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Phytochemical and physicochemical assessment of diversity in the leaves of *Glycosmis pentaphylla* (Retz.) DC

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Abstract
Traditional knowledge and ethno-botanical use of plants have been widely acknowledged all over the world. *Glycosmis pentaphylla* (Rutaceae) is a shrubby plant found all over India, used for the healing of wounds of livestock in Indian folk medicine. The present study involves preliminary phytochemical and physicochemical evaluation of diversity in ten accessions of *Glycosmis pentaphylla* collected from three different eco-geographical zones of Kerala, India. Significant differences were observed between the studied genotypes for most of the parameters under study. Variability of results of this secondary metabolite testing could be induced by several factors such as age, climate, habitat, plant part, season, chemical race of plants, etc. Thus, it is expected that the important physicochemical properties of this genotype will be very useful in the curing of various diseases.

Keywords: *Glycosmis pentaphylla*, genotypes, phytoconstituents, diversity, medicinal properties

1. Introduction
India has a rich source of biodiversity, due to its varied geographical and agro-climatic regions. Besides, it has a diverse cultural heritage where health care system consists of organized systems like Ayurveda, Siddha and Unani and unorganized systems like folk medicines. In the last few years, there has also been a growing interest in traditional and alternative systems of medicine in many developed countries. Medicinal herbs are the oldest known health-care products and their value is increasing, though its percentage varies depending on the ethnological, medicinal and historical background of a nation. In recent years, there has been a dramatic rise in use of herbal drugs in developed countries because of their easy availability and cost effectiveness besides having desired pharmacological effectiveness with high level of safety and low toxicity profile. It is estimated that world's one-fourth population i.e. 1.42 billion people are dependent on traditional herbal medicines for the treatment of various ailments [1]. However, the lack of documentation and stringent quality control procedures has hindered the easy acceptance of such plant drugs to be used as herbal medicine. According to the WHO, to ensure reproducible quality of herbal plants, physicochemical and phytochemical characterizations are required to be carried out for establishing their identity, purity, and quality standards [2]. Therefore, there is a need for documentation of standardization studies for profiling the quality control parameters of plant-derived crude drugs.
Glycosmis pentaphylla (Retz.) D.C, is a shrub or small (1.5–5.0 m) tree widely distributed in India, Malaysia, Southern China and the Philippine Islands where it occurs in tropical forests at low altitudes. This plant is thornless shrub or small tree. Leaves usually have 3 to 5 pinnately arranged leaflets, though these are sometimes reduced to one or two, all forms being often found on the same plant. Flowers are small, white, about 6 millimetres in diameter, borne in axillary, solitary or paired, interrupted, narrow, cymose panicles which are 5 centimetres long or less. Fruit is fleshy, pink or reddish, rounded, 1 centimetre in diameter, and contains a single nearly spherical seed which is about 4 millimetres in diameter. Root system is deep and spreading.
G. pentaphylla has a long history of usage in traditional medicine against various ailments around the world. In Ayurvedic and other traditional medicinal practices the plant has been used against diseases like bilious complaints, cough, worms, jaundice, fever, inflammation, rheumatism, anaemia and vermifuge. The plant is used in indigenous medicine for cough, jaundice, inflammation, rheumatism and anaemia.

The whole plant is bitter, astringent, vermifuge, anti-inflammatory and expectorant. The stems are widely used as a brush for cleaning the teeth [3]. The traditional healers in Gazipur district of Bangladesh utilize *G. pentaphylla* for prevention of all forms of cancer [4]. The root stem and leaf of this plant are used in folklore medicine in Kerala and Tamil Nadu to cure fever, rheumatism etc. [5]. Leaf juice is given with sugar in empty stomach in the morning to eradicate ascaris [6]. Crushed root piece mixed in water is administered in empty stomach in the morning to cure stomach pain. Juice of leaves is used in fever and liver complaints and as a vermifuge. Leaves are considered as good antidote for eczema and other skin troubles and applied in the form of paste.

As per our knowledge and literature review, there is a lack of information on the morphological, physiological and phytochemical analysis of different accessions of *G. pentaphylla* collected from various eco-geographical regions of Kerala. The objective of this study was to evaluate physicochemical and preliminary phytochemical profiling of *G. pentaphylla* accessions that can make possible to identify the plant and that can assist and provide the base in the preparation of monograph of this plant.

