International Journal of Molecular Biology
ISSN: 0976-0482 & E-ISSN: 0976-0490, Volume 3, Issue 3, 2012, pp.-71-76.
Available online at http://www.bioinfo.in/contents.php?id=34

PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT POTENTIAL, ANTIBACTERIAL ACTIVITY AND MOLECULAR CHARACTERIZATION OF *Clerodendrum species*

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Received: September 08, 2012; Accepted: November 20, 2012

Abstract- *Clerodendrum* is widely distributed plant in India and its medicinal use has been mentioned in traditional Indian systems. In the present study six species of *Clerodendrum* (*C. inerme, C. peniculatum, C. philippinum, C. phlomidis, C. serratum and C. villosum)* were screened for the presence of phytochemicals and were found positive for Glycosides, Terpeniods, Anthraquinones, Flavonoids, Saponins, Tannins, Lignin, Phenol and Alkaloids. All the species showed Antioxidant potential for all the Antioxidant Assays tested (DPPH Assay, Reducing Power Assay and Total Antioxidant Activity). For DPPH assay maximum antioxidant activity was seen for *C. inerme,* and *C. serratum* showed maximum activity in Reducing Power Assay and Total Antioxidant Activity. The plant species were also analyzed to check for their Antibacterial Activity. Among the solvents (Chloroform, Ethanol, Methanol, Iso-amyl Alcohol and Propanol) used for extraction, the Iso-amyl Alcohol extract was found to be most active compared to other solvent extracts. *B. subtilis* and *S. aureus* were most sensitive among the tested pathogens. Proteus sp exhibited sensitivity towards most of the plant sp and for almost all solvent extracts. These findings can be used as prerequisite for *Clerodendrum* plant screening for bioactive compound for medicinal purposes. Genomic DNA was extracted from the fresh leaves of selected cultivars and PCR was performed by using RAPD primers to check the genetic diversity among these species. From the PCR generated fingerprint, dendrogram was plotted by cluster analysis of similarity matrix. Dendrogram constructed by cluster analysis of RAPD markers showed that *C. inerme* and *C. serratum* are closely related. As very little work has been done on molecular characterization of *Clerodendrum sp.* by RAPD technique, our finding can be used as prerequisite for plant breeding activities as well as for conservation of genetic resources.

Keywords- *Clerodendrum*, Antibacterial, Antioxidant, Phytochemical, RAPD

Citation: Prasad M.P., Sushant S. and Chikkaswamy B.K. (2012) Phytochemical Analysis, Antioxidant Potential, Antibacterial Activity and Molecular Characterization of *Clerodendrum species*. International Journal of Molecular Biology, ISSN: 0976-0482 & E-ISSN: 0976-0490, Volume 3, Issue 3, pp.-71-76.

Introduction

Many plants are found to contain chemical compounds, which are used as natural medicines to treat common bacterial infections. These medicinal plants have been regularly used in various system of Indian medicine because of minimal side effect and cost effectiveness which provide scientific support to the therapeutic use of the plants in tribal medicine [1]. The genus *Clerodendrum* L. is widely distributed in tropical and subtropical regions of the world and is comprised of small trees, shrubs and herbs. Many species of the genus have also been documented in traditional systems of medicine practiced in countries like India, China, Korea, Thailand and Japan [2]. A number of species from the genus *Clerodendrum* are documented in ancient texts for their antimicrobial action. Clerodendrum species showed antifungal activity and also exhibited antibacterial activity against bacterial pathogens [3,4]. Methanolic fraction of *C. inerme* is effective against *S. faecalis* and *B. subtilis* which are gram positive and three of the gram negative bacteria (*K. pneumoniae, Proteus sp. and S. boydii*) [5]. Roots and leaf extracts of *Clerodendrum* have been used for the treatment of rheumatism, asthma and other inflammatory diseases [6-10] and also possess sedative, antihypertensive and antidiabetic properties [11,12]. The present investigation is conducted for screening antibacterial compounds from natural resources as the existing drugs are getting less effective due to increased tolerance of microorganisms.

