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Comparative phytochemical analysis of leaf, stem and callus extracts of *Solanum diphyllum* L.

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Abstract

Solanum spp. is well known for their wide range of therapeutic applications and has been recognized for various bioactivities. Therefore, it is time to explore the medicinal values and the phytochemical constituents present in stem, leaf, and callus extracts of *Solanum diphyllum* L. Hence, for the first time, the present study was conducted to evaluate the comparative phytochemical analysis between the solvent extracts of leaf, stem, and callus using four different solvents, *viz*., petroleum ether, chloroform, ethyl acetate, and methanol. Among the tried plant growth regulators, plentiful callus production was achieved on MS medium fortified with 2-4, D at 1.5 mg/l. Further, the qualitative phytochemical analysis revealed the presence of alkaloids, flavonoids, terpenoids, steroids, tannins, saponins, and glycosides, *etc*. Among the tested extracts, methanol extracts showed number of phytochemicals, followed by ethyl acetate in all the plant materials because of high polarity. The outcome of this study showed that thein *in vitro* derived callus extracts possess more number for phytochemicals than *in vivo* leaf and stem extracts. In view of the medicinal properties and increased demand for this plant in the pharmaceutical industry, we believe that further study is required to standardize the protocol for the production of secondary metabolites from callus cell suspension culture for large-scale production of desirable phytochemicals.

1. Introduction

The systematic scientific study of traditional medicinal plants for the discovery of new drugs through proper investigation concerned with medicinal plants is of great significance. In recent times, it was reported that the knowledge of the chemical study and bioactivity of the medicinal plant's species provides pilothints for an overall interpretation (Anand *et al*.,2019). It also providesa platform to investigate the occurrence of major classes of compounds in medicinal plants and their medicinal values (Ahmad *et al.*, 2021).

Abundant secondary metabolites are produced by most medicinal herbs in different environmental conditions. Some of them are not essential for their growth, or any primary metabolic pathways but are needed for the plant to interact with its surrounding environment and also with other organisms (Hussein and Anssary, 2019). In recent years, the development of the commercial importance of secondary metabolites attained great interest, particularly by altering the production of bioactive plant metabolites by employing tissue culture technology (Hussain, 2012).

Plant cell culture technologies is the conventional method performed under sterile conditions to enhance desirable secondary metabolites by using explants, such as plant leaves, stems, roots, meristems, *etc*. (Espinosa-Leal, 2018). The commercial *in vitro* production of secondary metabolites from plant cell suspension cultures has been reported in various medicinal plants by the application of bioreactors (Chandran *et al*., 2020). Suspension cultures are developed from friable callus cultures to obtain commercially essential secondary metabolites instead of using the whole plant (Khanpour-Ardestani *et al.*, 2015).

This research work was undertaken to carry out the comparative investigation of phytochemicals present in the stem, leaf, and callus of *S. diphyllum* (Figure 1). This plant belongs to the family Solanaceae; a potent medicinal herb frequently found in dry and deciduous land areas of the Eastern Ghat region of Karnataka (Paul and Biswas, 1995). *S. diphyllum* is also distributed in India, China, Srilanka, and Malaysia (Singh *et al.*, 2014; UF/IFAS, 2019).

This plant is considered a weed in many western countries and India as well because it is commonly grown in waste and unusual land areas (Meena and Rathi, 2021). It was commonly grown in shady places, and the leaves were simple and dark green, larger, entire, elliptic to oblong, sometimes broad, and widest at the center (Knapp, 2013). The plants produce profuse fruits and the fruits bearing shoots are slightly angled at each nodal region and arranged in a zigzagged manner (Brown *et al.*, 2020). Fruits-bearing shoots also contained simple leaves which are entire, smooth, and hairless, with a pair of unequally distributed on the same node. The cymose-type inflorescence contains numerous flowers, which produce plenty of immature fruit (Kalidass and Panda, 2019).