2. Materials and Methods

2. 1. Collection and Extraction of Plant Material

Fresh leaves of *Glycosmis pentaphylla* were collected from ten locations in Kerala from its natural habitat from three different eco-geographic zones such as coastline, hilly and planar regions of Kerala, India (Table 1). The botanical identities were verified by the Curator, Department of Botany, University of Kerala, taxonomical features were critically studied and confirmed with the Flora of the Presidency of Madras [7] and with other relevant available literatures. The collected plant leaves were shade dried, coarsely powdered (Sieve no. 40), and stored in a well closed airtight container. The fresh leaves were used for organoleptic evaluation and the dried powdered sample of leaves was employed for physicochemical characterization. As the previous reports showed high content of alkaloid in *G. pentaphylla*, the total alkaloid fraction from leaf was extracted with few modifications [8]. The powdered leaves were subjected to successive solvent extraction as follows: 10 gm of the powdered leaves was extracted successively by Soxhlation with 95% ethanol. The extracts were concentrated by distilling off the solvent and were subsequently evaporated to dryness and the dry residues were then used for physicochemical analysis. The percentage yield (w/w) of each extract was calculated in terms of the weight of initial air-dried plant material.

Table 1: List of Accessions and Geographic Co-ordinates

S. No.	Place of collection	Latitude	Longitude	Altitude	Code	
1	Parumala	9.3248°N	76.5367°E	27.00 m	GP01	Coastline
2	Pathanapuram	9.0927°N	76.8612°E	42.31 m	GP02	
3	Varkala	8.7379°N	76.7163°E	43.84 m	GP03	
4	Karamana	8.4814°N	76.9657°E	18.00 m	GP04	
5	Ambalappuzha	9.3773°N	76.3578°E	09.00 m	GP05	
6	Meppadi	11.8014°N	76.0044°E	27.00 m	GP06	Hilly
7	Thodupuzha	9.8930°N	76.7221°E	38.34 m	GP07	
8	Peruva	9.8282°N	76.5011°E	09.00 m	GP08	Planar
9	Vakathanam	9.5138°N	76.5691°E	26.22 m	GP09	
10	Thirumarady	9.8889°N	76.8612°E	12.00 m	GP10	

2.2. Chemicals and Reagents

All the chemicals, reagents, solvents used during the experimentation were of analytical grade (M/S Merck, Sigma, and Qualigens) purchased from local supplier.

2.3. Organoleptic evaluation

The fresh leaves of *Glycosmis pentaphylla* of all the accessions were visually examined and different organoleptic features such as colour, odour and taste were characterized [9].

2.4. Physicochemical analysis

Powder samples were subjected to determination of various physicochemical parameters such as moisture content (% LOD), ash constants viz. total ash, acid insoluble ash, water soluble ash and alcohol soluble ash and extractive values according to the methods specified in the Indian Pharmacopoeia. Each study was performed in triplicate; mean values with standard error of mean (SEM) were calculated.

2.5. Phytochemical screening

Preliminary phytochemical screening of all the extracts was carried out for detection of the presence of different phytochemicals such as alkaloids, glycosides, flavonoids, phenolic compounds, saponins, tannins etc. present in *G. pentaphylla* leaves. The qualitative chemical tests were performed according to the standard procedures [10, 11]. The total alkaloid fraction from leaf was extracted and the percentage yield was calculated.

2.6. Fluorescence Analysis

Fluorescent evaluation of *Glycosmis pentaphylla* dried leaf powder of all the ten accessions was examined both in daylight and short UV light (254 nm), and after treatment with different reagents like distilled water, acetone, ethanol, benzene and chloroform [12, 13]. The colour observed by the application of different reagents in different radiations was recorded and noted according to the colour chart [14].

2.7. Thin layer chromatographic (TLC) analysis

The chemical fingerprint of all the extracts were determined by thin layer chromatography using aluminium pre-coated silica gel 60 F254 (Merck, 0.2 mm thick, 20×20 cm) TLC plates. The plates were developed using solvent system (n hexane: ethyl acetate in 12:7 ratio) as mobile phase. One dimensional ascending method was used for the development of plates as per standard protocol [15]. The TLC plate was air dried and spots were visualized under ultraviolet light (254 & 365 nm). The Rf values of the spots were also recorded.

3. Results

3.1 Organoleptic Evaluation

Organoleptic analysis of the leaves of *Glycosmis pentaphylla* revealed an aromatic and slight bitter taste. The leaves of all accessions were impar pinnately compound, 3-5 foliate (Fig. 1). The accessions under study has sub-opposite leaflets, with the leaves being entire to sub- dentate to sub-crenate. The leaves were attenuate at base and acute to round at apex of the leaf. Venation was reticulate in all the accessions. The length of the leaf was highest for accession GP09 (15.63±0.00 cm) and accession GP10 has the highest laminar breadth (6.44±0.00 cm). The leaves were glandular on both sides and glabrous for all the accessions.