Antioxidants are compounds which quench molecular oxidation, and play a vital role in guarding the body defense mechanism against free radicals and reactive oxygen species, which are generated continuously in the body due to both normal metabolism and certain diseases [13,14]. Species like *C. inerme* have been used as antioxidant drugs in various indigenous systems of medicines [15]. When an imbalance occurs between oxidants and antioxidants in

favor of the oxidants, then oxidative stress sets up which may lead to the aging process as well as to chronic diseases such as cancer and coronary heart disease [16]. Interest in the role of antioxidants in human health has prompted research in the fields of food science and medicinal herbs to assess the role of herbs as antioxidants [17]. In the present study Antioxidant potential of six *Clerodendrum* species is analyzed for identifying new antioxidant compounds. Global interest in oriental medicine and production of medicinal plants has grown even over the recent years. Developing molecular markers could be important for quality assessment in the medicinal industry [18]. The study of genetic relationships is a prerequisite for plant breeding activities as well as for conservation of genetic resources [19]. The main advantage of RAPD-PCR is the use of unique and short arbitrary primer for PCR amplification, without prior information about the sequence of DNA. Besides, it is more advantageous with respect to other similar molecular marker techniques such as the more recently introduced AFLP [20]. In fact, RAPD-PCR technology is less time consuming, cost effective and for DNA fingerprinting it does not required any radioactive reagents [21]. Until now very little work has been done on molecular characterization of *Clerodendrum sp.* by RAPD technique, the purpose of this study is to find out phytochemical content of *Clerodendrum sp.* and molecular characterization of these species by RAPD technique.

Materials and Methods

Sample Collection

Plant samples such as *C. inerme, C. peniculatum, C. philippinum, C. phlomidis, C. serratum* and *C. villosum* were collected from University of Agriculture, Bangalore, India.

Solvent Extraction

Plant leaves were dried under shade and dried leaves were crushed using mortar and pestle. The crushed plant material (10 g) was kept on rotary shaker along with 100ml of different solvents like Chloroform, Ethanol, Methanol, Iso-amyl alcohol and Propanol for 48 hrs. The extract was concentrated by solvent evaporation and used for antibacterial activity.

Isolation of Microorganisms for Antibacterial Activity

The pathogenic microorganism were isolated from clinical samples and indentified on the basis of morphological, biochemical and physiological characteristics according to Bergey's manual of determinative bacteriology. The isolated microorganisms were found to be. *B. subtilis, Proteus sp., S. aureus, K. pneumoniae* and *S. typhi.*

Determination of Antibacterial Activity

The antibacterial activity was determined by the agar well-diffusion method. Overnight grown bacterial culture was transferred to sterile Petri plate with Mulleher Hinton agar medium (HiMedia Laboratories Limited, Mumbai, India) and was spread with sterile spreader to create a lawn. Wells of 6mm were punched into the previously seeded MH agar plates using sterile cork borer. About 50µl of the different solvent extract was placed in the wells and allowed to diffuse for 2 hrs. at 4ºC and the plates were incubated at 37°C for 24 hrs. The activity was determined by measuring the diameter of the inhibition zones for each well and expressed in millimeter.

Determination of Minimal Inhibitory Concentration (MIC)

The extracts that exhibited considerable activity were used for MIC determination. The extracts of the test samples were tested in four dose levels of 10µl to 40µl. The overnight grown bacterial culture was transferred on MH agar plate and wells were punched out using a sterile 6mm cork borer. Different concentration (10-40µl) of the extract was placed in separate wells and allowed to diffuse for 2 hrs. at 4°C and then the plates were incubated at 37°C for 24 hrs. The zone of inhibition was observed and the lowest amount of the test sample showing zone of inhibition was recorded as the MIC.

