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ORIGINAL PAPER

Phenolic and flavonoid contents and antioxidant activity of an endophytic fungus *Nigrospora sphaerica* (EHL2), inhabiting the medicinal plant *Euphorbia hirta* (dudhi) L.

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Abstract

Since endophytic fungi are pivotal sources of various bioactive natural compounds, the present study is aimed to investigate the antioxidant compounds of the endophytic fungus *Nigrospora sphaerica* isolated from a pantropical weed, *Euphorbia hirta* L. The fungus was fermented in four different media and each filtered broth was sequentially extracted in various solvents. Crude extracts collected from different solvents were subjected to phytochemical analysis and antioxidant activity. The total phenolic content (TPC) and total flavonoid content (TFC) were maximal in ethyl acetate crude extract (EtOAcE) of endophyte fermented in potato dextrose broth (PDB) medium (77.74 ± 0.046mgGAE/g and 230.59 ± 2.0 mgRE/g) with the highest 96.80% antioxidant activity. However, TPC and TFC were absent in hexane extract of Czapek Dox broth (CDB) medium exhibiting the lowest 4.63 ± 2.75% activity. The EtOAcE (PDB) showed a positive correlation between TFC and antiradical activity ($R^2 = 0.762$; $P < 0.05$), whereas a high positive correlation was noticed between TPC and antioxidant activity ($R^2 = 0.989$; $P < 0.05$). Furthermore, to determine the antioxidant activity, EtOAcE (PDB) was subjected to TLC bioautography-based partial purification, while GC/MS analysis of the partial purified extract was done to confirm the presence of phenolics along with antioxidant compounds that resulted in the detection of 2,4-Di-tert-butylphenol (13.83%), a phenolic compound accountable for the antioxidant potential. Conclusively, *N. sphaerica* is a potential candidate for natural antioxidant.

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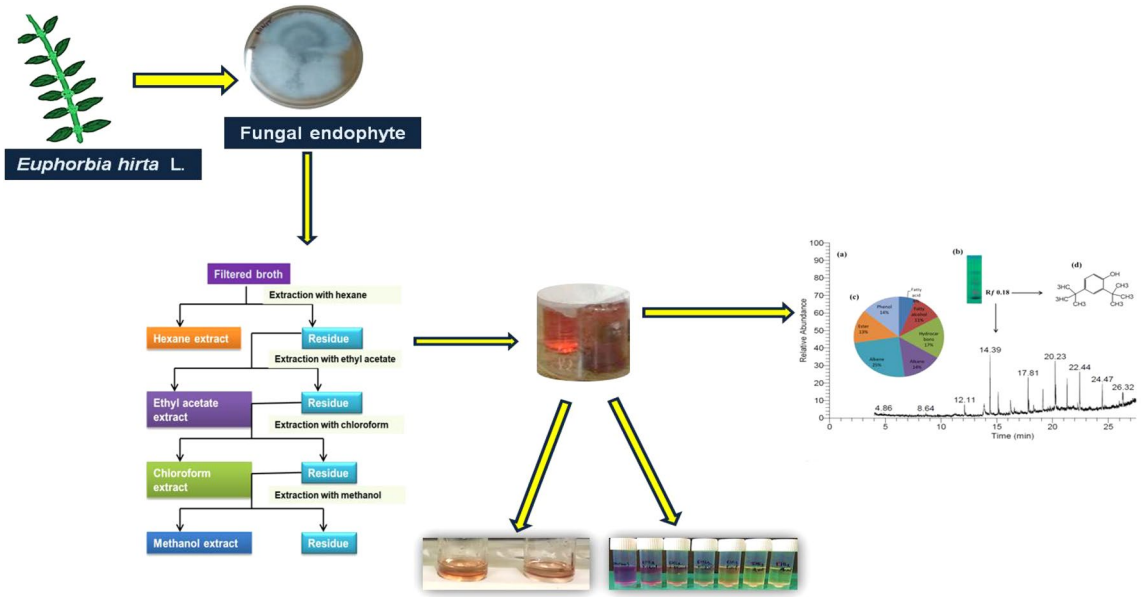
Graphical abstract



Keywords *N. sphaerica* · Phytochemical analysis · TLC bioautography · Compounds purification · Antioxidant activity · GC–MS analysis

Introduction

Oxidative stress/damage, caused by endogenous factors, such as reactive oxygen species (ROS) including the hydroxyl radical, superoxide anion, hydrogen peroxide, nitric oxide radical, singlet oxygen and hypochlorite radical, and exogenous factors, such as ionizing radiation, smoking, pollution, pesticides and organic solvents, can cause damage to biomolecules, like nucleic acids, lipids, proteins and enzymes, which may be the reasons for loss of structure and function, and eventually leading to several chronic diseases, such as cancer, diabetes, atherosclerosis, rheumatoid arthritis, cardiovascular disease, chronic inflammation, stroke, aging, septic shock and other degenerative disease in human (Fang et al. 2002). Natural antioxidant compounds play a significant role in reducing oxidative stress/damage and to improve the immune function via inhibiting diseases by scavenging radicals (Tan et al. 2018).

In the human body, there is a sort of balance between the number of free radicals generated and antioxidants (Mau et al. 2002). Excessive generation of ROS may result in oxidative damage, mutation, cytotoxicity and cell death due to the structural change of cellular molecules (Kohen and Nyska 2002). Various types of synthetic antioxidant compounds, like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ), have already been used as food preservatives or in food to prevent oxidation. However, the use of synthetic antioxidants in food is discouraged because of their carcinogenicity and toxicity (Sun and Fukuhara 1997). Hence, antiradical compounds isolated, especially from natural sources, may have potential application in preventing/curing diseases and making them capable of protecting ROS-mediated oxidative damage.

