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COMPARATIVE EFFICACY OF PHYTOCHEMICAL ANALYSIS AND ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF *CALOTROPIS GIGANTEA* AND *CALOTROPIS PROCERA*

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ABSTRACT

Calotropis species is a common wasteland weed and are widely used as alternative therapeutic tool for the prevention or treatment of many diseases. This study was designed to evaluate comparative antioxidant activity, metal chelating properties and larvicidal activity of methanolic extract of two common species of *Calotropis*, viz. *Calotropis gigantea* (Linn.) R.Br. and *Calotropis procera* (Ait.) R.Br. The total phenolic and flavonoid content was determined and expressed in term of gallic acid equivalent and quercetin equivalent respectively. In this study, antioxidant activity was measured by radical ($DPPH^{\bullet}$) scavenging, reducing power, *FRAP assay* and metal chelating activity assay. The leaves of *C. procera* were found to have higher antioxidant potential than flower and root extract with IC_{50} values of 0.21µg/ml for DPPH scavenging, 0.98 mg/ ml for metal chelating. *C. procera* significantly more potent in scavenging free radical and antioxidant activity than *C. gigantea.* Extract of all parts of both species however demonstrated similar IC_{50} value for metal chelating activity. *C. procera* possess comparatively higher antioxidant activity in reducing ferric ions than *C. gigantea*. The leaf methanolic extract showed a concentration dependent larvicidal activity with a lowest LD_{50} value of 387 mg/l compared to other extracts. There was no significant difference observed in the LC_{50} value for larvicidal activity of all parts between *C. procera* and *C. gigantea* which indicate both species exhibits same effect against *Ae. aegypti* larvae. Occurrence of more total phenols and flavonoids in all parts of *C. procera* as compared to *C. gigantea* correlates to its high antioxidant activity. The observations reported in this paper could be of applied value in utilization of *C. procera* for different clinical purpose which showed strong antioxidant potential rather than *C. gigantea*.

Key Words: *Calotropis*, Antioxidant activity, Larvicidal activity.

INTRODUCTION

Herbal medicine is becoming popular all over the world than the Allopathic medicine for medication. Much attention has been focused on assessment of antioxidant properties of various plants for finding new sources for natural antioxidants, functional foods and nutraceuticals. Natural antioxidants exhibits a wide range of biochemical activities, including inhibition of ROS generation, direct or indirect scavenging of free radicals and alteration of intracellular redox potential. Several medicinal plants have been screened based on the integrative approach on drug development from Ayurveda (Mukherjee and Wahile, 2006). The traditional and folk medicinal system uses the plant products for the treatment of various infectious diseases. The phytochemicals have been found to act as antioxidants by scavenging free radicals and may have therapeutic potential for free radical associated disorders (Hausladen and Stamer, 1999).

There are two common species of Calotropis, viz. *Calotropis gigantea* (Linn.) R.Br. and *Calotropis procera*

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(Ait.) R.Br. belongs to the family *Asclepiadaceae* (Singh, *et al.,* 1990) which are known for its pharmacological importance for centuries locally known as "Raktha Arka" and "Sweta Arka" respectively in India. It cultivated throughout India in warm dry places from Punjab to western, central and southern India and is a soft-wooded, evergreen, perennial shrub. The plant is reported to have diverse pharmacological actions (Sharma *et al*., 2011).

Various parts of this plant such as leaves, stem, flowers, and root bark are widely used in the folk medicine for the treatment of common disease such as fevers, rheumatism, indigestion, cough, cold, eczema, asthma, nausea, vomiting, and diarrhoea. It was reported that *C. procera* used in traditional medicine as a purgative, anthelmintic, anticoagulant, anticancer as well as antipyretic, analgesic (Sharma *et al.,* 2012; Basu & Chaudhury, 1991). The plant produces milky white latex that exhibits analgesic activity and wound healing properties in various animal models (Choedon *et al*., 2006). The leaves are useful in the treatment of paralysis, arthralegia, swelling and intermittent fevers. Methanolic and aqueous extracts of leaves of *C. procera* Linn, were subjected to the potential antioxidant and antibacterial activity (Kareem *et al.* 2008). Root bark of *C. gigantea* exhibits hepatoprotective activity, anticancer activity, antifertility, and anti-inflammatory activity. Cardiac glycosides from *C. gigantea* exhibit anticancer properties (Bhat & Sharma, 2013). The latex of *C. procera* has shown larvicidal efficacy against all three important vector species *viz, Ae. aegypti*, *An. stephensi* and *Cx. Quinqefaciatus* (Shahi *et al*., 2010). The hypoglycemic property of *C. procera* Linn was also assesse by an oral glucose tolerance test (OGTT) in STZ-diabetic rats (Singh *et al*., 2014). It is reported to contain cardiac glycosides, β-sitosterol, madrine, saponins, alkaloids, tannins, trisecharoides, calotropon and flavonols (Edman *et al*., 1983). *Calotropis is* one of such important traditional medicinal plants reported to be used for different clinical indication. The main objective of the present study is to investigate comparative efficacy of phytochemical analysis and antioxidant activity of methanolic extracts of *Calotropis gigantea* and *Calotropis procera*.

