DOI: 10.2478/s11686-014-0307-2

Acta Parasitologica, 2014, 59(4), 745–757; ISSN 1230-2821

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# A search for antiplasmodial metabolites among fungal endophytes of terrestrial and marine plants of southern India

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## Abstract

Eighty four different fungal endophytes isolated from sea grasses (5), marine algae (36) and leaves or barks of forest trees (43) were grown *in vitro* and the secondary metabolites secreted by them were harvested by immobilizing them on XAD beads. These metabolites were eluted with methanol and screened using SYBR Green I assay for their antiplasmodial activity against blood stage *Plasmodium falciparum* in human red blood cell culture. Our results revealed that fungal endophytes belonging to diverse genera elaborate antiplasmodial metabolites. A *Fusarium* sp. (580, $IC_{50}$: 1.94 μg $ml^{-1}$) endophytic in a marine alga and a *Nigrospora* sp. (151, $IC_{50}$: 2.88 μg $ml^{-1}$) endophytic in a tree species were subjected to antiplasmodial activity-guided reversed phase high performance liquid chromatography separation. Purification led to potentiation as reflected in $IC_{50}$ values of 0.12 μg $ml^{-1}$ and 0.15 μg $ml^{-1}$ for two of the fractions obtained from 580. Our study adds further credence to the notion that fungal endophytes are a potential storehouse for a variety of novel secondary metabolites vested with different bioactivities including some that can stall the growth of the malaria parasite.

## Keywords

Endophytes, antiplasmodial metabolites, *Plasmodium falciparum*, antimalarial compounds, marine-derived fungi

## Introduction

Malaria poses a major health threat in developing countries and annually more than 200 million new malaria cases are reported (WHO 2011), emphasising the urgent need for new drugs against malaria. In India, its management is challenging due to the country's huge population and large geographical area further compounded by rapid urbanisation (Shah *et al.* 2011) as well as the resistance developed by the malarial parasite to most of the existing classes of antimalarial drugs (Anderson 2009). Although artemisinin, a sesquiterpene lactone of the Chinese herb *Artemisia annua* L., and its synthetic analogues are very effective in controlling drug-resistant malaria (White 2008), a recent report indicates the emergence of artemisinin-resistant malarial parasite strains in western Cambodia and western Thailand (Cheeseman *et al*. 2012). Such wide spectrum drug resistance and the possibility of the existence of regional subpopulations of malarial parasites differing in sensitivity to different classes of drugs necessitates continuing the search for novel antiplasmodial pharmacophores for short- and long-term effective control of malaria (Anderson 2009). Natural products with antiplasmodial activity appear to be superior to purely synthetic ones because (a) natural metabolites have better prospects of becoming drugs (Ortholand and Ganesan 2004) and (b) the genes coding for these metabolites can be cloned and overexpressed to obtain appreciable quantities of chemically complex metabolites like artemisinin at affordable costs (Westfall *et al.* 2012; Paddon *et al.* 2013). In this context, endophytes, an ecological group of fungi may be good sources of antiplasmodial metabolites.

Endophytes are mostly ascomycetous fungi which reside inside living tissues of all major groups of plants without producing any visible disease symptoms. Endophytes of both land (Suryanarayanan *et al.* 2009; Weber 2009) and marine (Suryanarayanan *et al.* 2010; Aly *et al.* 2011) plants produce a vast array of secondary metabolites exhibiting diverse bioactivities including antimicrobial, antifungal, free radical scavenging, cytotoxic and anti-insect activities. Based on *ex vivo* inhibition of growth of *Plasmodium falciparum* in human red blood cell cultures, we report antiplasmodial activity ($IC_{50}$: <10 mg/ml) in 19 of the 84 endophyte isolates screened by us. Although some antimalarial compounds have been reported

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to be produced by a few free living marine fungi (Chinworrungsee *et al.* 2001; Wright and Lang-Unnasch 2005), here we report that marine endophytes are also a potential source of antiplasmodial compounds.