The literature survey reveals that the plant *Euphorbia hirta* contains several antioxidant compounds, such as gallic acid, quercetin, rutin and euphorbin-A,B,C,D (Muhammad et al. 2012). Endophytic fungi reside in a symbiotic manner inside its host plant, and can produce the same bioactive natural compounds for which the plant is known (Kumari et al. 2021). Several host origin compounds, such as azadirachtin, berberine, camptothecin, piperine, podophyllotoxin, rohitukine, vinblastine, vincristine and taxol, have been reported from fungal endophytes (Singh et al. 2021). The possibility of intergenic genetic exchange between a fungus and the host plant has been suggested. Such substitute sources of endophytic fungi may reduce the overexploitation of host medicinal plants and the cost of host mimetic natural compounds (Verma et al. 2009; Su et al. 2012). Recently, an antimicrobial compound "phomalactone" has been reported from a fungal endophyte, *Nigrospora sphaerica* isolated from *Adiantum philippense* (Ramesha et al. 2020). There are several reports that *Nigrospora* sp produces antimicrobial compounds, such as nigrosporins, nigrosporolides and

lactones. However, until date, little study has been done on the antioxidant natural compounds produced by *Nigrospora sphaerica*.

The present report narrates the isolation of a fungal endophyte, *Nigrospora sphaerica,* from healthy leaf tissues of the medicinal plant, *Euphorbia hirta,* with the aim of investigating the antioxidant compounds through TLC bioautography-based detection, and purification of antioxidant compounds, followed by identification of compounds using GCMS technique. Besides, phytochemical analysis of sequentially extracted crude metabolites and their correlation with antioxidant activity was also established. To the best of our knowledge, this is the first elaborative and exclusive report on antioxidant compounds produced by *N. Sphaerica* of *E. hirta* L.

TPC, total phenolic content: TFC, total flavonoid content: EtOAcE, ethyl acetate extract: PDB, potato dextrose broth: CDB, Czapek Dox broth: SDB, Sabouraud dextrose broth: CDYEB, Czapek Dox yeast extract broth: MEB, malt extract broth: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid): DPPH, 2,2-diphenyl-1-picrylhydrazyl:TLC, thin layer chromatography: GAE, gallic acid equivalent: RE, rutin equivalent: ITS, internal transcribed spacer: AA, ascorbic acid: QR, quercetin: GCMS, gas column mass spectroscopy.

Materials and methods

Isolation of fungal isolate

Mature, healthy and disease-free leaf samples of the medicinally important plant *Euphorbia hirta* were collected randomly from the Botanical Garden, Banaras Hindu University (BHU) Varanasi, India. To remove epiphytes, the samples were initially surface sterilized using standard protocol (Sharma et al. 2017) with certain modifications. The endophytic fungus was isolated from the leaves of *E. hirta*, a plant belonging to the family Euphorbiaceae. For surface sterilization, leaves were treated with 70% ethyl alcohol for 1 min, followed by immerging in 4% sodium hypochlorite solution for 1 min before ringing in 70% ethyl alcohol for 30 s. The sterilized samples were ringed in distilled water and allowed to dry between autoclaved blotting paper. The samples were cut into small pieces (0.5 × 0.5 cm squares), and placed onto Petri plates containing potato dextrose agar (PDA) medium supplemented with 100 µg/ml streptomycin. All the Petri dishes were sealed with Parafilm and incubated at 26 ± 2 °C in a BOD cum humidity incubator (Caltan, Narang Scientific Works, New Delhi), and monitored every day for fungal emergence for up to 20 days. The sterilized tissue imprinted control blank PDA plate and plates having tissue segments to check the probability of contamination throughout the incubation period were also incubated (Sharma et al. 2017). Endophytes emerging from explants that showed variation in morphology (colour, growth pattern) were transferred and maintained on freshly prepared PDA plates as axenic cultures.

Identification of fungal isolate by morphological and molecular method

Preliminarily, the fungal isolate was identified based on morphological features. Moreover, fungal identity was confirmed by molecular characterization after sequencing of the ITS-rDNA portion. For that, total genomic DNA was isolated from the freshly grown endophytic fungus, EHL2, using a standard method (Verma et al. 2014) with little modifications. The universal primers ITS1 (5′TCCGTA GGTGAACCTGCGG3′) and ITS4 (5′TCCTCCGCTTAT TGATATGC3′) were used to amplify the 5.8S rDNA and two ITS regions between the 18S and 28S rDNA in Mycycler unit (BioRad, United States) through polymerase chain reaction (PCR). The sample was amplified in total of 25 µL PCR reaction mixture containing 0.25 µL Taq polymerase enzyme, 1 µL of primer (ITS1 or ITS 4), 1 µL (120 ng/µL) DNA sample, 0.5 µL dNTPs, 2.5 µL 10 × PCR buffer with 25 mM MgCl2 and 18.75 µL milli Q water. Amplification was carried out under the following cycle profile: pre-denaturation at 95 °C (5 min), followed by 35 cycles of each denaturation at 94 °C (1 min), annealing at 54 °C (1 min), extension at 72 °C for 1.30 min and a final extension at 72 °C for 5 min. The quality and integrity of the resulting PCR products were tested on 1.5% agarose gel impregnated with EtBr (0.5 µgmL^{-1}) and visualized under an ultraviolet transilluminator. The amplified PCR amplicon was purified by HiYield PCR DNA mini kit (Real Biotech Corporation, India) through gel excision method. Purified ITS fragments were carried out for sequencing by Agri Genome Labs Pvt. Ltd., Kerala, India. Furthermore, the obtained sequence was submitted to the GenBank database of NCBI for accession number and identification.

Fermentation and sequential extraction of crude extract from fungal endophyte

Mycelial disc (5 mm) of freshly grown endophytic EHL2 was cultured in 500-ml Erlenmeyer flasks, each containing 200 ml of different media. All the five different culture media (Himedia), i.e. potato dextrose broth (PDB), Czapek Dox broth (CDB), Czapek Dox yeast extract broth (CDYEB), malt extract broth (MEB) and Sabouraud dextrose broth (SDB), were adopted for fermentation and incubated at 26 ± 2 °C. After 21 days of incubation, mycelia of all the cultures were filtered through muslin cloth. Each filtered broth was extracted thrice (1:1 ratio) in each organic solvent

sequentially using a separating funnel in the increasing order of relative polarity: hexane, ethyl acetate, chloroform and methanol, respectively. Each crude extract was concentrated using the rota evaporator (IKA RV 10, IKA, Staufen, Germany) on 65 rpm at 40 °C, dried slowly under a fume hood and stored at 4 °C until used for biological activity.

