PHYLOGENETICAL METABOLITE LOGICAL ALGORITHM: A TEST USING PHYLOGENETIC METABOLITE ANALYSIS USING TLC IN SOME CONVOLVULACEAE MEMBERS

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ABSTRACT: Chemical substances act as vital substances occurring in living organisms and can be studied through various biotechnological approaches. The characteristic metabolites of a plant species may reflect evolution towards the resistance produced to environment. *Evolvulus alsinoides*, *Evolvulus nummularius* and *Merrimia tridentata* belongs to convolvulaceae members has been taken as experimental plants in the present study. Thin layer chromatography (TLC) has been used as a data collection technique for identification of similar organisms by logical method, from various Rf values occurred by experimentation. Based on phylogenetic analysis using UPGMA method, *E.nummularius* has shown good matches with both *E.alsinoides* and *M.tridentata*. Hence *E.nummularius* may be showing evolutionary link between *E.alsinoides* and *M.tridentata.* Seventeen compounds are visualized with similar Rf values between all these plant members. Hence most of the data are observed to be having similar compounds, which makes to be placed in same family (convolvulaceae).

Key Words: Convolvulaceae, Thin layer chromatography, logical method, MEGA software, Phylogeny.

INTRODUCTION

Since the dawn of civilization, herbs have been used in almost all cultures as a source of medicine, since these compounds are devoid of side effects and cost effective (Jochen and Joseph 2006). The practice of using medicinal plants is to provide new and important leads against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, and pain **(**Marcy and Kinghom 2005). According to World Health Organization (WHO), more than 80% of the world's population depends on conventional medicine for their primary healthcare needs.

India has a rich history of using medicinal plants based on medicinal system in Ayurveda (Ishita et al., 2004). Cladistic analysis of the metabolite data, including evaluation of all equally or almost equally parsimonious cladograms, are not presently given in any of the research methodologies.

In the present decades, great consideration of plants has been given to the search for natural compounds or extracts for the purpose of medical use (Frantisek et al., 2008). *E. alsinoides* L. is used mainly in traditional medicine of East Asia contains alkaloids: betaine, shankhapushpine and evolvine (Amritpal Singh 2008). *Evolvulus alsinoides* (EA) is well known for its memory enhancement, antiepileptic and immunomodulatory properties in the traditional Indian system of medicine, Ayurveda (Kiran et al., 2005)**.**

Thin layer chromatography (TLC) is routinely used as valuable tool for qualitative determination of small amounts of impurities. Molecular markers generally refer to biochemical constituents, including primary and secondary metabolites and other macromolecules such as nucleic acids (Kalpana, et al., 2004). TLC has been used as a broad spectrum screen for detection of drug abuse. TLC results are only qualitative and cannot be quantified (Andrew et al., 1998; Jones and Gierasch 1994).

Evolvulus alsinoides is an herb perennial. Stems - several to numerous, prostrate or ascending, slender, with appressed and spreading hairs. Leaves petiolate or subsessile, 0.7-2.5 cm X 5-10 mm. Flora and fauna year round. *Evolvulus nummularius* is an herb perennial. Stems - several, rooting at nodes, prostrate, 20-40 cm, slender, ± villous or scabrous. Leaves distichous; petiole 2-4 mm; leaf blade nearly circular, 1.3-1.7 X 1.2-1.4 cm, glabrous or appressed pilose abaxially, base cordate to rounded, apex rounded or emarginate; lateral veins 2 or 3 pairs. *Merremia tridentata* is a climbing weed with slim, greyish leaves. (Fang and George, 1995)

MATERIALS AND METHODS

Collection of plant materials: The plant material is largely found in Gajuwaka region of Visakhapatnam District. Whole plant was collected from Visakhapatnam during rainy season and the experiments are conducted.

Preparation of plant extracts

Fresh plant materials were washed thoroughly under running tap water, shade dried and used for extraction. Plant parts such as fresh leaves, Dry leaves, root, flower, seed and *in vitro* grown leaves were homogenized separately, to a fine powder and stored in airtight bottles.

About 25 g of each powder separately were carefully transferred into round bottom flask of soxhlet extractor. The plant material was soaked in 2 liters of methanol for 24 hours at room temperature. The methanolic extracts of plants were extracted by using soxhlet extractor. The final extracts were filtered through whattman's filter paper no.1. The methanol present in the methanolic extract was evaporated under reduced pressure (Buchi vaporator) to yield the residue. The residue thus obtained was suspended in DMSO (Dimethylsulfoxide).

