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Molecular characterization and antioxidant activity of *Volkameria inermis* L.

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ABSTRACT

The present investigation aimed to study the genomic characterization and free radical scavenging potential of a traditional plant *Volkameria inermis*. A chloroplast genome rbcL was used in molecular characterization and it revealed the phylogeny and evolutionary affinities of *V. inermis* with the outgroups of the family through BLAST search based on Neighbor-Joining (NJ) analysis. The free radical scavenging potential of various extracts of *V. inermis* was observed at different concentrations (20, 40, 60, 80 and 100 µg/mL) in DPPH and ABTS *in vitro* models. Ascorbic acid (DPPH) and Trolox (ABTS) were used as standard. In DPPH assay, among the different extracts (hexane, chloroform, acetone, ethanol, methanol and water) tested, methanol showed maximum scavenging activity with the IC_{50} value of 28.33µg/mL followed by acetone with the IC_{50} value of 31.937µg/mL. The other extracts, ethanol and water extracts showed moderate activity with the IC_{50} value of 82.558 µg/mL and 81.758µg/mL; chloroform and hexane showed very lower antioxidant activity and the IC_{50} values are 518.776µg/mL, 1066.16µg/mL accordingly. In ABTS assay, methanol was observed as a highly active extracts with the IC_{50} value of 70.196µg/mL followed by ethanol extract (103.078µg/mL). The results showed that the methanol extract exhibits strong antioxidant activity in DPPH and ABTS assays. The scavenging efficiency showed dose dependent increase in concentration absorption compared to Ascorbic acid and Trolox. Our findings provide the molecular identification and evidence for the potential of *Volkameria inermis* as a source of natural antioxidants.

KEYWORDS: *Volkameria inermis* L., molecular characterization, phylogenetics and antioxidant potential.

INTRODUCTION

Medicinal plants are nature's gift providing medication to the people from the ancestors. Plants are the major resources of Indian traditional and modern systems of medicine, which constitute great strategic and economic value. According to WHO, more than 80% of the global population relies upon traditional medicinal plants for their treatment and betterment. About 30% of the pharmaceutical drugs were derived from green plants (Dobriyal *et al*., 2021). With the increasing demand for plant based treatments, authentication of medicinal plant materials is more important. In comparing the traditional identification methods (Macroscopic, microscopic and chemical profiling), the identification of plants at the DNA level (Molecular taxonomy) provides more reliability. DNA bar-coding is a revolutionary approach in taxonomy that allows identifying an organism at any stage of development from a very small tissue sample which may fresh or conserved many years ago. The plant DNA bar-coding is now performing the essence of species identification thereby serving in the molecularization of taxonomy. Molecular phylogenetic studies are profusing worldwide to provide greater resolution of phylogenetic relationships for inferring phylogenetic history. It ensures rapid, reliable and effective species identification (Floyd *et al*., 2002; Hebert *et al*., 2003). It can be a useful method to define the boundaries of species, flagging of new species and delimitation of species has been demonstrated recently on a large scale (Amin *et al*., 2020).

Antioxidant, free radical scavengers can prevent the pathogenic processes of cancer, cardiovascular disease, respiratory disorder, macular degeneration and cataracts (Farag *et al*., 2020). Plants are a rich source of natural antioxidants, which improve the immune system. The efficiency of antioxidant varies depends on the ecological features (Garcia & Castro, 2016). While analyzing the antioxidant activities of plant extracts by different methods, their reaction characteristics and mechanisms can show variations, and no universal assay is available that can accurately reflect all of the antioxidants in a complex system (Meng *et al*., 2012). Therefore, it is necessary to use at least two complementary methods to evaluate the antioxidant capacity *in vitro*. In this study, two antioxidant assays (DPPH and ABTS,) were applied to obtain more accurate evaluations of antioxidant activities. *Volkameria inermis* L. [= *V. neriifolia* Roxb., *Clerodendrum inerme* (L.) Gaertn., *Clerodendrum neriifolium* (Roxb.) Schauer] is an evergreen plant separated from the polyphyletic genera Clerodendrum L. and is transferred from Verbenaceae to Lamiaceae based on morphological and

