

Screening of Secondary metabolites produced from dry leaves of *Centella asiatica* and its isolated endophytic fungi

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Abstract

The new emerging investigation has challenged the scientific community to explore the efficacies of some medicinal plants and their associated microbial diversity. The fungal endophytes play a vital role in human life because these are the sources of drug for various deadly diseases like cancer, bacterial, viral fungal infections. Present work deals with biochemical screening for the presence of different types of secondary metabolites of *Centella asiatica* whole tissue extract as well as its isolated endophytic fungal extract. The endophytic fungi are present in almost all types of plants, which live as symbiont. Many studies revealed that most of the secondary metabolites are common in both endophytes as well as its host tissue *Centella asiatica*, which show a wide array of biological and pharmacological properties. Because of these properties the crude extract of many medicinal plants and their endophytes are investigated for their efficacies against different diseases. On my previous work the morphological and molecular characterization of isolated endophytic fungi from *Centella asiatica* was observed, which revealed the presence of *Penicillium*, *Aspergillus*, *Collectrotricum*, *Cladosporium*, *Curvularia*, *Alterneria* species, during present investigation methanolic extract of *Centella asiatica* was prepared for screening of secondary metabolites. Similarly methanolic extract of endophytic fungi isolated from *Centella asiatica* was carried out to observe the presence of secondary metabolites. For isolation of endophytic fungi, a definite protocol was followed and cultured.

Further it was investigated that endophytic fungi originated from *Centella asiatica* have the capability to produce same secondary metabolites which are produced by the whole tissue of *Centella asiatica*. In future the products from endophytic fungi will be a cheap

source for medical, agriculture and pharmaceutical industries. It is sure that the research on endophytic fungi will lead to isolate more novel compounds.

Keywords: Endophytes, secondary metabolites, novel drugs, pharmaceutical, symbiont.

I. Introduction:

In this study *Centella asiatica* (L.) was chosen as a host plant for endophytic fungi isolation which is abundant in the state Jharkhand. It belongs to Family-Apiaceae, it is commonly known as Gotu kola or Indian Pennywort, is a herbaceous plant with great medicinal value. Triterpenoid, saponins and sapogenins, particularly asiaticoside, madecassoside, asiatic acid and madecassic acid, are the chief bioactive molecules present in this plant (Mathur *et al.*, 2007; Randriamampionona *et al.*, 2007). Asiaticoside extracted from the aerial parts of the plant is known to exert strong diuretic, antispasmodic, circulation stimulatory and wound healing actions and is frequently used in the treatment of leprosy, ulcer, asthma, bronchitis, elephantiasis, eczemas, anxiety, mental disorders and urethritis (Mathur *et al.*, 2007; Gohil *et al.*, 2010). Asiaticoside derivatives are also widely used in the treatment of Alzheimer's disease as they can protect cells against b-amyloid induced cell death (Mook-Jung *et al.*, 1999). Recently, studying the possible mode of action of asiaticoside in wound healing and suppression of proliferation of keloid-derived fibroblasts and collagen production, Tang *et al.*, (2011) have demonstrated that asiaticoside suppresses collagen expression and TGF- β /Smad signalling by inducing Smad7 and inhibiting TGF- β receptors RI and RII in keloid fibroblasts to prevent hypertrophic scars and keloid formation. The pharmaceutical companies depend on the supply of this herb from its wild populations growing near the swampy ditches that are often contaminated with heavy metal pollutants and other harmful chemical residues that adversely affect the medicinal efficacy of its herbal formulations (McCaleb *et al.*, 2000).

Plant endophytes are rich and novel source of natural bioactive compounds. Medicinal plants provide a unique environment for endophytes and have been recognized as a repository of endophytes with novel metabolites of pharmaceutical importance (Tan & Zou, 2001, Strobel *et al.*, 2004). Endophytes are microorganism which resides inside the plant tissue. The term 'endophyte' includes all organisms that grow inside plant tissues without causing disease symptoms (Petrini, 1991 and Chanway, 1996). The plant-endophyte co-evolution hypothesis suggests that it might be possible for endophytic fungi to assist the plant in chemical defense by producing secondary metabolites (Ji *et al.*, 2009). There are some

different path-ways of chemo diversity generation in natural products and it is commonly accepted that different classes of compounds are produced by different biosynthetic pathways, for instance, the merging of different biosynthetic pathways into one compound, functional group modification, combination of monomers into dimers and occurrence of open chain and ring-closed products. Thousands of structurally differing secondary metabolites have apparently evolved during plant development to defend themselves against herbivores and against bacteria, fungi and viruses. The capacity to produce these metabolites by endophytes from the host is supported by the fact that these endophytes harbor similar biosynthetic pathway and genes encoding enzymes catalyzing various steps as that of the host (Chandra, 2012). The endophytic fungi producing such compounds have also shown the presence and expression of the similar homologous genes involved the biosynthesis of respective compounds in their host (Jennewein *et al.*, 2001).