Lyophilization

The residue obtained after extraction in chloroform was miscible in methanol solvent; the samples were lyophilized. Finally, the freeze-dried samples were dissolved in methanol for testing biological activity.

Qualitative phytochemical analysis of sequentially extracted crude metabolites of the fungal isolate

Sequentially extracted crude extracts of endophytic *N. sphaerica* were tested for the presence of the following secondary metabolites: phenolics (Devi et al. 2012), flavonoids (Tepal 2016), alkaloids (Mayer's reagent) (Ajuru et al. 2017), terpenoids (Abdel-Rahman et al. 2019) and glycosides (Keller-Kiliani test for digitoxose) with little modifications.

Determination of total contents of phenolic (TPC) and flavonoid (TFC)

The total phenolic content (TPC) of each extract was determined using Folin-Ciocalteu reagent (Siddhuraju and Manian 2007) with minor modifications. For this, 10 µL containing 1 mg of extract was mixed with 990 µL of double distilled water. Then, half a millilitre of 2 N-Folin–Ciocalteau phenol reagent was added to the vial, followed by the mixing of 2.5 mL of sodium carbonate (20%), and incubated in a dark room for 40 min. Absorbance of the solution was recorded at 725 nm using a UV–visible spectrophotometer against a reagent blank. Quantification of TPC was done using gallic acid standard curve. The results are expressed as gallic acid equivalent (mg GAE/g of crude extract). However, total flavonoid content (TFC) was evaluated applying the protocol of Zhishen et al (1999). For that, 0.5 mL, containing a concentration of 2 mg/mL of each extract, was taken in a separate plastic vial to which was added 2 mL of double distilled water, followed by subsequent well mixing of 150 µL of sodium nitrite (5%) solution. After 5 min, 150 µL of $AlCl_3$ (10%) solution was put in the vial, followed by the addition of 2 mL of sodium hydroxide (4%) solution. The final volume of the mixture was maintained to 5 mL by adding water. The mixtures were shaken well and allowed to stand at room temperature for 15 min. Absorbance was read at 510 nm. Rutin was used as a standard curve to quantify the TFC, and the results are expressed as (mg RE/g crude extract). All the experiments were performed in triplicate.

TLC bioautography-based detection and purification of antioxidant compounds

A TLC bioautography was performed to screen the antioxidant compounds present in the crude extract (Belaqziz et al. 2017). For this, 200 µg of EtOAcE (PDB) was separately applied on a chromatographic silica plate (5 cm × 10 cm, Merck, TLC grade) as a stationary phase. Ethyl acetate and hexane (1:1) were used as a mobile phase to perform the chromatography. Silica plate was observed pre and post derivatization (0.2 Mm DPPH solution) under visible, short UV light (254 nm) and long UV light (366 nm) in the UV–visible chamber. The mode of each separating spot of extract was expressed as a retention factor (R*f*) value, and R*f* was calculated as follows:

R*f* = Distance travel by solute from the centre of spot loading point/Distance travel by solvent front.

After partition on a silica plate, the antioxidant compound was demarcated in situ with 0.2 mM DPPH reagents (Belaqziz et al. 2017). Yellow spots marked areas against a purple background were considered as antioxidant compounds. This spot was scratched, centrifuged and collected for further study.

Antioxidant activity

Chemicals

Ascorbic acid (AA), quercetin (QR), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis (3-ethylbenzthiazoline-6sulphonic acid) ($ABTS^+$) were purchased from Sigma (Sigma-Aldrich). Rutin, gallic acid and Folin-Ciocalteu's reagents were obtained from Merk. All other chemicals and reagents were procured locally and were of analytical grade.

DPPH method

In vitro antioxidant activity of each crude extract and purified compounds was performed using standard methodology (Shen, et al. 2010) with slight modifications. In this method, an aliquot of 1 mL of DPPH solution (0.2 mM) prepared in methanol was contributed to a test tube having 3 mL of methanolic solution (containing 1000 µg crude extract, and purified compounds in the range of 12.5, 25, 50, 100, 200 and 400 µg). A percentage of antioxidant activity of purified compounds of EtOAcE (PDB) of EHL2 at different concentrations ranged from 12.5 to 400 µg were compared with ascorbic acid (AA) and quercetin (QR), the positive standard antioxidant compounds. After 30 min of incubation in the dark, optical density was measured at 517 nm against a methanol blank using a UV–visible spectrophotometer (U-2900, Hitachi). There is an inverse relation between optical density and DPPH free radical inhibition.

The effective concentration (EC_{50}) of purified compounds was also measured. The percentage of free radical inhibition (I) against control was calculated as [(Abs control − Abs test sample) ÷ Abs control × 100]. All the experiments were conducted in triplicates.

ABTS method

The ABTS assay is based on the ability of antioxidant compounds to scavenge 2,2-azino-bis (ethylbenzthiazoline-6-sulfonic acid ($ABTS^+$) free radical cations (Kaaniche et al. 2019). For this assay, a radical cation was prepared by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate in equal volume and leaving the mixture for 15 min at room temperature in the dark. The $ABTS^+$ solution was diluted with ethanol to obtain the absorbance of 0.700 ± 0.05 at 734 nm. In order to estimate the antioxidant potential of partial purified compounds of EtOAcE (PDB), 20 µL sample of various concentrations (12.5 to 400 µg) was added to the test tube containing 2 mL ethanol with 1 mL of $ABTS^+$ solution, and the mixture was vortexed for 30 s. Using a spectrophotometer, absorbance was recorded immediately at 734 nm. The IC_{50} value of the purified extract was measured. AA and QR were used as positive controls.

