

Antioxidant Activity and Phytochemical Screening of Crude Endophytes Extracts of *Tabebuia argentea* Bur. & K. Sch.

M. Govindappa^{1*}, R. Channabasava¹, K. R. Sunil Kumar², K. C. Pushpalatha²

¹Department of Biotechnology, Shridevi Institute of Engineering & Technology, Tumkur, India; ²Department of Studies in Biochemistry, Mangalore University, PG Center, Cauvery Campus, Madikeri, India.

Email: *dravideja07@gmail.com

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ABSTRACT

The aim of this study is to investigate *in vitro* antioxidant activities and the phytochemical screening endophytes. Seven different endophytic fungi were isolated from different parts of the plant and their extracts subjected to know antioxidant properties and phytochemical screening. Phytochemical analysis revealed the presence of tannins, flavonoids, steroids, alkaloids, phenols and proteins from different solvents extracts of different endophytes. The antioxidant activity was evaluated by six separated methods: scavenging of free radical DPPH, FRAP, TBA, superoxide radical, FTC and iron methods. All seven different endophytes yielded almost all phytochemicals in methanol extracts which were tested. The endophytes *A. niger*, *Penicillium* sp. and *Trichoderma* sp. have shown potential *in vitro* antioxidant activities. Further work is needful to isolate the exact compound which is responsible for antioxidant activity and biophysical characterization will be done in the future.

Keywords: *Tabebuia argentea*; Endophytes; Antioxidant Activity; Phytochemicals

1. Introduction

Tabebuia argentea (Bignoniaceae) is a large and yellow flowering tree. *Tabebuia* sp. has proven to be rich source of many organic compounds, especially, of phenolic and polyphenolic nature. Such substances have been classified as cytotoxic, antimicrobial and antifungal [1,2] by the presence of anthraquinone compounds. Many natural naphthoquinone and lapachol have extensively been used to control cancer and they have ability to interfere with topoisomerase enzymes, which are critical for DNA replication in cells [3]. The antitumor activity of lapachol may be due to its interaction with nucleic acids and the interaction of the naphthoquinone moiety between base pairs of the DNA helix occurs with subsequent inhibition of DNA replication and RNA synthesis [4]. Other biological activities of lapachol are antimetastatic activity [5], antimicrobial and antifungal activity [6], antiviral activity [7], anti-inflammatory [8], antiparasitic activity [4], leishmanicidal activity [9] and molluscicidal activity [10].

Increasing evidence indicates that Reactive Oxygen Species (ROS), (for example O₂ and OH) and free radical me-

diated reactions can cause oxidative damage to biomolecules (for example lipids, proteins and DNA), eventually contributing to aging, cancer, atherosclerosis and other neurodegenerative disorders [11]. Antioxidants are thought to be highly effective in the management of ROS mediated tissue impairments. Many antioxidant compounds possess anti-atherosclerosis, antitumor, antimutagenic, antibacterial, or antiviral activities to lesser or greater extent [12,13]. Many endophytic fungi have shown antioxidant activity. The present study was aimed to isolate different endophytes, identify phytochemicals from different endophytes and different endophytic extracts were used for antioxidant properties. The present investigation data was different from earlier works [14], we used other endophytes what they haven't noticed and we tried six different types of antioxidant activity methods for comparison.

2. Materials and Methods

2.1. Plant Material

Plant materials were collected from different places of Karnataka, India during April, 2011. The collected plant

*Corresponding author.

was authenticated from the Department of Botany, ManasaGangotri, University of Mysore, Mysore, Karnataka, India and Government Ayurvedic College, Mysore, and herbarium was prepared.

2.2. Isolation and Identification of Endophytic Fungi

The protocol for isolation follow methods used in other endophytestudy [15] with slight modifications. The plant tissues were washed in running tap water for one hour. Fifty segments of leaves from each plant were cut into 5 mm 2 pieces, including a vein (25 samples) and intervein (25 samples). 25 segments of branches were then cut randomly to a length of 5 mm. Endophytic fungi were isolated from the bark of the plant (25 segments). Twenty five segments (5 mm long) were cut from the stems and the roots. The total 150 segments of plant material were treated by triple surface sterilization techniques. Each piece was then placed on malt extract agar (malt extract 20 g/l), rose Bengal (0.033 g/l), chloromphenicol (50 mg/l; agar 15 g/l). All plates were incubated at $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$ until mycelium grew out; hyphal tips were cut and transferred to potato dextrose agar (PDA). Half strength PDA was used for subculture and stock culture. Identification was based on colony, hyphal morphology of the fungal cultures and characteristics of the spores [16,17].

2.3. Fungal Cultivation and Extraction of Metabolites

The fungal endophytes were cultivated on Potato Dextrose Broth by placing agar blocks of actively growing pure culture (3 mm diameter) in 250 ml Erlenmeyer flasks containing 100 ml of the medium. The flasks were incubated at $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 1 week, with periodical shaking at 150 rpm. After the incubation period, the cultures were taken out and filtered through sterile cheesecloth to remove the mycelia mats. The fungal metabolites from different endophytic mycelial mats were extracted by using ethyl acetate solvent extraction. Equal volume of the filtrate and solvents were taken in a separating funnel and was shaken vigorously for 10 min. The solution was then allowed to stand, the cell mass got separated and the solvent so obtained, was collected. All solvents were evaporated and the resultant compound was dried in vacuum evaporator using MgSO_4 to yield the crude extract [14].

2.4. Phytochemical Screening

Investigation on the phytochemical screening of *Tabebuia argentea* extract revealed the presence of saponins, steroids, terpenoids, glycosides, alkaloids, tannins, anthraquinone and flavonoids.

