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CHARACTERIZATION AND EXPLORING GENETIC POTENTIAL OF LANDRACES FROM WESTERN GHATS OF SATHYAMANGALAM WITH SPECIAL EMPHASIS ON PHYTOCHEMICAL CONTENT FOR BENEFACTION OF *EVOLVULUS ALSINOIDES* (LINN.) LINN

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ABSTRACT:

Evolvulus alsinoides (Linn.) Linn. is referred as "brain tonic" in Asia and America owing to its activity on the central nervous system. The plant is also found to possess ample of medical activities has been extensively used as traditional medicine in various culture aspects. In the present investigation, the impact of altitude and season of harvest on the phytochemicals and biological activities of E.alsinoides (Linn.) Linn. has been studied. The root, stem and leaves of the plant collected from different altitudes in the Western Ghats of Sathyamangalam, Tamilnadu during the month of July and December, 2016. The primary and secondary metabolites were analysed qualitatively and quantitatively using standard protocols. Minitab 17 Statistical software package was used for Statistical analysis. The altitude and month of plant harvest has an impact on the accumulation of the primary and secondary metabolite in the root, stem and leaves. The leaf samples are found to possess significant antioxidant activities which is further validated by the cytotoxicity activity on SH-SY5Y Neuroblastoma cell line with an IC50 of 119.783 ± 0.06 µg/ml. Isolation of individual molecules and its authentication on the biological activity could give prolific results.

Keywords: Evolvulus Alsinoides (Linn.) Linn., Trait Variation, Phytochemistry, Antioxidant, Tlc, Sh-Sy5y.

1. INTRODUCTION

The use of complementary and integrative medicines using biologicals have been increasing rapidly owing to its less frequent side-effects. Plants have been used to lessen various diseases in traditional medicine (Mohajer et al., 2016). Plants are the potent source of many therapeutics such as anticancer, antimicrobials, antidiabetic, antidepressants etc (Singhal and Chakraborthy, 2016). Temperature and UV-B radiation varying with altitude of habitats enforce significant effect on plant secondary metabolism (Zlatko S. Zlatev, 2012). However, an in-depth study on the altitudinal impact of chemical profile has not been studied with species of traditional medicine.

Evolvulus alsinoides (Linn.) Linn. is one among the Dashapushpam, literally meaning "ten flowers" referring to the ten species of plants that are being considered auspicious. *Evolvulus alsinoides* (Linn.) Linn. is considered very popular in Ayurveda for its powerful brain stimulant activity together with toning up effect on the intellectual powers (Vijayan et al., 2010). The plant is also endowed with potential anthelmintic property (Dash et al., 2002). hepatoprotective activity(Chander and Reddy, 2014) , antiinflammatory, antipyretic, and antidiarrhoeal (Lekshmi and Reddy, 2011) and to treat epilepsy (Sáenz et al., 2010). The phytochemical analysis has reported the existence of biomolecules such as β-sitosterol, scopolin, scopoletin, triacontane, umbelliferon, methyl-1,2,3,4-butaneterol, shankpushpine and betaine (Mehta and Shah, 1958). Phytochemical analysis and their extraction form the key principle in characterization of therapeutically active ingredients from plant sources (Ganie and Sharma, 2014).Owing to the widespread importance of *Evolvulus alsinoides* (Linn.) Linn. in traditional medicine, the natural habitats grown at varying altitudes was evaluated for seasonal variations in phytoconstituents reported from Western Ghats of Sathyamangalam, Tamil Nadu, for the first time. This sampling of populations from habitats with varying altitude can allow us to assess the intra-specific variations and main ecological trends of phytochemical accumulation in plants.

2. MATERIALS AND METHODS PLANT COLLECTION

The whole plant material (Figure 1) was collected from seven different places of Sathyamangalam Ghats in the months of July and December, 2016 from different altitude. The authentication of the plant was done at Botanical Survey of India (BSI/SRC/5/23/2016/Tech/1711 Dated 27.10.2016), Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Figure 1: Habitat of *Evolvulus alsinoides* (Linn.) Linn.

Geographical Location

Geographical Data of *Evolvulus alsinoides* (Linn.) Linn. growing places in Sathyamangalam Ghats are represented in Table 1 and Figure 2.

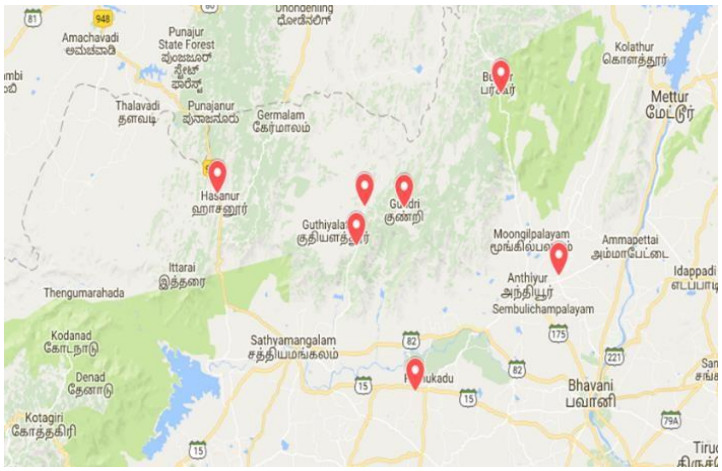


Figure 2: Map showing the location of plant collection

Table 1: Geographical Data and seasonal climatic conditions of *Evolvulus alsinoides (Linn.)* *Linn.* growing places in Sathyamangalam Ghats of Tamil nadu, India