S. diphyllum possesses various medicinal properties and used since ancient times in traditional medicinal practices to cure numerous diseases (Hamada, 2010). In many western countries, this plant was used frequently especially in Florida and China since time immemorial for medicinal purposes (Markle *et al.*, 2014). It was

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reported that this plant possesses anti-inflammatory, antiulcer, and hepatoprotective properties (Perkins and Payne, 1978). Pharmaceutical studies revealed that this plant contains secondary metabolites such as flavonoids, alkaloids, phenolic substances, *etc*., and they have played a crucial role (Singh and Singh, 2015). Because of the above facts, the species *S. diphyllum* was undertaken to evaluate the comparative phytochemical screening in various solvent extracts of the stem, leaf, and leaf callus. Therefore, we strongly believe that this work is useful to discover the presence of valuable phytochemicals present both *in vivo* and *in vitro* plants. It was also noted that the production of secondary metabolites from callus culture can be utilized for various medicinal purposes.

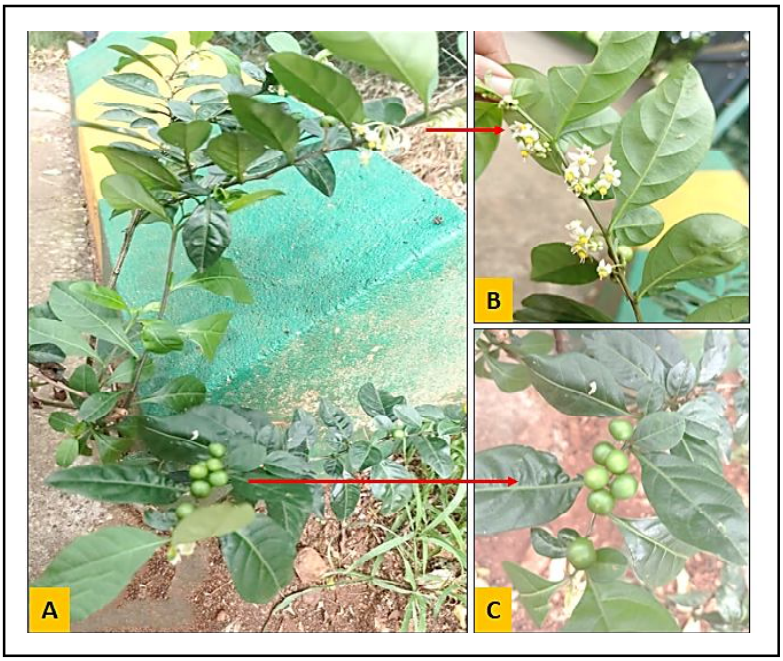

Figure 1: Habit of *S. diphyllum*: A. Whole plant, B. Inflorescence, C. Fruits.

2. Materials and Methods

2.1 Plant material collection

The fresh stem, leaf materials of *S. diphyllum* were collected in and around the Mysore district and brought to the lab, and identified with the help of a plant taxonomist. After identification, the plant herbarium was also submitted to the department for authentication (Herbarium Voucher number: UOMBOT22SD73) and further studies. Meanwhile, a few healthy plant saplings were also maintained at the Botanical garden and healthy explants such as stem and leaf were also collected and used for callus induction under *in vitro* laboratory conditions.

2.2 Induction of callus

Young and fresh healthy leaves of *S. diphyllum* were collected and washed with running tap water to remove the debris and then surface sterilized with 70% ethyl alcohol for 3 min. Followed by treatment with mercuric chloride (0.1%) for 4 min. Further, the explants were washed with sterilized distilled water consecutively three times, blot dried, and inoculated on MS medium supplemented with 2,4-D, NAA, BAP, 30 g/lsucrose, 9g/lagar, pH 5.8 was also maintained. Then, the inoculated cultures were incubated at 21°C ± 2 for a 16 h photoperiod. The induced callus was subcultured frequently onto fresh MS medium (Brown, 1990).