MATERIALS AND METHODS

Chemicals

1, 1- diphenyl -2- picryl hydrazyle (DPPH) and quercetin were procured from Sigma Chemical Co. (St. Louis, US); Gallic acid, ferrozine, TPTZ, DMSO, Folin Ciocalteu reagent, were purchased from Merck Ind. Ltd. All other chemicals and reagents were of analytical grade.

Collection of Plant material

Fresh parts including leaves, flower and root of *C. gigantea* and *C. procera* were collected from botanical garden of H. M. Patel Statue, Vallabh Vidhyanagar and the selected plant species ware identified by Dr. Rachana Dave, Botanist, M. B. Patel Science collage, Anand during December 2012. The plant parts were washed properly and dried in shade. Dried plant material was subjected to reduction to coarse powder using mechanical grinder, passing through sieve #40 and stored in a tight container

Preparation of plant extract

The dried coarse powder material (100 g) was subjected to Soxhlet's extraction separately and successively with methanol. The solvent was distilled under reduced pressure, controlled temperature (40-50°c) and the resulting semisolid mass was vacuum dried using rotary flash evaporator to yield a solid residue put in airtight container and stored in refrigerator. The following abbreviation has been used for each extract: *C. gigantea*: Leaves (CGL), Flower (CGF) and root (CGR) and *C. procera:* Leaves (CPL), Flower (CPF) and root (CPR).

Phyhytochemical analysis

Various phytochemical tests were carried out on the methanolic extract of all parts of both species of *Calotropis* using standard procedures to identify the phytoconstituents.

Total phenolics and flavonoids content

An amount of total phenolics content in the extract were determined using a series of gallic acid as standard solutions (0.05-0.35mg/ml) as described by Slinkard and Singleton (1977) but with some modifications. Each extract solution (0.1 mL) was mixed with 2 mL of a 2% (w/v) sodium carbonate solution and vortexed vigorously. After 3 minute, 0.1 mL of 50% Folin-Ciocalteu's phenol reagent was added and incubate for 30 min at room temperature and then absorbance was measured at 760 nm. All samples were analyzed in triplicates. Total phenolic contents of extracts were expressed as mg Gallic acid equivalent (GAE)/gm dry weight. All samples were analyzed in triplicates. The aluminium chloride colorimetric assay was used for total flavonoids determination, as described by Zhishen *et al.* (1999). Results were expressed as mg of quercetin equivalent / g extract.

In vitro antioxidant activity

The free radical scavenging activity of the methanolic leaf, flower and root extracts of *Calotropis* species was determined by using various *in vitro* assays such as $DPPH^{\bullet}$ assay, reducing power assay, FRAP assay and ferrous ion chelating activity.

Free radical scavenging activity

The free radical scavenging activity of each extract was determined by the method of Blois, 1958. The decrease in absorbance was measured at 517 nm and the percentage inhibition activity was calculated from; $[(A_0 – A_1)/A_0] \times 100$, Where A_0 is the absorbance of the control,

and A_1 is the absorbance of the extract/ standard. IC_{50} was calculated from equation of line obtained by plotting a graph of concentration versus % inhibition. Vitamin C was used as a positive control.

Reducing power

The reducing power is determined by the Fe^{3+} – Fe^{2+} transformation in the presence of extracts. Reducing power assay was determined according to the method of Yildirim *et al.,* (2001). Different concentrations of methanolic extracts of the study species were mixed with 1.0 ml of 200 mM sodium phosphate buffer (pH 6.6) and 1.0 ml of 1% potassium ferriccyanide followed by incubation at 50°C for 20 minutes. After adding 1.0 ml of 10% trichloro acetic acid, the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant was taken out and mixed with 2.0 ml of distilled water and 0.5 ml of 1% ferric chloride. After incubation for 10 minutes, the absorbance was measured at 700nm. Higher absorbance of the reaction mixture indicates reductive potential of the extracts. All the tests were performed in triplicates and ascorbic acid was used as reference standard BHT.