Preliminary Phytochemical Analysis

Phytochemical screening of plant extracts was done according to the standard procedure by Harbone [22]. All the prepared plant extracts were subjected to preliminary phytochemical screening for the presence of phenolic content, glycosides, anthraquinones, terpenoids, flavinoids, tannins, lignin and saponins.

Antioxidant Assay

Extract Preparation for Antioxidant Activity

For extraction, 5 g of the powdered sample was mixed with 50ml of methanol. The obtained extracts were filtered using whatman No.1 paper and the filtrate was collected, then methanol was removed by evaporation and residue was used for antioxidant assays.

DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay

Scavenging activity on DPPH was assessed according to the method reported by [23] with slight modification. Briefly, 0.5ml of extracts (200 to 1000µg/ml) was mixed with 3ml of methanolic solution of 0.1mM DPPH. Then the mixture was shaken thoroughly and incubated at room temperature for 30 min and absorbance was measured at 517nm in a spectrophotometer.

Reducing Power Assay

The reducing power was determined by following method, 0.5ml of extracts (200 to 1000µg/ml) was mixed with 0.5ml of 0.2 M phosphate buffer (pH 6.6) and 0.5ml potassium ferrocyanide (1%). After incubating the mixture at 50ºC for 20 min., 0.5ml of 10% trichloroacetic acid was added, centrifugation was carried out at 3000 rpm for 10 min. 1ml of supernatant was mixed with 1ml of distilled water and 0.2ml FeCl3 (0.1%) and the absorbance was measured at 700nm.

Total Antioxidant Capacity

The total antioxidant capacity was determined by the phosphormolybdenum method [24]. To 0.3ml of extract of different concentrations (200 to 1000µg/ml) was mixed with 3ml of reagent solution (0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The reaction mixture was incubated at 95°C for 90 min and cooled to room temperature. Absorbance was taken at 695nm using a spectrophotometer against blank (0.3ml distilled water in place of extract).

DNA Isolation and PCR Amplification

DNA was isolated from fresh leaf tissues as per the procedure

described previously [25]. The polymerase chain reaction was carried out in final volume of 25µl containing 100 ng DNA, 3 U of Taq DNA polymerase (Chromous Biotech, Bangalore), 2.5mM MgCl (Chromous Biotech, Bangalore), 2.5mM each dNTPs (Chromous Biotech, Bangalore) and 100 p mol of primers (GeNei, Bangalore). The DNA amplification was performed in the Corbett RG 6000 thermo cycler using the following conditions: complete denaturation (94°C for 5 min), 10 cycles of amplification (94°C for 45 sec, 35°C for 1 min and 72°C for 1.5 min) followed by 30 cycles of amplification (94°C for 45 sec, 38°C for 1 min and 72°C for 1 min) and the final elongation step (72°C for 5 min). All PCR products were separated on 2% (w/v) Agarose gel containing ethidium bromide (0.5 µg/ml). The gel was photographed with HP Alpha-imager.

Data Analysis

The RAPD profiles were analyzed based on the presence or absence of individual RAPD bands. The genetic distance was calculated by the coefficient of frequency similarity matrix. The matrix of genetic distance was used for grouping the *Clerodendrum* cultivars based on the dendrogram constructed by UPGMA (un-weighed pair group method with Arithmetic averages)

Results and Discussion

The antibacterial activity of six *Clerodendrum species* was determined in both gram positive and gram negative bacteria. The zone of inhibition produced by these extract against pathogenic bacteria is summarized in [Fig-1], [Fig-2], [Fig-3], [Fig-4], [Fig-5]. The isoamyl extract was most active against all the tested pathogens. Against *B. subtilis* highest zone of inhibition (38mm) was produced by isoamyl extract of *C. serratum.* Against *Proteus sp.* highest zone of inhibition (24mm) was produced by isoamyl extract of *C. serratum* and *C. philippinum.* Against *S. aureus* highest zone of inhibition (39mm) was produced by isoamyl extract of *C. villosum.* Against *K. pneumoniae* highest zone of inhibition (34mm) was produced by isoamyl extract of *C. villosum*. Against *S. typhi* highest zone of inhibition (38mm) was produced by isoamyl extract of *C. serratum.*