Fig 1: Habit of *Glycosmis pentaphylla* (Retz.) DC

3.2 Phytochemical Screening

The results of phytochemical screening are depicted in Table 2. All the accessions under study were characterized by the presence of carbohydrates, flavonoids, alkaloids, phytosterols, starch and amino acids. The total alkaloid fraction from leaf was extracted and the percentage yield was calculated. (Table 3). The accession GP07 (19.6 mg/g) showed the highest yield for total alkaloids, while the accession GP03 (9.53 mg/g) showed the least yield for the total alkaloid. From the results, it became clear that, accession GP07 can be used in pharmaceutical preparations.

Table 2: Phytochemical screening of *G. pentaphylla* leaves

Accessions	Carbohydrates P+Conc.H_2SO_4	Flavonoids P+ Mg HCl	Alkaloids P+ Dragendorff's Reagent	Phytosterols P+ Aqueous Sodium nitrite	Starch P+ Iodine	Amino Acids P+ Ninhydrin reagent	Oil P+ Spot test	Cardiac glycosides P+ Ammonia
GP01	+	+	+	+	+	-	-	-
GP02	+	+	+	+	+	-	-	-
GP03	+	+	+	+	+	-	-	-
GP04	+	+	+	+	+	-	-	-
GP05	+	+	+	+	+	-	-	-
GP06	+	+	+	+	+	-	-	-
GP07	+	+	+	+	+	-	-	-
GP08	+	+	+	+	+	-	-	-
GP09	+	+	+	+	+	-	-	-
GP10	+	+	+	+	+	-	-	-

P-Leaf powder '+' indicates presence '-indicates absence

Table 3: Percentage Yield for total alkaloid

Accession code	Percentage yield (mg/g)
GP01	15.61
GP02	18.75
GP03	9.53
GP04	13.09
GP05	12.03
GP06	15.48
GP07	19.6
GP08	15.56
GP09	10.71
GP10	17.24

3.3 Physicochemical Evaluation

Moisture Content: The moisture content of dried powdered accessions was determined by loss on drying method (Table 4). The highest moisture content was recorded for the accession GP08 (17.3±0.005 %) and lowest for the accession GP07 (19.8±0.004 %).

Table 4: Moisture content of different accessions

Accession code	Moisture Content (%)
GP01	18.8±0.001
GP02	18.4±0.002
GP03	18.3±0.002
GP04	18.6±0.002
GP05	19.6±0.004
GP06	17.4±0.004
GP07	19.8±0.004
GP08	17.3±0.005
GP09	17.6±0.004
GP10	18.1±0.004

Data were expressed as mean ± standard error done in triplicate.

Determination of pH: The pH value of 1 % and 10 % leaf powder solution was measured. The pH (6.0) recorded for accession GP07 indicated slightly acidic nature of the sample whereas pH (8.5) recorded for the accession GP10, indicated alkaline nature of the drug. The results were summarized in the Table 5. As acidic pH is preferred in pharmaceutical industry, accession GP07 proved as a good candidate.

Table 5: pH of different accessions

Accession code	pH
GP01	7.5
GP02	8.0
GP03	7.7
GP04	8.2
GP05	8.8
GP06	8.1
GP07	**6.0**
GP08	8.0
GP09	7.4
GP10	8.5

Data were expressed as mean ± standard error done in triplicate.

Ash Constants: In the present study, the ash content of *Glycosmis pentaphylla* of the accessions had significantly different ash value. The analytical result of ash constants is depicted in Table 6. From the results, total ash, acid soluble ash and acid insoluble ash was found to be the highest for the accession GP07 (12.7±0.08 %, 3.2±0.06 %, 9.8±0.04 % respectively).

