Sri Lankan Journal of Health Sciences

Article

Evaluation of Endophytic Fungal Extracts of *Mucuna pruriens* for the Presence of L-Dopa

Yalini Sivaladchanam[1,*], Chamila P.T. Liyanaarachchie[1]

[1] Department of Pharmacy, Faculty of Allied Health Sciences, University of Peradeniya, Sri Lanka

[*] Correspondence: yaallinisivaladchanam94@gmail.com

Abstract

Background: *Mucuna pruriens* (Fabaceae) is a natural source of levodopa (L-Dopa). Urbanization and overexploitation have made the plant scarce. Thus, investigations for alternative sources for natural L-Dopa are important. This study aimed to investigate the capability of the endophytic fungi present in *M. pruriens* plant for the production of L-Dopa. **Methods:** Compounds of air-dried plant parts were extracted with methanol. Endophytic fungi in fresh plant parts were isolated and grown to obtain metabolites. Plant extracts and the endophytic fungal metabolites were analyzed for the presence of L-Dopa with respect to the standard using thin-layer chromatography. **Results:** Nine endophytic fungi were isolated from different plant parts. Seed and pod extracts and their endophytic fungal metabolites contained L-Dopa. **Conclusion:** *M. pruriens* associated endophytic fungi could be excellent sources of natural L-Dopa.

Keywords: *Mucuna pruriens*; L-Dopa; Endophytic fungi; Parkinson's disease

Introduction

Medicinal plants are used extensively as a bioresource of natural products which used directly or indirectly in treating diseases. Demand for medicinal plants is increasing around the world as 80% of the total population in developing countries depend on medicinal plants for their primary healthcare (1). The extinction of medicinal plant species could result from increased use and overharvesting. Therefore, it is important to obtain the values of medicinal plants without disturbing their population and habitats. Nowadays, the focus of new drug sources has been shifted from plants to endophytes as endophytes that reside inside these plants are capable of producing compounds similar to the host plant (2). Therefore, endophytes present in medicinal plant species are alternative approaches for producing medicinal plant-derived biologically active substances (3).

Endophytes are a diverse group of microorganisms (4) that live below the epidermal cell layers of the tissues of plant parts without causing any negative consequences to the host plant. There can be one or more endophytes in an individual plant. The symbiotic relationship between the endophytes and their host plant stimulates the production of secondary metabolites of the host plant. The host plant can use these compounds for different purposes, including defense against pathogens and motivating plant growth. These compounds could be useful in the drug discovery process (5). Both fungi and bacteria live as endophytes, whereas fungi are the most often isolated endophytes. It has been found that endophytic

Citation: Sivaladchanam Y, Liyanaarachchie C. Evaluation of Endophytic Fungal Extracts of Mucuna pruriens for the Presence of L-Dopa. Sri Lankan J. Health. Sci. 2022, 1(1): 1-9.
Received: 16.01.2022; Accepted: 08.03.2022; Published: 06.07.2022

fungi are a promising source for generating metabolic products which can be used as drugs or function as lead structures in synthetic modifications (6).

Mucuna pruriens is a medicinal plant that belongs to the family Fabaceae. Its vernacular names are Cowitch/Velvet bean (English), '*Wanduru Me'* (Sinhala), and '*Punaikkali'* (Tamil) (6). It has been shown that all parts of the plant possess medicinal values, thus exerting a broader spectrum of pharmacological activities (7). *M. pruriens* is reported to have the highest content of natural L-Dopa (8). Due to nutritional, pharmaceutical, and cosmeceutical bioactive constituents (9) present in *M. Pruriens,* the global demand for *M. pruriens* is increasing.

L-Dopa is commercially synthesized from vanillin and hydantoin by a chemical process that involves eight steps. It is a time-consuming and very expensive process that involves several chemicals. Therefore, scientists have focused on plant-derived L-Dopa (10). Sharma *et al*. (2019) have isolated thirty-five different endophytic fungal species from different parts of *M. pruriens* plants collected at eight different geographical locations in India (11). However, endophytic fungi have not been isolated from the pods and seeds of *M. pruriens* plant. It was reported that the isolation of endophytic fungi from pods and seeds of *M. pruriens* plant is difficult (11).

Therefore, the current study was undertaken to isolate endophytic fungi from different parts of *M. pruriens* plant and screen the extracts of different parts of the plant and endophytic fungi isolated from different parts of the plant for the presence of L-Dopa.