GC/MS analysis

The compounds separated by TLC were detected and identified using gas chromatography mass spectrometry (GC–MS) analysis. The analysis was carried out in GCMS QP 2010 added to Shimadzu ultra-gas chromatograph coupled with mass selective detector, and the pressure was maintained at 90.4 kpa. Helium gas was used as a carrier with a flow rate of 1.0 mL/min and the pressure of the column head was maintained at 13.3psi. The injector temperature and injection volume were maintained at 260 °C and 1.21 ml/min, respectively. The column temperature was set at 100 °C, gradually increased to 280 °C at a rate of 5 °C/min for 5 min, further programmed to 320 °C for 19 min. The mass spectra were scanned from m/z 40 to 700 with a speed of 3333. The compounds were recognized based on the comparison of mass spectra in the National Institute of Standard and Technology (NIST) database.

Statistical analysis

All the values are expressed as means ($n = 3$) ± standard deviation (SD). Analysis of variance was carried out and differences between variables were tested by one-way ANOVA with Tukey post hoc test using the SAS 8.0 program. $P < 0.05$ was considered as suggestive of statistical significance. Correlation analysis was performed using the correlation and regression programme in the Microsoft EXCEL program.

Results

Isolation and identification of fungal isolate, EHL2

An endophytic fungus, EHL2, was isolated from the healthy leaf tissues of a medicinal plant, *Euphorbia hirta*, collected from the Botanical Garden of Banaras Hindu University (BHU), Varanasi, India. Preliminary characterization of EHL2 was done on its morphological features, including plate colony and growth pattern (Fig. S1a, b). Based on ITS 5.8S rDNA gene sequencing, followed by BLAST search tool with the NCBI database, it was identified as *Nigrospora sphaerica* EHL2 (code) with GenBank accession number MN726479.

Fermentation and sequential extraction of fungal crude extract

Sequentially isolated ethyl acetate extract of *N. sphaerica* fermented in PDB medium retained the maximum amount of crude extract (51.33 ± 0.57), while chloroform extract of the fungus fermented in MEB medium produced the minimal quantity of extract (11.33 ± 0.57). Moreover, PDB medium produced the largest amount of crude extract (167.66 ± 1.15), while it was the lowest (91 ± 1) in CDB medium (Table S1). Figure 1 shows the schematic diagram of sequential extraction of fungal endophyte fermented in various culture media.

Qualitative phytochemical analysis of the sequentially extracted crude extract of *N. sphaerica*

The results of qualitative phytochemical analysis of each crude extract are expressed as follows: + + + + = very heavily present, + + + = heavily present, + + = present, + = trace and — = absent. EtOAcE of PDB medium showed a very heavy presence of phenolics and a heavy presence of alkaloids. Methanol extract of PDB medium represents a very heavy presence of flavonoids, while chloroform extract of CDB medium indicates the heavy presence of terpenoid compounds. Preliminary phytochemical analysis of sequentially extracted crude metabolites of the fungus, *N. sphaerica,* fermented in different growth media is presented in Table1.

TPC and TFC determination of the sequentially extracted crude extract of *N. sphaerica*

As shown in Table 2, the amounts of TPC and TFC differed when *N. sphaerica* were cultured in different media

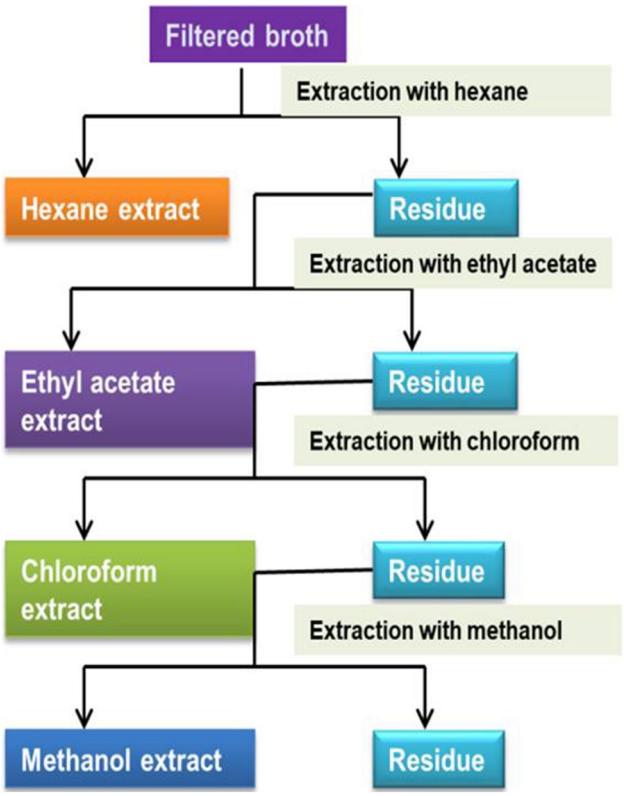


Fig. 1 Schematic diagram showing sequential extraction

and varied the extraction solvent. The level of TPC and TFC was the highest in EtOAcE (77.74 ± 0.04 mgGAE/g of crude extract for phenolic and 230.59 ± 2.0 mgRE/g extract for flavonoids) of fungus fermented in PDB medium with the maximal antioxidant potential (96.80%). However, the TPC and TFC were absent in the hexane extract of endophyte cultured in CDB medium, showing the lowest potential (4.63%) for free radical scavenging.

TLC bioautography-based detection and purification of antioxidants present in EtOAcE (PDB)

In order to separate the antioxidant component present in the crude extract, TLC was performed prior to the bioautography assay. The TLC Plate was removed when the solvent reached 8.8 cm from the original extract loaded position. After drying, two yellow spots (R*f* 0.18, 0.50) were visualized on the developed plate before derivatization (Fig. 2a). The number of spots varied in the post derivatization process depending on the wavelength of light: one spot (R*f* 0.18) was visualized under visual light (700–400 nm) (Fig. 2b), three spots (R*f* 0.18, 0.35, 0.50) under short UV light (254 nm) (Fig. 2c), and one spot (R*f* 0.18) under long UV light (366 nm) (Fig. 2d). The yellow colour reaction visualized at R*f* 0.18 in UV–visible light confirmed the presence of antioxidant compounds, which were collected for further study.