S.No.	Location	Sample Code	Collection date	Latitude (N)	Longitude (E)	Elevation (m)
1.	Gobichettipalayam	GCM	July 10, 2016	11.45	77.41	231.917
2.	GundriPirivu	GPU	July 9, 2016	11.66	77.34	669.131
3.	Kadambur	KDR	July 9, 2016	11.62	77.32	802.54
4.	Gundri	GDI	July 9, 2016	11.65	77.40	885.009
5.	Bargur	BGR	July 17, 2016	11.76	77.55	1019.095
6.	Hasanur	HSR	July 10, 2016	11.65	77.17	1225.371
7.	Patlur	PTR	July 17, 2016	11.58	77.63	221.648

Specimen Processing

After collection, the plant sample was washed once in running tap water and twice with double distilled water. The leaves were separated from the whole plant and dried in shade for 5-8 days until the moisture content becomes insignificant. Then the raw material was grounded to powder form and stored at room temperature in airtight container in dark until processed (Dash et al., 2002).

Qualitative Analysis of Phytochemicals

Phytochemical analysis of the root, stem and leaves was carried out using standard procedures as described by (Evans et al., 2009) Trease and Evans (1979), Harborne (1984) and Sofowara (1993). The phytochemical analysis was done for the grounded raw material (50mg) and the ethanol extract (50mg/ml) of the raw material prepared using maceration method (Hussain and Kumaresan, 2014).Electronic Weighing Balance (Readability 0.1mg) "Mettler Toledo", AB-265-S was used for measuring the weight of the samples and the chemicals. The phytochemicals analysed are Carbohydrates (Barfoed's test, Molish' s test, Benedict' s test), Proteins (Biuret test), Flavonoids, Alkaloids, Phenolic compounds, Tannins, Saponins, Glycosides, Steroids, Cardiac glycosides, Phlobatannins (Auwal et al., 2014), Phytosterols (Altemimi et al., 2017).

Quantitative screening

The quantitative analysis of the biomolecules in the leaf, stem and root were analysed as triplicates. 50mg of the test sample was utilized for the biochemical assays. Double beam, UV-VIS Spectrophotometer (Make: Perkin Elmer & Model: Lambda35) was used tomeasure the absorbance of the complexes being formed.Quantitative analysis was performed for carbohydrates (Dey, 1990) and proteins (Esen, 1978) .

Phenolics

The total phenolics in the test sample were estimated using spectrophotometric assay (Barreira et al., 2008) . Gallic acid was used for constructing the standard curve (20–100 µg/mL, $Y = 6.720x + 0.036$, $R^2 = 0.994$) and the results were expressed as mg of gallic acid equivalents/mg of extract (GAEs).

Flavanoids

The flavonoid content in the plant samples was determined using Aluminium Chloride Colorimetric Method. The absorbance was read at 510 nm using Quercetin standard with the equation of $y = 0.012x - 0.104$, $R^2 = 0.995$. The flavonoid content was quantified as mg Quercetin equivalent per g dry weight (Masoko and Nxumalo, 2013) .

DPPH radical screening activity

The ability of the biomolecules to scavenge DPPH radicals was assessed. 500 µL of 0.2 mmol/L of alcoholic DPPH reagent was added to 50 mg/ml extract, shaken dynamically and allowed to stand at room temperature in dark for 30 mins. The absorbance of the sample was read using UV-Vis spectrophotometer at 517 nm (Gomathi et al., 2014). A lower absorbance value indicates higher radical scavenging activity.

Total antioxidant activity (FRAP Assay)

The stock solutions used for FRAP assay were 300 $mmol \cdot L^{-1}$ acetate buffer at pH 3.6, 10 $mmol \cdot L^{-1}$ 2,4,6-tripyridyl-*S*-triazine in 40 $mmol \cdot L^{-1}$HCl and 20 $mmol \cdot L^{-1}$ $FeCl_3 \cdot 6H_2O$ solution. 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3 \cdot 6H_2O$ were mixed together to get the working solution. The temperature was increased to 37℃ prior to use (Ahmed et al., 2015) . 50 mg/ml extract was made to react for 30 min with 2850 µL FRAP solution in the dark. Ferrous tripyridyltriazine complex formed is filtered and its absorbance was read at 593nm.

