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Qualitative analysis and TLC screening of variousfruit extract of medicinally important climber Diplocyclos palmatus (L) C. Jeffrey

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ABSTRACT

Diplocyclos palmatus (L) C. Jeffrey is a climber from family Cucurbitaceae. Traditionally used in treatment of various life-threatening diseases. This plant also known for presence of various important bioactives.The present study deals with qualitative analysis of secondary metabolites from fruits of D. palmatus. Different extracting solvents (chloroform, petroleum ether, ethanol, methanol and water) were treated with various reagents. On the basis of colour/precipitation intensity secondary metabolite content was assessed. Further by using thin layer chromatography (TLC) phenolics and terpenoids were separated. Rf values of plant extract was compared with standard phenolics and terpenoids. Qualitative screening of D. palmatus demonstrated the existence of various bioactive compounds, including terpenoids, alkaloids, phenols, flavonoids, tannins, sterols, anthraquinones, cardiac glycosides, saponins, volatile oils, and more.Among the various extracting solvents, the aqueous extract was found to be the most effective, as it contained a higher concentration of secondary metabolites.Conversely, petroleum ether was found to be less effective in the extraction of bioactive secondary metabolites.The TLC analysis showed the formation of reddish-brown and yellowish-brown spots, indicating the presence of terpenoids and phenolics respectively in the tested plant extract.In conclusion, D. palmatus fruits found to be important source of bioactive rich secondary metabolites and water proving to be most efficient for extraction.

Keywords: D. palmatus, Secondary metabolites, TLC, Phenolics, Terpenoids.

I. INTRODUCTION

Diplocyclos palmatus (L) C. Jeffrey is member of family Cucurbitaceae. From very long back this family was consumed by mankind for food purpose. In word this family represent 125 genera containing 960 species (Jeffery, 2005), while in India 31 genera containing 94 species are found (Renner and Pandey, 2013). Literature survey reported that genotypes from the family used tom treat malaria, epilepsy, diarrhea, leprosy, diabetes, boils, diuretic, snakebites, dysentery, eczema, cough, asthma, antioxidant etc (Rolnik and Olas, 2020). Classical evidences denoted that Diplocyclos is native to Australia, Malaysia, Papua New Guinea and Tropical Africa. In these regions it is reported from slightly warmer area. In India this species found very widely in wild and along the roadside. In vernacular language it is called as Shivlingi; as upper surface of its seeds contain marking and morphology similar to 'Shivling' which is icon of Lord Shiva (Chauhan and Dixit, 2010).In English known as Lollipop Climber or Striped Cucumber. Morphologically this plant is annual climbing herb, having tendrils which are slender and bifid. Leavers are five-lobed, glabrous. Male flowers and female flowers are separate. Male flowers are found in small fascicles of 3-6 peduncles, and female flower single, axillary. After attending the maturity, fruit colour becomes red with white lines on the upper surface (Chauhan and Dixit, 2010).

Literature review denoted that this plant is used in curing of different life threatening diseases. Along with that historical ayurvedic books such asRajanighantuand Nighantu ratnakarareported applicability of this plant (Vadnere et al., 2013). Seeds are commonly used to improve sexual behavior and general tonic. Seeds are also used in treating female and male infertility, impaired spermatogenesis, asthenozoospermia, teratospermia, constipation, obesity, weight loss, hyperglycemia and diabetes(Sud and Sud, 2017). Leaf decoctions also found effective against inflammations, impotency, malarial fever and chronic colitis (Pushpangadan and Atal, 1984).

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Tribal peoples and local tribal informants from Aravali ranges suggested the use of 10 to 12 number of fruits boiled and fried with edible oil that served as curing agent against liver complaints (Punjani and Kumar, 2003). Different hidden compounds in plants, possesses foetid smell, acrid, depurative, tonic and acts as good alterative. Due to versatile nature of plant various important biochemical activity denoted by D. palmatus. It possesses several activities such as gynaecological, anti-asthmatic, anti-convulsant, anti-venom, anti-inflammatory, androgenic and antioxidant (Patel et al. 2020; Attar and Ghane, 2017a, 2017b).