2.4.1. Test for Saponins

1 ml aliquots of the various plant extracts were combined with 5 ml water which is 60°C , then, shaken for 2 min, as saponins are known to possess frothing activity, the volume of froth produced in this experiments was observed and recorded every 10 min for a period of 30 min [18].

2.4.2. Test for Steroids

1 ml of the respective plant extract was treated with three drops of acetic anhydride and one drop of concentrated sulphuric acid. A colour change from deep green, turning to brown indicated the presence of sterols [18].

2.4.3. Test for Anthraquinones

The Borntrager test was used for the detection of anthraquinones. 2 ml of the test sample was shaken with 4 ml of hexane. The upper lipophilic layer was separated and treated with 4 ml of dilute ammonia. If the lower layer changed to violet pink, it indicates the presence of anthraquinones [18].

2.4.4. Test for Cardiac Glycosides

1 ml of the sample solution was mixed with 1 ml of glacial acetic acid then treated with one drop of 5% methanolic ferric chloride solution. 1 ml of concentrated sulphuric acid was carefully poured down the sides of test tube. The appearance of brownish ring between the two layers with lower layer turning blue green upon standing indicates the presence of cardiac glycosides.

2.4.5. Test for Tannins

1 ml of the sample solution was mixed with 1 ml vanillin hydrochloride reagent. Appearance of purple colour indicates the presence of tannins.

2.4.6. Test for Flavonoids (Magnesium Hydrochloride Reduction Test)

To the methanol extract, added 5 ml of 95% ethanol and few drops of conc. HCl. To this solution 0.5 g of magnesium turnings were added. Observance of pink coloration indicated the presence of flavonoids.

2.4.7. Test for Lapachol Identification

Dried endophytic extract and flower extract of *T. argentea* was extracted with ethyl acetate. 1 g of the endophytic and flower extract was re-crystallized in petroleum ether and benzene (80:60) and heated at 139°C to 140°C for 5 min. 2 ml of ferric chloride solution was added and observed for the color change [19].

2.5. Antioxidant Activities of the Endophytic Extracts

2.5.1. FRAP Assay

FRAP reagents was freshly prepared by mixing 25 ml

acetate buffer (300 mM, pH 3.6), 0.5 mL 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ) solution (10 Mm TPTZ in 40 Mm/I HCL) and 2.5 mL FeCl₃ (2 Mm) water solution. Each sample (150 µl) (0.5 mg/ml) dissolved in methanol was added to 4.5 ML of freshly prepared FRAP reagent and stirred. After 5 min, absorbance was measured at 593 nm, using FRAP working solution as blank [20]. A calibration curve of ferrous sulphate (100 to 1000 µmol/l) was used and results were expressed in µmol Fe²⁺/mg dry weight extract. The relative activity of the sample was compared to L-ascorbic acid.

2.5.2. DPPH Radical Assay

The hydrogen atom or electron donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of purple coloured methanol solution of DPPH. This spectrophotometric assay uses stable radical diphenylpicrylhydrazyl (DPPH) as a reagent [21] (Gulluce *et al.*, 2006). Fifty microlitres of various concentrations of the extracts in methanol was added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm. Inhibition free radical DPPH in percent (I%) was calculated in the following way:

$$I\% = A \text{ blank} - (A \text{ sample}/A \text{ blank}) \times 100,$$

where A blank is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound. Extract concentration from the graph plotted inhibition (IC₅₀) was calculated from the graph plotted against extract concentration. Synthetic antioxidant reagent butylatedhydroxytoluene (BHT) was used in triplicate.

2.6. Superoxide Radical Scavenging Activity

Superoxide radical (O₂⁻) was generated from the photoreduction of riboflavin and was deducted by nitro blue tetrazolium dye (NBT) reduction method. Measurement of superoxide anion scavenging activity [22]. The assay mixture contained sample with 0.1 ml of Nitro blue tetrazolium (1.5 mM NBT) solution, 0.2 ml of EDTA (0.1 M EDTA), 0.05 ml riboflavin (0.12 mM) and 2.55 ml of phosphate buffer (0.067 M phosphate buffer). The control tubes were also set up where in DMSO was added instead of sample. The reaction mixture was illuminated for 30 min and the absorbance at 560 nm was measured against the control samples. Ascorbate was used as the reference compound. All the tests were performed in triplicate and the results averaged. The percentage inhibition was calculated by comparing the results of control and test samples.

2.7. Iron Chelating Activity

The principle is based on the formation of O-Phenanthroline-Fe²⁺ complex and its disruption in the presence of chelating agents. The reaction mixture containing 1 ml of 0.05% O-Phenanthroline in methanol, 2 ml ferric chloride (200 µM) and 2 ml of various concentrations ranging from 10 to 1000 µg was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator [23]. The experiment was performed in triplicates.

2.8. Ferric Thiocyanate (FTC) Method

Different extracts (4 mg) and standards (4 mg; BHT, vitamin C and vitamin E) were mixed with 4 ml of absolute ethanol, 4.1 ml of 2.52% linoleic acid in absolute ethanol, 8 ml of 0.02 M phosphate buffer (pH 7.0) and 3.9 ml of distilled water. The mixture was placed at 40°C (0.1 ml) and was then mixed with 9.7 ml of 75% (v/v) ethanol and 0.1 ml 30% ammonium thiocyanate. Three minutes after adding ferrous chloride (0.1 ml of 2 × 10⁻² M ferrous chloride), the absorbance was measured at 500 nm in a spectrophotometer. This step was repeated every 24 h until the control reached its maximal absorbance value. The mixture without added sample was used as a control [24]. The inhibition of lipid peroxidation (%) was estimated by the following formula:

$$\% \text{Inhibition} = 100 - [(A1 - A0) \times 100],$$

where A0 is the absorbance of the control and A1 is the absorbance of the sample extracts [25].