Thin layer Chromatography

The methanol extract of the leaf samples collected from the study area were analyzed using thin layer chromatography. Pre-coated and pre-activated TLC Silica gel 60 F_{254} plates used

in the study was procured from Merck Specialities Private Limited. The samples were loaded on the TLC plate 0.50 cm above its bottom using glass capillary tubes. After the application of the sample, the plates werekept in solvent saturated TLC glass chamber for the elution of the molecules (Harbourne, 1998). The solvent system used for fingerprinting analysis is Chloroform:Methanol (15:1).

Liquid - Liquid Extraction

Sequential solvent extraction coupled Cold maceration method was used for extracting the phytochemicals present in the leaf of the plant using organic solvents of different polarity as solvents used in the soaking process play a critical role in extracting the phytochemicals (Nn, 2015) . The solvents used for the extraction are hexane, chloroform, ethanol, ethyl acetate, methanol and water (Nahata et al., 2009) . The fractions obtained was concentrated using Rotary Evaporator (Make: Cyber lab, USA & Model: CR2000) under reduced pressure and freeze dried (Khan et al., 2012) using Freeze Dryer (Make: Martin Christ – Germany, Model: Alpha 1 – 2 LD Plus, P/No. 101525) and stored in air tight container at 4℃. The residue was dried at room temperature to remove residual solvent and then added with 200 ml of chloroform and the process was repeated as done with hexane. The residue was added with solvents of increasing polarity and the solvent soluble fraction was concentrated and dried as done for hexane fraction.

SH SY5Y (Human, Neuroblast) cytotoxicity assay

The hydroalcoholic extract of the *Evolvulus alsinoides* (Linn.) Linn. leaves were assessed for its cytotoxicity activity on SH-SY5Y, neuroblastoma cell lines. The cells were treated with 30–250 µg/mL of leaf extract. 3-(4, 5-dimethylthiazolyl)-2,5 – diphenyl – tetrazolium bromide (MTT) colorimetric assay was used to study the cytotoxicity of the extracts. The absorbance was read at 540nm. The percentage cell viability was calculated and concentration of extract needed to inhibit cell growth by 50% values were generated from the dose-response curves (Kurokawa et al., 2016).

Statistical analysis

Statistical analysis was done by Analysis of Variance (ANOVA) using Minitab-17 Statistical Software Package. All the experiments were carried out in triplicates. Statistical significance was determined at $p< 0.001$.

3. RESULTS AND DISCUSSION PHYTOCHEMICAL ANALYSIS

The phytochemical analysis of primary and secondary metabolites (Table 2 and 3) in the leaf samples reveal the existence of major phytochemical constituents both in the whole sample (RM) and the ethanol extract (EXT). The concentration of alkaloids in the leaf, stem and root had significant differences (Table 4) with changes in altitude. Leaves were found to possess more alkaloid content than the stem and root, the intensity of the complex formed with the reagents is also more for the samples collected at higher altitudes. Studies have shown that phenol content is affected directly by sunlight and climate. *Evolvulus alsinoides* (Linn.) Linn. is very polymorphic with altitudinal and seasonal variations. The results obtained with the phytochemical studies are in comparable with the reports of (Amin et al., 2014) and (Gupta et al., 2014) in the aqueous and alcoholic extracts. (Sullivan et al., n.d.) 2011) also reported the effect of climate and location on the phenol content. (Gordon and An, 1995) reported the antidiarrheal activity of tannins by its action on peristaltic movement and intestinal secretion. Saponins have been studied to be expectorants with cardiotonic activity and hypoglycemic with anti-diabetic effects by (Auwal et al., 2014) . The presence of tannins and saponins at

Table 2: Phytochemical analysis of whole leaf and ethanol extract for primary metabolites in *Evolvulus alsinoides (Linn.) Linn.*

S.No.	Sample Code	Carbohydrates						Proteins	
		Molish Test		Barfoed Test		Benedict Test			
		RM	EXT	RM	EXT	RM	EXT	RM	EXT
1	PTR	+++	+++	+++	+++	+++	+++	+++	+++
2	HSR	+	+	+	+	+	+	+	+
3	GDI	+	+	+	+	+	+	+	+
4	KDR	++	++	++	++	++	++	++	++
5	GCM	+	+	+	+	+	+	+	+
6	BGR	+	+	+	+	+	+	+	+
7	KDM	++	++	++	++	++	++	++	++

Table 3: Phytochemical analysis of whole leaf and ethanol extract in *Evolvulus alsinoides (Linn.) Linn.*

S.No.	Sample Code	Flavanoides		Phenolic compounds		Tannins		Saponins		Cardiac glycosides		Steroids		Phlobatannins		Phytosterols	
		RM	EXT	RM	EXT	RM	EXT	RM	EXT	RM	EXT	RM	EXT	RM	EXT	RM	EXT
1	PTR	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-
2	HSR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	GDI	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
4	KDR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	GCM	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+

6	BGR	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+
7	KDM	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+

significant levels recommends the plant for the treatment of diarrhoea and diabetes.