Ferrous ion chelating activity

The chelating of ferrous ions by methanolic extracts of the study species was estimated by the method of Singh and Rajini (2004). The different concentrations of methanolic extracts (leaf, flower and root) were mixed with 100µl of 2.0 mM ferrous sulphate solution and 300µl of 5.0 mM ferrozine. The mixture was incubated at room temperature for 10 minutes. The absorbance of the solution was measured at 562 nm. EDTA was used as standard. Percentage of inhibition was calculated by using this formula, Percentage of inhibition = Absorbance of control – Absorbance of test x 100 Absorbance of control

Ferric Reducing Antioxidant Power (FRAP)

In the FRAP assay, blue colored Fe^{II}-tripyridyltriazine compound is formed from the colorless oxidized Fe^{III} form by the action of electron donating antioxidant. The change in absorbance was measured at 593 nm. (Benzie and Strain, 1999) Briefly, 30µl standard (Ferrous sulfate) or 50 µl sample was added to 1.5 ml freshly prepared FRAP reagent (300mM acetate buffer, pH 3.6;10 mM TPTZ in 40 mM HCL and 20mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1). After 10 min incubation at 37°C, the absorbance was read against at 593 nm. Results are expressed as µM ferrous sulfate equivalent per gram of sample.

Larvicidal bioassay

The larvicidal bioassay was done following the standard World Health Organization protocols (WHO, 2005)**.** Insectory reared *Ae. aegypti* larvae were used for the study. Test concentrations of methanolic extract ranging from 100-1000 mg/l were prepared in Dimethyl Sulphoxide (DMSO) and final volume of 250ml was made by tap water. A batch of 25 larvae was used for each test and the tests were performed in replicates. Mortality was recorded after 24 hours. Dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region. The experiments were conducted under laboratory conditions at 25–30°C and 80–90% relative humidity. The 50% lethal Dose (LD_{50}) was calculated.

Statistical analysis

Significant differences of the data among the parameters were calculated by performing ANOVA test with the help of SPSS and means were compared by least significant difference (LSD). The values $P < 0.05$ were regarded as significant and the values $P<0.01$ were considered as highly significant.

RESULTS & DISCUSSION

The present investigation provides a comprehensive profile of the antioxidant activity of extracts of different plant parts of an important medicinal plant, *Calotropis* species, with respect to its phenols and flavonoids content. Our data shows significant antioxidant potential exists, more importantly in the *C. procera* compared to *C. gigantea*.

Phytochemical analysis

Phytochemical compounds were screened in methanolic extract of leaves, flower and root of both species *C. gigantea and C. procera* through qualitative method presented in Table 1. Flavonoids and phenolic compounds have been reported to possess antioxidant activity in all parts of both *Calotropis* species. Form the result of phytochemical analysis, it is indicated that *C. procera* showed the positive result for the presence of cardiac glycosides but not *C. gigantea* except leaves. Table 1 show the presence and absence of various pharmacological active chemical constitute including alkaloids, saponins, phenols, sterols, flavonoids and terpenoids. Previous phytochemical studies on the aerial parts of the plant showed the presence of alkaloids, cardiac glycosides, flavonoids, sterols and/or triterpenes reported in *C. procera* as well as in *C. gigantea* (Mossa *et al*., 1991; Joshi *et al*., 2010).

Total phenolics and flavonoids content

The total phenolic content was calculated from standard curve of gallic acid with R^2 value 0.98. The results showed remarkably higher total phenolic content in the leaves than flower and root extract in both of the species of *Calotropis*. Root of *C. gigantea* (19.63 ± 1.3) *and C. procera* (21.32 ± 1.85) *shows* similar total phenolic content. Flower and leaves of *C. procera* represent significantly higher phenolic content than *C. gigantea.* Fig. 1 represents total phenolic and flavonoids content of

leaves, flower and root of selected species of *Calotropis.* The root extract of C. *procera* (28.13 ± 1.23) had shown highest flavonoids content compared to leaves and followed by flower of *Calotropis* species. Significant higher flavonoids content has been observed in *C. procera* compared to *C. gigantea* in all selected part of this plant. Phenolic compounds are known as powerful chain breaking antioxidant. Several studies have described the antioxidant properties of medicinal plants which are rich in phenolic compounds (Pietta, 2000). Flavonoids are capable of effectively scavenging the reactive O_2 species because of their phenolic hydroxyl groups and so they are potent antioxidants (Cao *et al*., 1997).