Materials and Methods

Plant materials

Leaves, seeds and pods of *M. pruriens* plant were collected from a home garden in Gampola, Central Province, Sri Lanka. A herbarium sheet was prepared, and the plant was authenticated by the National Herbarium, Royal Botanical Gardens, Peradeniya, Sri Lanka.

Chemicals, reagents and solvents

Methanol, acetone, chloroform, 1-butanol, glacial acetic acid, ethyl acetate, ethanol, ninhydrin, iodine, silica gel, and dextrose used in the study were of analytical grade. Commercially available L-Dopa tablets (TIDOMET FORTE, Torrent PHARMA, India) were purchased from University Rajya Osusala, Peradeniya, Sri Lanka.

Other materials

TLC plates (Sigma) were purchased from Aldrich, Germany. Chlorex (CLOROX, Malaysia) and potatoes were purchased from a commercial food stall in Peradeniya, Sri Lanka.

Equipment

Grinder (Waring, USA) Rotary evaporator (Buchi, India), Laminar flow cabinet (SRCJ.15), Autoclave (ALP, Japan), Water bath (Bioer, China), Centrifuge (Gemmyco, Taiwan), UV illuminator (VL-4.Lc, France), Sonicator (MRC, China), Shaker (Memmert, Germany), Analytical balance (VWR, Italy) were the equipment used in the study.

Separation of L-Dopa and carbidopa from commercially available L-Dopa-carbidopa tablets

L-Dopa-carbidopa tablets (TIDOMET FORTE, Torrent PHARMA, India) were weighed and crushed to obtain a fine powder. The crushed powder was then dissolved in a mixture of water:methanol (7:3 V/V ((Nail and Vega, 2007)), 10 ml), followed by sonication for 20 min. The solution was

centrifuged at 25,000 rpm for 15 min. The supernatant was separated from the precipitate. The solvent of the supernatant was evaporated at 45 ºC using a rotary evaporator. Obtained solid residue (considered as standard L-Dopa-carbidopa) was transferred into a clean universal bottle and stored in a refrigerator (2 – 8 ℃) until used in TLC analysis.

Selecting a suitable mobile phase

TLC was developed for the standard L-Dopa-carbidopa and methanolic extract of seeds using acetone:chloroform:1-butanol:glacial acetic acid:water in volume based solvent ratios; 6:4:4:4:3.5 (12) and 6:4:4:4:3.8. After the development, plates were sprayed with ninhydrin. TLC patterns and Rf values were compared to select the best mobile phase.

Preparation of plant extracts (Direct extraction)

A part of the collected plant parts was shade dried for four weeks inside a room and processed separately for direct extraction. Dried plant samples (leaves, seeds, and pods) were ground separately using a mechanical grinder to obtain coarse powders of respective plant parts and processed separately. Powdered plant materials (each 25 g) were sonicated with methanol (200 ml) and filtered through Whatman filter paper. The filtrate was collected. The procedure was repeated twice for the solid residue. Filtrates were combined. The solvent of the resulting extract was evaporated to dryness under pressure at 40 ℃ using a rotary evaporator. Crude solid extracts were stored in the freezer until they were used.

Preparation of endophytic fungal extracts

Isolation and growth of endophytic fungi

Fresh plant samples (leaves, seeds, and pods) were subjected to surface sterilization by the following procedure. Initially, plant materials were washed several times under running tap water, followed by washing in sterile distilled water. Surface sterilization was then carried out by subsequently rinsing the plant materials with 70% ethanol for 30 seconds, then with chlorex for 2–3 min, followed by rinsing in 70% ethanol for nearly 2 min, and finally with sterile distilled water for 2 minutes. Plant samples were then dried between folds of sterilized steri tissue papers. Then, surface sterilized plant samples were cut into small segments (approx. 5 mm×5 mm) using a sterile scalpel. Small segment samples were placed on Petri dishes containing potato dextrose agar (PDA) supplemented with gentamicin (320 µg/ml). Plates were then labeled properly and incubated at room temperature inside a clean box until fungal growth was initiated. When the fungal growth was initiated, another two days were allowed for further growth. Using a sterile cork-borer, growing tips of fungal mycelia were punched from the agar plate and transferred to a new PDA plate supplemented with gentamicin (320 µg/ml) for pure culture. After that two new PDA plates were inoculated from each pure fungal culture, and incubated in the dark at room temperature for two weeks.