The comparative analysis of the amino acids, lipids, Carbohydrates and secondary metabolites present in parts of field grown plants and leaves of plants grown *in vitro* of *Evolvulus alsinoides, Evolvulus nummularius* and *Merremia tridentata* were performed by Thin Layer Chromatography (TLC). Various parts from taxas are qualitatively analyzed for presence of various primary and secondary metabolites.

REAGENTS USED

REAGENTS FOR AMINO ACIDS

Silica gel, Solvent mixture: (Butanol: Acetic acid: Water = 4:1:1), Spraying reagent: 0.3% Ninhydrin in Butanol containing 3 ml of Acetic acid, Amino acids standards.

REAGENTS FOR LIPIDS

Silica gel, Solvent mixture: Chloroform: methanol: Water = 65:25:4, Spraying reagent: 30% concentric sulphuric acid, Lipid standards.

REAGENTS FOR CARBOHYDRATES

Silica gel, Solvent mixture: (Ethyl acetate: Isopropyl alcohol: water = 130:57:2.3), Spraying reagent: Aniline diphenyl Amine reagent: Mix 5 volumes of 1% Aniline in Acetone and 5 volumes of 1% Diphenyl amine (DPA) in Acetone with 1 volume of 85% hydrogen phosphate, Carbohydrate standards.

REAGENTS FOR SECONDARY METABOLITE(S)

Silica gel, Solvent mixture: Chloroform: Acetone: Ammonium hydroxide = 30:70:2, Spraying reagent: 5% concentrated H_2SO_4 (in ethanol).

PROCEDURE

Preparation of thin layer

Plates were made with silica gel by mixing about 30 gm of Silica gel (G60) with 60ml of double distilled water and the contents are vigorously mixed until the gel is uniformly dispersed, within 4 minutes after addition of water. Then the slurry is pour into Stahl's mechanical spreader adjusted at 0.2 mm thickness. Using spreader the gel is layered on the glass plate and dried at room temperature for few minutes. Subsequently the plates are placed in an oven, kept at 100°C for 30 minutes and allowed to cool. (Stahl 1969)

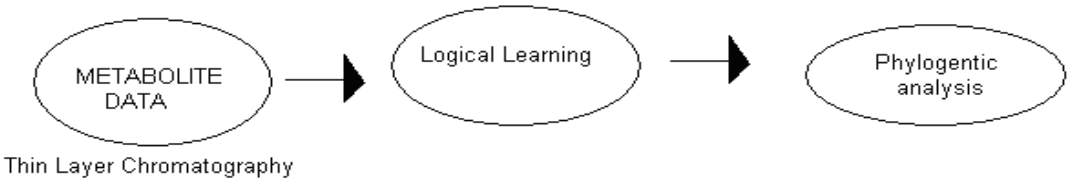
Development of Chromatogram:

Into respective developing chambers, solvent mixture was poured to a depth of about 10 mm. The chamber should be made wet with the solvent system (or solvent mixture) used in that chamber. The lid should be closed and the tank is left for 10-15 minutes for saturation with solvent vapours. TLC plate should be marked with a pencil line, about 1.5 cm from the bottom. Subsequently a mark should be drawn, every 1.5 cm from left to right. These marks will be used to apply standards and samples. The micropipette should be holded vertically and should touch the filled end to the pencil mark line. The samples prepared from field grown plant parts and *in vitro* grown leaves (methanoic extract) were applied on the TLC plate in 10µl quantities. Liquid should flow from the micropipette to the plate to form a spot. Similarly standards are also applied on TLC plate in 10µl quantities. After loading the samples and standards, the plates are allowed to air dry. Once the spots have dried, lower the plate carefully into the chamber. The lid should be closed and monitor the movement of solvent up the plate. When the solvent has advanced at least 15 cm, the plate should be removed from the chamber, mark the solvent front with a pencil, and allow all solvents to evaporate. The plate should be sprayed with spraying reagent, allow it to dry, and heat the sprayed plate inside the oven at 110 °C for 15 minutes. The plates are removed and then calculate the Rf (Retardation Factor) values based on equation:

$$Rf = \frac{\text{Distance moved by compound}}{\text{Distance moved by Solvent system}}$$

Logical algorithm

Step 1: Collect the Convolvulaceae members data (Rf values) obtained from TLC.
Step 2: Transfer the data into string (Aligned as A/ Unaligned as B, C and D) .
Step 3: Run sequence alignment from MEGA software.
Step 4: Run Construct phylogeny using UPGMA method from MEGA.