cytological variation (Yuan *et al*., 2010). Verbenaceae shows a close relationship with Lamiaceae and is evident from numerous shared characters (Wagstaff & Olmstead, 1997). *Volkameria inermis* often a scrambling or scandent shrub that can reach up to 3 m tall but sometimes a liana and measures up to 13 m long. The plant is native to the coastal area of India, Sri Lanka, Burma, Malaya, tropical Australia, Polynesia and the Philippine Islands. Traditionally the plant is used in the treatment of fever, cough, scrofulous infection, venereal infection and skin diseases in folklore medicine (Kanchanapoom *et al*., 2001). The plant exerts many pharmacological effects such as anti-inflammatory, analgesic, antipyretic, neural and smooth muscle effects, antimicrobial, antidiabetic, antioxidant, antiparasitic, insecticidal, anti-allergic, anticancer, protective and many other pharmacological effects (Chethana *et al*., 2013). On the basis of ethnomedicinal application the present investigation was aimed at systematic study and to evaluate its antioxidant potential associated with the reduced risk of cancer and many chronic diseases.

MATERIALS AND METHODS

Selection, Identification and Collection of Selected Taxon

The fresh leaves of *Volkameria inermis* L. were collected from Thiruverumbur, Tiruchirappalli district, Tamil Nadu, in the month of March 2021. Juvenile leaf material of healthy plant with flower and fruit were identified and the plant was authenticated at Rapinat Herbarium, St. Joseph's College, Tiruchirappalli. The voucher specimen was prepared and submitted with the voucher number: 2991 to the respected department for further reference. The fresh leaf material of *Volkameria inermis* (Figure 1) was stored at -80°C. for molecular (identification) studies.

Molecular Taxonomy

Genomic DNA isolation, PCR amplification of rbcL gene and sequencing

The genomic DNA was extracted from the young leaf tissues of V. *inermis* using plant DNA extraction kit (NucleoSpin® Plant II Kit (Macherey-Nagel) following the protocol established by Kumar *et al*. (2003), with slight modifications. The quantity (ng/µl) and quality (A260/A280 ratio) of extracted DNA was determined by measuring the absorbance using spectrophotometer. The quality of DNA was further accessed by fractioning them on a 0.8% agarose gel in 1× TBE buffer and stained with ethidium bromide. To amplify the *ribulose1-5-bisphosphate carboxylase/oxygenase* (*RBCL*) gene, a pair of RBCL F ATGTCACCACAAACAGAGACTAAAGC and RBCL R TCGCATGTACCTGCAGTAGC, oligonucleotide primers were designed. A 10 µL of PCR amplification reaction contains 2×Phire Master Mix (Thermo Fisher Scientific, USA), 0.25µL of both forward and reverse primers, and 1µL of genomic DNA and the reactions were carried out in a thermal cycler (Gene Amp PCR System 9700, Applied Biosystems). The PCR amplification included one cycle at 98 °C for 30 s, followed by 40 cycles of 98 °C for 5 s, 58 °C for 15 s and 72 °C for 15 s and a final cycle at 72 °C for 60 s. The gene specific PCR amplification of the *rbcL* fragment was confirmed by resolving on a 1.2% agarose gel. Then the amplified PCR products were purified using ExoSAP-IT (GE Healthcare) kit. The purified PCR products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in the presence of 1 µL PCR product, following the manufactures protocol. The sequencing PCR products were further purified using BigDye × Terminator kit (Thermo Fisher Scientific®, Waltham, MA, USA) and then subjected to sequencing in ABI 3500 DNA Analyzer (Applied Biosystems). The results were analyzed withSequence Scanner Software v1 (Applied Biosystems) for sequencing quality. The obtained DNA sequences were further compared with already reported sequences in Genbank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast). The required sequence editing and alignments were performed using BioEdit v. 7.2 (https://bioedit.software.informer.com).

Figure 1: Morphology of *Volkameria inermis*

Phylogenetic Analysis

Phylogenetic analysis was performed to determine the genetic relationship and genetic distance of selected taxa. DNA barcoding sequence of *Volkameria inermis* and their outgroups (*Tetraclea coulteri*, *Rotheca serrata* and Lamiaceae) were searched in NCBI's GenBank, which includes deposited data of other researchers and research institutions. We collected all the sequences in NCBI BLAST searches, organized and aligned them using BioEdit ver. 7.2 sequence alignment editor and multiple alignments program Clustal W (Thompson *et al*., 1994). The phylogeny was inferred with the Neighbor-Joining method by using Kimura-2-parameter model (Kimura, 1980) through MEGA 5 (Tamura *et al*., 2011). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary relationship of the taxa analyzed (Felsenstein, 1985). Branches having less than 50% bootstrap values were merged into higher valued branches.