In vitro antioxidant activity

The DPPH radical scavenging activity is a sensitive method for the antioxidant screening of plant extract. Extent of DPPH radical scavenged was determined by the decrease in intensity of violet colour in the form of IC_{50} values. In the present study, leave extract of *C. procera* (84.23 ± 3.69) showed highest free-radical scavenging activity in term of % inhibition of DPPH radical compared to *C. gigantea* leaves (68.52 ± 2.71), but root extract of both *Calotropis* species showed very little free-radical scavenging activity (Fig. 2). Lower IC_{50} value represents higher antioxidant activity. The IC_{50} values of CPF, CGF, CGR, CPR, CGL, and CPL were found to be 0.68, 0.56, 0.42, 0.31, 0.32, and 0.21µg/ml respectively in DPPH assay represent in Fig 3. Vitamin C used as a positive control and found to have an IC_{50} value 0.044 µg/ml. The leaves extract (CPL) of *C. procera* has lowest IC_{50} value among the extracts and hence maximum antioxidant activity. Antioxidant activity of ethanolic extract of *Calotropis gigantea* has been reported in vitro by reducing power, DPPH and nitric oxide method (Joshi *et al*., 2010).

Significant lower IC_{50} value of DPPH radical scavenging activity in *C. procera* than *C. gigantea* indicates more potent in scavenging free radical and antioxidant activity might be due to higher content of phenolic and flavonoid compound present in *C. procera*. Most of the tannins and flavonoids are phenolic compounds and may be responsible for antioxidant properties of many plants (Larson, 1988).

The metal chelating ability of the methanolic extracts was measured by the formation of ferrous ion ferrozine complex. Each of the extract interfere with the formation of Ferrous ion (Fe^{+2}) and ferrozine complex which suggests its metal chelating activity. IC_{50} for metal chelating activity of leaves (0.98 and 1.24 mg/ ml), flower (2.5 and 2.9 mg/ ml) and root (2.38 and 2.98 mg/ ml) of *C. procera* and *C. gigantea* were reported respectively, which was higher than the positive control EDTA with IC_{50} 0.111 µg/ml (Fig. 3). Lower IC_{50} value represents higher antioxidant activity. The leaves extract (CPL) of *C. procera* has lower IC_{50} value among the extracts and hence maximum metal chelating ability. There was no significant difference in the IC_{50} value for metal chelating activity of all parts between *C. procera* and *C. gigantea*. Both species exhibits chelating agents are effective as secondary antioxidants, because they reduce the redox potential there by stabilizing the oxidized form of the metal ion (Duh *et al.*, 1999).

The total FRAP activity of leaves, flower and root extract of both *Calotropis* species exhibits in Fig 4. FRAP activity of *C. procera* leave extract was 784.34 ± 22.81 mM FeSO4/ g powder which indicates the strong antioxidant activity of the plant extract. Extract of the flower also shown the higher activity of FRAP compared to root extract. The root extract of *C. gigantea* demonstrate lowest FRAP activity compared to all other extract of the plant. Fig 4 indicate that *C. procera* possess comparatively higher antioxidant activity in reducing ferric ions than *C. gigantea.* The reasons accounting for higher antioxidant activity of methanol extract of C. *procera* compared to *C. gigantea* in present investigation, might be due to enhanced production of biologically active compounds like; alkaloids, tannins, saponins, and flavonoids etc. in this particular species (Elakkiya & Prasanna, 2012).

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. There was concentration dependent increase in the absorbance of reaction mixture for all the extracts and standard. The results of reducing power assay represented in form of EC_{50} value indicate conentration at which it produced 50% absorbtion at 700 nm. CPL and CGL has shown the maximum absorbance and hence maximum reducing power among the extracts. Lower EC_{50} value of C. *procera* vs *C. gigantea* of root (0.84 vs 1.02), leaf (0.42 vs 0.5) and flower (0.71 vs 0.87) extract showed that C. *procera* have more potent reducing capacity (Fig. 5). The significant linear correlation was confirmed between total phenolic, flavonoid content and FRAP, DPPH and reducing activity (Khasawneh *et al*., 2011).