Table 4: Test for alkaloids in *Evolvulus alsinoides (Linn.) Linn.*

Sample Code	Part	Mayer's	Hager's
GDI	Root	+	+
	Stem	++	++
	Leaf	+++	+++
GPU	Root	+	+
	Stem	++	++
	Leaf	+++	+++
KDR	Root	+	+
	Stem	++	++
	Leaf	+++	+++
BGR	Root	+	+
	Stem	++	++
	Leaf	+++	+++
HSR	Root	+	+
	Stem	++	++
	Leaf	+++	+++
PTR	Root	-	-
	Stem	+	+
	Leaf	++	++
GCM	Whole Plant	++	++

Quantitative analysis

Total carbohydrates and Proteins

The total carbohydrates and proteins are found to be more in the leaf samples than in stem and root. Substantial variances in the concentration of carbohydrates (Figure 3,5) and proteins (Figure 4) is detected in the leaf samples with the changes in altitude. The percentage of crude protein content differs between roots, stem and leaves with altitude. Leaves had more protein than roots and Figure 6 shows the maximum protein content found in the plant sample collected during winter. The carbohydrate content had a positive correlation with net photosynthetic capacity. (Gruber et al., 2011) have reported the spatial and seasonal variations in carbohydrates with altitude with higher concentration being in the leaf mass. The changes in protein content with altitude may be due to the annual rainfall, soil salinity and climatic condition in the locality (Abbas, 2005) .

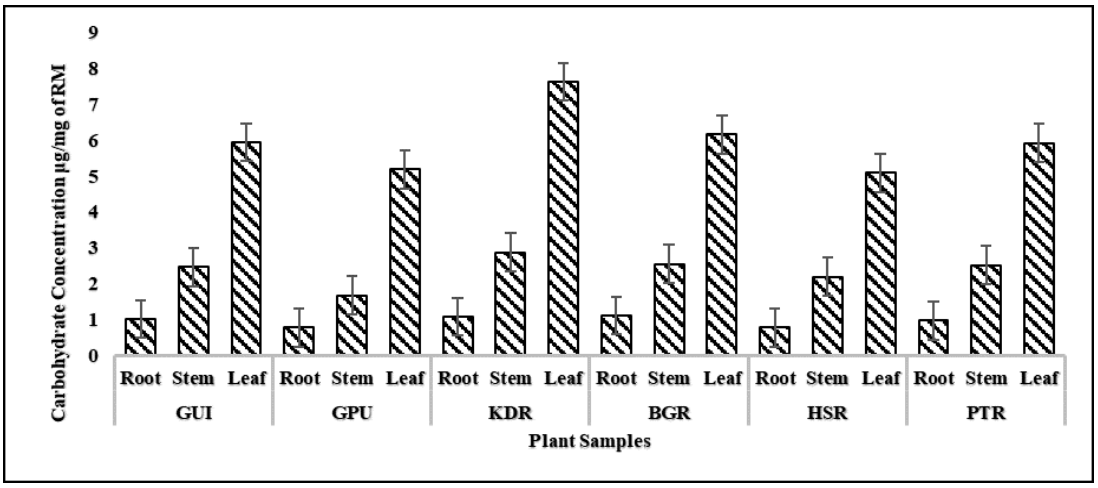
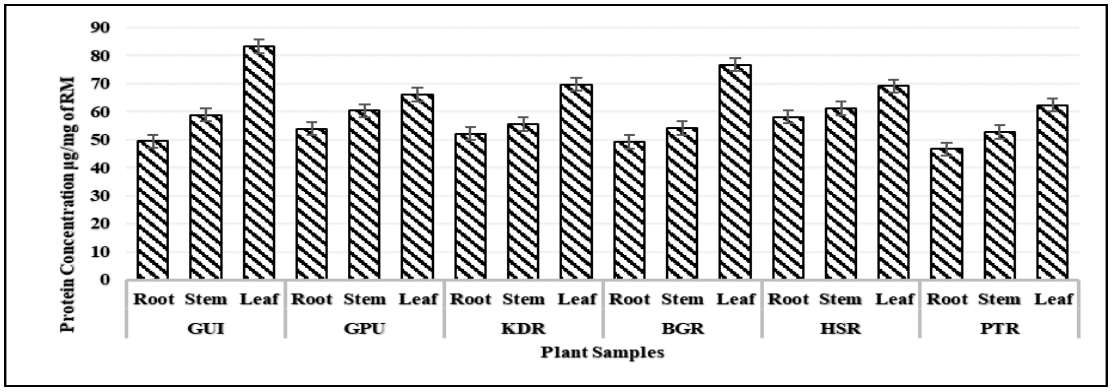


Figure 3: Quantitative analysis of Carbohydrates in *Evolvulus alsinoides* (Linn.) Linn.

Figure 4: Quantitative analysis of Proteins in *Evolvulus alsinoides* (Linn.) Linn. Figure



5: Seasonal Variation in Carbohydrate Content among *Evolvulus alsinoides* (Linn.) Linn.