Preparation of Plant Extracts

The healthy leaves of V. *inermis* were collected, washed and shade dried for about a week; the dried leaves were grounded

coarsely using an electrical grinder and stored in air tight container. Based on the polarity index (Harborne, 1973; Trease & Evans, 1987) the solvents of n-hexane, chloroform, acetone, ethanol, methanol and aqueous were selected for extraction using the Soxhlet apparatus. The extracts were concentrated in Rotary evaporator and stored at 4°C.

Antioxidant Activity

DPPH free radical scavenging activity

In the presence of antioxidant potential, plant extracts can donate an electron to DPPH and reduction in the DPPH free radicals measured at 517 nm. Various concentrations of plant extracts (20, 40, 60, 80 &100 µg/mL.) were added to 3 mL of 0.3mM solution of DPPH in methanol/ethanol. The absorbance was measured after a 30-min reaction under darkness at room temperature. Ascorbic acid was used as standard. Measurements were taken in triplicate. The radical scavenging activity of plant extracts was calculated using the following formula (Brand-Williams, 1995).

$$\%\ \text{of Radical scavenging activity} = \frac{A0 - Al}{A0} \times 100$$

Whereas, A_0 = absorbance of the control and A_1 = absorbance of the test sample/standard. The results were reported as IC_{50} values and ascorbic acid equivalents (AAE, mg/g) of *V. inermis* leaf extracts.

ABTS radical cation scavenging activity

Scavenging activity of ABTS+ radical was conducted following Chang *et al.* (2007) with minor modifications. ABTS + cation radical was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1), stored in the dark at room temperature for 12-16 h before use. The ABTS radical solution was diluted with methanol to an absorbance of 0.75 ± 0.05 at 734 nm. $ABTS^+$ solution was then diluted with methanol to obtain an absorbance of 0.700 at 734 nm. Each 5 µl of plant extracts was diluted with 3.995 mL of $ABTS^{\cdot+}$ solution, the absorbance was measured after 30 min of initial mixing. An appropriate solvent blank was run in each assay and Trolox was used as the standard substance. Measurements were taken in triplicate. The percentage of inhibition was calculated using the formula-

$$\%\ \text{of Scavenging Activity} = \frac{A0 - Al}{A0} \times 100$$

Where A_0 was the absorbance value of the control or blank sample and A_1 was the absorbance value of ABTS radical$^+$ sample/standard. The results were reported as IC_{50} values and Trolox equivalents TE, (mg/g) of *V. inermis* leaf extracts.

Statistical Analysis

The experimental results were expressed as the means ± SD of triplicate values. Where applicable, the data were subjected to one way analysis of varience (ANOVA) and significance of difference between the sample were calculated by Duncan's Multiplication Range test using Graph Pad Prism software version 5.0. P values < 0.005 were regarded as significant.

RESULTS AND DISCUSSION

Standardization of Barcode Genes

Taxonomically identified *Volkameria inermis* L. was subjected to further molecular study. DNA barcodes enable rapid and accurate species identification using short, standardized genomic regions as internal species tags (Yang *et al.*, 2019). The results of the DNA isolation from young leaves sample of *V. inermis* showed good quality and the determination of the quality was carried out through electrophoresis using 0.8% agarose. The quality of DNA is indicated by its thickness and brightness of DNA bands. Universal barcode genes (i.e. *rbcL*) were amplified in PCR, while satisfactory amplification was obtained with the *rbcL* (aF & aR). A single discrete PCR amplicon band of ~661 bp was observed when resolved on agarose (Figure 2). DNA sequences can easily extract with *rbcL* primers from a wide range of tropical plant species (Lahaye *et al.*, 2008). The results of the present study clearly supported that the most common gene used to provide sequence data for plant phylogenetic analyses is the plastid-encoded *rbcL* gene. The Consortium for the Barcode of Life (CBOL) plant working group has also recommended that, *rbcL* will be the standard genes for the barcoding of land plants (Kress & Erickson, 2007; CBOL Plant Working Group, 2009; Dhakad *et al.*, 2020). However, using more than one primer pair can be time consuming as well as costly and is often complex for large-scale projects (Heckenhauer *et al.*, 2016).