Table 6: Ash constants

Accessions	Total Ash (%)	Acid Souble Ash (%)	Acid Insoluble Ash (%)	Water Soluble Ash (%)	Water Insoluble Ash (%)
GP01	12.24±0.10	2.7±0.09	6.8±0.12	3.5±0.21	8.7±0.05
GP02	10.15±0.07	2.0±0.05	8.0±0.05	1.2±0.04	8.9±0.07
GP03	7.15±0.07	1.9±0.04	5.1±0.07	0.1±0.02	7.1±0.07
GP04	7.18±0.07	1.3±0.05	5.8±0.10	2.2±0.12	5.0±0.06
GP05	10.7±0.04	2.7±0.11	9.0±0.06	0.2±0.30	12.5±0.04
GP06	7.7±0.06	2.5±0.04	5.2±0.06	1.9±0.02	5.8±0.10
GP07	12.7±0.08	3.2±0.06	9.8±0.04	1.1±0.01	8.1±0.05
GP08	8.7±0.05	1.6±0.02	7.1±0.05	5.1±0.07	3.6±0.12
GP09	8.8±0.09	1.9±0.06	6.9±0.08	5.6±0.04	3.2±0.03
GP10	11.4±0.05	2.6±0.03	8.8±0.06	2.3±0.03	9.1±0.08

Data were expressed as mean ± standard error done in triplicate.

Extractive Value: The extractive values in water, alcohol, chloroform and ethyl acetate was reported highest in accession GP07 (0.12±0.004 %, 0.17±0.004 %, 0.07±0.006 %, 0.09±0.002 % respectively). The results were summarized in Table 7.

Table 7: Extractive Values

Accessions	Alcohol (mg)	Chloroform (mg)	Ethyl Acetate (mg)	Distilled water (mg)
GP01	0.06 ±0.002	0.05 ±0.027	0.03 ±0.002	0.06 ±0.002
GP02	0.09 ±0.004	0.06 ±0.024	0.08 ±0.003	0.09 ±0.003
GP03	0.04 ±0.002	0.05 ±0.025	0.06 ±0.004	0.05 ±0.004

GP04	0.05 ±0.004	0.01 ±0.027	0.03 ±0.003	0.12 ±0.007
GP05	0.14 ±0.006	0.08 ±0.006	0.07 ±0.004	0.09 ±0.004
GP06	0.05 ±0.002	0.01 ±0.004	0.03 ±0.003	0.08 ±0.003
GP07	0.17 ±0.004	0.08 ±0.006	0.09 ±0.002	0.06 ±0.002
GP08	0.03 ±0.004	0.01 ±0.004	0.04 ±0.004	0.07 ±0.003
GP09	0.05 ±0.004	0.02 ±0.002	0.04 ±0.004	0.05 ±0.004
GP10	0.17 ±0.004	0.05 ±0.003	0.06 ±0.005	0.08 ±0.003

Data were expressed as mean ± standard error done in triplicate

Fluorescence Analysis of Leaf Powder: The characteristic colour behaviour of dried powdered drug dissolved in organic solvents like distilled water, acetone, ethanol, benzene and chloroform was observed both under visible and UV light. The colour reactions of these drug solutions thus emitted fluorescence light are summarized in Table 8. The powdered drug solutions exhibited a wide range of fluorescence colours under the UV and visible light.

Table 8: Fluorescence Analysis of Leaf Powder

Treatment	GP01		GP02		GP03		GP04		GP05	
	Short UV	Ordinary Light	Short UV	Ordinary Light	Short UV	Ordinary Light	Short UV	Ordinary Light	Short UV	Ordinary Light
Powder (P) as such	Fern Green	Coriander Brown	Leek Green	Brown	Fern Green	Coriander Brown	Ivy Green	Sung Green	Sage Green	Fern Green
P +Distilled Water	Pea Green	Mimosa Yellow	Cyprus Green	Mimosa Yellow	Agathia Green	Mimosa Yellow	Pea Green	Primrose Yellow	Pea Green	Mimosa Yellow
P+Acetone	Cyprus Green	Primrose Yellow	Veronese Green	Uranium Green	Veronese Green	Dresden Yellow	Pea Green	Agathia Green	Veronese Green	Primrose Yellow
P+Ethanol	Veronese Green	Canary Yellow	Veronese Green	Chartreuse Green	Chartreuse Green	Canary Yellow	Agathia Green	Dresden Yellow	Sap Green	Dresden Yellow
P+Benzene	Agathia Green	Dresden Yellow	Veronese Green	Straw Yellow	Pea Green	Primrose Yellow	Pea Green	Primrose Yellow	Agathia Green	Canary Yellow
P+Chloroform	Fern Green	Primrose Yellow	Sap Green	Pea Green	Agathia Green	Sap Green	Agathia Green	Dresden Yellow	Pea Green	Uranium Green