Phytochemically, the plants have been investigated for cardenolides, cardiac glycosides from the latex and leaves, triterpenoids, anthocyanins from flowers and pentacyclic triterpenes, alkaloid, cardinolides phytosterols and triterpenoid saponins from the root. C. *procera* and *C. gigantea* exhibit diversity in their chemical constitute and secondary metabloites even witnin differernt part of the plant may be responsible for differnce in their antioxidant potential (Khairnar *et al*., 2012).

Larvicidal activity

The results of larvicidal activity against *Ae. Aegypti* larvae are shown in Fig. 6 in the form of LC_{50} value. The root and flower extract showed to be less toxic than leaves extract of C. *procera* and *C. gigantea* against *Ae. aegypti*. The LC_{50} values were 387 and 456 mg/l for leaves extract of C. *procera* and *C. gigantea*, respectively

(Fig. 6). Overall, *Ae. aegypti* larvae were more susceptible towards the leaves extract than flower or root extract of *Calotropis* species. Earlier studies of ethanolic extract of *C. gigantea* have shown larvicidal activities on *Ae. aegypti* larvae with LD_{50} value of 351.43ppm. The active principles in the *Calotropis* which might be have insecticidal property (Shreya *et al*., 2012). Calotropin and Calotoxin might be main component responsible for the larvicidal activities exhibits by *Calotropis* species (Dubey *et al.*, 2007). There was no significant difference observed in the LC_{50} value for larvicidal activity of all parts between *C. procera* and *C. gigantea* which indicate both species exhibits same effect in against *Ae. aegypti* larvae. An advantage of using botanicals as mosquito larvicide is due to its photodegradative property as compared to chemical insecticides which causes toxicity in non-target organisms and also causes environmental contamination. From the present it is clear that the leaves of both species of Calotropis formulation are highly effective as larvicide.

Fig 1. Total phenolic and flavonoid content present in two species of *Calotropis procera and Calotropis gigantean*

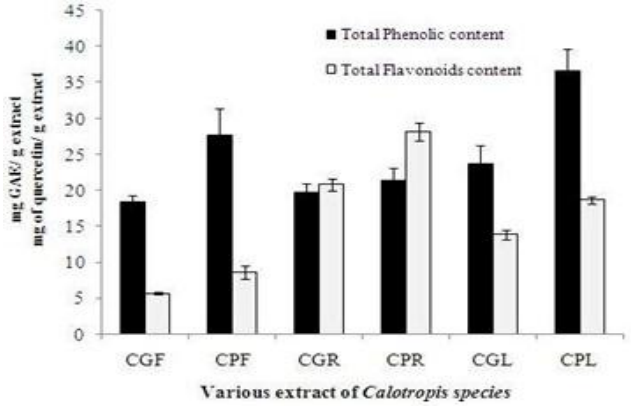

Fig 2. Percentage Inhibition of DPPH radicals and metal chelating activity by various extract of both *Calotropis* species

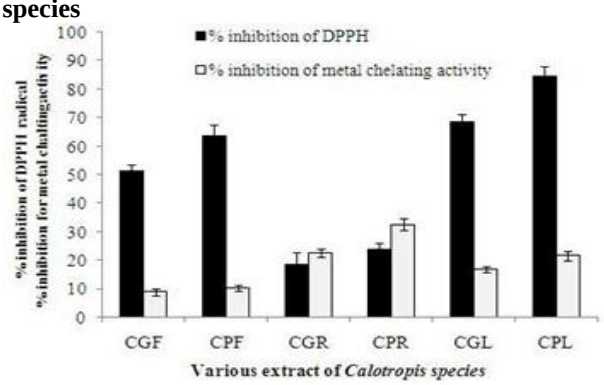

Fig 3. Inhibitory concentration IC_{50} value obtained from DPPH assay and metal chelating of various parts of *Calotropis procera and Calotropis gigantea*