Identification of species and nucleotide sequences

The DNA nucleotide sequence determined in the present study was identified as *Volkameria inermis*. The result of molecular identification was compared with a level of identity (homology) of 98%-100% (Table 1). The molecularly identified nucleotide sequence was submitted in NCBI with the accession number:

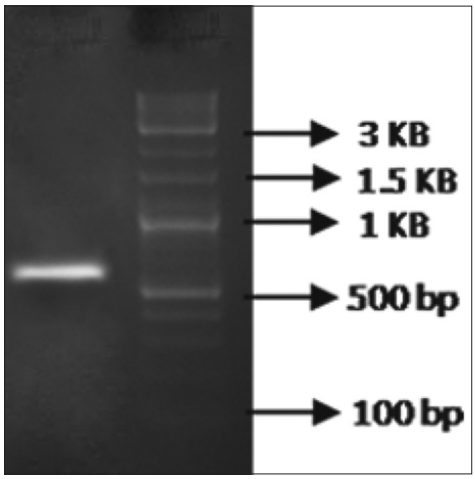

Figure 2: Genomic DNA Ladder of *Volkameria inermis*

Table 1: BLAST results of *Volkameria inermis* sequences showing similarity with the other species NCBI database

Description	Max Score	Total score	Query cover	Identity	Accession
Volkameria inermis isolate Cine5 ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1216	1216	100%	99.85%	MK331784.1
Volkameria inermis isolate Cine4 ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1216	1216	100%	99.85%	MK331783.1
Volkameria inermis isolate Cine3 ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1216	1216	100%	99.85%	MK331782.1
Volkameria inermis isolate Cine9 ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1216	1216	100%	99.85%	MK331760.1
Volkameria inermis isolate Cine8 ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1216	1216	100%	99.85%	MK331759.1
Volkameria inermis isolate Cine7 ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1216	1216	100%	99.85%	MK331758.1
Volkameria inermis isolate Cine6 ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1216	1216	100%	99.85%	MK331757.1
Volkameria inermis isolate Cine2 ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1216	1216	100%	99.85%	MK331756.1
Volkameria inermis isolate Cine1 ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1216	1216	100%	99.85%	MK331755.1
Volkameria inermis isolate Cine10 ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1216	1216	100%	99.85%	MK331761.1

MN901637 and can be used further in research as *Volkameria inermis*.

Molecular Phylogenetics

Results of the BLAST search shows, sequences producing significant alignments. The first ten closest sequences of *Volkameria inermis* based on nucleotide homology, producing significant sequence alignments with the NCBI database were presented in Table 1. Sequences producing significant alignments give us an idea about the phylogenetic closeness of *Volkameria inermis* to *Clerodenrum inerme*, *Tetraclea coulteri*, *Rotheca serrata*, *Clerodenrum indicum*, *Clerodendrum thomsoniae*, *Clerodendrum quadriloculare*, *Tetraclea* sp. belongs to Lamiaceae. Neighbor-Joining analysis of the *rbcL* sequence of *Volkameria inermis was* inferred for molecular phylogenetics with the closely related 100 sequences of different species (Figure 3). The phylogenetic analysis between the target sequence and closely related NCBI sequences showed 99-100% sequence percentage alignment for *rbcL* genes. Branch length between species shows the difference in the rate of evolution and their nucleotide bases. The longer branches indicated the species has a greater DNA base difference than other species. Conversely, the shorter a branch shows the difference in nucleotide bases and the less evolutionary rate (Nurilmala *et al*., 2020).

In Vitro Antioxidant Activity

DPPH free radical scavenging activity

The various solvent extracts of *Volkameria inermis* tested for its antioxidant potential against ascorbic acid the standard drug. The results of DPPH assay reveals effective and concentration dependent (20, 40, 60, 80 and 100 µg/mL) scavenging potential. The IC_{50} values of various tested extracts were found to be 65.507µg/mL, 1066.16µg/mL, 440, 518.776µg/mL, 31.937µg/mL, 82.558µg/mL, 28.33µg/mL and 81.758µg/mL for the ascorbic acid, hexane, chloroform, acetone, ethanol, methanol and water respectively. From the results it is observed that methanol and acetone extracts of *V. inermis* showed comparatively higher scavenging activity with the IC_{50} value of 28.33 µg/mL and 31.937 µg/mL than the standard drug ascorbic acid. However, ethanol and water extracts of *V. inermis* showed moderate; chloroform and hexane extracts showed the lowest scavenging activity against the DPPH radicals. Scavenging potential of plant extracts showed reduction in DPPH, it becomes visible by the change of purple color to yellow (Musa *et al*., 2016; Dehariya et al., 2020). Intensity of the yellow color proportionate to scavenging potential and it is increased with the increase concentration of plant extracts and standard. The results (Figure 4) of present study acknowledge that, majority of the tested leaf extracts of *V. inermis* show scavenging activity against DPPH radical. Methanol extracts of *V. inermis* exhibited a remarkable twofold higher scavenging activity than that of standard ascorbic acid. Similar observations with the increased scavenging activity with the increased concentration were reported in *Severinia buxifolia* leaves by Truong *et al*. (2019) and in the whole plant of *Sphaeranthus indicus* by Tandon and Gupta (2020) in methanol extracts.