2.9. Thiobarbituric Acid (TBA) Method

Extracts (2 ml) and standard solutions (2 ml) on the final day (day 8) of the FTC assay were added to 1 ml of 20% aqueous trichloroacetic acid and 2 ml of 0.67% aqueous thiobarbituric acid. After boiling for 10 min, the samples were cooled. The tubes were centrifuged at 3000 rpm for 30 min. Absorbance of the supernatant was evaluated at 532 nm in a spectrophotometer [26]. The antioxidant activity was calculated by percentage of inhibition in this method as follows:

$$\% \text{Inhibition} = 100 - [(A1 - A0) \times 100]$$

where A0 is the absorbance of the control and A1 is the absorbance of the sample extracts [25].

2.10. Free Radical Scavenging Activity

The ability of methanol and chloroform extracts of MC to scavenge 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radicals was estimated [27]. MC extracts (3 ml) with six

different concentrations (15.62, 31.25, 62.5, 125, 250 and 500 µg/ml) were mixed with 1 ml of a 0.1 mM methanol solution of DPPH. The absorbance was measured by a spectrophotometer at 517 nm at 30 min intervals against a blank (pure ethanol). The percentage of radical scavenging activity was calculated using the following formula:

$$\text{Radical scavenging \%} = 1 \left[\frac{(A_0 - A_1)}{A_0} \times 100 \right]$$

where A₀ is the absorbance of the control and A₁ is the absorbance of the sample extracts [25].

Lower absorbance values show higher free radical scavenging activity. Ascorbic acid was used as a reference standard in different concentrations (1.56, 3.12, 6.25, 12.5, 25 and 50 µg/ml). The 50% inhibitory concentration value (IC₅₀) is indicated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radicals [27].

2.11. Determination of Total Phenolic Content

Total Phenolic Content (TPC) in endophytic extracts was determined using Folin-Ciocalteu's colorimetric method. To 5 ml of 0.3% HCl in methanol/deionised water (60:40, v/v), 100 mg of the ethanol extract was added. From the resulting mixture (100 µl) was added to 2 ml of 2% aqueous sodium carbonate. The mixture was incubated for 2 min. To that 100 µl of 50% Folin-Ciocalteu's reagent was added and incubated for 30 min, absorbance was measured at 750 nm against blank. The content of total phenol was calculated on the basis of the calibration curve of gallic acid and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract [28].

2.12. Flavonoid Determination

The fungal extract (250 µl) was mixed with distilled water (1.25 ml) and NaNO₂ solution (5%, 75 µl). After 5 min the AlCl₃ H₂O solution (10%, 150 µl) was added. After 6 min, NaOH (1M, 500 µl) and distilled water (275 µl) were added to the mixture. The solution was mixed well and the intensity of the pink color was measured at

510 nm against blank. The content of flavonoid was calculated on the basis of the calibration curve of quercetin and the results were expressed as mg of quercetin equivalents per g of extract [29].

2.13. Statistical Analysis

Analysis of variance (ANOVA) was used to determine the significance of difference between treatment groups ($p < 0.05$). Means between treatment groups were compared for significance using Duncan's new Multiple Range post test.

3. Results

3.1. Identification of Endophytes

The plant materials were collected from six different places of Tumkur district of Karnataka, India (**Table 1**). Seven different fungal species were reported from all incubated parts (leaves, bark, stem and root). *A. niger*, *A. flavus*, *Penicillium* sp., *Rhizopus* sp. and *Fusarium* sp. were presented in sample 1, 2, 3, 4, 5 and 6, where *Rhizopus* sp. was not reported from sample 5 and 6. The *Alternaria* sp. has reported from sample 3, *Trichoderma* sp. has showed presence in sample 4 and 5 (**Table 2**).

3.2. Phytochemical Screening

Phytochemical analysis of methanol solvent extracts of endophytes revealed the presence of saponins, phenol compounds, anthraquinones, flavonoids, steroids, cardiac glycosides and tannins. Extracts from cultures of all (seven different fungal species) endophytic fungi gave a wide variety of biological activities. Endophytes, *A. niger*, *Penicillium* sp., *Trichoderma* sp. and plant extracts has showed presence of all compounds (of saponins, phenolic compounds, anthraquinones, steroids, cardiac glycosides and tannins), whereas other endophytes also have all the phytochemical except tannins. The presence of anthraquinones was observed in only three endophytes (*A. niger*, *Penicillium* sp. and *Trichoderma* sp.) and flower extract (**Table 3**).

Table 1. Collection of plant material from different place.

| Plant Sample | Collected Place |
|--------------|---------------------------------------|
| Sample 1 | SIET Campus, Sira Road, Tumkur |
| Sample 2 | Behind SIET Campus |
| Sample 3 | DevarayanaDurga, Tumkur |
| Sample 4 | Sira Gate, Tumkur |
| Sample 5 | Doddabele, Nelamanga Taluk, Bangalore |
| Sample 6 | SIT Campus, Tumkur |

Table 2. Isolation of endophytes from *Tabebuia argentea* on PDA media.

| Samples | Endophytes | | | | | | |
|----------|-----------------|------------------|------------------------|---------------------|---------------------|-----------------------|------------------------|
| | <i>A. niger</i> | <i>A. flavus</i> | <i>Penicillium</i> sp. | <i>Rhizopus</i> sp. | <i>Fusarium</i> sp. | <i>Alternaria</i> sp. | <i>Trichoderma</i> sp. |
| Sample 1 | + | + | + | + | + | - | - |
| Sample 2 | + | + | + | + | + | - | - |
| Sample 3 | + | + | + | + | + | + | - |
| Sample 4 | + | + | + | + | + | - | + |
| Sample 5 | + | + | + | - | + | - | + |

+ = Presence, - = Absent, Experiments were repeated thrice for each sample.