There are over one million fungal endophytes existed in the nature (Petrini 1991). They are the second largest group after insects and key component of tropical ecosystems throughout the world. The endophytic fungi are present in almost all plant parts, especially the leaves, where the tissue is apparently healthy. Compare to other endophytic microorganisms, fungal endophytes produce large number of secondary activities (Zhang *et al.*, 2006). Endophytes are rich source of secondary metabolites with multifold importance (Strobel and Daisy 2003). Endophytes isolated from medicinal plants possess strong fungicidal, bactericidal and cytotoxic metabolites (Wang *et al.*, 2007). It produces enzymes which are used for various applications like degradation and biotransformation of organic compound (Firáková *et al.*, 2007; Pimentel *et al.*, 2011). The metabolic derivatives are used in biotechnological applications (Tomita 2003). These metabolites have a wide significance in pharmaceutical science because of its antimicrobial, anticancer and antiviral activities. (Selim *et al.*, 2012).

Endophytic fungi are an important source for novel, potential and active metabolites. Plant-endophyte interaction and endophyte-endophyte interactions study provide insights into mutualism and metabolite production by fungi. Bioactive compounds produced by endophytes help the host plants to resist external biotic and abiotic stress, which benefit the host survival in return. However, in the recent years, it is gradually recognized that endophytic fungi or endophytes have played a very important role in affecting the quality and quantity of the crude drugs through a particular fungus-host interaction, indicating that more

understanding on the particular relationships between endophytic fungi and medicinal plants is required for promoting crude drug production (Faeth and Fagan, 2002). Endophytes are rich source of natural products displaying broad spectrum of biological activities like anticancer, antibacterial, antiviral, immunomodulatory, antidiabetic, antioxidant, anti-arthritis and anti-inflammatory.

Present study focuses on the screening of bioactive compounds present on endophytic fungi and its host *Centella asiatica*. The investigation revealed the presence of alkaloid, phenolic compounds, terpenoides, saponin, tannins in both. These metabolites can be an important constituents of a novel drugs for new emerging diseases to combat this global community threat.

II. Material and Methods:

2.1 Collection and identification of plant materials

The plant *Centella asiatica* was collected from the campus of Ranchi Women's College, Ranchi, Jharkhand, India. The authenticity of the plant was confirmed by University Professor Dr.Kunul Kandir, P.G. Department of Botany Ranchi. Fresh leaves were washed properly under running tap water, shade dried for a week. By means of mixer grinder powdered sample was prepared and stored in plastic bags.

2.2 Screening of Secondary metabolites produced from dry leaves of *Centella asiatica*.

Centella asiatica (CA) leaves were air dried at room temperature (Tegelbergetal.,2018),with relative humidity < 20% for 4-5 days. Then it was grinded and powdered by using 2mm to 3mm sieve was chosen for the grounded particles. The powdered samples were used for the process of *Centella asiatica* extract (CAE).

2.3 Leaf extracts preparation:

The air dried *Centella asiatica* powdered leaves were extracted with methanol in 10:1 ratio in conical flasks, plugged with cotton wool and then kept on a rotary shaker at 190-220 RPM for agitation for 24 hours. After 24 hours of continuous agitation samples were filtered and fill rate of each solvent was used as crude extracts. The powdered leaves were extracted with methanol. For extraction the powdered pack material was air dried and then used. The solvents of the extracts were reduced under room temperature and stored at 4°C for future use.

2.4 Qualitative phytochemical screening of Leaf tissue extract of *Centella asiatica*:

Preliminary qualitative phytochemical analysis was carried out to identify the secondary metabolites like alkaloids, flavonoids, saponins, steroids, tannins, phenols, terpenoids and triterpenoids were made by following standard procedures, present in the methanol extract of leaf of *Centella asiatica*. The presence of different metabolites was qualitatively screened by using the standard procedures to identify the constituents (Harborne, J.B 1998; Solohokara *et al.*, 2015; Mehta A 2017).