ABTS radical cation scavenging activity

The scavenging activity of ABTS radical by the leaf extracts of *V. inermis* was found to be higher than the standard (Trolox) used in this study. A blue chromophore formed in the reaction between ABTS and potassium persulfate was reduced based on the efficacy of the plant components (Wang *et al*., 1998). The results showed that the concentration dependent (20, 40, 60, 80 and 100 µg/mL) scavenging ability of the leaf extracts against the ABTS radicals. The IC_{50} values of various extracts were found to be 616.648µg/mL, 165.39µg/mL, 103.078µg/mL, 70.196µg/mL, 545.361µg/mL and 106.75µg/mL for chloroform, acetone, ethanol, methanol, water and Trolox respectively. Methanol and ethanol extracts of *V. inermis* showed remarkably higher activity

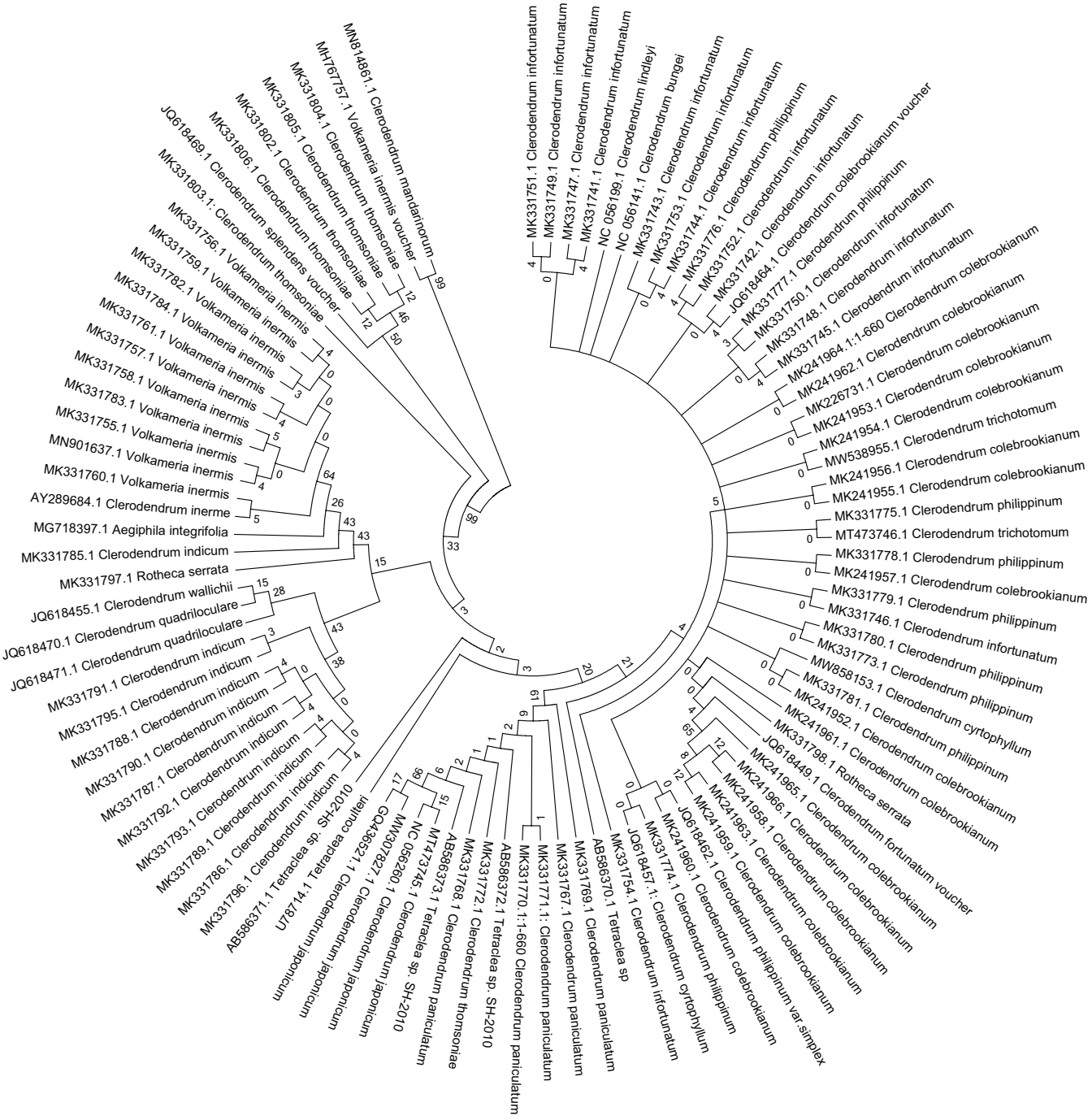

Figure 3: Phylogenetic relationship of *Volkameria inermis* with their similar sequences through BLAST search based on Neighbor-Joining (NJ) analysis of *rbcL* sequence

compared to the standard Trolox. However, acetone shows moderate activity and water and chloroform extracts exhibits lower activity against ABTS radicals. There are no scavenging reactions were observed in various concentration of hexane extracts of *V. inermis* (Figure 5). Similar results were reported by Adebiyi *et al*. (2017) in stem extracts of *Grewia carpinifolia*. Wang *et al*. (1988) reported that the compounds which have the ability to scavenge ABTS radical did not show DPPH scavenging activity. In contrast, the present results showed the capability of *V. inermis* extracts to scavenge different free radicals in different systems, indicating the presence of strong phenolic