenolic Contents of CoEAE, CoMeE and CoAqE

Total phenolic contents of three different extracts of *Chromolaena odorata* was determined by using Folin-ciocalteu method. The results were expressed in mg Galic acid Equivalents/gm extract (GAE/gm) by using Galic acid standard calibration curve. By the results obtained in the present study ethyl acetate extract showed maximum phenolic contents (313.31±2.88) when compared with methanolic (246.16±1.05) and aqueous extracts (125±2.18).

### Total Antioxidant Activity of CoEAE, CoMeE and CoAqE

Total antioxidant activity of three different extracts was determined by phosphomolybdenum method. The total antioxidant activity was calculated as mg of Ascorbic acid Equivalents/gm of extract by using calibration curve of standard Ascorbic acid. Methanolic extract showed highest total phenolic contents (180.18±1.23), where as

ethyl acetate and aqueous extract showed 142.33±0.98 and 52.72±1.42 respectively.

### DPPH Radical Scavenging Activity CoEAE, CoMeE and CoAqE

DPPH radical scavenging activity of three extracts was observed to be increase with increase of doses (12.5-100 µg/ml) were represented in the graph. The methanolic extract showed least $IC_{50}$ value (12.28µg/ml) than ethyl acetate (24.47µg/ml) and aqueous extracts (170.71µg/ml). Methanolic extract has maximum DPPH activity but less than standard Ascorbic acid ($IC_{50}$-08.72 µg/ml).

### $H_2O_2$ Scavenging Activity CoEAE, CoMeE and CoAqE

$H_2O_2$ scavenging activity of three extracts was observed to be increase with increase of doses (25-100 µg/ml) were represented in the graph. The methanolic extract showed least $IC_{50}$ value (42.98/ml) than ethyl acetate (49.14µg/ml) and aqueous extracts (134.26µg/ml). Methanolic extract has maximum $H_2O_2$ scavenging activity but less than standard Ascorbic acid ($IC_{50}$-30.11 µg/ml).

### Ferric Reducing Activity of CoEAE, CoMeE and CoAqE

The ferric reducing activity determination of three extracts (CoEAE, CoMeE, CoAqE) of *Chromolaena odorata* showed lowest absorbance (0.56±0.09, 0.65±0.02 & 0.15±0.03) at 25 µg/ml and highest absorbance (1.89±0.07, 2.14±0.03 & 0.77±0.08) at 100 µg/ml. The absorbance increased with increase of concentrations (25-100 µg/ml). At high concentration the absorbance of CoMeE was somehow equal to the standard (2.34±0.07).

### Phytochemical Screening for CoMeE and CoEAE

The phytochemical screening for methanolic and ethyl acetate extracts revealed positive result for saponins, tannins, triterpenoids, phenols. Alkaloids and carbohydrates were not present in ethyl acetate extract (Table 3).

## DISCUSSION

Phenolic acids and flavonoid aglycons reported from *chromoleana odorata* were already proved to have powerful protection against cultured skin cells from oxidative damage.[19] Daily supplement of polyphenolic compounds (1gm) inhibit mutagenesis and carcinogenesis. Contribution of extract's antioxidant activity was mainly due to the phenolic compounds.[20] The natural flavonoids and its derivatives are proved to be effective against inflammatory, cancer and allergic diseases where ROS plays major role.[21] Flavonoids can also effectively acts as antioxidants by scavenging oxidizing molecules including singlet oxygen and various free radicals.[22] The phenolic compounds depart antioxidant activity by different mechanisms like reduction (donating electrons), hydrogen donation and by quenching the singlet oxygen or decomposing peroxides was reported already.[23] The hydroxyl group of phenolic compounds plays an important role in free radical scavenging activity. In the present study total phenolic contents were determined by using Folin-ciocalteu method and were expressed in mg of GAE/gm of extract. Among three plant extracts CoEAE has maximum number of phenolic and secondly in CoMeE. Where as in CoAqE has very less quantity of phenolics. Strong and effective antioxidant activity of phenolic compounds and their vital role as potential therapeutics against oxidative damages[24] were reported to be present in different parts of plants.[8] Total antioxidant activity in the present used method was based on measurement of formed green phosphate (reduced form-Mo(V) from Mo(IV)) that has maximum absorbance at 695nm due to redox potential of antioxidants.[25] Among the three different extracts CoMeE showed maximum antioxidant activity than CoEAE and CoAqE. The order of antioxidant activity of three extract was CoMeE > CoEAE > CoAqE. Phenolics were familiar for their hydrogen donating property and can inhibit the chain initiation. Even though there was less number phenolics in CoMeE showed highest antioxidant activity and maximum number of phenolics containing CoEAE showed moderate antioxidant activity. The differences in