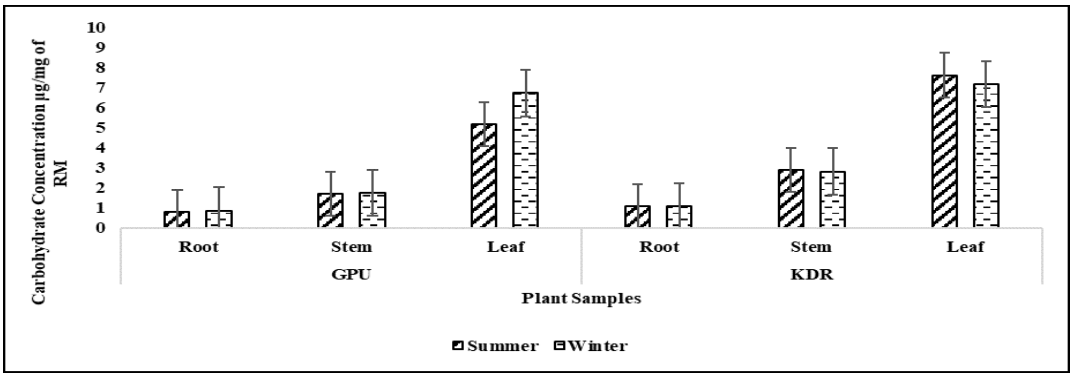
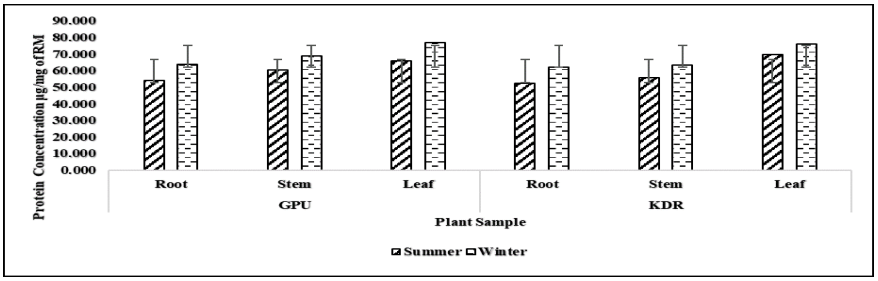


Figure 6: Seasonal Variation in Protein Content among Evolvulus alsinoides (Linn.) Linn.



Total phenolic content

Figure 7 represents the concentration of phenolics in the populations being studied. The leaf sample of GDI recorded the highest content of about 98.92 mg/mL in terms of Gallic acid equivalent. The next highest was observed in HSR sample. The least observed sample was the one collected from PTR of about 21.41 mg/ml. Significant variation of the total phenolic content was observed between summer and winter and are depicted in Figure 8. Apart from the two locations encoded as GPU and KDR, plants were not found grown in other places taken for study. Phenolic compounds serve as defense against pathogens and environmental stress such as heat, moisture, UV radiation (Dey and Harborne, 1989). The differences in the concentration of phenolics could be justified considering the variations in climatic, temperature, biotic and environmental conditions at the localities in different seasons (Treutter, 2001) . The impact of temperature at the particular season correlates with the results of (Generalić et al., 2012) . (Ncube et al., 2011) have reported the higher amount of phenolics in winter and autumn, which implies that the production of secondary metabolites depends on the favourable stimuli.

Figure 7: Total phenolic content (GAE mg/ml) in *Evolvulus alsinoides* (Linn.) Linn.