Spectroscopy-assisted in vitro antioxidant assay of each crude extract and purified compounds

DPPH method

In order to establish a relationship among each crude extract's TPC, TFC and antioxidant activity, statistical analysis was performed and correlated with its significant or insignificant values (Table 2). Antioxidant activity evaluated by the DPPH method was observed as a shift of the DPPH colour from purple to yellow. The free radical scavenging activity of partial purified compounds of EtOAcE (PDB) at 12.5, 25, 50, 100, 200 and 400 µg concentrations was 10.09, 23.28, 37.76, 43.54, 72.83 and 91.18%, respectively (Fig. S2a). The activity of the purified compound was less compared to that of the AA and QR at identical concentrations; however, the activity was almost equal to QR at 400 µg/mL concentration. Antioxidant activity of the purified compounds increased with the increasing concentration of metabolites (Fig. 3b). The calculated IC_{50} value of the purified compound was 148.5 µg/mL.

ABTS method

The reduction of $ABTS^{\bullet+}$ was done by reacting with low redox potential bearing phenolic compounds. The colour changed from blue-green to colourless. Activity of the purified crude compounds increased with the increasing concentration of extract. The free radical inhibition values of purified compounds of EtOAcE (PDB) at the concentrations of 12.5, 25, 50,100, 200 and 400 µg/mL were 16.96, 46.81, 66.05, 73.93, 86.43 and 92.11%, respectively (Fig. 2b). At equal concentrations, the antioxidant potential of the compounds was found to be relatively lower than that of AA and QR, whereas at 400 µg/mL concentrations, the activity of the purified compounds increased rapidly (92.11%), while QR and AA exhibited 94.69 and 98.02% antioxidant potential, respectively (Fig. 3a). The calculated IC_{50} value of the purified extract was 32.44 µg/mL.

GC–MS analysis of partial purified compounds of EtOAcE (PDB)

Based on the MS library of NIST, several peaks were detected. All the major peaks were resolved and presented in the form of a chromatogram (Fig. 4a). A total of 13 peaks were observed from the GC–MS analysis. Table 3 shows the number of peaks or identified compounds, the retention time, the name of the compounds along with their molecular weight and molecular formula, and the percent area and

Table 1 Qualitative phytochemical analysis of sequentially extracted crude extract of *N. sphaerica* fermented in various culture media

Solvents/Media	Phenolics	Flavonoids	Terpenoids	Alkaloids	Glycosides
Ethyl acetate					
PDB	+ + + + +	+ +	+	+ + +	–
CDB	–	+	–	–	–
CDYEB	+ +	+	+	+	+
MEB	+	+	+	+ +	–
SDB	–	–	–	–	–
Chloroform					
PDB	–	+	+ +	–	+
CDB	–	–	+ + +	–	+
CDYEB	–	–	–	–	+
MEB	–	–	–	–	+
SDB	–	–	+	–	+
Methanol					
PDB	–	+ + + +	–	–	–
CDB	–	–	–	–	–
CDYEB	–	–	–	–	–
MEB	–	–	–	–	–
SDB	–	–	–	–	–
Hexane					
PDB	–	–	+	–	–
CDB	–	–	–	–	–
CDYEB	–	–	–	–	–
MEB	–	–	–	–	–
SDB	–	–	–	–	–

– Not detected

activity of the compounds. GC–MS analysis of the purified extract showed the highest peak area at 20.23 RT with the presence of 9-hexacosene (13.83%), followed by 2,4-Di-tert-butylphenol (14.83%) at RT 14.39, whereas 1-undecanol (0.73%) showed the lowest % composition at RT 8.65 and others (Table S2). Alkene grabbed the highest area of the chromatogram at 25%, representing the dominance in purified crude extract, followed by hydrocarbons (17%), phenolic compounds (14%), alkane (14%), ester (13%), fatty alcohol (11%) and fatty acid (6%) (Fig. 4c).

Discussion

Based on morphological structure and molecular characterization, the endophytic fungus EHL2, isolated from the healthy leaf tissues of *Euphorbia hirta*, was identified as *Nigrospora sphaerica*. The fungus, *N. sphaerica,* has also been documented in a huge range of hosts, viz, *Moringa oleifera*, *Ginkgo biloba*, *Saccharum arundinaceum* and *Phoenix dactylifera*. Several antimicrobial bioactive compounds, such as nigrosporins, nigrosporolides and lactones, have previously been reported from *Nigrospora* sp. (Ramesha et al. 2020). However, information available on the antioxidant compounds of the species is scanty. In this study, *N. sphaerica* has been isolated for the first time as an endophyte from the healthy and disease-free leaves of *E. hirta* in search of prominent endophytic fungus, which could produce the antioxidant compounds. A variety of procedures and different media were used to obtain the maximum number of endophytes from unripe fruits and roots of *Azadirachta indica* (Verma et al. 2011). Unlike the previous study, various culture media were used for fungal fermentation in this study, and the effect of the media on various phytochemical groups was assessed using biochemical tests. Besides, sequential extraction of all filtrates was done to recover the total crude metabolites, which are left after extraction in a single solvent. In the phytochemical groups, phenols and terpenes are the chief biochemical constituents accountable for reducing lipid peroxidation, and that is why they act as primary and secondary antioxidants (Hijdu et al. 2007). The extract exhibited antiradical activity even after being low in the concentration of TPC and TFC. This might be because of the presence of other phytochemical groups, such as tannins, saponins and terpenoids, which could not be quantified in this experiment. Ethyl acetate extract of *Penicillium* sp. isolated from *Mussaenda luteola* exhibited both TPC and TFC, which were 24.38 mg GAE/g and 15.62 mg

Table 2 Total phenolic, flavonoid contents and DPPH scavenging potential of different crude extracts of *N. sphaerica*