Phytochemicals are naturally occurring non-nutritive substances found in plants. While plants also produce primary metabolites, which are vital for their own growth, development, and reproduction, these primary metabolites are essential for the survival of all living organisms. Examples of primary metabolites include sugars, amino acids, proteins, lipids, and polysaccharides. Apart from primary metabolites, plants also synthesize a wide range of organic compounds known as secondary metabolites (Guerriero et al., 2018). These secondary metabolites are unique to certain species or groups of organisms and are a reflection of the individual characteristics of those species. Various researchers have documented that this category includes phytochemicals such as tannins, phytosterols, saponins, terpenoids, carotenoids, resins, and carbohydrates (Rajasree et al., 2016). The initial stage in obtaining natural products from raw materials is the extraction process. Different techniques are employed for this purpose, such as solvent extraction, distillation, pressing, and sublimation, each based on distinct extraction principles. Among these methods, solvent extraction stands as the most commonly utilized approach. During extraction first solvent penetrate into matrix, followed by solute dissolved in solvent, later on extracted solutes were collected. Choosing the right solvent is a critical aspect of solvent extraction. The selection should take into account factors such as selectivity, solubility, cost, and safety (Zhang et al. 2018).

In present paper different extraction solvents were used for extraction of bioactive compounds. Theses extracts were tested for various phytochemical test. Chloroform, petroleum ether, ethanol, methanol, and water were used as extracting solvents.

II. MATERIALS AND METHODS

2.1. Collection and extraction:

Fresh fruits of Diplocyclos palmatus were collected in polythene bags, from locality Panhala; (N16˚48’540” latitude and E74˚07’757” longitude). Fruits were brought to laboratory and then washed with running tap water to remove dirt. After washing, water was blotted by using blotting paper. Further, fruits were cut into small pieces and placed in hot air oven at constant 60 ^{0}C temperature for a period of 72 h. Dried material was then ground into fine powder by using electric mill and stored at 4˚C for further use.

The 2 g of fruit powder was homogenized with 40 mL of one of the extracting solvents (chloroform, petroleum ether, ethanol, methanol, and water). This mixture is prepared in a 100 mL conical flask. The reaction mixture in the conical flask is placed on an orbital shaker and agitated at 140 revolutions per minute (rpm) for a duration of 12 hours. This step is crucial for allowing the solvent to extract compounds from the fruit powder effectively. After the 12-hour extraction period, the reaction mixture is centrifuged at 6000 rpm for 10 minutes. Centrifugation separates the solid particles and any unwanted material from the solvent and extracted compounds. The supernatant obtained after centrifugation is carefully separated and placed in a petri plate.The supernatant is allowed to evaporate, which removes the solvent and leaves behind the extracted compounds. After complete evaporation, the residue is redissolved in a known volume of the respective solvent used for extraction. This step is essential for concentrating the extracted compounds. The redissolved solution is then placed in glass vials and stored at a temperature of 4°C. These vials are used for further analysis.

2.2. Test for Phytochemicals:

Qualitative analysis of fruit extract was performed by using following table

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Table 1: Phytochemical Screening of secondary metabolites