MEGA v2.0

The Molecular Evolutionary Genetics Analysis (MEGA) software is a desktop application designed for comparative analysis of homologous gene sequences either from multigene families or from different species with a special emphasis on inferring evolutionary relationships and patterns of DNA and protein evolution. A phylogenetic tree, using logical method of analysis (aligned as A unaligned or nonaligned sets as B/C/D) expressed in this work, was drawn using the **MEGA software** version 2.0.

RESULTS AND DISCUSSION

The Convolvulaceae members are mostly twining herbs or shrubs, comprising about 85 genera and 2,800 species that are further characterized by almost always having the flowers solitary or in terminal or axillary dichasia.

The qualitative analysis of various primary and secondary metabolites was analyzed by TLC results. The Results of TLC were tabulated and listed in **Tables 1 to 4. Figure 1** provided the logical conversion of TLC data and drawing phylogenetic tree using MEGA v2.0, a phylogenetic analysis software.

E.alsinoides

The methanolic extract of I*n vivo* plant parts has shown various spots on silica gel TLC plates. The *In vivo* grown leaf extracts are also experimented by TLC.
Based on standards used, the fresh leaf extract shown four aminoacids - Histidine (0.28), Unknown (0.40), Valine (0.55) and Glutamic acid (0.60). Dry leaf showed five aminoacids - Unknown (0.04), Lysine (0.17), Histidine (0.28), Glutamine (0.50) and Unknown (0.79). Root shown three aminoacids- Unknown (0.05), Lysine (0.17), Histidine (0.28). Flower shown six aminoacids - Unknown (0.04, 0.08), Aspergine (0.15), Histidine (0.28), Serine (0.47) and Leucine (0.65). Seed had shown two amino acids - Lysine (0.17) and Histidine (0.28). *In vitro* grown leaves shown five amino acids – Histidine (0.28), Unknown (0.40), Glysine (0.50), Threonine (0.57), Aspartic acid (0.68)
All the standards not matched with samples used. Fresh leaves shown one unknown lipid, dry leaf had shown three lipids, root shown two unknown lipids, lipids absent in flower and two unknown lipids in seeds. *In vitro* grown leaves extract shown 2 lipids.

TABLE 1 : TLC - Amino acids

Sample	*E.alsinoides*		*E.nummularius*		*M.tridentata*		Standard	Rf
	Spots	Rf	Spots	Rf	Spots	Rf		
In Vivo leaf extract	1	0.28	1	0.28	1	0.28	A ALA	0.46
	2	0.40	2	0.40	2	0.40	C CYS	0.51
	3	0.55	3	0.55	3	0.53	D ASP	0.68
	4	0.60	4	0.60	4	0.60	E GLU	0.60
			5	0.77	5	0.75	F PHE	0.87
					6	0.85	G GLY	0.35
In Vitro leaf extract	1	0.28	1	0.28	1	0.28	H HIS	0.28
	2	0.40	2	0.40	2	0.41	I ILE	0.95
	3	0.50	3	0.51	3	0.53	K LYS	0.17
	4	0.57	4	0.68	4	0.55	L LEU	0.65
	5	0.68	5	0.74			M MET	0.89
Dry leaf	1	0.04	1	0.68	1	0.78	N ASN	0.15
	2	0.17	2	0.80	2	0.85	P PRO	0.53
	3	0.28					Q GLN	0.50
	4	0.50					R ARG	0.37
	5	0.79					S SER	0.47
Root	1	0.05	1	0.60	1	0.78	T THR	0.57
	2	0.17	2	0.89			V VAL	0.55
	3	0.28					W TRP	0.92
Flower	1	0.04	1	0.89	1	0.78	Y TYR	0.85
	2	0.08						
	3	0.15						
	4	0.28						
	5	0.47						
	6	0.65						
Seed	1	0.17	1	0.85	1	0.78		
	2	0.28						

Fresh leaves shown six sugars - Starch (0.05), Unknown (0.22), Unknown (0.30), Sucrose (0.42), Galactose (0.54) and Unknown (0.66). Dry leaf had shown two sugars – Starch (0.05) and Unknown sugar. Root shown two sugars - Starch (0.05) and Galactose (0.54). Flower shown four sugars-Starch (0.05) and three Unknown carbohydrates (0.85, 0.90 and 0.97). Seed had shown three sugars – Starch (0.05), Xylose (0.70) and Unknown (0.95). *In vitro* grown leaves shown five sugars - Starch (0.05), Unknown (0.27), Sucrose (0.44), Unknown (0.60) and Unknown (0.73) sugars.