2.3 Preparation of plant extracts

The collected stem and leaves were washed thoroughly in running tap water and then shade dried. The profusely induced matured callus was harvested and dried at 50°C and all the materials were dried properly and powdered using a mechanical blender. 30 g of each plant material were taken in thimbles extracted with petroleum ether, chloroform, ethyl acetate, and methanol successively based on their polarity using a Soxhlet extractor. The obtained concentrated extracts were stored at 4°C in air-tight vials for further experiment.

2.4 Phytochemical screening

Each solvent extract of *S. diphyllum* stem, leaf, and callus extracts was subjected to comparative phytochemical screening to reveal the presence of various secondary metabolites. The phytochemical analysiswas carried out by the method described by Trease and Evans (2002).

2.5 Statistical analysis

All the experiments were repeated thrice and data were expressed as mean ± SE. The data were analyzed statistically by one-way analysis of variance, followed by a Duncan Multiple Range Test (DMRT) using SPSS software. Probability values $p<0.05$ were considered significant.

3. Results

3.1 Callus induction

The establishment of callus from leaf explants of *S. diphyllum* was successfully achieved on MS medium supplemented with various types of growth hormones *via* 2, 4-D, NAA, and BAP were tried individually in the range of 0.5-2.5 mg/l concentration. Initially, callus started to develop on the surface as well as at the cut ends of the leaf

explants one week after inoculationon MS medium. The leaf explants are more suitable for callusing than other explants due to fleshy texture and their responding quickly on MS medium. The results obtained from this study are clearly stated in Table 1. The nature of the callus developed from the leaf explants at each concentration of the plant growth regulators was also recorded at different time intervals (Figure 2A). Among the different growth regulators, 2, 4-D, offered a maximum callusing of 93.6 % at 1.5 mg/l. In the course of time, the callus development also differs between the plant growth regulators. However, the nature of the callus also varied in each concentration such as creamish and light brown, and so on, and it was recorded clearly and mentioned. The amalgamation of the plant growth regulators that were tried for callogenesis was not efficiently inducing the callus in *S. diphyllum*. The results indicate that among the tried hormones, the 2, 4-D, was the best-suitable hormone for maximum callus induction in *S. diphyllum* leaf explants, and callus induction declined consecutively at higher concentrations. A result of the fact that the overall study of the investigation shows that higher as well as lower concentrations of hormones did not give any significant results. Further, the multiplication of callus was successfully achieved to get a sufficient amount of callus for further experiments. The friable callus was subcultured frequently on MS medium supplemented with 2, 4-D at 1.5 mg/l concentration alone (Figure 2B).

Table 1: Effect of plant growth regulators on callus induction from leaf explants of *S. diphyllum*

Hormones	Concentrations (mg/l)	The intensity of callus (%)	Nature of callus
2,4-D	0.5	00.0 ± 0.00^{h}	No callus
	1.0	84.1 ± 0.54^{b}	Whitish, fragile
	1.5	93.6 ± 0.48^{a}	Creamish, compact
	2.0	58.5 ± 0.61^{f}	Whitish
	2.5	49.3 ± 0.21^{g}	Whitish
2,4-D + BAP	0.5 + 0.5	00.0 ± 0.00^{h}	No callus
	1.0 + 0.5	59.1 ± 0.15^{f}	Brownish, fragile
	2.0 + 0.5	71.5 ± 0.68^{c}	Light brownish
NAA + BAP	0.5 + 0.5	00.0 ± 0.00^{h}	Light yellowish
	1.0 + 0.5	69.1 ± 0.78^{d}	Light brown
	2.0 + 0.5	61.1 ± 0.74^{e}	Brown

Note: Values represented mean ± SE: followed by the same letter within columns are significantly different (p<0.05) according to Duncan's Multiple Range Test.