Group	Test	Procedure	Observation	Reference
Alkaloid	Dragendroff test	Extract+Dragandroff reagent **Reagent-** A) 0.6 g Bismuth subnitrate1.7 g + 2 mL HCL + 10 mL water GAA 20 ml + Water 80 mL + 50% potass (B) 6 g potassium iodide in 10 mL water A+B-7mL conc. HCl + 15 mL water - Diluted to 400mL with water	orange brown ppt = presence of alkaloid	Aziz, 2015
	Wagner's test	Extract treated with Wagners reagent **Reagent:** 2 g iodide + 6 g KI in 100 ml water	Reddish brown ppt	Aziz, 2015, Sasidharan et al. 2011
	Mayer's test	Extract + Mayers reagent **Reagent:** 1.358g $HgCl_2$ + 60 mL water + 5 g KI in 10 mL water -Final volume 100 mL with water	White or pale ppt	Aziz, 2015
Phenols	Ferric chloride test	Extract + 2mL water + 10 % aqueous ferric chloride	Blue or green colour	Kumar et al. 2007, Aziz, 2015
	Elagic acid test	Extract + Few drops of 5% GAA + 5% $NaNO_2$ solution	Muddy or Niger brown ppt	Kumar et al. 2007, Aziz, 2015
Flavonoids		5 mL extract + Few drops NaOH- Yellow PPT form. Add few drops of dil H_2SO_4colour changes to colourless/PPT disappear	Flavanoid present	Kumar et al. 2007
		Extract + 10% Ammonium hydroxide	Yellow fluorescence-Flavanoid present	Kumar et al. 2007
Tannins	Lead acetate test	Extract + Few drops of 1% lead acetate	Yellow or red ppt	Parekh and Chanda, 2007
	Ferric chloride test	Extract + 2mL $FeCl_3$ (1%)	Blue or Black ppt	Sasidharan et al. 2011, Kumar et al. 2007
	Alkaline reagent test	Extract + Sodium hydroxide solution	Yellow to red ppt	Parekh and Chanda, 2007
	Braemer's test	10% Alcoholic ferric chloride + 2-3 mL extract	Dark blue or greenish grey coloration of solution	Sasidharan et al. 2011, Parekh and

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				Chanda, 2007
Terpenoids		Extract + 2 mL Chloroform + 1 M Conc. H_2SO_4	Reddish brown colour	Sasidharan et al. 2011
		Extract + 1mL 2-4-Dinitrophenyl hydrazine (1%) in 2M HCl	Yellow orange colour	Aziz, 2015
	Libermann Burchard test	Extract + Few drops acetic anhydride + Boil and cool + conc. Sulphuric acid added from side wall	Brown ring at junction upper layer dead red	Sasidharan et al. 2011, Kumar et al. 2007
	Salkowaski test	Extract + Chloroform + Few drops Conc. Sulphuric acid –Shake well and allow to stand	Formation of yellow coloured lower layer	Edeoga et al, 2005, Sasidharan et al. 2011
Sterols	Salkowaski test	2mL extract + 2 mL chloroform + 2 mL Conc. H_2SO_4 – shake well	Chloroform layer did not appear red and acid layer florescent greenish yellow	Edeoga et al, 2005, Sasidharan et al. 2011
Anthraquinone	Borntrager's test	Extract + 1mL 10% Ferric chloride + 1 mL Hydrochloric Acid- cool the extract and filter + Add equal amount of Diethyl ether + extract with strong ammonia	Pink or deep red colouration of aqueous layer	Kumar et al. 2007
	Borntrager's test	1mL Dil. 10% Ammonia + 2mL Extract	Pink red colour in ammonia layer	Onwukaeme et al. 2007
Cardiac Glycosides	Kellar-Kiliani test	2mL extract + 1 mL GAA + 1mL Ferric chloride + 1mL Conc. Sulphuric acid	Green blue colouration	Parekh and Chanda, 2007
	Kellar-Kiliani test	50mg extract + 2 mL Chloroform + H_2SO_4	Brown ring at interphase	Onwukaeme et al. 2007
Phalobatannin		2mL extract + 2mL 1% HCl -boil	Formation of red PPT	Kumar et al. 2007
Saponin	Frothing test/ Foam test	0.5mL Extract + 5 mL distilled water –shake well	Persistence of frothing	Parekh and Chanda, 2007
Volatile oil		2mL Extract + 0.1mL Dil NaOH + Dil. HCl – shake well	Formation of white PPT	Sasidharan et al. 2011

2.3. Thin Layer Chromatography (TLC):

2.3.1. TLC plate activation

In the experiment, 5 × 10 cm silica gel 60 F_{254} aluminum-backed TLC plates (Merck, Darmstadt, Germany) were utilized. These TLC plates were marked 2 cm from the bottom using a pencil as a reference point. By using a capillary tube or a micropipette, each sample and standards

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were applied separately to a plate. During this process, samples as well as standards were kept at the same distance from one another. Spots were allowed to dry and repeatedly loaded in order to obtain a higher concentration sample/standard spot. Air dryer (Phillips, India) was used to remove excess solvent during sample and standard loading.