## Materials and Methods

### Endophyte isolates used

We have used for our investigations of endophytes, five isolates recovered from seagrasses, 36 isolates from marine algae (both from the coast of Rameswaram, Tamil Nadu) and 43 isolates from leaves or barks of trees growing in the forests of the Western Ghats. All these isolates were maintained in Vivekananda Institute of Tropical Mycology (VINSTROM), Ramakrishna Mission Vidyapith, Chennai. The endophytes were isolated by surface sterilising the plant tissues with ethanol and sodium hypochlorite (Suryanarayanan *et al.* 2010, 2011; Murali *et al.* 2013). The following isolates have been deposited at the National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune [*Sordaria* sp. 1 (65) – Accession Number (AC) 10342, *Curvularia lunata* (93) – (AC) 10334, *Pestalotiopsis* (455) – (AC) 10336, *Curvularia tuberculata* (516) – (AC) 2434] and Institute of Microbial Technology (IMTECH), Chandigarh, India [*Nigrospora oryzae* (151) – (AC) 8660, *Pithomyces maydicus* (152) – (AC) 8657, *Chaetomium* sp. 1 (154) – (AC) 8658, *Aspergillus terreus* (543) – (AC) 10346, *Phomopsis* sp. (562) – (AC) 10345, *Curvularia* sp. (566) – (AC) 10337, *Trichoderma* sp. (568) – (AC) 10344, *Drechslera papendorphii* (589) – (AC) 10338, *Colletotrichum* sp. (599) – (AC) 1033 and *Curvularia tuberculata* (606) – (AC) 10340].

### Extraction of secondary metabolites

Each fungus was grown as a static culture in 60 ml potato dextrose broth contained in a 250 ml flask along with XAD™ 16 amberlite™ resin (Rohm and Hass, Philadelphia, PA, USA) (400 mg /flask) at 26°C for 21 days. XAD-16 amberlite is a polystyrene resin that adsorbs small molecular weight metabolites produced by fungi and facilitates their easy extraction (Suryanarayanan *et al*. 2010). Three replicates were maintained for each fungus. After incubation, the mycelium was filtered and the XAD from all the three flasks was combined, washed in distilled water and air dried. These dried XAD beads were shaken with 12 ml methanol (in a 15 ml Falcon tube) for 45 min at 25°C. Aliphatic alcohols like methanol exhibit unique abilities to extract diverse molecules of widely different polarities. Thus methanol can extract polar, amphipathic and also fairly non polar molecules. Methanol's economics and relatively low boiling point also encourages the use of this solvent for the first round of extraction whose aim is to capture molecules of diverse polarities. Supernatants were collected by decantation. This process was repeated thrice resulting in a total volume of 36 ml per sample. Methanol was evaporated at 40°C in a Labconco RapidVap parallel evaporation system. The residue was dissolved in 0.2 to 0.4 ml of methanol, transferred to pre-weighed microfuge tubes and subjected to evaporation to dryness.The weight of each elute was determined (Table I) before making stock solutions (25 mg/ml) in dimethyl sulfoxide (DMSO).

### *In vitro* cultivation of *Plasmodium falciparum*

Chloroquine-sensitive 3D7 strain of *P. falciparum* (*Pf*3D7) was maintained in continuous culture following the method of Trager and Jensen (1976) with minor modifications. Cultures were maintained in fresh $O^{+ve}$ human erythrocytes suspended at 4% (v/v) haematocrit in complete medium {16.2 g/L RPMI 1640 (Sigma) containing 25 mM HEPES, 11.11 mM glucose, 0.2% sodium bicarbonate (Sigma), 0.5% Albumax I (Gibco), 45 µg/litre hypoxanthine (Sigma) and 50 µg/litre gentamicin (Gibco)} and incubated at 37°C in a gas mixture consisting of 5% $O_2$, 5% $CO_2$, and 90% $N_2$. The spent medium was replaced with fresh complete medium every day to propagate the culture. Giemsa-stained blood smears were examined microscopically to monitor parasitemia. Synchronized ring stage parasite was obtained by 5% sorbitol (w/v) treatment (Lambros and Vanderberg 1979).

### Drug/Crude extract dilutions

Stock solutions of XAD extracts were prepared in DMSO and that of Chloroquine phosphate (CQ) (Sigma) was prepared with Milli-Q grade water. The required drug concentrations were achieved by diluting the stocks with culture medium. The solutions of drugs and extracts were placed in 96-well flat bottom tissue culture grade plates (Corning).

### Assay for anti-plasmodial activity

For drug screening, SYBR green I based fluorescence assay was used (Smilkstein *et al.* 2004, Bennet *et al*. 2004). The ability of SYBR green to give strong fluorescence only in the presence of DNA forms the basis for its use to assess cell proliferation. The absence of nucleus in human red blood cells where the malarial parasite proliferates allows the use of SYBR green for the specific monitoring of the growth of malarial parasite. However, caution is required since this fluorescence-based method is prone to quenching effects from the test samples which yield (antiplasmodial) false positives. In order to be not misled by such effects, each of the extracts which showed promise in the SYBR green assay was validated using Giemsa stained microscopy based analysis. Conversely, fluorescent molecules in test extracts can in principle lead to enhanced fluorescence even as these extracts may be antiplasmodial in action. However such cases of false negatives