Media/ Solvent	Total phenolics (mg GAE/ g crude extract)	Total flavonoids (mg RE/g crude extract)	DPPH scavenging activity (%)
Ethyl acetate			
PDB	77.74 ± 0.046^{a}	230.59 ± 2.00^{a}	96.80 ± 0.24^{a}
CDB	22.09 ± 0.06^{e}	88.73 ± 2.31^{d}	$21.29 \pm .04^{e}$
CDYEB	53.04 ± 0.04^{b}	123.53 ± 3.06^{c}	68.69 ± 0.75^{b}
SDB	33.32 ± 0.03^{d}	31.19 ± 3.06^{e}	33.32 ± 0.24^{d}
MEB	45.06 ± 0.46^{c}	134.23 ± 4.01^{b}	50.86 ± 0.43^{c}
Chloroform			
PDB	3.32 ± 0.03^{d}	–	29.85 ± 0.66^{b}
CDB	7.43 ± 0.06^{c}	–	35.79 ± 0.24^{a}
CDYEB	7.32 ± 0.01^{c}	–	35.35 ± 0.25^{a}
SDB	10.28 ± 0.03^{b}	–	16.22 ± 0.25^{d}
MEB	13.45 ± 0.06^{a}	–	23.32 ± 0.24^{c}
Methanol			
PDB	34.88 ± 0.03^{b}	24.90 ± 3.06^{b}	85.50 ± 0.25^{a}
CDB	2.10 ± 0.01^{d}	15.54 ± 2.00^{c}	21.15 ± 0.66^{c}
CDYEB	14.88 ± 0.01^{c}	–	62.45 ± 0.24^{b}
SDB	52.26 ± 0.03^{a}	54.34 ± 3.06^{a}	86.37 ± 0.50^{a}
MEB	14.77 ± 0.30^{c}	–	87.82 ± 0.43^{a}
Hexane			
PDB	–	44.98 ± 3.06^{a}	72.60 ± 0.60^{c}
CDB	–	–	4.63 ± 2.75^{e}
CDYEB	–	–	75.35 ± 0.25^{b}
SDB	–	6.18 ± 3.06^{b}	78.83 ± 0.25^{a}
MEB	–	–	61.58 ± 0.24^{d}

Data are expressed as mean of three replicates ± standard deviation. Means within each column with different letters (a–e) differ significantly ($P < 0.05$); –not detected

RE/g, respectively (Gunasekaran et al. 2017). Ethyl acetate extract of another endophytic fungus, *Achaetomium* sp. isolated from *E. hirta*, was estimated to have 218.16 mg RE/g TFC (Anitha and Mythili 2017). Ethyl acetate solvent is the most efficient, and selectively extracts the high molecular weight polyphenols and low molecular weight phenolic components (Garcia et al. 2012; Scholz and Rimpler 1989). Most of the pigmented parts of culture media are soluble in this solvent and allow the highest phenolic content and permit selective removal of non-phenolic compounds (Anitha and Mythili 2017). The results indicated that the sequentially extracted EtOAcE (PDB) contains significantly more phenolic and flavonoid contents than that of some important endophytic fungi. Previous studies have ratified that there is a linear correlation between TPC and antioxidant capacity (Liu et al. 2007; Sultana et al. 2007). Similarly, there is a direct correlation between TFC and antioxidant activity of any sample (Fitriansyah et al. 2018). In the correlation analysis, it was found that there was a positive correlation between TFC and antioxidant activity, $R^2 = 0.762$ ($P < 0.05$) of sequentially extracted EtOAcE (PDB), whereas a high positive correlation was noticed between TPC and free radical scavenging potential, $R^2 = 0.989$ ($P < 0.05$) of the same extract (Table 2). However, no correlation was found among phenolic content, flavonoid content and antiradical activity of other crude extracts. The extracts that have shown an unclear relationship among TPC, TFC and antioxidant potential may be due to the crucial role of some compounds, like alkaloids and terpenoids, which were not estimated in this study. The antioxidant activity of crude extract is dependent on the concentration, along with the structure and interaction among the phenolic compounds.

The DPPH (0.2 mM) solution was sprayed in situ, and a yellow colour reaction visualized at R*f* 0.18 under UV–visible light indicated the presence of phenolics, flavonoids and terpenoids, which could be responsible for the antioxidant activity (Fig. 4b). Spraying the plate with DPPH is more stable than that of ABTS and gives a more homogeneous colouring to the spots (Dewanjee et al. 2015). The TLC bioautography assay is the easiest screening method of antioxidant compounds due to several advantages that include simplicity, flexibility and high throughput. Since this technique provides a great platform by which antioxidant compounds can be detected in a cost-effective manner, several

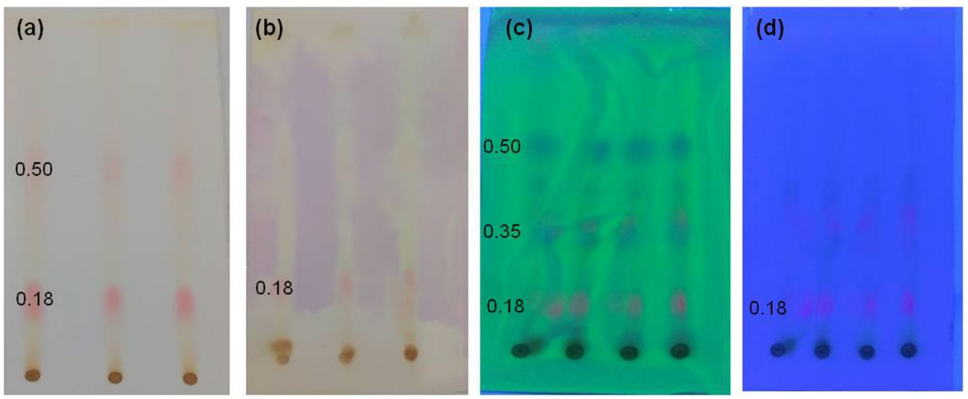


Fig. 2 TLC plates of ethyl acetate extract (PDB) of *Nigrospora sphaerica* visualized under visible light before and after derivatization (0.2 Mm DPPH solutions), UV light at 254 nm (green panel) and 366 nm (blue panel)