2.3.2. TLC plate development

In the experiment, the TLC plates, after the application of standards and samples, were placed inside a glass chamber that had been saturated with a mobile phase. For the separation of phenolic compounds, the mobile phase used was acetic acid: chloroform (1:9), as described in Sadasivam and Manickam (2011). Similarly, terpenes were separated using a mobile phase consisting of toluene: ethyl acetate (6:4), following the method outlined by Oleszek et al. (2008). The mobile phase was allowed to move over the plates, (approx.11 cm.) To visualize and identify phenolic compounds, a Folin-Ciocalteau reagent, diluted in a 1:1 ratio with water, and a 20% Na_2CO_3 solution were sprayed onto the plates. This resulted in the appearance of yellowish-brown colored spots for the separated phenolic compounds. A similar approach was used to identify terpenes, but in this case, a vanillin-phosphoric acid reagent (prepared by mixing 1 g of vanillin with 100 ml of 50% phosphoric acid) was used. After heating the plates at 100°C for 10 minutes on a hot plate, reddish-brown patches became visible. Prior to spraying these reagents, the TLC plates were exposed to UV light with a wavelength of 365 nm, which produced reddish or blue spots. To quantify and analyze the separated compounds, the distance from the bottom of the TLC plates was measured, and all visible spots were marked with a pencil. The Rf (retention factor) values for both standards and samples were calculated using a specific formula. These Rf values are essential for determining the relative migration of compounds on the TLC plate.

$$\text{Rf value} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

The presence of certain compounds was shown by comparing the R_f values of the standard and samples.

III. RESULT AND DISCUSSION

3.1. Qualitative analysis of secondary metabolites:

Phytochemicals encompass a group of compounds developed in the normal metabolic processes of plants. Additionally, various stress conditions can lead to the synthesis and accumulation of compounds in plants, often referred to as secondary metabolites (Lucky et al., 2012; Attar and Ghane, 2017). These secondary metabolites, whether individually or in combination, play a crucial role in reducing the risk of various life-threatening diseases, including cancer, stroke, heart diseases, and urinary tract infections (Lucky et al. 2012). The distribution of phytochemicals in plants is influenced by factors such as age, geographical location, developmental stage, plant parts, and genotype (Aziz, 2015). Screening for phytochemicals is a fundamental step in identifying the bioactive components present in plants. Limited information is available on the constituent profiling of Diplocyclos, so efforts have been made to study the yield and qualitative analysis of various metabolites (phenols, flavonoids, tannins, terpenes, alkaloids, sterols, anthraquinones, cardiac glycosides, saponins, and volatiles) present in different solvents.

The results of the qualitative analysis are presented in Table 2. The highest extraction yield (0.469 g) was obtained from the aqueous extract, followed by methanol (0.057 g), ethanol (0.048 g), petroleum ether (0.019 g), and chloroform (0.04 g) extracts. The qualitative screening of D. palmatus revealed the presence of bioactive metabolites such as terpenoids, alkaloids, phenols, flavonoids, tannins, sterols, anthraquinones, cardiac glycosides, saponins, and volatile oils, among others (Table 2). Alkaloids, anthraquinone, and cardiac glycosides were found in all the tested extracts. Phytochemicals like phenols, flavonoids, and tannins were most abundant in aqueous, methanol, and ethanol extracts, while alkaloids were detected in trace amounts in the chloroform and petroleum ether extracts. Sterols were present in moderate quantities in the ethanol and methanol extracts. Moderate to high levels of volatile oils were found in the chloroform, ethanol, and methanol extracts. The Phalobatannin test yielded negative results in all the tested solvents (Table 2).