antioxidant potential vary with the polarity of solvents used.[14] This may also be due to the variations in the position of hydroxyl group or their number on the aromatic ring of phenolic molecule and other factors like glycosylation of aglycons. Flavonoid aglycons have high antioxidant activity than their glycosides. The correlation between chemical structure and activity of some phenolic compounds were also studied and reported.[26] DPPH is a stable free radical can readily accept electrons or hydrogen to become a stable diamagnetic molecule (non radical). In this process the purple colour of DPPH in methanol after accepting hydrogen from donor converts to yellow colour (diphenyl-2-picryl hydrazine) was measured at 515 nm.[14] In the present study among three extracts CoMeE extracts showed highest activity while ethyl acetate has moderate and aqueous extracts has less activity. The activity was compared with standard Ascorbic acid ($IC_{50}$-08.72 µg/ml). Free radicals scavenging activity of present extracts may be due to the hydrogen/electron donating ability of polyphenolic compounds. Thus reduction of reactive free radicals to non reactive forms can inhibit chain initiation and break chain propagation that leads to lipid peroxidation.[27]

Hydrogen peroxide generated *in vivo* can be converted into water by the enzymes Catalase and glutathione reductase in normal physiological condition.[28] During pathological conditions (imbalance between free radicals produced and in vivo antioxidants) increase concentration of $H_2O_2$. Though $H_2O_2$ was a weak oxidizing agent can inactivate enzymes by oxidizing essential thiol (SH-) groups. It can rapidly transverse through cell membrane and interacts with $Fe^{2+}$ and $Cu^{2+}$ to form hydroxyl radicals, which is harmful to the cell.[29] In the present method when $H_2O_2$ was incubated with antioxidants, decomposition of hydrogen peroxide was measured spectrophotometrically.[30] Thus removing of $H_2O_2$ is very important throughout the food system. The maximum activity was observed in the CoMeE and moderate activity in CoEAE, were less when compared with standard ascorbic acid. $H_2O_2$ scavenging activity of both extracts was in dose dependent manner. The order of $H_2O_2$ scavenging activity was CoMeE > CoEAE > CoAqE. The phenolics present in the extract can donate electrons to $H_2O_2$, neutralizing them to form water.[31] Presence of phenolic in the extract was observed in preliminary phytochemical screening may attribute for hydrogen peroxide scavenging activity or other antioxidants in the extract may also contribute.

In the present used method reducing activity was determined by the ability of antioxidants to reduce ferric 2, 4, 6-triperidyl-s-triazine complex to blue colored ferrous complex in acidic medium. If the redox potential of antioxidants in extract is lower than redox pair $Fe^{3+}$/ $Fe^{2+}$ can reduce $Fe^{3+}$ to $Fe^{2+}$ .[20] and extent of reduction was measured (absorbance) at 593 nm. The reducing power of CoMeE, CoEAE and CoAqE increased with dose dependent manner and maximum FRAP activity was found to be in CoMeE, which was more or less similar to the standard ascorbic acid. Increase in absorbance indicates increase in the reducing property[32] was observed in the present plant extracts also. Thus antioxidants in *Chromolaena odorata* root bark was proved to have electron donating capacity that can reduce or scavenge the free radicals. The phytochemical screening revealed the presence of saponins, tannins, triterpenoids, phenolics and flavonoids in common for CoMeE and CoEAE. In the present study it was proved that CoEAE had maximum quantity of phenolics. Fractionation of CoEAE by using varying polarity containing solvents can show maximum antioxidant activity. Investigation for active fractions and single compound was going on in our laboratory.

## CONCLUSION

From the above results *Chromolaena odorata* root bark was reported to contain phenolic compounds that have effective antioxidant activity. Thus the plant extract can be used against the common causative agents (ROS) that are associated with many of degenerative and deadly diseases. In *vivo* toxic studies should be evaluated for further clinical use.

## CONFLICTS OF INTEREST

All authors of this research declare that there is no conflict of interest.

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**Table 1:** Total phenolic contents results of different extracts of *Chromolaena odorata* root bark

| S. No. | Extracts | Total phenolic contents expressed in mg of galic acid equivalents/gm of extract |
|---|---|---|
| 1. | CoEAE | 313.31±2.88 |
| 2. | CoMeE | 246.16±1.05 |
| 3. | CoAqE | 125±2.18 |

CoEAE: *Chromolaena odorata* ethyl acetate extract.
CoMeE: *Chromolaena odorata* methanolic extract.
CoAqE: *Chromolaena odorata* aqueous extract.