All the six species of *Clerodendrum* exhibited diverse antimicrobial activity in the MIC assay at varied concentrations as summarized in [Table-1]. The Iso-amyl Alcohol extract was most active compared to other solvent extracts and *B. subtilis* and *S. aureus* were most sensitive among tested microorganism. For overall result, antibacterial activity of nearly all plant for almost all solvent extract has been seen for *Proteus sp*.

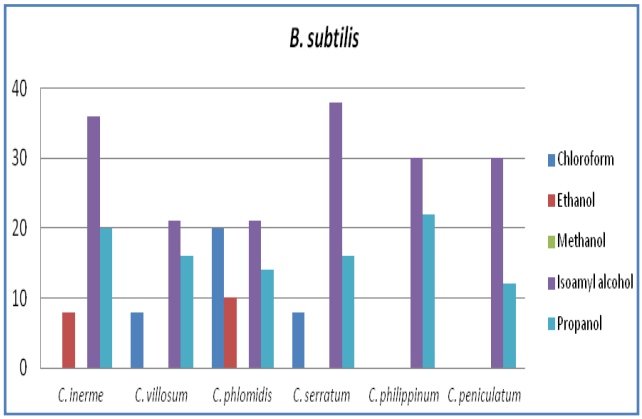

Fig. 1- Antibacterial of *Clerodendrum* against *B. subtilis.*

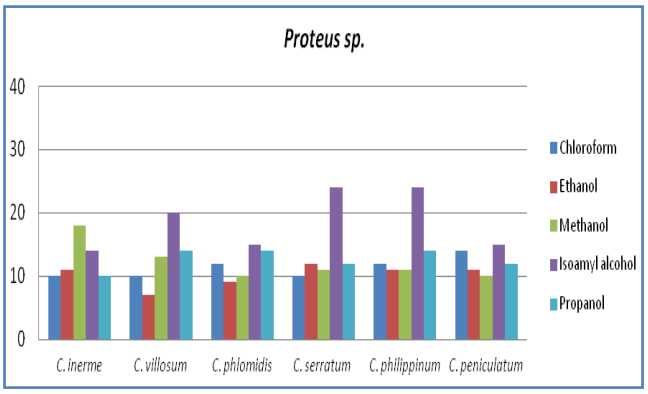

Fig. 2- Antibacterial of *Clerodendrum* against *Proteus sp.*

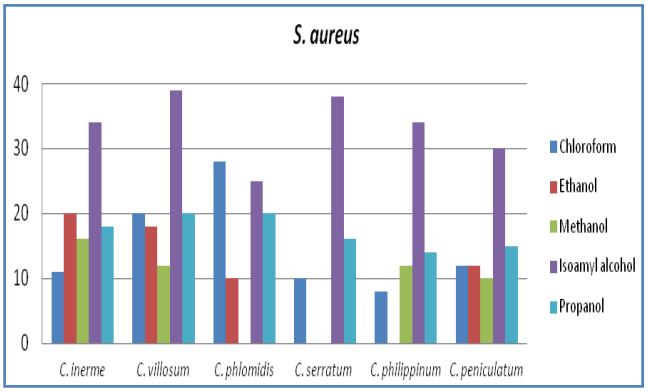

Fig. 3- Antibacterial of Clerodendrum against *S. aureus*.