Preparation of fungal extracts

New pure fungal cultures on PDA plates were cut into small segments using a scalpel and transferred to a sterile 250 ml Erlenmeyer flask containing potatoes dextrose broth (PDB) (150 ml). One Erlenmeyer flask containing PDB was kept without adding any fungal extracts. The mouth of each Erlenmeyer flask was quickly closed with a pre-sterilized cotton plug. Then the neck of the flask and the cotton lid was covered with a pre-sterilized aluminium foil. These starter endophytic fungal cultures were then placed on an orbital shaker (shaking at 120 rpm for 20 min). After that, they were removed from the shaker and allowed to continue growing in the dark for two weeks with intermittent shaking. The content of each Erlenmeyer flask was filtered through a funnel with a Whatman filter paper followed by a collection of filtrates. Filtrate (aqueous) from each Erlenmeyer flask was partitioned with ethyl acetate (100 ml). Organic and aqueous phases were evaporated separately under vacuum at 40 ºC and 44 ºC, respectively.

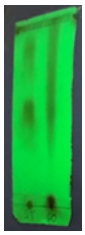
TLC analysis

TLC was developed for the solid extracts of plant materials (leaves, seeds, and pods); organic and aqueous phases of endophytic fungal extracts; and standard L-Dopa-carbidopa using acetone:chloroform:1-butanol:glacial acetic acid:water (6:4:4:4:3.8 V/V/V/V/V) as the mobile phase. Air dried TLC plates were analyzed by three methods. They were observed under UV light (254 nm) applied with ninhydrin (0.5%) in methanol and kept in an iodine chamber to visualize the spots. Retardation factor (Rf)s of solid extracts of plant materials (leaves, seeds, and pods) and endophytic fungal extracts were compared with standard L- Dopa-carbidopa to identify any similarities.

Results

Table 1 shows the results of the TLC analysis carried out to evaluate the presence of L-Dopa in methanolic extracts of leaves, seeds, and pods of *M. pruriens* with respect to standard L-Dopa.

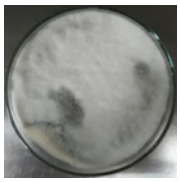
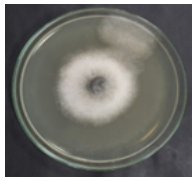
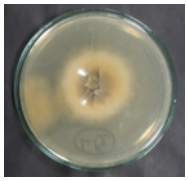
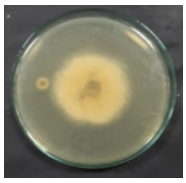
Table 1. Results of TLC analysis on methanolic extracts of leaves, seeds and pods of *M. pruriens*

***M. pruriens* plant extract**	**TLC plate observed under UV light, 254 nm***	**TLC plate after spraying ninhydrin and drying***	**TLC plate after vaporizing iodine***
Leaf extract			
Seed extract			
Pod extract			

*The left most spot in each TLC plate was of the standard L-Dopa-carbidopa

A total of nine endophytic fungal species were isolated from *M. pruriens* plant materials. Among the nine endophytic fungi, three were isolated from leaves (LF1-LF3), five were from seeds (SF1-SF5), and one was from pods (PF) and shown in Tables 2-4, respectively.

Table 2. Endophytic fungi from leaves of *M. pruriens*

Leaf fungi	Front view	Back view	Streak plate
LF1			
LF2			
LF3			

LF1, 1st leaf fungus; LF2, 2nd leaf fungs; LF3, 3rd leaf fungus

Discussion

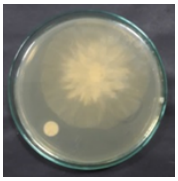
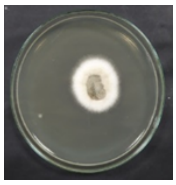
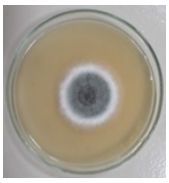
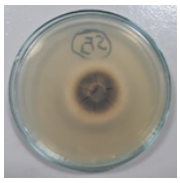
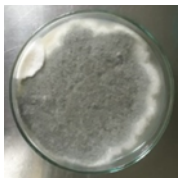
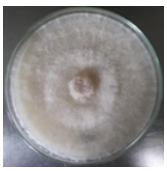
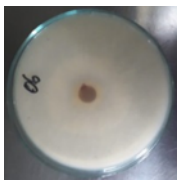
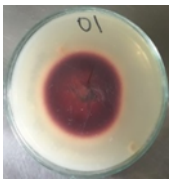
The unavailability of standard L-Dopa was rectified using the commercially available L-Dopa-carbidopa in 10:1 w/w ratio, respectively in tablet dosage form Acetone:chloroform:1-butanol:glacial acetic acid:water (6:4:4:4:3.8 V/V/V/V/V) solvent system was chosen as the mobile phase due to the better separation observed for the L-Dopa and carbidopa. A quantitatively larger spot with a 0.54 Rf value was obtained for L-Dopa, whereas carbidopa Rf was 0.84, which was a clear distinction from that of L-Dopa.