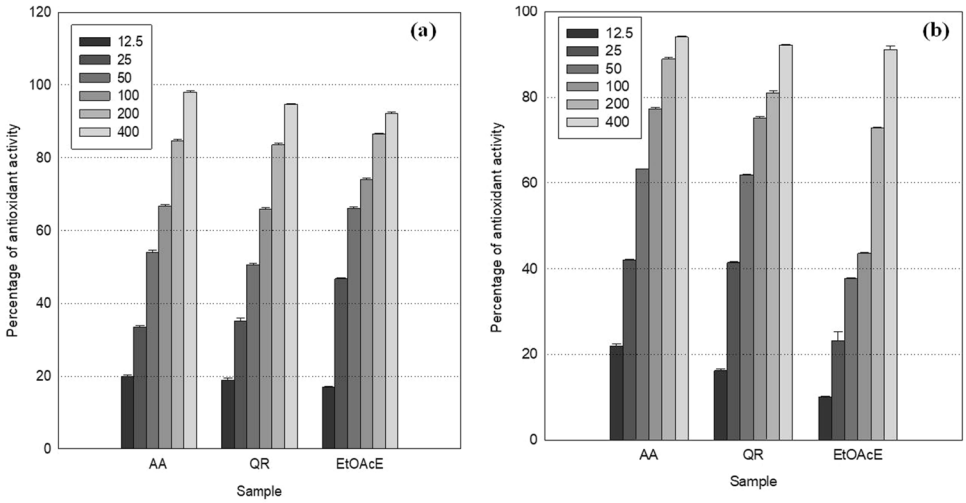


Fig. 3 **a** ABTS free radical inhibiting potential of purified ethyl acetate extract (PDB) of *Nigrospora sphaerica* and positive control using different concentrations (12.5–400 µg) and **b** DPPH free radical scavenging potential of purified ethyl acetate extract (PDB) of *N. sphaerica* and positive control using different concentrations (12.5–400 µg)

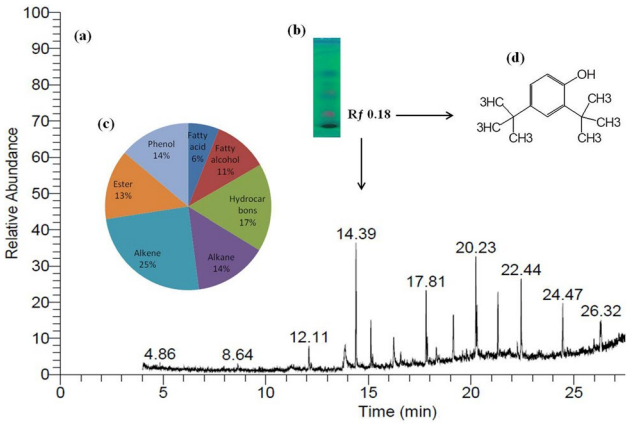


Fig. 4 **a** Chromatogram of GC–MS analysis, **b** TLC bioautography-based purification of compounds, **c** presence of different chemical groups in the partial purified ethyl acetate extract (PDB) and **d** depicting chemical structure of 2,4-Di-tert-butylphenol

antioxidant compounds have been identified, such as isoorientin and isoorientin 2″-O-α-L-rhamnoside (Wang et al. 2012). The DPPH-dependent antioxidant assay is based on the reduction of DPPH in the presence of hydroxyl groups of antioxidant compounds due to the synthesis of the non-radical form DPPH-H (Shon et al. 2003). The antioxidant activity of *N. sphaerica* isolated from *Artemisia herba alba* and *Chiliadenus montanus* at 1000 µg/mL concentrations was 14% and 13%, respectively (Selim et al.2018). However, antioxidant activity of EtOAcE (PDB) of the fungus *N. sphaerica* isolated from *E. hirta* at the same concentration (1000 µg/mL) was 96.80% (Table 2), while activity of the partial purified extract at the concentration of 400 µg was 91.18% (Fig. 3b). Thus, the antioxidant scavenging potential of the crude extract is comparatively lower than that of the purified extract due to the less availability of antioxidant compounds in the crude of EtOAcE (PDB). In the present study, the IC_{50} value of the purified compounds was 148.5 µg/mL, which was comparatively lower than that of the endophytic fungus *Cytospora rhizophorae*, obtained from *R. stylosa*, which had performed the most potent antiradical activity with an IC_{50} value of 330 µg/mL (Zhou et al. 2018). A low IC_{50} value reveals high antioxidant capacity. Therefore, the antiradical potential of the fungus *N. sphaerica* EHL2 is remarkably high compared to those of the previously reported endophytic fungus, and confirms that the antiradical nature of crude metabolites of endophytic fungi varies among fungi. When estimated by the ABTS method, the fungal endophyte *Neopestalotiopsis protearum* exhibited a lower IC_{50} value (1240 µg/mL) compared to that of the DPPH method (1800 µg/ml) (Zhou et al. 2018). The IC_{50} value observed in this study (32.44 µg/mL) was relatively lower than the above result estimated by the ABTS method, which has great antioxidant potential. During the reaction, antiradical molecules changed the blue-green colour of $ABTS^{\bullet+}$ to a colourless neutral form, and the reduction of blue-green $ABTS^{\bullet+}$ is studied by the suppression of its long wavelength absorption spectra. Thermodynamically, a compound may reduce $ABTS^{\bullet+}$ if it has a redox potential lower than that of ABTS (0.68 V) (Antolovich et al. 2002; Apak et al. 2013). Several phenolic compounds react with $ABTS^{\bullet+}$ due to low redox potential. $ABTS^{\bullet+}$ can be used to determine the antioxidant activity of both hydrophilic and lipophilic compounds, because it is soluble in both inorganic and organic solvents. When a single method is selected to test antioxidant potential, the results may alter according to the time of reaction, solvent's choice, pH and standard employed (Alam et al. 2013).