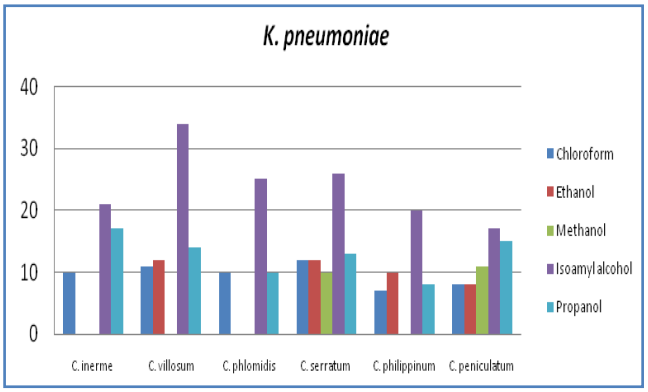

Fig. 4- Antibacterial activity of *Clerodendrum* against *K. pneumoniae*

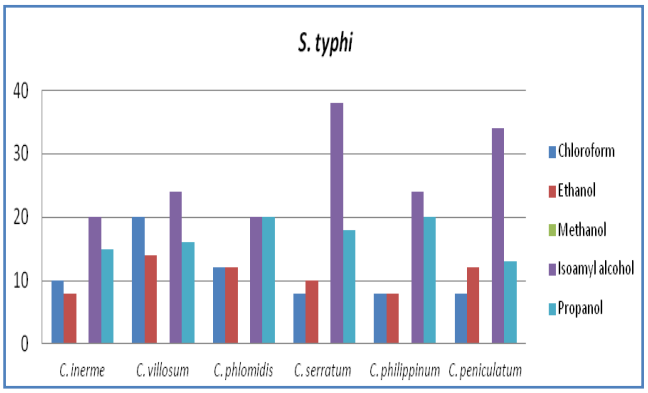

Fig. 5- Antibacterial of *Clerodendrum* against *S. typhi.*

Table 1- Minimal Inhibitory Concentration of Clerodendrum Species

Extraction Solvent and Plant Sample	Minimum Inhibitory Concentration (Pathogen)				
	B. subtilis	*Proteus sp.*	*S. aureus*	*K. pneumoniae*	*S. typhi*
Chloroform Extract of					
C. inerme	-	40	40	-	-
C. villosum	-	40	40	-	40
C. phlomidis	20	30	30	40	-
C. serratum	-	40	-	-	-
C. philippinum	-	30	-	-	-
C. peniculatum	-	30	-	-	-
Ethanol Extract of					
C. inerme	-	40	30	-	-
C. villosum	-	-	30	40	40
C. phlomidis	-	-	-	-	40
C. serratum	-	40	-	40	-
C. philippinum	-	40	-	-	-
C. peniculatum	-	40	40	-	40
Methanol Extract of					
C. inerme	-	30	30	-	-
C. villosum	-	40	40	-	-
C. phlomidis	-	-	-	-	-
C. serratum	-	40	-	-	-
C. philippinum	-	40	40	-	-
C. peniculatum	-	-	-	-	-
Iso-amyl Alcohol Extract of					
C. inerme	10	30	10	20	20
C. villosum	30	20	10	10	20
C. phlomidis	10	30	20	20	30
C. serratum	10	10	10	20	10
C. philippinum	10	10	10	20	10
C. peniculatum	10	30	10	20	10
Propanol Extract of					
C. inerme	30	-	30	30	40
C. villosum	40	40	30	40	40
C. phlomidis	40	40	30	-	30
C. serratum	40	40	40	40	30
C. philippinum	30	40	40	-	30
C. peniculatum	40	40	40	40	40

Preliminary phytochemical screening of *C. inerme, C. peniculatum, C. philippinum, C. phlomidis, C. serratum and C. villosum* showed the presence of Glycosides, Terpeniods, Anthraquinones, Flavonoids, Saponins, Tannins, Lignin, Phenol and Alkaloids which account for their usefulness as medicinal plants. Flavonoids, Saponins, Lignin and Phenol were present in all six species; whereas, Glycosides, Terpeniods, Anthraquinones, Tannins and Alkaloids were present in most of the species but not all. The detailed description of phytochemical distribution among each of the *Clerodendrum sp.* is shown in [Table-2].