Treatment	GP06		GP07		GP08		GP09		GP10	
	Short UV	Ordinary Light	Short UV	Ordinary Light	Short UV	Ordinary Light	Short UV	Ordinary Light	Short UV	Ordinary Light
Powder (P) as such	Pea Green	Fern Green	Verdigris	Willow Green	Coriander Brown	Jade Green	Parsely Green	Carnation Green	Sung Green	Ivy Green
P+Distilled Water	Sap Green	Primrose Yellow	Pea Green	Mimosa Yellow	Pea Green	Primrose Yellow	Pea Green	Primrose Yellow	Agathia Green	Mimosa Yellow
P+Acetone	Agathia Green	Mimosa Yellow	Veronese Green	Uranium Green	Chartreuse Green	Dresden Yellow	Pea Green	Agathia Green	Primrose Yellow	Sap Green
P+Ethanol	Pea Green	Agathia Green	Pea Green	Chartreuse Green	Sap Green	Mimosa Yellow	Agathia Green	Pea Green	Pea Green	Chartreuse Green
P+Benzene	Pea Green	Dresden Yellow	Agathia Green	Straw Yellow	Veronese Green	Aureolin	Pea Green	Cyprus Green	Agathia Green	Sap Green
P+Chloroform	Fern Green	Sap Green	Fern Green	Pea Green	Agathia Green	Sap Green	Agathia Green	Pea Green	Pea Green	Canary Yellow

*According to wilson colour chart

3.4 Thin Layer Chromatographic Analysis

Analytical TLC of the leaves on silica gel revealed the presence of certain prominent spots (Fig. 2) which could be used for studying the variations existing among different accessions. The results exhibited a greater number of spots for accession GP07. The Rf values of the spots were given in Table 9.

Table 9: Thin Layer Chromatography

Accession code	No of bands in long UV	R_f value
GP01	3	0.33, 0.35, 0.85
GP02	3	0.33, 0.35, 0.85
GP03	3	0.33, 0.35, 0.89
GP04	3	0.33, 0.35, 0.89
GP05	3	0.33, 0.35, 0.89
GP06	3	0.33, 0.35, 0.85
GP07	4	0.33, 0.35, 0.85, 0.89
GP08	2	0.33, 0.85
GP09	3	0.33, 0.35, 0.85
GP10	3	0.33, 0.35, 0.85

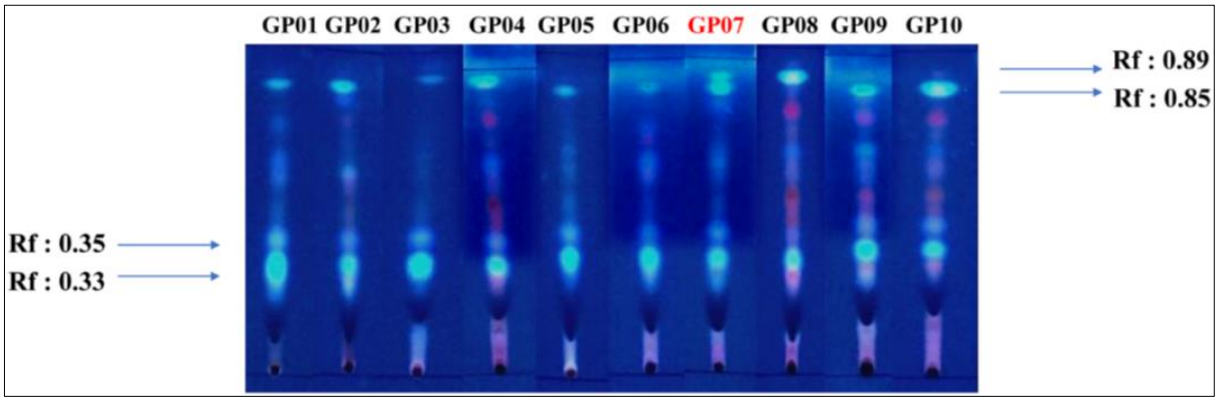

Fig 2: Thin Layer Chromatography

4. Discussion

Glycosmis pentaphylla is an ethnobotanically important plant in traditional medicine and provides an important health care service to persons both with and without geographic or financial access to allopathic medicine. Realizing this potential, the World Health Organization has encouraged and supported its member states in incorporating traditional and complementary or alternative medicine in national health care systems. Due to the high demand and utilization of Ayurvedic preparations and medications, proper identification and genetic documentation has become increasingly important in this medicinal plant.

