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Research Article

Quantitative phytochemical analysis of the fungus endophytic extracts isolated from *Azadirachta indica* using gas chromatography- flame ionization detector

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Abstract

Background information: The phytochemicals of endophytes have generated substantial interest in drug discovery programs because they offer the possibility of discovering new biologically active molecules. **Objective:** The objective of this study was to quantify and analyze the phytochemical profile of the fungus endophytic extracts isolated from Azadirachta indica leaves, which have been used ethnobotanically for treating malaria and bacterial infections. **Methods:** Endophytic fungi were isolated, solid-state fermentation of rice medium was performed, and secondary metabolites were extracted according to standard techniques. An analytical system that uses gas chromatography and flame ionization detection (GC-FID) was used to determine the phytochemical constituents contained in the endophytes. **Results:** The results of GC-FID analysis showed the presence of Ribalinidine, Naringenin, Sparteine, Phenol, Steroids, Kaempferol, Flavone, Oxalate, Catechin, Tannin, and Rutin at different concentrations. **Conclusion:** This study reveals the promising ability of the endophytic fungi of *A. indica* as a foundation of naturally occurring bioactive. The quantitative phytochemical assessment of the endophyte extracts from the leaves of *A.indica* showed that endophyte extracts from the plant are rich in both alkaloids and flavonoids (Phenolics).

Keywords: GC-FID, Phytochemical, Endophytes, Secondary metabolites, Fermentation.

INTRODUCTION

Phytochemicals of endophytes have generated significant interest in drug discovery programs due to their immense potential to discover new biologically active molecules. Natural products are chemical or bioactive compounds obtained from living microorganisms such as plants which are the most notable sources of phytochemicals. Studies have so far reported many important phytochemical compounds isolated from endophytes belonging to several classes such as quinines, alkaloids, terpenoids, peptides, phenols, steroids, and flavonoids have exhibited various activities such as antimicrobial, among others [1]. Drug discovery experts often refer to these promising compounds as "leads," and chemicals with desirable properties in-vitro are called lead compounds. Phytochemicals are chemicals without nutritional value secreted by plants, which possess bioactive properties[2]. They also serve as defense systems against pathogens and animals [3]. There are different classifications of phytochemical compounds, including carbohydrates, terpenoids, tannins, alkaloids, lipids, phenolic compounds, etc., [4]. The term endophytes simply mean microorganisms that grow intercellularly and asymptomatically within living tissues establishing a mutual relationship with the main (host) plant [5]. Endophytes are viewed in recent times as an outstanding source of bioactive phytochemicals because of their ubiquitous presence in unique biological niches growing in so many unusual environments, including plants hence conferring on them inherent biological activities. *Azadirachta indica*, commonly known by its common name neem tree, has been credited with a wide range of medicinal properties. In addition to *Azadirachta indica* being regarded as a wonder plant (tree) because of its abundance of bioactive compounds in all aspects of its plant life - leaves, bark, flowers, fruits, seeds, roots - it has a wide range of applications. A large number of

different chemical constituents belonging to notable bioactive compounds such as ketones flavonoids, steroids alkaloids, phenolics, triterpenoids, etc., have been extracted from *Azadirachta indica*. Hence, this study was aimed to quantitatively analyze the phytochemical extracts of endophytic fungus isolated from the plant; *Azadirachta indica* using gas chromatography- flame ionization detector (GC-FID).

MATERIALS AND METHOD

Isolation and purification of endophytic fungi

The leaves were thoroughly washed with sterile water in the laboratory to remove dust and debris before being immersed in 70% ethanol for about 3 minutes and 2% sodium hypochlorite for 2 minutes for disinfection. In a lamina flow cabinet, they were then rinsed with sterile distilled water and blotted dry with sterile blotting paper. The leaf blade and midrib were cut with a sterile scalpel approximately 1cm in length. On malt extraction agar media (MEA) that has been prepared with 250 mg chloramphenicol per liter to suppress bacteria, about 5 - 6 segments were placed aseptically. Leaf-blades and midribs were positioned so that they were in contact with the media. Afterward, the plates were incubated at 28^{0}C for five days and monitored daily. To test the efficacy of surface sterilization, the sterilized segments were applied to the surface of the MEA medium and immediately removed. Incubation results indicated that there were no signs of fungi growth on the surface of any of the mediums [6]. A variety of different pure colonies were obtained by sub-culturing hydra tips from different colonies arising from leaf parts.