Secondary metabolite analysis shown two spots in fresh leaves, one spot in dry leaf, three spots in root, two spots in flower, one spot in seed and two spots shown in *in vitro* grown leaves.

E.nummularius

Based on standards used, the fresh leaf extract shown five aminoacids - Histidine (0.28), Unknown (0.40), Valine (0.55), Glutamic acid (0.60) and Unknown (0.77). Dry leaf shown two aminoacids - Aspartic acid(0.63) and Unknown (0.80).

Root shown two aminoacids- Glutamic acid (0.60) and Methionine (0.89). Flower shown one aminoacid-Methionine (0.89). Seed had shown one amino acid-Tyrosine (0.85). *In vitro* grown leaves shown five amino acids-Histidine (0.28), Unknown (0.40), Cystine (0.51), Aspartic acid (0.68) and Unknown (0.74) sugar.

TABLE 2 : TLC - Lipids

Sample	*E.alsinoides*		*E.nummularius*		*M.tridentata*		Standard	Rf
	Spots	Rf	Spots	Rf	Spots	Rf		
In Vivo leaf extract	1	0.90	1	0.87	1	0.91	Tween 60	0.41
In Vitro leaf extract	1	0.68	1	0.76	1	0.92	cholesterol	0.73
	2	0.90	2	0.89			Cederwood oil	0.84
Dry leaf	1	0.69	1	0.65	1	0.66	Coconut oil	0.17
	2	0.71	2	0.73	2	0.72		
	3	0.88	3	0.81	3	0.82		
			4	0.88	4	0.90		
Root	1	0.86	1	0.73	1	0.67		
	2	0.93	2	0.89	2	0.80		
			3	0.96	3	0.94		
Flower	Nil	Nil	1	0.61	1	0.67		
			2	0.81	2	0.81		
			3	0.85	3	0.90		
			4	0.92				
Seed	1	0.69	1	0.85	1	0.67		
	2	0.88			2	0.80		
					3	0.87		

All the standards not matched with samples used. Fresh leaves shown one unknown lipid, dry leaf shown four lipids, root shown three unknown lipids, flower shown four lipids and one unknown lipid in seeds. *In vitro* grown leaves extract shown two lipids.

Fresh leaf had shown six sugars -Starch (0.05), Unknown (0.17), Unknown (0.26), Sucrose (0.42), Galactose (0.54) and Unknown (0.66). Dry leaf had shown three sugars –Starch (0.05) and Unknown sugars (0.75, 0.92). Root had shown one Unknown sugar (0.49). Flower shown four sugars-Starch (0.05), Unknown (0.68), Xylose (0.70) and Unknown (0.77) sugar. Seed shown two sugars – Starch (0.05), Xylose (0.70) and Unknown (0.77) sugar. *In vitro* grown leaves shown five sugars - Starch (0.05), Unknown (0.26), Glucose (0.45), Unknown (0.61) and Unknown (0.75) sugar.

Secondary metabolite analysis shown two spots in fresh leaves, three spots in dry leaf, two spots in root, one spot in flower, three spots in seed and two spots shown in *In vitro* grown leaves.

M.tridentata

Based on standards used, the fresh leaf extract shown six aminoacids – Histidine (0.28), Unknown (0.40), Proline (0.53), Glutamic acid (0.60), Unknown (0.75) and Tyrosine 0.85). Dry leaf had shown two aminoacids – Unknown (0.78) and Tyrosine (0.85). Root shown one aminoacid - Unknown (0.78) amino acid. Flower shown one aminoacid - Unknown (0.78) amino acid. Seed shown one unknown (0.78) amino acid. *In vitro* grown leaves shown four amino acids-Histidine (0.28), Unknown (0.41), Proline (0.53), Threonine (0.55) and Valine (0.55).

TABLE 3 : TLC - Carbohydrates

Sample	*E.alsinoides*		*E.nummularius*		*M.tridentata*		Standard	Rf
	Spots	Rf	Spots	Rf	Spots	Rf		
In Vivo leaf extract	1	0.05	1	0.05	1	0.45	Glucose	0.45
	2	0.22	2	0.17	2	0.66	Fructose	0.36
	3	0.30	3	0.26	3	0.76	Sucrose	0.42
	4	0.42	4	0.42			Galactose	0.54
	5	0.54	5	0.54			lactose	0.10
	6	0.66	6	0.66			xylose	0.70
In Vitro leaf extract	1	0.05	1	0.05	1	0.45	Starch	0.05
	2	0.27	2	0.26	2	0.60		
	3	0.42	3	0.45				
	4	0.60	4	0.61				
	5	0.73	5	0.75				
Dry leaf	1	0.05	1	0.05	1	0.05		
	2	0.30	2	0.75	2	0.81		
			3	0.92	3	0.93		
Root	1	0.05	1	0.49	1	0.05		
	2	0.54			2	0.60		
Flower	1	0.05	1	0.05	1	0.05		
	2	0.85	2	0.68				
	3	0.90	3	0.70				
	4	0.97	4	0.77				
Seed	1	0.05	1	0.05	1	0.05		
	2	0.70	2	0.77	2	0.54		
	3	0.95			3	0.70		