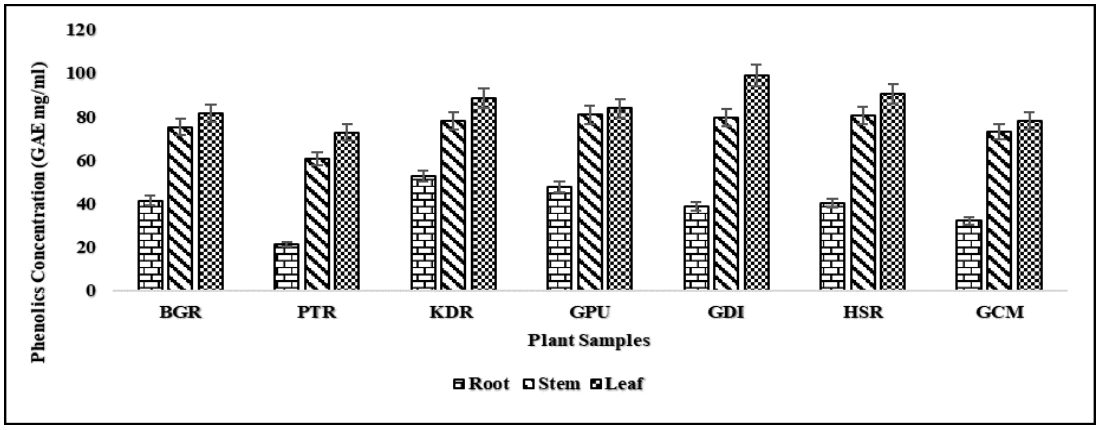
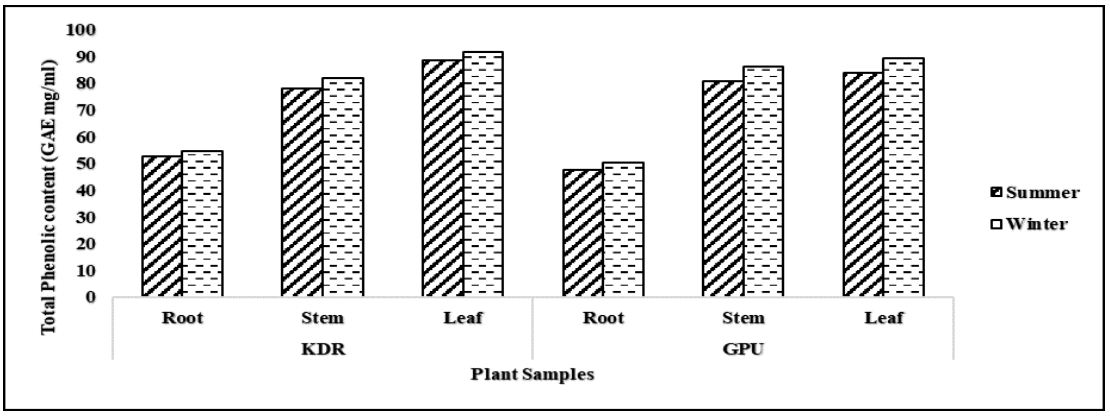


Figure 8: Seasonal variation of total phenolic content among *Evolvulus alsinoides* (Linn.) Linn.



Total flavonoid content

The impact of altitude and seasonal variation on the flavonoid content in the populations is given in Figure 9 and 10 respectively. The sample collected from Gundri (6.12 mg/g) had recorded the highest content for flavonoid too, then stood the sample collected from Patlur (4.78 mg/g) and Kadambur (4.45 mg/g) extracts. The samples collected during winter possess higher flavonoid concentration than that of summer which shows the dependence of climate

on the flavonoid concentration. Flavanoides can directly react with superoxide anions and lipid peroxyl radical and consequently inhibit/break the chain of lipid peroxidation (Torel et al., 1986) . The active biomolecules present in the plant are flavonoid glycosides. The flavonoids possess high antioxidant potential and can be used in anticancer studies (Ren et al., 2003) . High concentrations of flavonoids have also been reported in winter in most of the plant species (Ncube et al., 2011) . The higher levels of flavonoid during winter season can be possibly due to cold and water stress during the period. Metabolic cost gets abated during leaf senescence that appears in winter (Reimberg et al., 2009; Zietz et al., 2010) . The concentrated and retrieved metabolites from senescing tissues might have contributed for the observed trend in the differences in flavonoids in the plant samples.

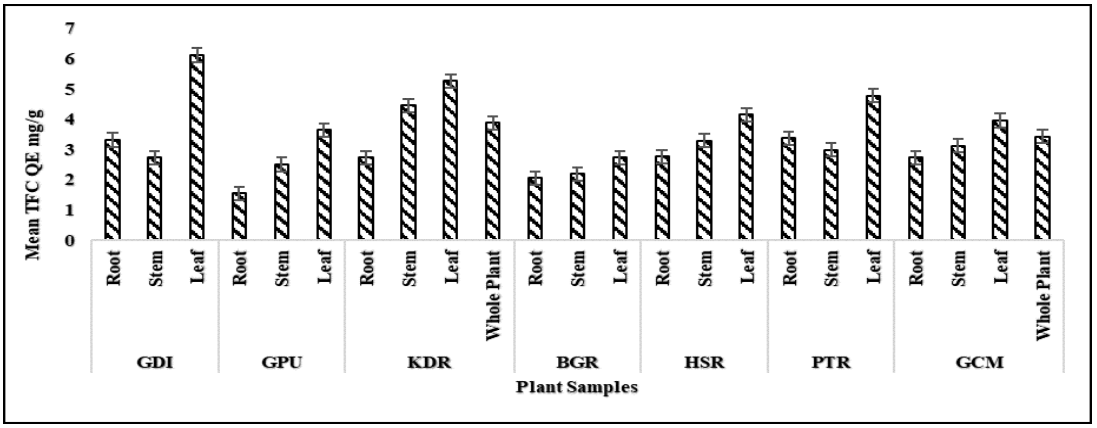


Figure 9: Variation in Total Flavanoids Concentration (QE mg/g) among *Evolvulus alsinoides* (Linn.) Linn.