Table 3. Phytochemical analysis of endophytic extracts.

| Endophytes | Carbohydrates | Proteins | Tannins | Steroids | Flavonoids | Alkaloids |
|------------------------|---------------|----------|---------|----------|------------|-----------|
| <i>A. niger</i> | + | + | + | + | + | + |
| <i>A. flavus</i> | + | + | + | + | + | + |
| <i>Penicillium</i> sp. | + | + | + | + | + | + |
| <i>Fusarium</i> sp. | + | + | + | + | + | + |
| <i>Rhizopus</i> sp. | + | + | + | + | + | + |
| <i>Alternaria</i> sp. | + | + | + | + | + | + |
| <i>Trichoderma</i> sp. | + | + | + | + | + | + |
| Plant Parts | + | + | + | + | + | + |

+ = Presence, - = Absent, repeated the experiments three times for each replicates.

3.3. Lapachol Identification Test

1 g of the endophyte and flower extract was recrystallized in petroleum ether and benzene (80:60) and heated at 139°C to 140°C for 5 min. 2 ml of ferric chloride solution was added and observed for colour change. Yellow colour confirmed the presence of quinoned compounds (Naphthoquinone). Naphthoquinone was identified in *A. niger*, *Penicillium* sp. and *Trichoderma* sp. and in plant extract. Other endophytes did not show the presence of naphthoquinone presence (Table 4).

3.4. In Vitro Antioxidant Activity

Table 5 shows the absorbance and percentage of inhibition of all endophytic samples. The absorbance values of methanol extracts of *A. niger*, *Penicillium* sp. and *Trichoderma* sp. were significantly ($P < 0.05$) higher than the absorbance value of BHT and vitamin E. The percentage inhibition of methanolic extracts of *A. niger*, *Penicillium* sp. and *Trichoderma* sp. were significantly ($P < 0/05$) lower than BHT and Vitamin E.

Superoxide is highly reactive molecule that reacts with various substances produced through metabolic processes. Superoxidase dismutase enzymes present in aerobic and

anaerobic organisms catalyses the breakdown of superoxide radical. Percentage scavenging of superoxide anion examined at different concentrations of methanol extract of three endophytes (125, 250, 500, and 1000) was depicted in Table 6. The percentage scavenging of superoxide radical surged with the enhanced concentration of endophytic extract. The maximum scavenging activity of endophytes extract and quercetin at 1000 µg/ml was found to be 96.48 ± 0.014 (*A. niger*), 83.84 ± 0.009 (*Penicillium* sp.) and 80.97 ± 0.022 (*Trichoderma* sp.) respectively. Superoxide scavenging ability of plant extract might primarily be due to the presence of flavonoids.

Iron is essential for life because it is required for oxygen transport, respiration and activity of many enzymes. However, iron is an extremely reactive metal and catalyzes oxidative changes in lipids, proteins and other cellular components. It causes lipid peroxidation through the reaction and decomposes the lipid hydroxide into peroxy and alkoxy radicals that can perpetuate the chain reactions. Iron binding capacity of methanol extracts three different endophytes and metal chelator EDTA at various concentrations (125, 250, 500, 1000 µg/ml) were examined and the values (Table 7). Maximum chelating of metal ions at 1000 µg/ml for endophytes extracts and

Table 4. Identification of lapachol from different endophytes using different procedures.

| Endophytes | Test 1 | Test 2 |
|------------------------|--------|--------|
| <i>A. niger</i> | + | + |
| <i>A. flavus</i> | - | - |
| <i>Penicillium</i> sp. | + | + |
| <i>Fusarium</i> sp. | - | - |
| <i>Rhizopus</i> sp. | - | - |
| <i>Alternaria</i> sp. | - | - |
| <i>Trichoderma</i> sp. | + | + |
| Plant Parts | + | + |

Test + = Presence, - = Absent, repeated the experiments three times for each replicates.

Table 5. Total antioxidant activity of endophytes by TBA method.

| Extracts | Absorbance (nm) | % of inhibition |
|------------------------|----------------------------|-----------------------------|
| control | 1.23 ± 0.067 ^a | 0 |
| BHT | 0.05 ± 0.05 ^e | 96.24 ± 8.435 ^a |
| Vit E | 0.45 ± 0.080 ^f | 63.51 ± 66.424 ^b |
| Vit C | 1.04 ± 0.038 ^b | 16.75 ± 4.578 ^f |
| <i>A. niger</i> | 0.96 ± 0.041 ^{bc} | 36.62 ± 4.412 ^e |
| <i>Penicillium</i> sp. | 0.85 ± 0.056 ^d | 34.13 ± 4.643 ^d |
| <i>Trichoderma</i> sp. | 0.83 ± 0.056 ^{dc} | 31.62 ± 4.389 ^e |

Repeated the experiments three times for each replicates. Data represents mean ± SD. Different lowercase and upper case letters in the same column indicate significant difference at P < 0.05.