The presence of L-Dopa in the seeds of *M. pruriens* has been evaluated by several study groups (8, 9, 13). The present study also confirmed the presence of L-Dopa in seeds of *M. pruriens* regardless of geographical differences. According to the results shown in Table 1, methanolic extracts of seeds and pods obtained a dark blue spot. The respective Rf values were matched exactly with the standard L-Dopa (Rf, 0.54) spot. In comparison, a dark blue/Ruhemann's purple spot did not appear in the methanolic extract of leaves. The findings of this study imply that the dried seeds and pods of the *M. pruriens* plant contain L-dopa, whereas L-Dopa is not present in the leaves of *M. pruriens* plant. It has been reported that fresh leaves of *M. pruriens* plant contain 1 % of L-Dopa (13). However, there were no records on L-Dopa isolated from the pods of the *M. pruriens*.

Endophytic fungi isolated from leaves, seeds and pods of *M. prriens* were not identified but separately screened for the production of L-Dopa. For that, well grown fungi broths were separately extracted to organic and aqueous solvents.

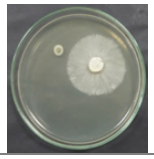
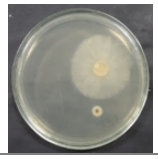
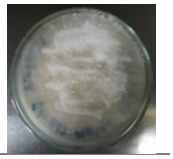
The broths containing SF1 and SF5 fungal cultures turned yellow to orange-red. This could be due to the increased broth incubation period, production of tyrosinase enzyme lead to the conversion of L-Dopa into melanin (14). As a result, the yield of L-Dopa could be low. Further studies are needed in this area for confirmation of the proposed idea.

Table 3. Endophytic fungi from seeds of *M. pruriens*

Seed fungi	Front view	Back view	Streak plate
SF1			
SF2			
SF3			
SF4			
SF5			

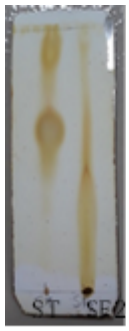
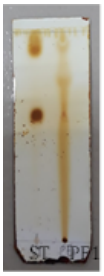
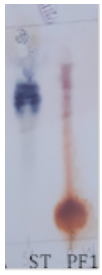
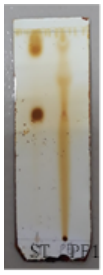
SF1, 1st seed fungus; SF2, 2nd seed fungus; SF3, 3rd seed fungus; SF4, 4th seed fungus; SF5, 5th seed fungus; SF6, 6th seed fungus

Table 4. Endophytic fungi from pods of *M. pruriens*

Pod fungus	Front view	Back view	Streak plate
PF			

PF, Pod fungus

Table 5. TLC analysis of water fraction of the SF1, SF2, PF endophytic fungal extracts

Endophytic fungal extract	TLC plate observed under UV light, 254 nm*	TLC plate after spraying ninhydrin and drying*	TLC plate after vaporizing iodine*
SF1			
SF2			
PF			

*The left most spot in each TLC plate was of the standard L-Dopa-carbidopa; SF1, 1st seed fungus; SF2, 2nd seed fungus; PF, Pod fungus

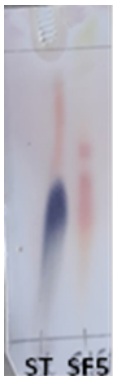


Figure 1. TLC analysis of ethyl acetate fraction of SF5 endophytic fungal extract of *M. pruriens* plant (The left most spot in the TLC plate was of the standard L-Dopa-carbidopa)

Furthermore, a previous study reported that the seeds of *M. pruriens* plant possess Proline as a pharmaceutical constituent. It may be assumed that SF1 and SF5 fungi produce proline or hydroxyl proline as one of the spots was visually observed as yellow after ninhydrin spraying and drying. However, no firm conclusion could be drawn due to the limited data available. As ninhydrin reacts with an amine functional group of alpha-amino acids to form purple-coloured compounds, Only L-Dopa gives a purple-colored compound. Carbodopa does not produce a purple-coloured compounds with ninhydrin. Therefore, It can be concluded that the purple-coloured compound is L-Dopa.