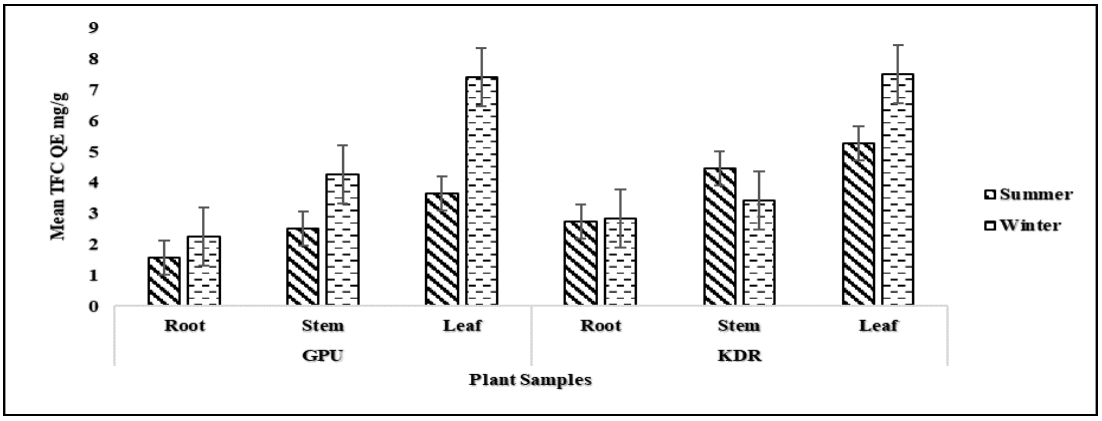


Figure 10: Seasonal Variation in Total Flavanoids Concentration (QE mg/g) among *Evolvulus alsinoides* (Linn.) Linn.

DPPH Scavenging activity

The leaf samples are found to contain more flavonoid content and it confirms that the leaves possess higher antioxidant potential than the root and stem. DPPH scavenging activity of the leaf extracts as reported in figure 11 shows the higher scavenging potential of KDR leaf. The ability of a compound to scavenge DPPH radicals is dependent on their ability to pair with radical containing unpaired electrons (PARK et al., 2004).

Total antioxidant activity (FRAP Assay)

The variations in ferric reducing anti-oxidant power of the leaf extracts is reported in figure 11. It can be determined that *E. alsinoides* extracts may act as free radical scavenger. The FRAP assay computes the ability of an antioxidant compound to reduce a ferric oxidant (Fe^{3+}) to a ferrous complex (Fe^{2+}) by electron-transfer. This indicates the capacity of the compound to reduce reactive species (Benzie and Strain, 1999). The positive correlation of hydrogen peroxide scavenging activity of *E. alsinoides* (Linn.) Linn. whole plant ethanolic extract and its concentration has been by (Gomathi et al., 2014). Significant antioxidant activity with varying solvents has been reported by (Nahata et al., 2009). (Soni and Sosa, 2013) has also reported the impact of seasonal discrepancy on the important constituents like alkaloids, glycosides, polyphenol and flavonoids.

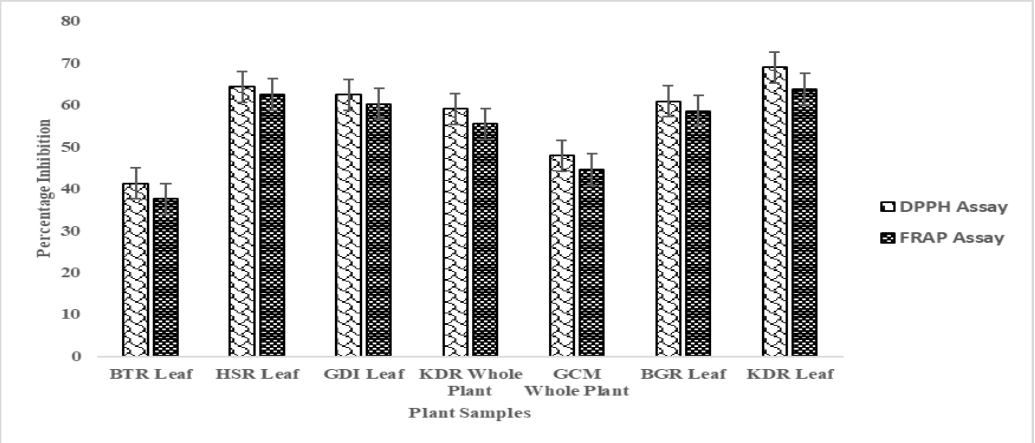


Figure 11: Variation in DPPH Scavenging activity of *Evolvulus alsinoides* (Linn.) Linn.

Thin Layer Chromatography

Figure 12 illustrates the fingerprint of metabolites present in the leaf extracts. The biomolecules with significant Rf value appears in all the extracts. Thin layer chromatography aids in better identification of therapeutically active compounds. The TLC profiling of the leaf extracts shows the presence of diverse metabolites such as alkaloids, flavonoids, phenols and tannins. The Rf values of the molecules provides an evidence about the polarity that shall aid in selecting the particular solvent system for extended studies on the characterization of metabolites using spectroscopic and chromatographic techniques (Dey et al., 2003) . Low polarity molecules shows high Rf value in less polar solvent system whereas high polarity molecules has a low Rf value (Cieśla and Waksmundzka-Hajnos, 2009).