**Table 2:** Total antioxidant activity results of different extracts of *Chromolaena odorata* root bark.

| S. No. | Extracts | Total antioxidant activity expressed in mg of Ascorbic acid equivalents/gm of extract |
|---|---|---|
| 1. | CoEAE | 142.33±0.98 |
| 2. | CoMeE | 180.18±1.23 |
| 3. | CoAqE | 52.72±1.42 |

**Table 3:** Phytochemical screening results of *Chromolaena odorata* root bark methanolic and ethyl acetate extracts.

| S. No. | Chemical constituent | Methanolic extract | Ethyl acetate extract |
|---|---|---|---|
| 1. | Alkaloids | + | - |
| 2. | Flavonoids | - | - |
| 3. | Triterpenoids | + | + |
| 4. | Saponins | + | + |
| 5. | Tannins | + | + |
| 6. | Phenols | + | + |
| | Carbohydrates | + | - |
| 7. | Amino acids | - | - |

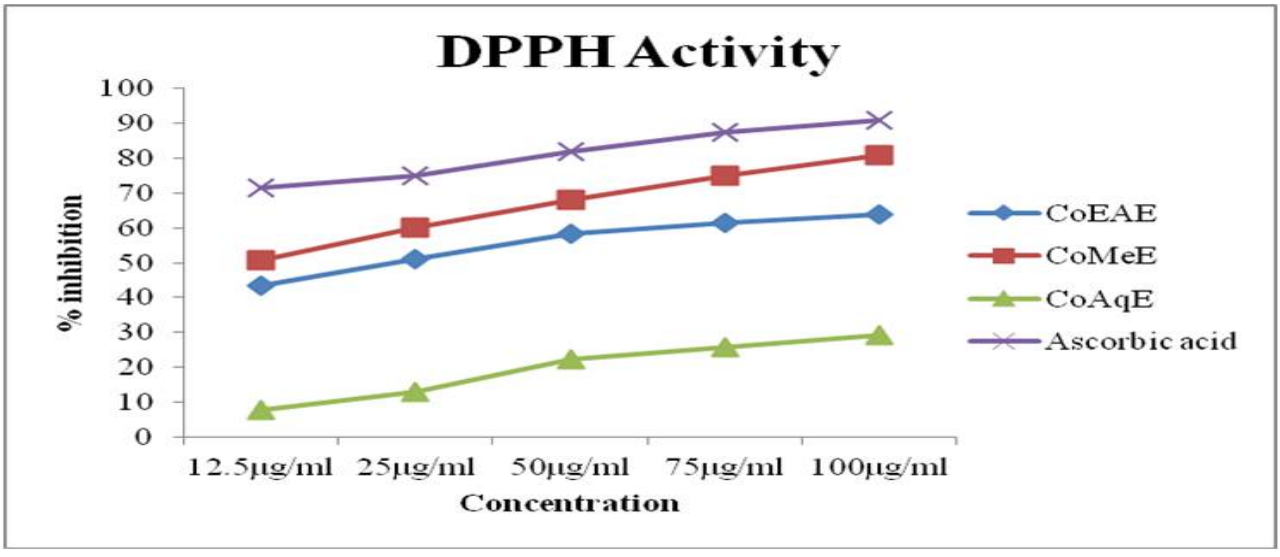


**Figure 1:** DPPH radical scavenging activity results of different extracts of *Chromolaena odorata* root bark

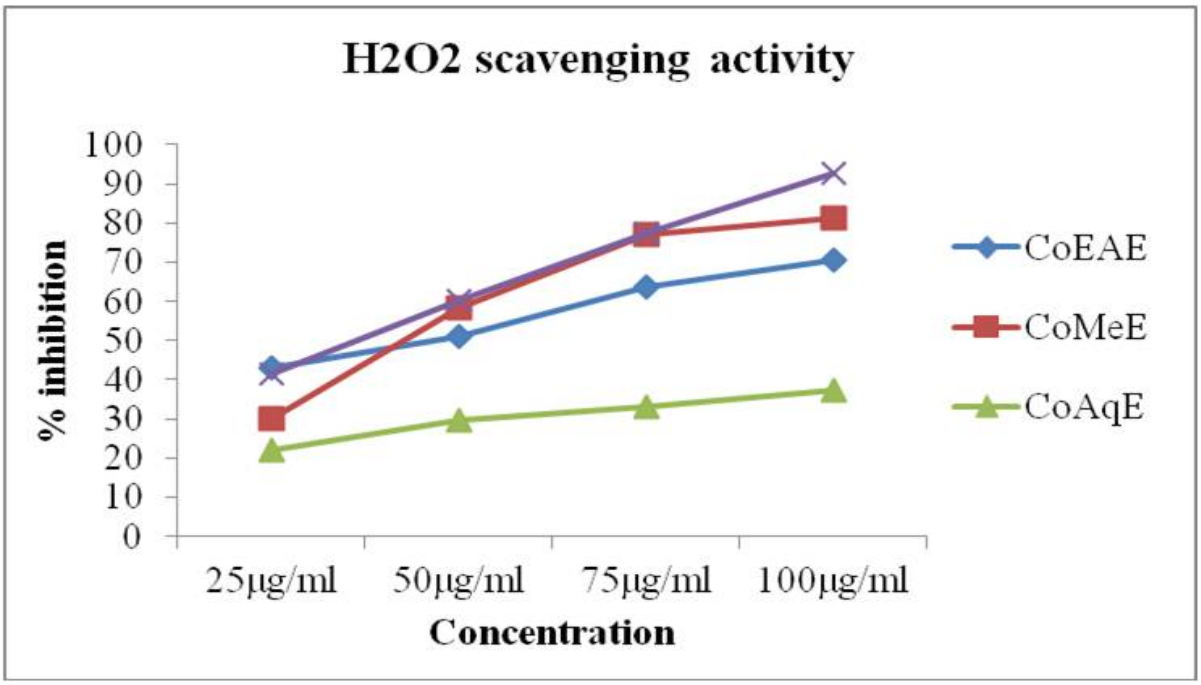


**Figure 2:** $H_2O_2$ scavenging activity results of different extracts of *Chromolaena odorata* root bark

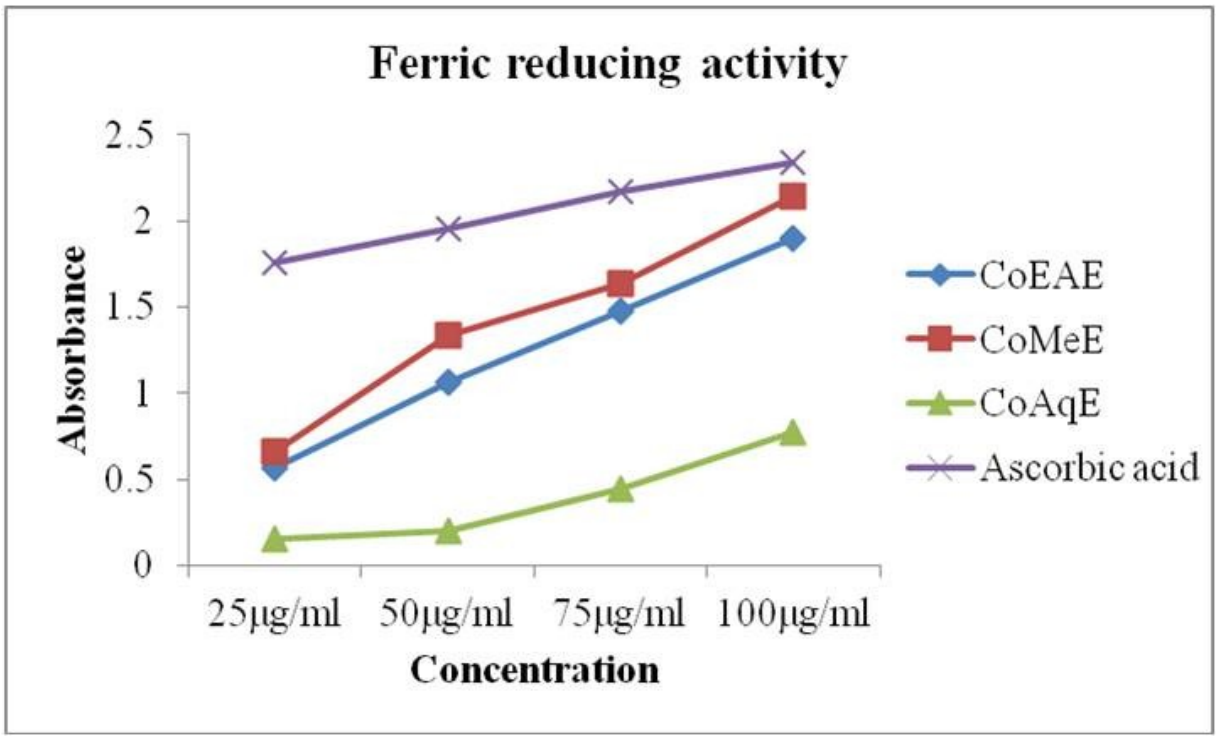


**Figure 3:** Ferric reducing activity results of different extracts of *Chromolaena odorata* root bark

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