Table 6. Effect of endophytes extract on super oxide radical scavenging activity.

| Conc (µg/ml) | Extracts | | | |
|--------------|----------------------------|----------------------------|----------------------------|----------------------------|
| | <i>A. niger</i> | <i>Penicillium</i> sp. | <i>Trichoderma</i> sp. | quescertin |
| 125 | 34.51 ± 0.016 ^d | 31.12 ± 0.036 ^d | 27.66 ± 0.031 ^d | 73.88 ± 0.006 ^d |
| 250 | 44.76 ± 0.033 ^c | 40.57 ± 0.038 ^c | 38.31 ± 0.052 ^c | 91.38 ± 0.012 ^c |
| 500 | 91.75 ± 0.012 ^b | 79.63 ± 0.013 ^b | 77.64 ± 0.014 ^b | 93.41 ± 0.026 ^b |
| 1000 | 96.48 ± 0.014 ^a | 83.84 ± 0.009 ^a | 80.97 ± 0.022 ^a | 98.31 ± 0.013 ^a |

Repeated the experiments three times for each replicates. Data represents mean ± SD. Different lowercase and upper case letters in the same column indicate significant difference at P < 0.05.

Table 7. Effect of endophytes extracts on iron chelation.

| Conc (µg/ml) | Extracts | | | |
|--------------|-----------------|------------------------|------------------------|---------------|
| | <i>A. niger</i> | <i>Penicillium</i> sp. | <i>Trichoderma</i> sp. | Std EDTA |
| 125 | 31.43 ± 0.039 | 29.47 ± 0.018 | 23.63 ± 0.031 | 58.68 ± 0.007 |
| 250 | 58.92 ± 0.007 | 43.83 ± 0.031 | 41.71 ± 0.031 | 65.87 ± 0.019 |
| 500 | 87.54 ± 0.037 | 56.14 ± 0.023 | 53.91 ± 0.024 | 83.83 ± 0.013 |
| 1000 | 96.19 ± 0.020 | 63.37 ± 0.017 | 59.11 ± 0.031 | 97.90 ± 0.019 |

Repeated the experiments three times for each replicates. Data represents mean ± SD. Different lowercase and upper case letters in the same column indicate significant difference at P < 0.05.

EDTA was found to be 96.19 ± 0.020 (*A. niger*), 63.37 ± 0.017 (*Penicillium* sp.), 59.11 ± 0.022 (*Trichoderma* sp.) and 97.90 ± 0.019 (EDTA).

As shown in **Table 8**, the absorbance of methanol extracts of three endophytes at 7 days was significantly (P < 0.05) decreased when compared with absorption of extracts at 6 day. In contrast, endophyte *A. niger* extract had a significantly (P < 0.05) higher than *Penicillium* sp. and *Trichoderma* sp. The absorbance of endophytic methanol extracts at 7 and 8 day was markedly lower than the absorbance of vitamin C and higher than the vitamin

E. In contrast, the *A. niger* methanol extract had a significant higher absorbance at 6 and 7 day when compared to vitamin C and vitamin E. The control had a highest value throughout the study. The highest absorbance value was recorded at 6 day, then dropped at 7 day due to malonaldehyde (MDA) compounds from linoleic acid oxidation, in which peroxidase reacts with ferrous chloride to form reddish ferric pigment (**Table 9**).

The percentage of inhibition of methanol extract of *A. niger* (74) followed by *Trichoderma* sp. (66) and *Penicillium* sp. (62) was significantly higher than the percentage

Table 8. Antioxidant activity of standards and different endophytes of *T. argentea* by FTC method.

| Samples | Percent inhibition | | | | | | | |
|------------------------|--------------------|-----|-----|-----|-----|------|------|------|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| BHT | 0 | 0 | 0 | 0 | 0.3 | 1.2 | 1.7 | 1.52 |
| EAF | 0 | 0 | 0 | 0 | 0.2 | 0.87 | 1.6 | 1.4 |
| <i>A. niger</i> | 0 | 0 | 0 | 0 | 0.1 | 0.71 | 1.3 | 1.26 |
| <i>Penicillium</i> sp. | 0 | 0 | 0 | 0 | 0.1 | 0.53 | 1.1 | 0.83 |
| <i>Trichoderma</i> sp. | 0 | 0 | 0 | 0 | 0.1 | 0.46 | 0.9 | 0.81 |
| control | 0 | 0.1 | 0.3 | 0.5 | 0.5 | 1.43 | 1.93 | 1.6 |

Repeated the experiments three times for each replicates. Data represents mean \pm SD. Different lowercase and upper case letters in the same column indicate significant difference at $P < 0.05$.

Table 9. Percentage of inhibition of linoleic acid and peroxidation by endophytic extracts in FTC method.

| Sample | Percent inhibition | | | | | | | | |
|------------------------|--------------------|----|----|----|----|------|------|------|------|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| BHT | 96 | 97 | 99 | 98 | 99 | 99.4 | 99.8 | 99.6 | 99.9 |
| EAF | 96 | 97 | 98 | 91 | 83 | 79 | 77 | 74 | 64 |
| <i>A. niger</i> | 96 | 97 | 98 | 90 | 83 | 79 | 76 | 74 | 64 |
| <i>Penicillium</i> sp. | 96 | 97 | 80 | 77 | 76 | 75 | 73 | 62 | 60 |
| <i>Trichoderma</i> sp. | 96 | 97 | 78 | 75 | 72 | 70.4 | 68 | 66 | 59 |
| control | 96 | 97 | 94 | 92 | 92 | 78 | 48 | 38 | 35 |
| VitE | 96 | 81 | 79 | 77 | 76 | 75 | 73 | 64 | 61 |
| vitC | 96 | 97 | 88 | 87 | 86 | 76 | 52 | 37 | 34 |

Repeated the experiments three times for each replicates. Data represents mean \pm SD. Different lowercase and upper case letters in the same column indicate significant difference at $P < 0.05$.

of vitamin C (37) at 7 and 8 with vitamin E (Table 9).