Table 2- Phytochemical screening of leaf of Clerodendrum Sp.

	C. inerme	*C. peniculatum*	*C. philippinum*	*C. phlomidis*	*C. serratum*	*C. villosum*
Cardiac Glycosides	+ve	-ve	-ve	-ve	+ve	-ve
Terpeniods	-ve	-ve	-ve	-ve	+ve	+ve
Anthraquinones	+ve	+ve	+ve	+ve	-ve	-ve
Proteins	+ve	+ve	+ve	+ve	+ve	+ve
Flavonoids	+ve	+ve	+ve	+ve	+ve	+ve
Saponins	+ve	+ve	+ve	+ve	+ve	+ve
Tannins	+ve	+ve	-ve	-ve	+ve	+ve
Lignin	+ve	+ve	+ve	+ve	+ve	+ve
Phenol	+ve	+ve	+ve	+ve	+ve	+ve
Alkaloids	-ve	-ve	+ve	+ve	+ve	-ve

In the DPPH method the absorbance was measured and the percent inhibition of the DPPH radical by *Clerodendrum* was calculated based on the measured absorbance. Antioxidant capacities in series of concentrations for *Clerodendrum* was used for calculating the percentage of antioxidant activity as showed in [Fig-6]. All species of *Clerodendrum* showed good antioxidant activity. In the Reducing Power assay, *C. inerme* showed maximum antioxidant activity followed by *C. serratum* as compare to others [Fig-7]. In Total Antioxidant Capacity assay [Fig-8], the maximum antioxidant activity is shown by *C. serratum.*