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Table 2: Preliminary phytochemical screening in D. palmatus fruit extract in various solvents

Group	Test	Chloroform	Pet ether	Ethanol	Methanol	Water
Alkaloid	Dragendroff test	-	-	-	-	++
	Wagners test	++	+	-	-	+++
	Mayers test	-	-	-	-	-
Phenols	Ferric chloride test	-	-	+	++	+++
	Elagic acid test	-	-	-	-	+++
Flavonoids		-	-	++	++	+++
		+	-	-	+	+++
Tannins	Lead acetate test	-	-	+	+	+++
	Ferric chloride test	-	-	+	++	+++
	Alkaline reagent test	-	-	+	+	+++
	Braemer's test	-	-	-	-	+++
Terpenoids		+++	+	++	+++	++
		+	++	+	+	+++
	Libermann Burchard test	++	++	+	+	+++
	Salkowaski test	+++	+++	++	++	+++
Sterols	Salkowaski test	-	-	+++	+++	-
Anthraquinone	Borntrager's test	+	+	+	+++	+
	Borntrager's test	+	-	+	+	++
Cardiac Glycosides	Kellar-Kiliani test	-	-	-	-	-
	Kellar-Kiliani test	++	+	++	++	+++
Phalobatannin		-	-	-	-	-
Saponin	Frothing test/ Foam test	-	+++	-	-	+++
Volatile oil		++	-	+	+	-

3.2. Thin Layer Chromatography:

Thin-layer chromatography (TLC) is a widely used method for separating mixtures and detecting compounds due to its speed, reliability, and simplicity. In TLC, compounds are separated based on their relative affinities for the stationary phase and the mobile phase. Compounds that have a stronger affinity for the stationary phase will travel more slowly with the mobile phase (Masoodi et al., 2021; Santiago and Strobel, 2013). TLC can be applied to various purposes, including the separation and detection of multiple compounds in a mixture, assessing the purity of standard compounds, monitoring the progress of a chemical reaction, and analyzing fractions obtained from various separation techniques (Santiago and Strobel, 2013). In the present analysis, TLC was used for the separation and detection of compounds analyzed from the extract, as indicated in Figure 2. This technique allows for the visualization and identification of the compounds present in the sample, making it a valuable tool in phytochemical and chemical analyses.

Formation of pinkish brown spots under UV and reddish-brown spots when exposed to vanillin phosphoric acid reagent were observed. In the present investigation, separation of terpenoids was noted (cucurbitacin I Rf= 0.125; and Ursolic acid = 0.35). All standards reacted rapidly with vanillin phosphoric acid reagent; hence, remarkable spots were noted when heated at 100 °C. In the fruit sample, total five spots appeared (Rf= 0.10, 0.18, 0.27, 0.72 and 0.97). Rf value of spot 1 and cucurbitacin I, were very near to each other (Rf = 0.10); therefore presence of cucurbitacin in D. palmatus fruit sample was confirmed. Wutsqaet al., (2021) detected terpenoids and steroids from Lindsaeaobtusausing TLC. TLC plate of phenolics after spreading the reagent revealed yellowish brown spot was observed. These results confirmed