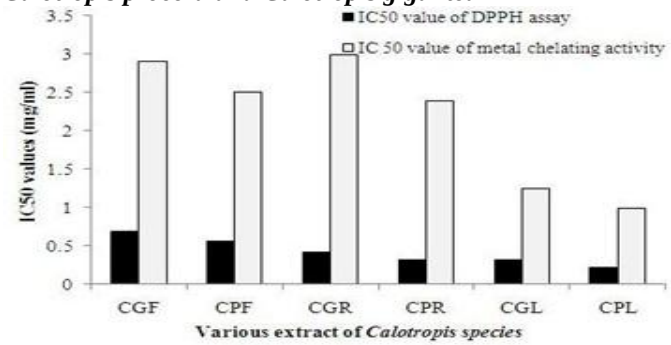

Fig 4. Ferric Reducing Antioxidant Power of methanolic extract of all studies parts of *C. procera and C. gigantea*

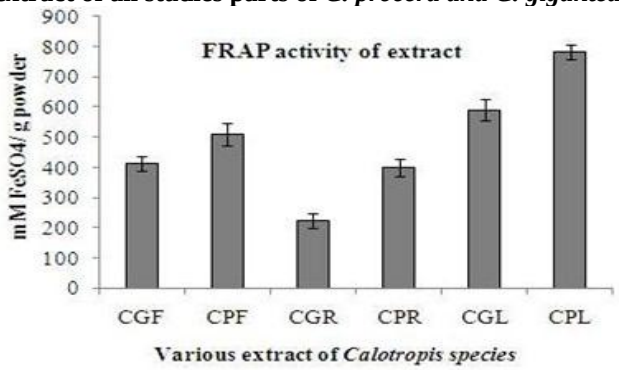

Fig 5. Reducing ability of different extract of leaves, flower and root of both *Calotropis* species in terms of EC_{50} value

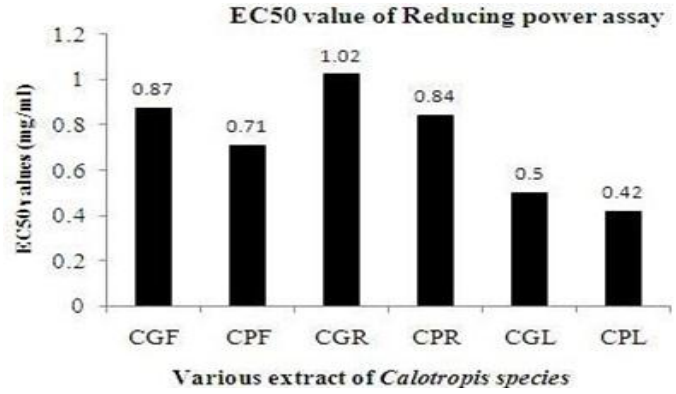

Fig 6. LC_{50} value for larvicidal activity of various parts of both *Calotropis* species

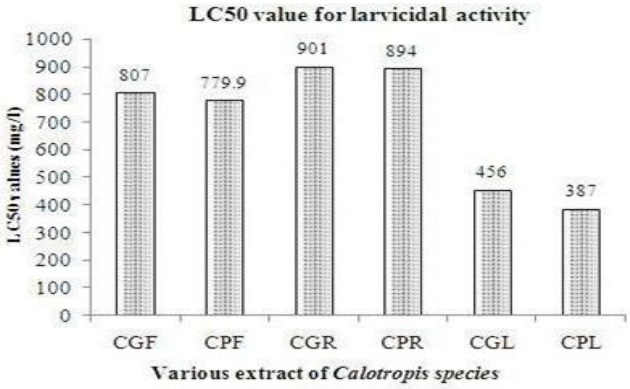

Table 1. Phytochemical screening of *Calotropis gigantea and Calotropis procera*

Phytochemical screening	Tannins	Saponin	Flavonoids	Alkaloids	Cardiac glycosides	Terpenoids	Steroids
Calotropis gigantea							
Flower	-	-	+	+	-	-	-
Leaves	+	-	+	+	+	+	+
Root	-	+	+	+	-	-	+
Calotropis procera							
Flower	-	-	+	+	+	+	-
Leaves	+	-	+	+	+	+	+
Root	+	+	+	+	+	+	+

CONCLUSION

The plant extract enriched with phenolic and flavonoids can be used in routine life to treat various diseases which are due to free radicals generation in our body. On the basis of the results obtained in the present study, it was concluded. Methanolic leaf extract of *C. procera* possess maximum total antioxidant activity and larvicidal activity than *C. gigantea*. The present investigation presents the first report on comparative analysis of antioxidant potential of extracts from its leaf, root and latex of *C. procera* and *C. gigantea* plants.

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