components. Plant phenolics confer scavenging ability because of their hydroxyl groups (Gangwar *et al*., 2014). The present results were in accordance with Gangwar *et al*. (2014) and says that *V. inermis* leaf extract contains active phenolic constituents that are capable of donating hydrogen atom to a free radical in order to remove odd electron which is responsible for radical's reactivity. The total phenolic content of *V. inermis* leaf was determined as 558.94±1.527µg/g in earlier reports by Shanthi *et al*. (2020). The result of the present studies on molecular

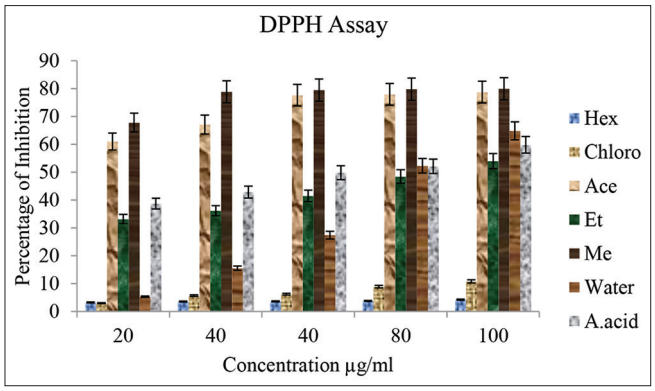

Figure 4: Free Radical Scavenging activity of *Volkameria inermis* using DPPH assay

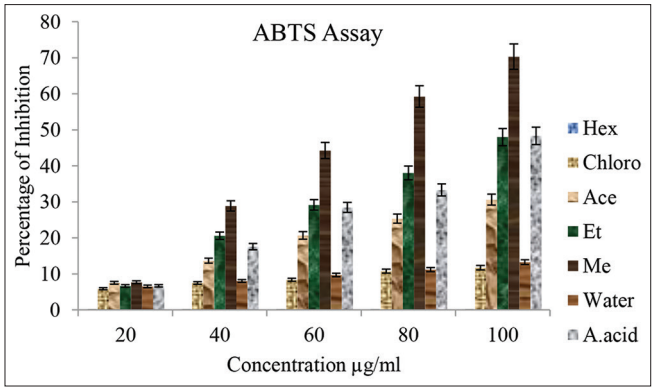

Figure 5: Free Radical Scavenging activity of *Volkameria inermis* using ABTS assay

characterization and the free radical scavenging activity will be of immense value in determining the identity, purity and efficacy of the plant *V. inermis* as a potent drug. Molecular bar-coding is an advanced identification method of medicinal plants, providing their substitutes and adulterants at the genus and species level. DNA bar-coding assists plant taxonomy by studying the characteristic variations for each species in widely recognized genomic loci. The antioxidant determination of indicates that the plant *V. inermis* is a significant natural antioxidant, which might be helpful to avert the progress of various oxidative stresses. Consumption of this plant leaves may prevent human disorders such as cancer, cardiovascular disease and pre-mature ageing, in which free radicals play major role.

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