All the standards not matched with samples used. Fresh leaves shown one unknown lipid, Dry leaf shown four lipids, root shown three unknown lipids, flower shown three unknown lipids and three unknown lipids shown in seeds. *In vitro* grown methanolic leaf extract shown one lipid.

Fresh leaf had shown three sugars -Glucose (0.45), Unknown (0.66) and Unknown (0.76) sugar. Dry leaf had shown three sugars – Starch (0.05) and Unknown (0.81 and 0.93) sugars. Root shown two sugars - Starch (0.05) and Unknown (0.60) sugar. Flower shown one sugar - Starch (0.05). Seed had shown three sugars - Starch (0.05), Galactose (0.54) and Xylose (0.70). *In vitro* grown leaves shown two sugars - Glucose (0.45) and Unknown (0.60) sugar.

Secondary metabolite analysis shown one spot in fresh leaves, one spot in dry leaf, one spot in root, no secondary metabolites in flower, one spot in seed and one spot shown in *in vitro* grown leaves.

CONCLUSION

Most of the *E.nummularius* compounds are shown relationship with *E.alsinoides* and *M.tridentata* based on TLC data and logical analysis. This is a preliminary work and should carry further studies with other species and families for understanding phylogeny.

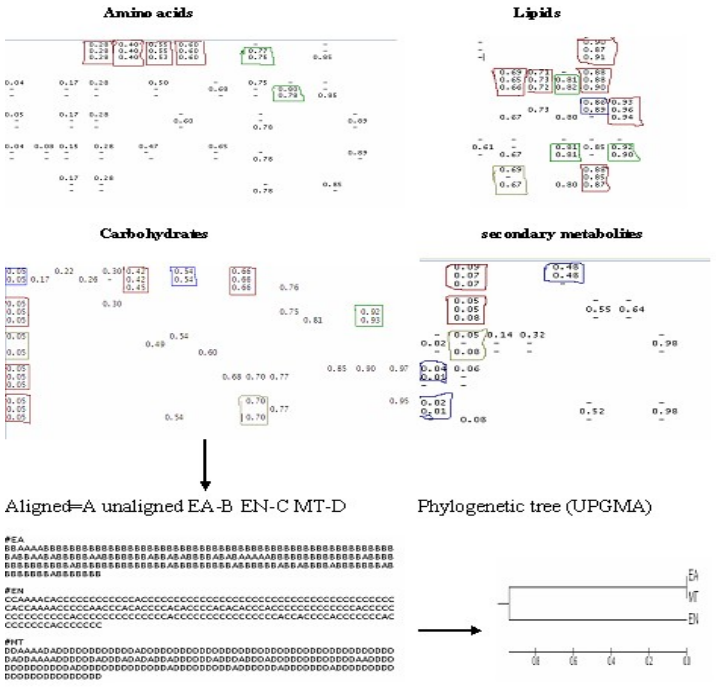
ACKNOWLEDGEMENT

I am thankful to management and staff of GITAM University, Visakhapatnam, India for helping in bringing out the above literature and experimentation

TABLE 4 : TLC - Secondary Metabolites

Sample	*E.alsinoides*		*E.nummularius*		*M.tridentata*	
	Spots	Rf	Spots	Rf	Spots	Rf
In Vivo leaf extract	1	0.09	1	0.07	1	0.07
	2	0.48	2	0.48		
In Vitro leaf extract	1	0.08	1	0.08	1	006
	2	0.44	2	0.44		
Dry leaf	1	0.05	1	0.05	1	0.08
			2	0.55		
			3	0.64		
Root	1	0.05	1	0.02	1	0.08
	2	0.14	2	0.98		
	3	0.32				
Flower	1	0.04	1	0.01	------	nil
	2	0.06				
Seed	1	0.02	1	0.01	1	0.08
			2	0.52		
			3	0.98		

Figure 1: Logical design and phylogenetic analysis of strings based on alignment of biological compounds from TLC Data



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