- **Test for Alkaloids (Wagner's Test):** 1ml of plant extract was taken and 3-5 drops of Wagner's reagent were added and observe for the formation of a reddish brown precipitate or coloration.
- **Test for Flavonoids (Alkaline reagent Test):** 1ml of extract was taken and treated with 3-5 drops of 20% NaOH solution. Formation of intense yellow color was observed, which became colorless on the addition of 0.5ml dilute HCl indicates the presence of flavonoids.
- **Test for Phenols (Ferric Chloride Test):** 1ml of extract was taken 5-6 drops of 5% aqueous ferric chloride solution were added and observed in the formation of deep blue or black color for positive result.
- **Test for Saponins (Foam Test):** 1ml of extract was taken and 5ml of distilled water was added and shaken vigorously. Formation of persistence foam was observed for 10-15 min that confirms the presence of saponins.
- **Test for Tannins (Braymer's Test):** 1ml of extract was taken and treated with 1ml of 10% alcohol ferric chloride solution and observed for formation of blue or greenish color for positive results.
- **Test for Terpenoids (Salkowski's Test):** 1ml of extract was taken and 0.5 ml of chloroform was added along with 3-5 drops of conc. H₂SO₄. The reddish brown precipitate was observed, which was produced immediately.
- **Test for Quinones:** 1ml of extract was taken and treated with 0.5ml of conc. HCl and observed for formation of yellow precipitate or coloration for positive results.
- **Test for Resins:** 1 ml of extract was taken and 5ml of distilled water was added and observed for the turbidity.

- **Test for Coumarins:** 1 ml of extract was taken and 1.5ml of 10% NaOH was added and then observed in the formation of yellow color which indicates the presence of coumarins.

2.5 Endophytic Fungal Culture and extraction of fungal metabolites:

2.5.1 Surface sterilization of *Centella asiatica* leaves for fungal culture:

The plant samples were rinsed gently in running water to remove adhered dust and debris. Then the leaves were cut into 2 mm segments. Which were surface sterilized with 70% ethyl alcohol for 1 min. The segments were soaked in 0.2% mercuric chloride solution for 1 min. and rinsed with 70% ethyl alcohol for 1 min. Finally the traces of mercuric chloride were removed by rinsing with autoclaved distilled water three times and blotted dried on sterile filter paper followed by drying under laminar airflow chamber. The leaves were cut into small segments (2mm X 2mm) using a sterile scalpel.

2.5.2 Media preparation:

Potato Dextrose Agar (PDA) medium was used for the growth of endophytic fungi in which 200 grams of potato, 20 gm of dextrose, 20 gm of agar were dissolved in 1000ml of sterile distilled water, the media was autoclaved in 15psi pressure and the temperature was maintained at 121°C, pH was maintained at 5.6. Before autoclaving the PDA medium is supplemented with streptomycin to suppress the growth of bacteria.

2.5.3 Inoculation and Incubation

The plant segments were inoculated into potato dextrose agar (PDA) plates which were supplemented with antibiotics specially to suppress unwanted bacterial growth at $25 \pm 2^\circ\text{C}$ for 5-7 days in dark. The growth of endophytic fungi from the plant segments should be observed once a day. Hyphal tips growing out of the cultured segments were transferred into a fresh PDA using the hyphal tip method (Kirsop and Doyle, 1991) and incubated at $25 \pm 2^\circ\text{C}$ for 7 days to obtain pure cultures. For the mass cultivation of fungus, agar blocks of actively growing pure culture (3 mm in diameter) were placed on 100 ml Potato dextrose broth in 250 ml Erlenmeyer flask. Each flask was incubated at $25 \pm 2^\circ\text{C}$ for 3 weeks with periodical shaking at 150 rpm in Incubator Shaker.

2.5.4 Sub culture of Endophytic Fungi

For the extraction of secondary metabolites, the fungi were sub-cultured on Potato Dextrose Broth by inoculating selected fungal cultures in 250ml Erlenmeyer flask containing 100ml of the medium. The flask was incubated at 28°C for 2 weeks with periodical shaking at 150rpm in an incubator shaker.