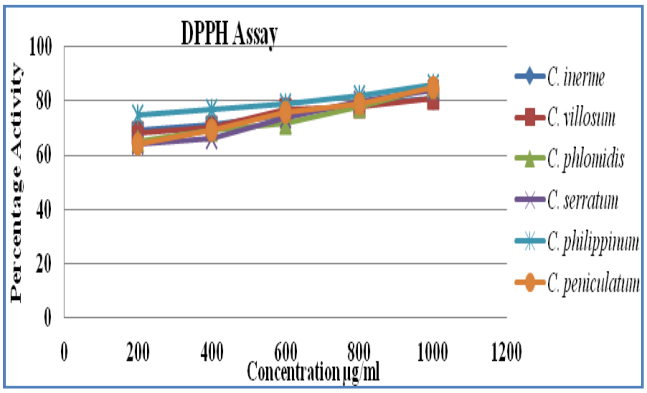

Fig. 6- Antioxidant Activity of *Clerodendrum* by DPPH Assay

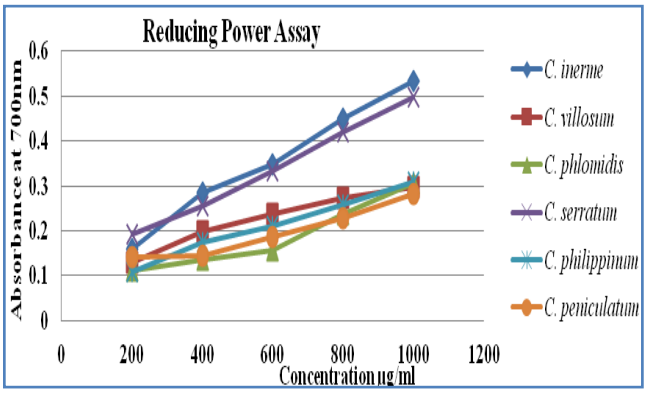

Fig. 7- Antioxidant Activity of *Clerodendrum* by Reducing Power Assay

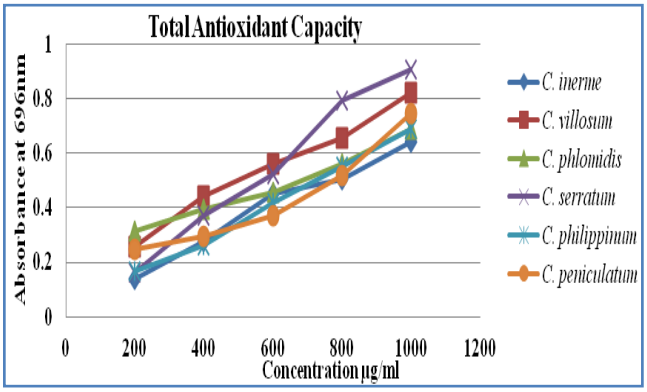

Fig. 8- Antioxidant Activity of *Clerodendrum* by Total Antioxidant Capacity

Free radicals causes major health problems such as Cancer, Alzheimer's disease, Cardiovascular Diseases, Cataract, Rheumatoid

Arthritis and other degenerative diseases associated with aging, therefore Antioxidants can be beneficial to neutralize free radicals before they can attack cells and hence prevent damage to cell proteins, lipids and carbohydrates.

To determine genetic diversity, 10 primers (GeNei RPI-1 to GeNei RPI-10) were used. Among ten primers used, two primers (GeNei RPI-3 and GeNei RPI-4) gave the best distinguishable band pattern as shown in [Fig-9a], [Fig-9b].

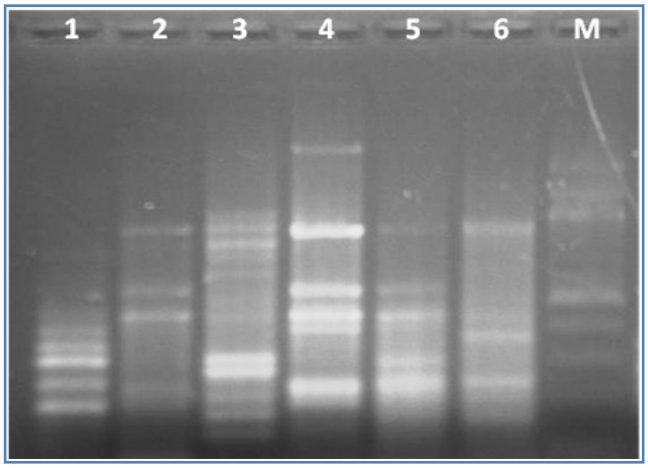

Fig. 9a- RAPD profiles of six selected cultivars obtained with GeNei RPI-3

1. *C. inerme,* 2. *C. peniculatum,* 3. *C. philippinum,* 4. *C. phlomidis,* 5. *C. serratum,* 6. *C. villosum* and M. 100bp marker. A. GeNei RPI-3 B. GeNei RPI-4.

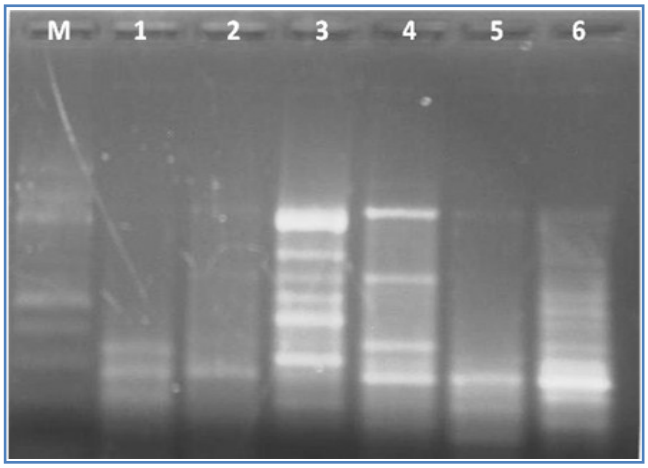

Fig. 9b- RAPD profiles of six selected cultivars obtained with GeNei RPI-4

1. *C. inerme,* 2. *C. peniculatum,* 3. *C. philippinum,* 4. *C. phlomidis,* 5. *C. serratum,* 6. *C. villosum* and M. 100bp marker. A. GeNei RPI-3 B. GeNei RPI-4.