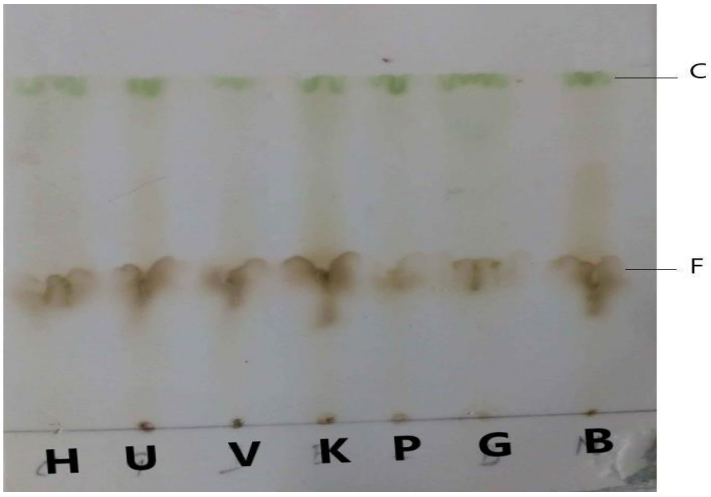


Figure 12: TLC finger printing of *Evolvulus alsinoides* (Linn.) Linn. Leaf extracts

H – Hasanur Sample; U – Gundri Sample; V – Gundripirivu Sample; K – Kadambur Sample; P – Patlur Sample; G – Gobichettipalayam Sample; B – Bargur Sample
TLC Spots: F – Flavanoid; C – Chlorophyll

SH SY5Y (Human, Neuroblast) cytotoxicity assay

The leaf extract showed potent ($p < 0.05$) cytotoxic effect against SH SY5Y Human, Neuroblastoma cell lines at a concentration (IC_{50}) of 119.783 ± 0.06 µg/mL (Figure 13). LD_{50} values lower than 1000 µg/ml is considered to be cytotoxic in the toxicity evaluation of plant extracts (Suffness and Pezzuto, 1999) . The ethanol extract of the plant leaves should be possessing anti-cancerous components that inhibits the growth of SH-SY5Y cell lines.

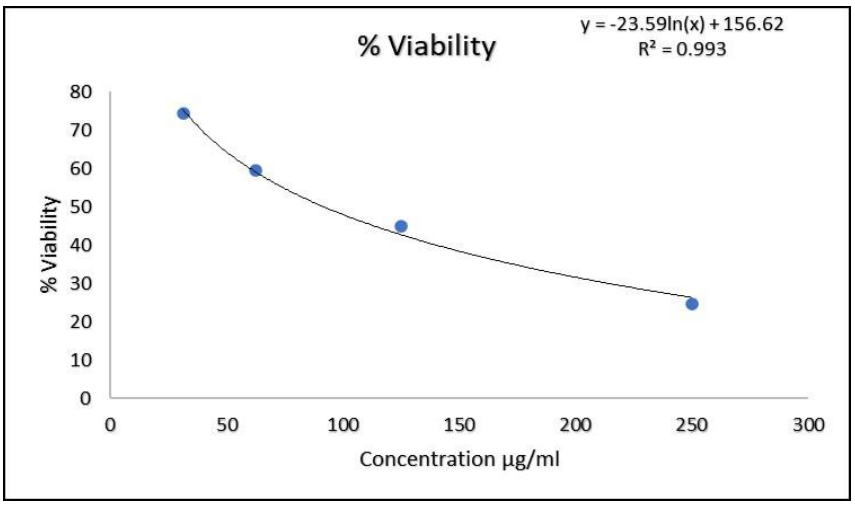


Figure 13: Cytotoxic Study of Evolvulus alsinoides (Linn.) Linn. Leaf extract

4. CONCLUSION

The present results imply significant phytochemical variations among *Evolvulus alsinoides* (Linn.) Linn. found in the Sathyamangalam Ghats. The variability of the constituents among the populations might be due to variances in geographic origins and the same should be considered while dealing with the plant material for pharmaceutical purpose. The presence of various biomolecules with significant differences in their polarity has been identified through TLC. The chromatogram analysis confirms the presence of both polar and non-polar compounds in the plant with therapeutic importance. The finding of this study suggests that leaves of *Evolvulus alsinoides* (Linn.) Linn. are eventual source of natural antioxidant having great importance as therapeutic agents in thwarting and decelerating the progress of ageing and degenerative diseases. In spite of various studies signifying the importance of seasons on the production and accumulation of secondary metabolites, documentary reports representing appropriate period for harvesting plants with pharmaceutically important constituents remains unreported.

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Author’s contributions
Both the authors are involved in laboratory determinations, interpretation of data and drafting the manuscript.

Compliance with ethical standards Conflict of interest
Authors declare that there is no conflict of interest regarding the publication of this article.

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