Organoleptic evaluation is based on the study of morphological and sensory profiles of whole drugs [16]. It is therefore considered as a primary screen in the qualitative assessment of crude drugs. The parameters such as the structure of leaves, the colour, the typical tongue sensation and the odour are some important diagnostic as well as qualitative organoleptic indicators of leaf drugs. For example, the characteristic aroma of leaves is a true indicator of the presence of volatile active principles. Among all the accessions studied, similar sensations were reported. Hence, organoleptic studies conformed the relationship between ten accessions of *G. pentaphylla*.

Phytochemical characters are the indicators of chemical constituents of plant. The study of phytochemical variation is very important in medicinal plants since medicinal property of any plant is the result of action of such chemicals. Analysis of phytochemical variability between accessions will help to identify them in terms of their difference in phytochemical constitution [17]. Previous reports showed that among the phytochemicals studied, alkaloids constitute a major content in *G. pentaphylla*. Hence, our study focussed on the extraction of alkaloids. The total alkaloid fraction from leaf was extracted and the percentage yield was calculated. The accession GP07 (19.6 mg/g) showed the highest yield for total alkaloids. From the results, it became clear that, accession GP07 contained a greater number of alkaloids than from others.

Pharmacogenetic studies are pivotal in herbal technology as it will help in authentication of the plants and ensures reproducible quality of herbal products which will lead to safety and efficacy of natural products. Similar work has been reported by other researchers in leaf powder from many plants like *Nelumbo nucifera* [18], *Terminalia catappa* [19]. Moisture content of drugs might be at minimum level to dispirit the reduction of bacteria, yeast or fungi through storage. In our present study, accession GP07 showed low moisture content, indicating that it can prevent bacterial and fungal growth in comparison with others. The ash content gives an idea about the inorganic content of powdered leaves under investigation and thus the quality of the drugs can be assessed. It indicates presence of various impurities like carbonate, oxalate and silicate. The water-soluble ash is used to estimate the amount of inorganic compound present in drugs. The acid insoluble ash consist mainly silica indicate contamination with earthy material. The extractive values are useful to evaluate the chemical constituents present in the crude drug and helps in the estimation of specific constituents soluble in a solvent. High water-soluble extractive value indicates the presence of water soluble components such as sugar, acids and inorganic compounds etc.; and high alcohol soluble extractive value indicates the presence of polar constituents like phenols, alkaloids, flavonoids etc. Thus, the results of physicochemical analyses of all the accessions, especially accession GP07 lie within the acceptable limit, which in turn ascertains the quality as well as purity of leaf drugs. It was also revealed that the ash constants values showed a significant increase, whereas the soluble extractive value with solvents such as alcohol, chloroform, ethyl acetate and water showed a decrease in values when compared earlier reports.

The fluorescence character of powdered drug plays a vital role in the determination of quality and purity of the drug material. Fluorescence studies of powder with Acetone, Ethanol, Benzene, Chloroform and aqueous extract revealed the presence of green fluorescence under UV light. Several previous studies also reported similar results which are in close association to the present study [20]. If substance themselves are not fluorescent, they may often be converted into fluorescent derivatives or decomposition products by applying different reagents. Hence crude drugs are often assessed qualitatively in this way and it is an important parameter for pharmacogenetic evaluation of crude drugs [20].

TLC profiling of plant extracts in different solvent system confirms the presence of diverse group of phytochemicals. In our present study accession GP07 revealed the presence of 4 compounds having Rf values of 0.33, 0.35, 0.85 and 0.89 whereas others showed only the presence of 3 compounds, in GP08 only 2 compounds were observed. Similar results were reported in *G. pentaphylla* [21]. Phytochemical analysis in the case of above 10 accessions revealed differential levels of chemical affinities, very often associated with population distances and differences. Such variation can also be exploited both for commercial purposes. So, from our studies, it has been observed that different accessions of *G. pentaphylla* have a different genetic pool and does not share non- significant characteristics.

5. Conclusion

Diversity analysis in *Glycosmis pentaphylla* using phytochemical and physicochemical markers revealed that, the *Glycosmis* genotypes showed diversity among accessions. Phytochemical analysis of leaf extracts of *G. pentaphylla* showed the presence of various bioactive compounds in all the extracts. The results of phytoconstituents of different extracts of *G. pentaphylla* accessions documented that, accession GP07 showed variation which might be useful to supplement information for future studies on leaves. TLC profiles were done to identify chemical constituents of the plant. The results revealed that the total crude alkaloid extract and characteristic spots were maximum in accession GP07. It was concluded that, the accession GP07 resolved a greater number of compounds, compared to that of other accessions. Hence, the accession GP07 was phytochemically distinct and can be used for further analysis and characterization.

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