In the present experiment, lipid peroxidation was elevated through 8 days (optical density of the control was maximal at 7 day). The control had increasing absorbance values from 0 day until the absorbance reduced the maximum level at 7 day and the absorbance value dropped at 8 day. Table 9 shows the absorbance and percentage of inhibition of all samples. The absorbance value of endophytic methanol extract were significantly ($p < 0.05$) higher than the absorbance value of BHT and vitamin E. Among endophytes, *Trichoderma* sp. the absorbance value was significantly ($P < 0.05$) lower than the BHT and vitamin E. The TBA results were in agreement with FTC results.

The antioxidant activity of the methanol extract was measured by the ability to scavenge DPPH free radicals, was compared with the standards Butylated Hydroxy

Toluene (BHT). It was observed that methanol extract of the *Aspergillus niger*, *Penicillium* sp. had higher activity than that of *Trichoderma* sp. At a concentration of 0.1 mg/ml, the scavenging activity of ethanol extract of the *A. niger* and *Penicillium* sp. reached 88.61% and 86.72% respectively while at the concentration, that of *Trichoderma* sp. was 51.66%. Though the DPPH radical scavenging abilities of the extract were less than those of (97.8%) at 0.1 mg/ml, the study showed that the extracts have the proton donating ability and could serve as free radical inhibitors or scavenging, acting possibly as primary antioxidants (Figure 1). The performance of methanol extract of endophytic fungi, *Phyllosticta* sp. was higher than that the standard μ -tocopherol [28] and from different endophytes [30]. ABTS a stable free radical with the characteristic absorption at 734 nm was used to study the radical scavenging effect of endophytic extract reacted with ABTS at different concentration ranging from 100, 200, 400, 800 and 1600 μ g/ml respectively and readings were observed by measuring the reduction of radical cation generated by ABTS⁺ at 734 nm (Figure 2).

The methanol extract of *A. niger* and *Penicillium* sp. showed maximum decolouration of 1600 μ g/ml with the EC_{50} 587.06 \pm 0.74 and 566.71 \pm 45 and the other endophyte showed less decolouration (Table 10). ABTS assay is an excellent tool for determining the antioxidant activity of phytochemicals [31]. The edible basidiomycetes and endophytes assayed against ABTS radical and reported to have scavenging ability against these radicals [28]. The reducing ability of the endophytic extracts was in the range of 448.26 - 1266.14 μ m Fe (II)/mg (Table 11). The antioxidant potentials of the ethanol extracts of *A. niger* and *F. oxysporum* were estimated from their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II). The FRAP values for the ethanol endophytic extracts significantly lower that of ascorbic acid but higher that of BHT. Antioxidant activity increased proportionally to the polyphenol content. According to recent reports, a highly positive relationship between total phenol and antioxidant activity appears to be the trend in many plant species [32]. Phenolic and flavonoid compound seems to have an important role in stabilizing lipid oxidation, associated with antioxidant activity [33]. Total phenol found to be in *Aspergillus niger* and *Penicillium* sp. of 19.20 and 18.23 mg/GAE/g dry weight and flavonoid content of *Aspergillus niger* and *Penicillium* sp. of 7.25 and 6.41 μ g/mg equivalent respectively (Table 12). The present investigation results reveal that ethanol extract of *Aspergillus niger* and *Penicillium* sp. contains significant amount of phenols and flavonoids. Results of our findings confirmed the use *Aspergillus niger* and *Penicillium* sp. extract can be as traditional medicine. We found strong antioxidants activities specifically in the ethanol

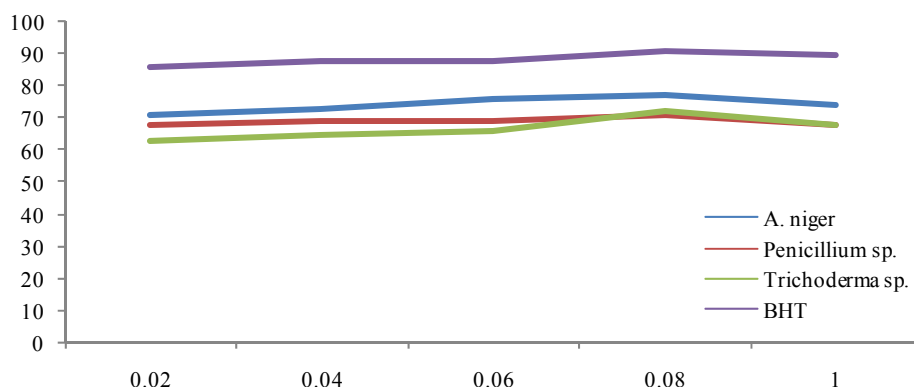


Figure 1. DPPH scavenging activities of methanol endophytes extract.