Identification of fungi isolates

To characterize the isolates, lactophenol cotton blue reagent was used to stain slide preparations from cultures, and phase-contrast and bright-field microscopes were used to observe them. The morphological identification of the fungal isolates was according to the standard taxonomic key, which includes colony diameter, texture, color, margin character, and the dimensions, colony reverse, and microscopic characteristics, including conidiophore structure of hypha and conidia [7].

Fermentation and extraction of secondary metabolites

Solid-state fermentation was conducted as formerly described by Okoye *et al.,* [8], in 1000 ml Erlenmeyer flasks containing 100 g of rice media (200 ml of water was added to the rice and then autoclaved at 121^o C at 15 psi for 30min). After inoculation with fungi endophyte-containing agar blocks of 3 mm diameter, the flasks were incubated at 28°C for 21 days. Ethyl acetate was used to extract the culture media and separate the mycelia. A rotary vacuum evaporator was used to extract the organic phase under reduced pressure at 40°C.

Quantification by Gas-Chromatography- Flame Ionisation Detector (GC-FID)

The procedure used was modified from Elkin et al., (1985). In a 10 x 5-mm tube, 50 µL of the sample solution was pipetted and dried at 65 °C in vacuo. After adding 30 µL of methanol-water-trimethylsilyl (2: 2: 1 [v/v]) to the residue, it was removed by vacuum at 65 °C. In the next step, 30 mL of the derivatizing reagent methanol-water-trimethylsilyl (7:1:1:1 [v/v]) were added, and the tube was shaken for about 20 minutes. As a final step, the solvents used were removed using a nitrogen stream, and the tube was sealed, awaiting analysis, in a 4°C freezer. A diluent containing 5 mM sodium phosphate and 5% acetonitrile was added to each tube [9], 150 l before injection.

BUCK M910 GC equipped with a flame ionization detector was used for the quantification of the phytochemicals present in the endophytes of *A. indica*. A total of 2 liters of the sample was injected at 280^{0}C at a linear velocity of 30 cm-1 using a splitless injector at a temperature of 280^{0}C. The carrier gas was helium 5. 0 pa.s with a flow rate of 40 ml min^{-}1. The oven operated from a temperature of 200^{0}C until it heated to 330^{0}C at a rate of 3^{0}C min^{-}1. This temperature was maintained for 5 min, and the detector worked at a temperature of 320^{0}C. The concentration of the different phytochemicals was expressed in µg g^{-}1 [10].

RESULTS AND DISCUSSION

Endophytic fungus isolated from the leaves of *A. indica* was used for this study. The GC-FID results showed a wide range of phytochemical compounds that had been previously stated to have very high antimalarial and antimicrobial activity. In this study, *A. indica* endophytic fungi were identified and their potential was explored for its potential to produce bioactive compounds with pharmaceutical applications. *Azadirachta indica* leaves were quantitatively screened for the phytochemical composition of endophyte extracts - and both flavonoids and alkaloids (Phenolics) were found. The extract is rich in flavonoids, mostly phenols, and polyphenols from the GC – FID results. These flavonoids have served various functions in human cells, including antioxidant, antimalarial, and antimicrobial activities. Alkaloids, tannins, saponins, flavonoids have been shown to possess a wide range of pharmacological actions causing some physiological changes and are involved as active drug candidates in producing medicines. The phytochemical constituents found in *A. indica* indicate that the plant has high therapeutic activity. The quantitative phytochemical content of the endophyte from the leaves of *A. indica* is shown in Fig. 1.