To investigate the chemical composition of purified compounds of EtOAcE (PDB) and to validate the results of phytochemical analysis and TLC bioautography, the GC–MS technique was applied. In this technique, first, the sample mixture vapourizes and then separates its components, which

Table 3 Phytochemical-compounds present in the TLC based purified extract of EtOAcE (PDB) determined by GC–MS analysis

RT	Name of compound	MF[a]	MW[b] g/mol	% Area	Category	Biological activity	Reference
8.65	1-Undecanol	$C_{11}H_{24}O$	172.3	0.73	Fatty alcohol	Antibacterial activity	Togashi et al. (2007)
12.11	6-Dodecene	$C_{12}H_{24}$	168.31	2.68	Unsaturated aliphatic hydrocarbon	–	–
13.86	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy) tetrasiloxane	$C_{18}H_{52}O_7Si_7$	577.2	9.67	Alkane	Like CNS stimulant	Khalid et al. (2019)
14.39	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206.32	13.83	Alkylated phenol	Antioxidant and cytotoxic activity	Zhao et al. (2020)
15.13	Cyclotridecane	$C_{13}H_{26}$	182.35	4.56	Alkane	–	–
16.23	2,4-Dihydroxybenzoic acid, 3TMS derivative	$C_{16}H_{30}O_4Si_3$	370.66	6.04	Fatty acid	–	–
17.81	Heptacos-1-ene	$C_{27}H_{54}$	378.7	9.8	Alkene	–	–
19.14	Phthalic acid, 5-methylhex-2-yl isobutyl ester	$C_{19}H_{28}O_4$	320.4	6.54	Ester	–	–
20.23	9-Hexacosene	$C_{26}H_{52}$	364.7	14.83	Alkene	Analgesic, Antiinflammatory, Antinociceptive	Kuriuki et al. (2012)
21.31	Vinyl 2-ethylhexanoate	$C_{10}H_{18}O_2$	170.25	7.06	Ester	–	–
22.44	n-Tetracosanol-1	$C_{24}H_{50}O$	354.7	9.87	Fatty alcohol	Anticancer	Vergara et al. (2015)
24.46	Octatriacontyl pentafluoropropionate	$C_{41}H_{77}F_5O_2$	697	6.55	Hydrocarbons	Anti covid 19	H Elwakil et al. (2021)
26.33	17-Pentatriacontene	$C_{35}H_{70}$	490.9	7.85	Hydrocarbons	Anticancer Antibacterial	Kumar et al. (2018)

[a]Molecular formula

[b]Molecular weight

are ionized and move through mass analysers. GC–MS is an excellent technique for identifying molecules/compounds, especially of low molecular weight that are both thermally stable and sufficiently volatile in nature.

GC–MS analysis of the purified compounds ratified the presence of various phytochemical groups with reported anticancer, antibacterial, anti-COVID-19 and other activities. The purified extract's highest chemical group was alkene (25%), followed by hydrocarbons (17%), alkane (14%), phenol (14%), ester (13%), fatty alcohol (11%) and fatty acid (6%) (Fig. 4c).

The most important compound detected from the purified extract of EtOAcE (PDB) of EHL2 was 2,4-di-tert butyl phenol (2,4-DTBP) (Fig. 4d), a volatile phenolic compound (VPC). Compounds having aromatic rings in combination with tert-butyl groups, like 2,4-di-tert butyl phenols, are rather rare and have considerable antioxidant, and remarkable cytotoxic activity (Varsha et al. 2015). Besides, it is widely utilized in pharmaceuticals, fragrances and the plastic industry (Dembitsky 2006). 2,4-DTBP is reported to be present in fruits and seeds, which are responsible for antioxidant properties and act as an antioxidant agent (Varsha et al.2015). Moreover, 2,4-DTBP isolated from a fungal endophyte, *Daldinia eschscholtzii*, inhibits the quorum-sensing activity of *Pseudomonas aeruginosa* (Mishra et al.2020).

A recent review advocates that 2,4-DTBP is a common toxic compound that is produced by at least 169 species of microorganisms, plants and animals, including fungi (11 species), two of which are *Colletotrichum gloeosporioides* and *Fusarium tricinctum* (Zhao et al. 2020). Generally, anticancer activity in the cell line can be correlated with the antioxidant activity of the tested compounds (Grigalius and Petrikaite 2017). In this order, 2,4-DTBP displayed considerable anticancer activity with 10 µg/mL IC50 value against HeLa cells (Zhao et al. 2020). Based on the present and earlier reports, 2,4-DTBP has been proven to be a natural source of an antioxidant compound with very good activity.

Conclusion

The results presented in this study have demonstrated that the EtOAcE (PDB) exhibited the highest amount of TPC and TFC and was positively correlated with antioxidant activity. The antioxidant activity of the purified extract estimated by ABTS model reveals the presence of some phenolic compounds that have almost similar activity to quercetin, which

has been ratified by the TLC bioautography result at R*f* 0.18. GCMS analysis of the purified extract detected only a single antioxidant compound, 2,4-DTBP, and it may be due to the limitation of GCMS, which is able to detect predominantly non-polar compounds. For the complete detection of antioxidant compounds present in the purified extract, LCMS analysis can be done in future research. The toxicity of the purified extract with its antioxidant activity should be tested to corroborate its safety for use as a food additive. However, the work on complete purification, identification and mechanism of antioxidant compounds present at R*f* 0.18 is in progress. The results suggest that *Nigrospora sphaerica* is a potential source of natural antioxidants. This is the first elaborative and exclusive report on endophytic *N. sphaerica* isolated from *Euphorbia hirta.*

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Author contributions VSG conceived the research, analysed the data and wrote the manuscript, and all the co-authors (AS, PK, JHN, JK, MY, RB, PP) contributed to conceptualization, experimental work, data collection and manuscript preparation, while RNK helped in conceptualization, provided the consumables and corrected the manuscript.

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Availability of data and material All data associated with the work are mentioned in the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Consent for publication All the authors read and are aware of publishing of the manuscript.

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