According to the results shown in Table 5, low-intensity blue/Ruhemann's purple colour spots were obtained on TLC plates with water fractions of SF1, SF2 and PF fungal extracts. A similar spot was obtained by ethyl acetate fraction of SF5 fungal extract, as shown in Figure 1. This reveals that endophytic fungi isolated from the seeds and the pods of *M. pruriens* produce low-yield L-Dopa.

Conclusion

The dried seeds and pods of *M. pruriens* plants could be a potential sources of natural L-Dopa. The findings provide the evidence that endophytic fungi present in the *M. pruriens* plant produce L-Dopa similar to those found in the host plant.

Author Contributions: Conceptualization, CPTL; methodology, CPTL, YS; analysis, YS; investigation, CPTL, YS; writing—original draft preparation, YS; writing—review and editing, CPTL; supervision, CPTL; project administration, CPTL. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: Authors are thankful to Dr. (Mrs.) R.A.S.W. Ranasinghe, Deputy Director, National herbarium, Royal botanical gardens, Peradeniya, who has confirmed the authentication of the plant sample and to Mr. Bandara Warakagoda, Mr. R. G. K. S. Deshappriya, and Mrs. W.D.M.P. Wasana, who have provided us with the assistance in obtaining the plant sample.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Chen RC, Chang SF, Su CL, et al. Prevalence, incidence, and mortality of PD: A door-to-door survey in Ilan County, Taiwan. Neurology. 2001, 57(9): 1679-86.
2. Venieraki A, Dimou M, Katinakis P. Endophytic fungi residing in medicinal plants have the ability to produce the same or similar pharmacologically active secondary metabolites as their hosts Bioactive secondary. Hell Plant Prot J. 2017, 51-66.
3. Chen S. L et al. Conservation and sustainable use of medicinal plants : problems , progress , and prospects. Chin Med. 2016, 11(37):1-10.
4. Anyasi RO, Atagana HI. Review Article Endophyte : understanding the microbes and its applications. Pak J Biol Sci. 2019, 22(4):154-167.
5. Ka S, Aa E, Tm A, et al. Biology of endophytic fungi. Curr. Res. Environ. Appl. 2012, 2(1):31-82.
6. Divya B. J, Suman B, Venkataswamy M, et al. The traditional uses and pharmacological activities of Mucuna. Indo Am J Pharm Res. 2017, 7(01):7516-7525.
7. Lampariello L, Cortelazzo A, Guerranti R, et al. The magic velvet bean of *Mucuna pruriens*. J Tradit Complement Med. 2012, 2(4):331-39.
8. Cassani E, Cilia R, Laguna J, et al. Mucuna pruriens for Parkinson's disease: low-cost preparation method, laboratory measures and pharmacokinetics profile. J Neurol Sci. 2016, 365:175-80.
9. Natarajan K, Narayanan N, Ravichandran N. Review on "Mucuna" - The wonder plant. Int J Pharm Sci Rev Res. 2012, 17(1):86-93.

10. Lee WW, Jeon BS. Clinical spectrum of dopa-responsive dystonia and related disorders. Curr Neurol Neurosci Rep. 2014, 14(7):1-13.
11. Sharma S, Srivastava A, Dharmil N, et al. Diversity of endophytic fungi from the medicinal plant Mucuna pruriens Diversity of endophytic fungi from the medicinal plant Mucuna pruriens. Int. J. Basic Appl. Res 2019, 9(5):1119-1128.
12. Nail M, Vega M. Short communication quantitative determination of L-DOPA in tablets by high performance thin layer chromatography. J Sep Sci. 2007, 30:1893-1898.
13. Fujii Y, Shibuya T, Yasuda T. Allelopathy of Velvetbean : Its Discrimination and identification of L-Dopa as a candidate of allelopathic substances. Jpn Agric Res Q. 1992, 25: 238–247.
14. Neelgund Y. F, Thakur M.S, Krishnaveni.R, et al. Transformation of L-Tyrosine to L-Dopa by a novel fungus, Acremonium rutilum, under submerged fermentation. Curr Microbiol. 2009, 58:122-128.