2.5.5 Fungal extract Preparation for Screening of Secondary metabolites:

After the incubation period, the fermentation broth of the fungus was homogenized by adding 10% methanol. Alcohols (EtOH and MeOH) are universal solvents in solvent extraction for phytochemical investigation. Metabolite was extracted by solvent extraction procedure using methanol as organic solvents. The broth culture was filtered to separate the mycelia and filtrate. To the filtrate equal volume of solvents were added, mixed well for 10 min and kept for 5 min till the two clear immiscible layers formed. The upper layer of methanol containing the extracted compounds was separated using separating funnel. Solvent was evaporated and the resultant compound was dried in rotator vacuum evaporator to yield the crude metabolite (Bhardwaj *et al.*, 2015).

2.5.6 Preliminary qualitative biochemical screening of fungal metabolites produced by endophytic fungi

Chemical prospecting in methanolic extract of endophytic fungi were performed to observe the presence of important metabolites. For this preliminary biochemical analysis of the crude extracts of fungi was carried out to determine the presence of the following secondary metabolites such as alkaloids (Mayer reagents), saponins, terpenoids, flavonoids, steroids, phenols, and tannins (reaction with FeCl_3) using standard methods with minor modification (Devi *et al.*, 2012; Bhardwaj *et al.*, 2015) and were evaluated. For the biochemical screening of fungal extract following test were done.

- **Alkaloids (Mayer's reagent):** The fungal crude extract was dissolved in 2N HCL solutions. The mixture was treated with a few drops of Mayer's reagent (3 ml of potassium iodide solution mixed with 2 ml mercuric chloride solution). The creamish precipitate indicates the presence of alkaloids.
- **Flavonoids:** In the test tube, containing 1 ml of fungal crude extract a few drops of 20% NaOH solution was added. A change to yellow colour was observed which on

addition of 0.5 ml HCl, changed to colourless solution depicted the presence of flavonoids.

- **Phenols:** The fungal extract was dissolved in 5 ml of distilled water. To this few drops of neutral 5% ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds.
- **Saponins:** The presence of saponins was determined by frothing test. The fungal extract was vigorously shaken with distilled water and was allowed to stand for 10 min. Formation of a fairly stable emulsion indicated the presence of saponins.
- **Steroids:** Libermann-Burchard reaction was performed to assess the presence of steroids. The crude fungal extract was added in 1 ml of chloroform solution. The mixture was treated with acetic anhydride and few drops of concentrated H₂SO₄ were added. A blue green ring indicated the presence of steroids.
- **Tannins:** The fungal crude extract was treated with alcoholic FeCl₃ reagent. A bluish black colour, which disappears on addition of a little dilute H₂SO₄, was followed by the formation of yellowish brown precipitate indicated the presence of tannins.
- **Terpenoids:** 1 ml of fungal crude extract was mixed in 2 ml of chloroform. 3 ml of concentrated H₂SO₄ was then added to form a layer. A reddish-brown precipitate coloration at the interface formed indicated the presence of terpenoids.

III . OBSERVATIONS:



Fig. 1



Fig. 2



Fig. 3

- Fig. 1** *Centella asiatica* (L.)
- Fig. 2** Fresh leaves of *Centella asiatica* (L.)
- Fig. 3** Powdered leaves of *Centella asiatica* (L.)
- Fig. 4&5** Mixed Endophytic Fungi Culture
- Fig. 6** Suspension culture of Endophytic Fungi



Fig. 4



Fig. 5



Fig. 6



Fig. 7



Fig. 8



Fig. 9



Fig. 10



Fig. 11



Fig. 12



Fig. 13

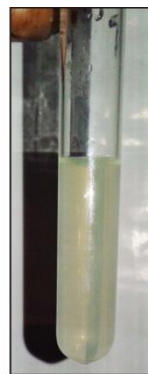


Fig. 14

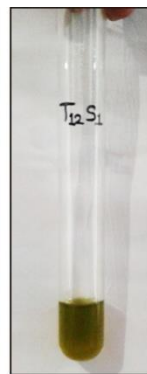


Fig. 15

Screening of *Centella asiatica* (L.) secondary metabolites

- | | | |
|------------------------|-------------------------|---------------------------|
| Fig. 7 Alkaloid | Fig. 8 Flavonoid | Fig. 9 Phenols |
| Fig. 10 Saponin | Fig. 11 Tannins | Fig. 12 Terpenoids |
| Fig. 13 Quinone | Fig. 14 Resins | Fig. 15 Coumarins |