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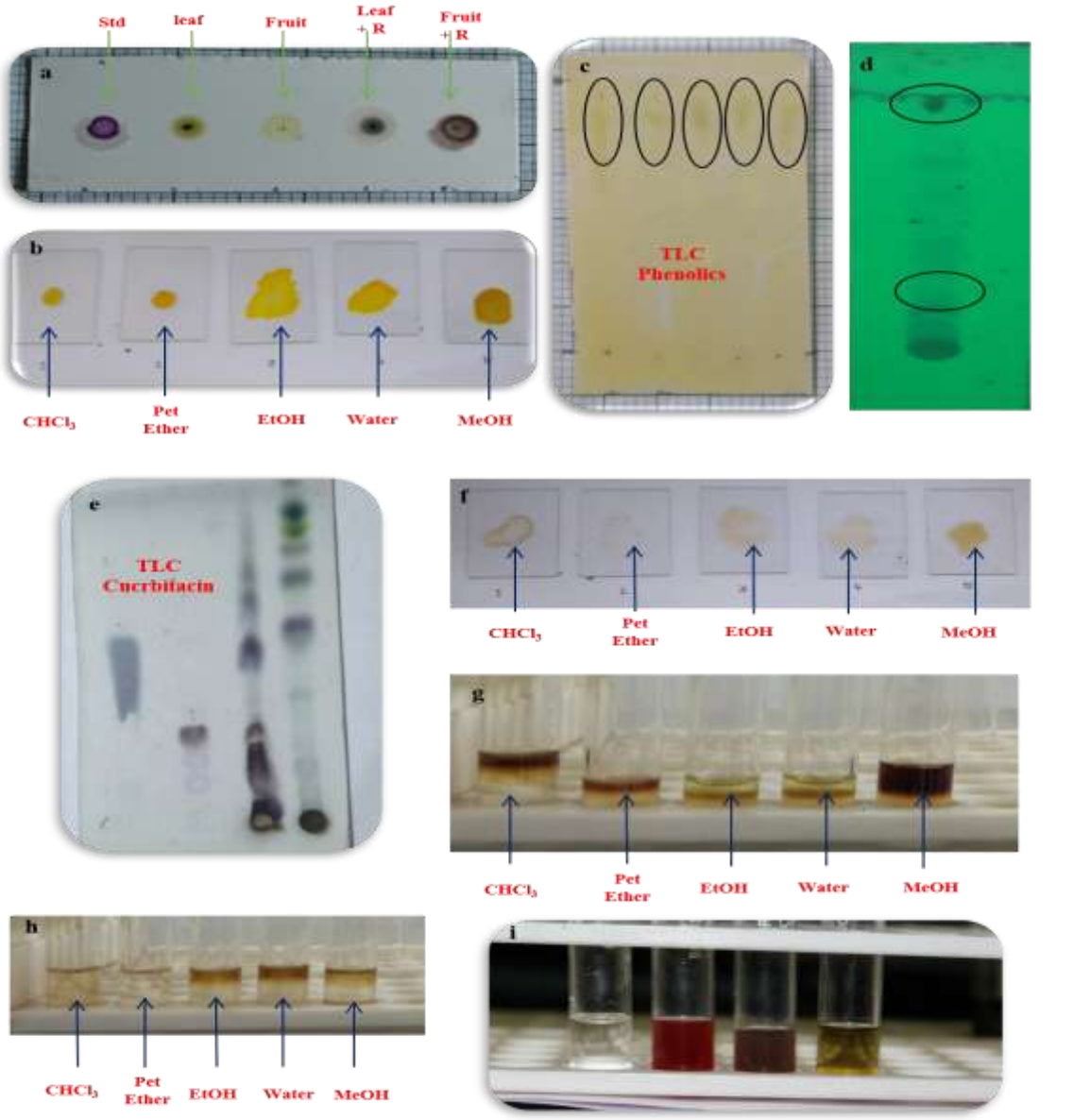
the presence of phenolics in fruit extract of D. palmatus. Males˘ and Medic´ -Saric, (2001) also reported phenolic acids and flavonoids from Helleborus atrorubens. Sharma et al. (2013) also rereported tannic acid from Bryophyllumpinnatum.

IV. CONCLUSION

Diplocyclos palmatus fruits were chosen as sources of secondary metabolites like phenolics, flavonoids, terpenoids, tannins, steroids, cardiac glycoside etc these are used for antioxidant, antibacterial, antifungal, anti-inflammatory, anticancer activities. The screening of phytochemicals in medicinal plants is of paramount importance, carrying considerable economic implications in the development of novel therapeutic remedies for a range of illnesses. These bioactive components assume a pivotal role in the endeavors of research and pharmaceutical industries, and the development of formulations containing these constituents is integral to the management of diverse diseases.



Qualitative analysis: a) Elagic acid test, b) Ferric chloride test, c) & d) TLC of phenolic compounds, e) TLC of Terpenoids, f) Test for Terpenoid, g) Libermann Burchard test, h) Salkowaski test, i) Test for terpenoids from fruits of *D. palmatus*.

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