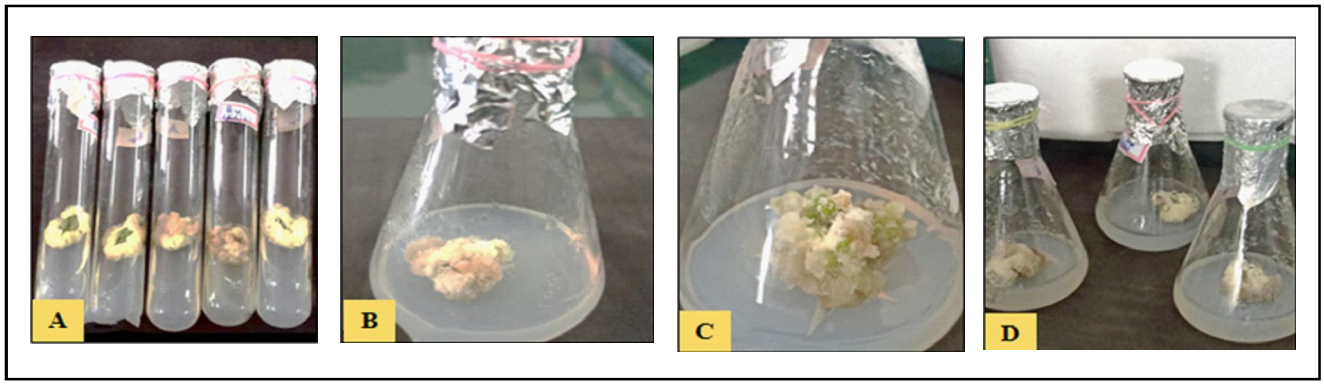

Figure 2: Various stages of callus development from leaf explant of *S. diphyllum*.A. Two weeks old callus from leaf explants, B. Sub-cultured callus (2 weeks old), C. 4 week's old callus, D. Fully matured callus (two months old).

3.2 Preliminary phytochemical analysis

Every medicinal plant has its own chemical constituents. The presence of secondary metabolites in medicinal plants often play an essential role in the defense mechanism against foreign invaders such as a microbes, pests, insects, *etc*., besides that, these phytochemicals also provide life saving drugs for many diseases. In such cases, we need to investigate the presence of phytochemicals to explore their biological assets. The plant material such as stem, leaf and callus were extracted sequentially with Soxhlet extraction by using organic solvents, *viz*., petroleum ether, chloroform, ethyl acetate, and methanol based on theirpolarity index. Each extract was subjected to preliminary phytochemical screening and the results revealed that each tested samples showed both positive and negative inference towards the presence of some active secondary metabolites. The results of the phytochemical screening indicated the presence of different types of active phytoconstituents such as alkaloids, carbohydrates, flavonoids, glycosides, protein, resins, phenols, saponins,sterols, tannins, and triterpenes, *etc*., and the results were presented in the Table. 2. The test revealed that among

the tested solvent extracts, *in vitro* derived callus showed maximum positive tests compare to stem and leaf samples. Likewise, proteins is present only in methanol extracts of all three samples and petroleum ether of all the extracts showed negative results.

Table 2: Preliminary phytochemical analysis of stem, leaf and callus extracts of *S. diphyllum*

	Callus				Leaf				Stem			
	PE	CL	EA	ME	PE	CL	EA	ME	PE	CL	EA	ME
Sterols	-	-	+	+	-	-	+	+	-	-	+	+
Triterpenes	-	+	+	+	-	+	+	+	-	+	+	+
Phenols	-	-	+	+	-	-	-	-	-	-	-	-
Saponins	-	+	-	+	-	-	-	+	-	-	-	-
Alkaloids	+	+	+	+	+	+	+	+	-	+	-	-
Tannins	-	-	+	+	-	-	+	+	-	-	-	+
Flavonoids	+	+	+	-	+	-	-	-	-	-	-	-
Carbohydrates	-	-	-	+	-	-	-	-	-	-	+	+
Resins	-	-	+	+	-	-	+	+	-	-	-	-
Proteins	-	-	+	+	-	-	+	+	-	-	+	+
Glycosides	-	+	+	+	-	+	+	+	-	+	-	-

Note: "+" Presence, "–" Absence, PE: Petroleum ether, CL: Chloroform, EA: Ethyl acetate, ME: Methanol.