Dendrogram analysis of the DNA fingerprint generated by GeNei RPI-3 and GeNei RPI-4 primer is shown in [Fig-10], [Fig-11] respectively. Similarity Matrix showed that *C. inerme* and *C. serratum* are closely related. *C. inerme* and *C. serratum* showed 100% similarity for primer GeNei RPI-4. *C. peniculatum* and *C. phlomidis* are more closely related based on the similarity matrix by GeNei RPI-3 primer.

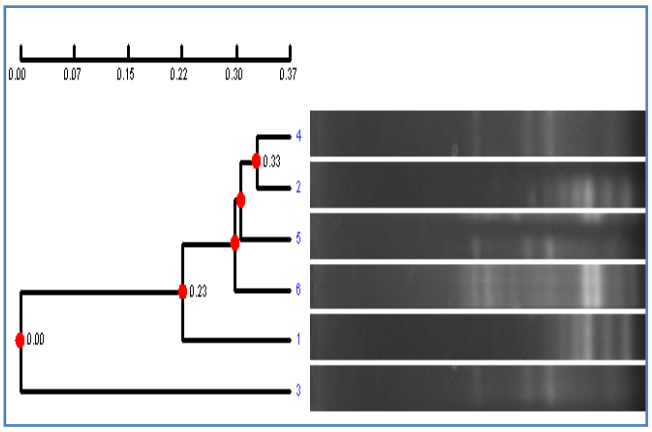

Fig. 10- Dendrogram of six samples for primer GeNei RPI-3

1. *C. inerme,* 2. *C. peniculatum,* 3. *C. philippinum,* 4. *C. phlomidis,* 5. *C. serratum,* 6. *C. villosum*

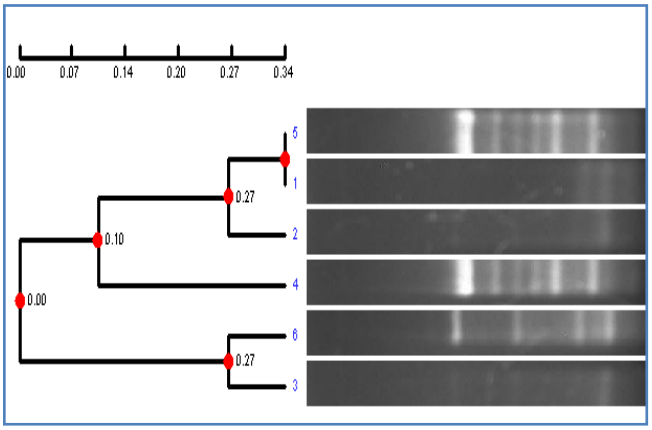

Fig. 11- Dendrogram of six samples for primer GeNei RPI-4

1. *C. inerme,* 2. *C. peniculatum,* 3. *C. philippinum,* 4. *C. phlomidis,* 5. *C. serratum,* 6. *C. villosum*

Conclusion

The selected *Clerodendrum sp*. contains phenolic compounds, glycosides, anthraquinones, terpenoids, flavinoids, tannins, lignin and saponins. The potential for developing Antioxidants from *Clerodendrum* plants appear rewarding as it may lead to the development of novel phytomedicine. Also, screening of *Clerodendrum* can make a way for identification of new important antimicrobial components. Further study can be done for determination of toxicity, side effects and pharmaco-kinetic properties of isolated antimicrobial compounds. Molecular characterization by RAPD PCR revealed that *C. inerme* and *C. serratum* are closely related and share more common DNA sequence among all six species. Molecular study data can be served as prerequisite for plant breeding activities as well as for conservation of genetic resources. From the current phytochemical data it can be conclude that *Clerodendrum sp.* may be utilize as a source of ingredient to the pharmaceutical industries for the development of new bioactive compound.

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