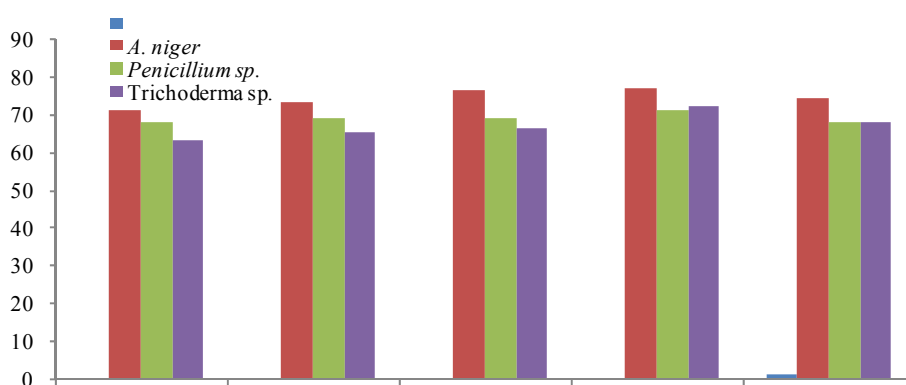


Figure 2. Free radical scavenging effect of different endophytes extracts against ABTS.

Table 10. Total antioxidant activity of the different endophytes and plant extract of *T. argentea*.

| Total antioxidant activity | FRAP | PAC |
|----------------------------|-------------|------------|
| <i>A. flavus</i> | 5276 ± 0.01 | 3.8 ± 0.01 |
| <i>Penicillium sp.</i> | 4988 ± 0.03 | 3.1 ± 0.02 |
| <i>Trichoderma sp.</i> | 4896 ± 0.03 | 2.8 ± 0.02 |
| Plant leaf extract | 5644 ± 0.03 | 2.6 ± 0.02 |
| Ascorbic acid | 4401 ± 0.03 | 2.7 ± 0.02 |

Data is three replicates of each sample. Phenolic antioxidant coefficient calculated as the ratio FRAP (µM/l)/total phenols (µMCE/l).

Table 11. Determination of phenols and flavonoid from endophytes extract of *Tabebuia argentea*.

| Samples | Phenol (mg/g) | Flavonoid (mg/g) |
|------------------------|---------------|------------------|
| <i>A. niger</i> | 20.17 ± 0.03 | 7.56 ± 0.07 |
| <i>Penicillium sp.</i> | 19.34 ± 0.07 | 6.71 ± 0.07 |
| <i>Trichoderma sp.</i> | 18.47 ± 0.07 | 5.94 ± 0.07 |
| Plant Leaf Extract | 18.92 ± 0.03 | 6.72 ± 0.07 |

Repeated the experiments three times for each replicates. According to Duncan's Multiple Range Test (DMRT), values followed by different subscripts are significantly different at p < 0.05, SE—standard error.

Table 12. Table of EC₅₀ values of endophytic extracts.

| Sample | EC ₅₀ (µg/ml) | |
|------------------------|--------------------------|----------------|
| | ABTS | DPPH |
| <i>A. niger</i> | 594.09 ± 0.69 | 2114.42 ± 0.69 |
| <i>Penicillium sp.</i> | 561.24 ± 0.69 | 2007.14 ± 0.69 |
| <i>Trichoderma sp.</i> | 552.76 ± 0.69 | 1899.43 ± 0.69 |
| Plant Leaf Extract | 556.97 ± 0.69 | 2078.49 ± 0.69 |

Repeated the experiments three times for each replicates. According to Duncan's Multiple Range Test (DMRT), values followed by different subscripts are significantly different at p < 0.05, SE—standard error.

Aspergillus niger and *Penicillium sp.* endophytic stem extracts. High phenol and flavonoid values found in ethanol *Aspergillus niger* and *Penicillium sp.* extracts imply the role of phenol compounds in contributing these activities. Plant phenol compounds have been found to possess potent antioxidants [34]. The flavonoids from endophytic extracts have been found to possess antioxidants properties in various studies. The terpenoids act as antioxidant [35]. Strong presence of tannins and saponins in all extracts may explain its potent bioactivities are known to possess potent antioxidants [36]. The present investigation has shown that the endophytic extracts have active

phytochemicals and exhibited strong antioxidant properties. These activities may be due to strong occurrence of polyphenolic compounds such as flavonoids, tannins, terpenoids phenols and saponins. The antioxidant activity was comparable with standard ascorbic acid and BHT. These findings provide scientific evidence to support endophytic fungal medicinal uses and indicate a promising potential for the development of an antioxidant agent plant. These endophytic fungi by *in vitro* results appear as interesting and promising and may be effective as potential sources of novel antioxidant drugs.

4. Discussion

Endophytic fungi are reported ubiquitously from each and every higher plant, which has been investigated for their antimicrobial complement [37]. The endophytes have made greater interest in the use reservoir of natural bioactive compounds that they (host) produced [38]. Endophytic fungal species are now considered as exciting novel sources for obtaining new bioactive compounds and have been reported from several hosts [39]. Investigations on the phytochemical screening of the *Tabebuia argentea* revealed the presence of saponins, glycosides, alkaloids, flavonoids etc. The presence of phytochemical within endophytes can be potential source for medicinal and industrial use. The presence of phytochemicals in the endophytes can be potential source of precursors in the development of synthetic drugs [14].