Fig. 16

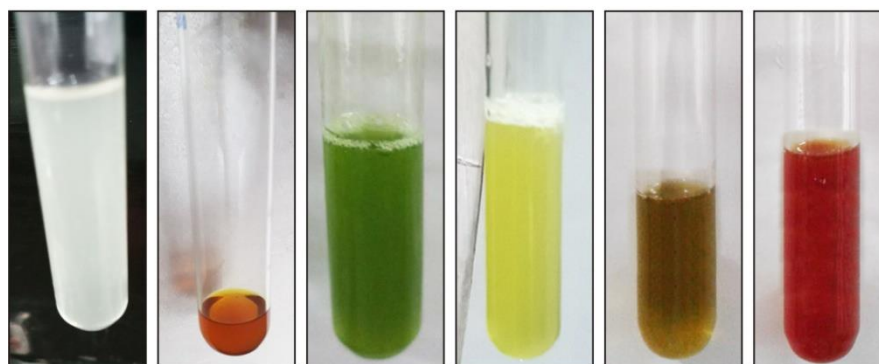


Fig. 17

Fig. 18

Fig. 19

Fig. 20

Fig. 21

Fig. 22

Fig. 16 Methanolic Extract of Fungal Metabolites

Screening of Fungal Metabolites

Fig. 17 Alkaloid

Fig. 18 Flavonoid

Fig. 19 Phenols

Fig. 20 Saponin

Fig. 21 Tannins

Fig. 22 Terpenoids

Following metabolites are present in the endophytes and its host plant *Centella asiatica*:

Phytochemicals	Centella leaf tissue	Endophytic Fungi
Alkaloids	++	++
Flavonoids	++	++
Phenols	++	++
Saponins	++	++
Tannins	++	++
Terpenoids	++	++
Quinones	++	-----
Resins	++	-----
Coumarin	++	-----

Table-1: Phytochemical Screening of *Centella asiatica* leaf tissues and its endophytic fungi.

IV. Result and Discussion:

Endophytic fungi isolated from the plant *Centella asiatica* would be a promising source for many pharmaceutical ingredients as some of which have valuable biological activities (Bills & Polishook 1991). Present work was focused on bioactive compounds produced in endophytic fungus found in leaves of *Centella asiatica*. The healthy leaves were selected for the study because, according to Arnold *et al.*, (2000), endophytes are especially rich and abundant in leaves. Isolating a compound from the plants and its large scale production is expensive and time consuming. The endophytic fungi are cheap and rich source of bioactive compounds and can be easily cultured. The large scale production of these metabolites is also possible through fermentation process. From the study it was revealed that in methanolic extract of endophytic fungus of *Centella asiatica* produces alkaloids, flavonoids, saponin, tannin, terpenoids and phenols. Presences of similar metabolites were also found in ethyl acetate extract which was also reported by previous workers. Presences of these metabolites have the capability to produce valuable compounds (Table - 2). These bioactive compounds can be used as efficient antibiotic, antiviral, anticancerous, antioxidant etc. Many workers have demonstrated that the endophytes isolated from medicinal plants are excellent producers of strong fungicidal, bactericidal and cytotoxic metabolites (Wang *et al.*, 2007). In future the products formed from the metabolites of endophytic fungi will be a cheap source for medical, agriculture and other industries. It is sure that the research on endophytic fungi will lead to isolate more novel compounds.

Table -2 : Effect of individual metabolites on human health.

Metabolites	Effect
Alkaloids	Antimalarial, antiasthma, anticancer, antibacterial
Flavonoids	Neuro protective, suppress neuro inflammation,
Phenol	antioxidants
Saponin	lowers cholesterol levels, anticancer, lowers blood glucose
Tannins	anticarcinogenic, antimutagenic, antioxidant
ETerpenoids	anti-inflammatory, anti-tumorigenic, chemotherapeutic agents

V. Conclusion:

From the study it was observed that diverse and complex compounds are present in both centella and endophytic fungus these bioactive compounds are alkaloids, phenolic compounds (flavonoids, tannins) Phenols, Saponins and terpenoids. Steroids are absent in both plant and its endophytes. The finding proved that same type of bioactive compounds are result of co-evolution of plants and microbes (Darwin 1859; Wade 2007; Kutschera & Briggs 2009). The presence of metabolites indicates that these endophytic fungi can produce potential drugs against deadly diseases.

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