Naringin and Resveratol were reported to possess impressive antimicrobial properties [12,13], while Proanthocyanin and Epicatechin were previously named as antimalarials [11,12]. The GC-FID analysis showed these compounds to be present in reasonable amounts. During this study, the endophytic fungi associated with *Azadirachta indica* were investigated and were determined to be Aspergillus species, we have yet to explore other species. It provides a starting point for future research, allowing us to isolate and identify phytochemicals in the leaves of this plant that are associated with endophytes. The endophyte extract of the plant shows a great reservoir of bioactive secondary metabolic, which may have conferred therapeutic activities on the plant through synergistic interactions between the endophytes and the plant. Folkloric usage of *A. indica* has been justified by the results observed, as an antimalarial and antimicrobial agent, and has further established the fact that endophyte extract of the plant could have even more promising activities since these endophytes co-exist within the plant. Considering endophytes and plants share the same nutrients, their secondary metabolites might be similar. According to our GC-FID results, the majority of the bioactive compounds were phenols, which have been reported to have different physiological functions, including antimalarial activity [14]. The production of medicine relies heavily on phytochemicals. As a result of their many pharmacological activities, they could also be used as precursors to developing new drug candidates due to their variety of pharmacological properties.

Figure 1: An illustration of the chromatography of phytochemical constituents of the endophytic extract of *A. indica*

Sparteine is a quinoline alkaloid that has been reported to have antimalarial and antimicrobial properties [15]. Rutin is digested in the body and converted to quercetin as a flavonoid with reported anti-carcinogenic, anti-inflammatory, antiviral, and antioxidant activities [16,17]. Flavan-3-ol was also found to be very high in the endophyte extract of *A. indica,* which confirms its folkloric claim of being used as an antimicrobial agent [18]. Kaempferol has been reported to have both antimicrobial and antimalarial activities [19,20]. Phytochemicals used as an antimalarial agent over the years were also present and had been written by scientists to be an effective antimalarial agent [21]. Therefore, it can be concluded that the phytoconstituents found in the plant extracts are also very present in the endophyte extracts. The appreciable amounts of the phytoconstituents observed and literature reports have justified the biological activities of the plant *A. indica.*

Table 1: Phytochemical components identified in the endophytic extract of *A. indica* by GC-FID

Component	Retention time (mins)	Area	Height	Concentration µg/g
Rutin	1.006	6380.1854	163.578	29.8096
Ribalinidine	9.146	13608.6972	348.088	5.4572
Naringenin	12.016	4411.7218	113.050	2.1392
SparteinSpartein	14.310	4131.5482	105.883	0.8513
Phenol	20.116	13866.9043	354.001	1.6407
Steroids	25.573	12104.6302	309.637	10.1968
Kaempferol	29.456	10545.2078	268.425	7.9189
Flavone	**32.263**	**17262.2381**	**440.771**	**80.6529**
Oxalate	35.140	4459.4490	114.282	8.7105
Catechin	40.080	3868.8293	99.130	19.7452
Tannin	45.223	7406.4114	220.012	14.6283
		103778.6064		185.7040

CONCLUSION

The quantitative phytochemical analyses of the endophytic extract of this plant have shown the abundant reservoir of alkaloids and flavonoids in the endophytic extract of the plant *Azadirachta indica.* These phytochemicals have contributed to important metabolic roles, which led to the physiological and pharmacological activities of the plant. The GC-FID analysis of the phytochemical compounds showed that the endophytic extract of *Azadirachta indica* could represent a potential source of lead molecules for developing novel products to treat various diseases. The abundance of these alkaloids and flavonoids (phenolic compounds) supports the use of the endophyte extract as an antimalarial and antimicrobial agent.

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CONFLICT OF INTEREST STATEMENT

Conflicts of interest are not declared by the authors.

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