Based on the above results, endophytes, *Aspergillus niger*, *Penicillium* sp. and *Trichoderma* sp. of *Tabebuia argentea* yielded medically important phytochemical compounds, may be due to these endophytes possess potent antioxidant potentials of methanol extracts in all *in vitro* study. In addition, all endophytic extracts were found to possess significant amount of total phenolic content. Different ingredients of endophytes, *Aspergillus niger* and *Penicillium* sp. of *Tabebuia* sp. have been claimed in different studies to possess biological properties related to antioxidant mechanisms. Hence, the significant antioxidant activity of methanol extract in the present study may be attributed to these aforementioned potent antioxidant ingredients of endophytes, *Aspergillus niger* and *Penicillium* sp. The ferric thiocyanate (FTC) results showed that methanolic extract had greater antioxidant activity. Previous studies have claimed that, the aqueous extract of herbal plants has high antioxidant activity against lipid peroxidation. The reaction in the FTC method is due to the malondialdehyde (MDA) compounds from the linoleic acid oxidation in which, peroxide reacts with ferrous chloride to form a reddish ferric chloride pigment [40]. In this study, however, the high antioxidant activity of *Aspergillus niger* and *Penicillium* sp. methanol extract may be due to the majority of the active com-

pounds being dissolved in the methanol solvent (strong polar). In this study, the results of the thiobarbituric acid (TBA) test were confirmed with the FTC data. Based on these results, endophytic methanol extract had higher antioxidant activity. Therefore, the higher antioxidant activity found from the ferric thiocyanate method indicated that, the amount of peroxide in the initial stage of lipid peroxidation was greater than the amount of peroxide in the secondary stage. Thus, these data suggest that methanol extract has a better beneficial effect against lipid peroxidation when compared with chloroform extract. The DPPH assay is one of the most common and relatively quick methods used for testing radical scavenging activity of various plant extracts [25]. The results of this study indicated that, the IC₅₀ in methanol extract was significantly lower than the IC₅₀ in chloroform extract suggesting that the methanol extract had better scavenging activity than the chloroform extract. In this study, we found a dose dependent relationship in the DPPH assay. The activity increased as the concentration increased for both extracts. The increased formation of free radicals was associated with the increase in lipid peroxidation. One of the important roles of antioxidants is to inhibit the chain reaction of lipid peroxidation. We conclude that, the methanol extract (polar extract) with maximal inhibition of free radicals is a more potent extract when compared with the chloroform extract (non-polar).

The present study estimated the phenol and flavonoid contents of methanol extract and chloroform extract of *Tabebuia argentea*. Our results showed that methanol extract contained significantly higher phenol (approximately 15 fold) and flavonoid (approximately 19 fold) contents than chloroform extract. Phenol and flavonoid contents in methanol extracts (polar solvent) of endophytic *Xylaria* sp. are higher than the contents in hexane extracts (nonpolar solvent) [41]. Because of the hydroxyl groups in the phenol compounds, they may directly contribute to the antioxidant activity and have a critical role in scavenging free radicals [25]. Recent studies have shown that, fruit and vegetable phenols and polyphenols such as flavonoids are one of the major groups that indicate a large spectrum of biological activities that are principally ascribed to their antioxidant property. They prevent free radical damage and lipid peroxidation. The high content of total phenol components in the methanol extract may have led to the better results found in the total antioxidant activity and free radical scavenging ability when compared with the chloroform extract. In addition, the differential activities may have been due to the higher solubility of phenol components in the methanol solvent than in the chloroform solvent. In this study, the radical scavenging ability was significantly increased in methanol extract when compared with chloroform extract. The reason for the increase in radical scavenging ability may

have been due to the increase in total phenol compounds. Thus, it was important to calculate the correlation between the total phenol contents and total antioxidant activity. A good correlation was found between the total phenol contents and DPPH scavenging ability for methanol extract and chloroform extract. Our results are in accordance with previously published results that indicated a high correlation between antioxidant activity and total phenols. Thus, this indicates that the antioxidant activity of many plant extracts is related to their phenol components. Furthermore, the methanol extract containing the higher concentration of phenols had significant free radical scavenging activity in this study. The results suggest that, phenols are important components of endophytes and that some of its pharmacological effects may be attributed to the presence of these valuable constituents.

Superoxide is a highly reactive molecule that reacts with various substances produced through metabolic processes. Superoxide dismutase enzymes present in aerobic and anaerobic organisms catalyses the breakdown of superoxide radical. Superoxide scavenging ability of plant extract might primarily be due to the presence of flavonoids.

Iron is essential for life because it is required for oxygen transport, respiration and activity of many enzymes. However, iron is an extremely reactive metal and catalyzes oxidative changes in lipids, proteins and other cellular components. It causes lipid peroxidation through the lipid hydroxide into peroxy and Alkoxy radicals that can perpetuate the chain reactions.

Based on the above results indicated, the methanol extract of *Aspergillus niger* and *Penicillium* sp. of *Tabebuia argentea* was found to most effective in exhibiting *in vitro* antioxidant activity in various method. The results indicted the endophytes, *Aspergillus niger* and *Penicillium* sp. possess iron binding capacity which might be due to the presence of polyphenols that averts the cell from free radical damage by reducing of transition metal ions. Various plant extracts were proved to be good chelators and correlation exists between phenols, flavonoids and chelating activity. Further *in vivo* experiment is needed to recommend the endophytic extracts can be used as antioxidant drug.

5. Conclusion

In this study, all antioxidant methods (ferric thiocyanate, thiobarbituric acid, and DPPH) showed that the methanol endophytic extracts of *Tabebuia argentea* contain more antioxidant activities than the chloroform extract. Moreover, this study demonstrated that, endophytes *Aspergillus niger* and *Penicillium* sp. are an important source of phenol compounds, which are a good source of antioxidant activity. The phenol component has a high inhibi-

tory effect that prevents lipid peroxidation. However, the solvent type has an important role in detecting phenol compounds and antioxidant factors. Thus, we concluded that endophytes *Aspergillus niger* and *Penicillium* sp. react via its free radical scavenging to prevent lipid peroxidation. Therefore, natural antioxidants and phenol compounds in endophytes *Aspergillus niger* and *Penicillium* sp. have the capability to be used medically and in food systems to preserve food quality.

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