4. Discussion

Nowadays, plant cell culture technologies offered the best conventional method to enhance desirable secondary metabolites by using explants, such as plant leaves, stems, roots, meristems, etc., performed under sterile conditions (Espinosa-Leal, 2018). The *in vitro* production of secondary metabolites from cell suspension cultures has been reported long back by the application of bioreactors using various medicinal plants (Chandran *et al.*, 2020). The present study we standardize the suitable protocol for best callus induction using leaf explants in *S. diphyllum*. This can be more useful for further extraction of desirable phytochemicals through suspension culture. Suspension cultures are developed from friable callus cultures to obtain commercially essential secondary metabolites instead of using the whole plant (Khanpour-Ardestani *et al.*, 2015). Similar findings were reported in *S. tuberosum* by Khalafalla *et al.* (2010). In addition to this, the fully matured callus was harvested after two months when the matured callus turned brownish**.** This result is also in agreement with the earlier reports of Kumar and Kaur (2017), wherein the usage of optimum level of 2, 4-D was found to be the most effective for callus induction in *S. lycopersicon.* The results presented here is contrary to the earlier reports of Qin *et al.* (2017), wherein 100% callogenesis was achieved in *S. torvum* at 1 mg/lof 6-benzyl adenine + 0.5 mg/lof NAA. The plant growth regulators have played a crucial role in callogenesis as well as in organogenesis and in several cases the explants were not responding to higher as well as lower concentrations of hormones and in due course, the explants become senescence (Abdelmaksood *et al.*, 2017).

The presence of secondary metabolites in medicinal plants often play an essential role in the defense mechanism against foreign invaders such as a microbes, pests, insects, *etc*., besides that, these phytochemicals also provide lifesaving drugs for many diseases (Chew *et al.*, 2011). Every medicinal plant has its own chemical constituents. In such cases, we need to investigate the presence of phytochemicals to explore their biological assets. Several recent reports have confirmed that phytochemicals including alkaloids, glycosides, terpenoids, saponins, phenols, and steroids have enormous antioxidants properties and responsible defense system. It was realized that the results of this study were somewhat in accordance with the observation obtained by Rani *et al.*, (2019) who reported that, the phytochemical screening of the crude extract of *S. nigrum* revealed the presence of alkaloids, reducing sugars, tannins, flavonoids, phlobatannis and steroids. Our study also suggest that the methanol is the best solvent for the extraction of phytochemicals in the tested samples due to its high polarity and used in wide range for extraction process. So far, no previous report of comparative phytochemical screening was available and hence our study might provide scientific platform to support the traditional claims this valuable plant for future pharmaceutical implications.

5. Conclusion

Medicinal plants are a potent source for human health due to the presence of phytochemical constituents that are responsible for various pharmacological activities. We standardize the best protocol for the induction of profuse callus from leaf explants of *S. diphyllum* on MS medium. Based on the results obtained in this study, the comparative phytochemical analysis revealed significant variations in the presence of phytochemicals in stem, leaf and its callus extractsin *S. diphyllum*. Therefore, our study suggest that *in vitro* callus extracts offered more number of phytochemicals than *in vivo* extracts of leaf and stem. Callus cultures offers numerous advantages as a model system for the production of desirable phytochemicals. We believe that further studies are required for the production of secondary metabolites from callus cell suspension culture and it may enrich the required phytochemicals by providing a suitable medium. In view of the medicinal properties and increased demand of this plant in the pharmaceutical industry, our study offers a simple protocol for mass callus production of this important medicinal plant.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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