**Table I.** Antiplasmodial potencies, mammalian cell cytotoxicities and selectivity indices of endophytic fungal secretions

| Fungus | Extract ID | Strain isolated from # | Recovery (mg/400 mg XAD) | *Pf3D7* $IC_{50}$ µg $ml^{-1}$ | HeLa $TC_{50}$ µg $ml^{-1}$ | Selectivity Index $IC_{50}/TC_{50}$ |
|---|---|---|---|---|---|---|
| Uninoculated growth medium | Control | | 18 | >100 | | |
| *Alternaria* sp. | 572 | *Sargassum wightii* (A*) | 16.53 | 5 | 44 | 8.8 |
| *Alternaria* sp. | 590 | *Turbinaria* sp. (A*) | 16.52 | 5.2 | 67 | 12.9 |
| *Alternaria* sp. | 592 | *Turbinaria* sp. (A) | 31.47 | 16 | | |
| *Aspergillus terreus* | 526 | *Caulerpa sertularioides* (A) | 40.54 | 43 | | |
| *Aspergillus terreus* | 543 | *Ulva lactuca* (A) | 46.01 | 37 | | |
| *Aspergillus niger* | 544 | *Ulva lactuca* (A*) | 17.64 | 1.2 | 25 | 20 |
| *Aspergillus* sp. | 551 | *Halimeda macroloba* (A) | 14.59 | 18 | | |
| *Aspergillus* sp. 2 | 650 | *Padina tetrastromatica* (A) | 34.51 | 100 | | |
| *Aspergillus janus* | 581 | *Sargassum wightii* (A) | 14.49 | 24 | | |
| *Aspergillus* sp. 4 | 597 | *Turbinaria* sp. (A) | 26.41 | 26 | | |
| *Chaetomium* sp. | 542 | *Gracilaria edulis* (A*) | 11.25 | 2.6 | 35 | 13.5 |
| *Chaetomium* sp. | 548 | *Ulva lactuca* (A*) | 19.78 | 4.8 | 77 | 16 |
| *Chaetomium* sp. | 558 | *Padina tetrastromatica* (A) | 6.91 | 85 | | |
| *Chaetomium* sp. | 628 | *Grateloupia lithophila* (A) | 3.65 | 15 | | |
| *Cladosporium* sp. | 520 | *Caulerpa racemosa* (A) | 7.97 | >100 | | |
| *Cladosporium* sp. | 564 | *Portieria hornemannii* (A) | 7.98 | 21 | | |
| *Curvularia* sp. | 566 | *Sargassum wightii* (A) | 31.01 | 40 | | |
| *Curvularia lunata* | 602 | *Sargassum* sp. (A) | 12.76 | 14 | | |
| *Curvularia tuberculata* | 606 | *Turbinaria* sp. (A) | 18.81 | 44 | | |
| *Colletotrichum* sp. | 599 | *Sargassum* sp. (A) | 4.66 | 45 | | |
| *Drechslera* sp. | 589 | *Turbinaria* sp. (A) | 11.46 | 39 | | |
| *Drechslera* sp. | 610 | *Turbinaria* sp. (A) | 6.71 | 40 | | |
| *Emericella nidulans* | 560 | *Halymenia* sp. (A) | 9.36 | 36 | | |
| *Fusarium* sp. | 580 | *Sargassum wightii* (A*) | 42 | 2 | 72 | 36 |
| *Memnoniella* sp. | 648 | *Portieria hornemannii* (A) | 26.39 | 60 | | |
| *Nigrospora* sp. | 546 | *Ulva lactuca* (A) | 15.65 | 17 | | |
| *Nigrospora* sp. | 594 | *Turbinaria* sp. (A) | 2.9 | 13 | | |
| *Paecilomyces* sp. | 534 | *Caulerpa scalpelliformis* (A*) | 14.73 | 9.7 | 70 | 7.2 |
| *Penicillium* sp. | 536 | *Caulerpa scalpelliformis* (A) | 51 | 43 | | |
| *Penicillium* sp. | 552 | *Halimeda macroloba* (A*) | 8.43 | 4.8 | >100 | >20 |
| *Pestalotiopsis* sp. | 574 | *Sargassum wightii* (A) | 7.54 | 31 | | |
| *Phoma* sp. | 605 | *Sargassum* sp. (A) | 8.56 | 25 | | |
| *Phomopsis* sp. | 562 | *Portieria hornemannii* (A) | 12.58 | 39 | | |
| *Pithomyces* sp. | 604 | *Sargassum* sp. (A) | 11.74 | 46 | | |
| *Trichoderma* sp. | 568 | *Sargassum wightii* (A*) | 25.13 | 1.56 | 100 | 64.1 |
| UNH-1 | 649 | *Padina* sp. (A) | 23.73 | 34 | | |
| *Alternaria* sp. | 105 | *Bridelia retusa* (E) | 25.97 | 21 | | |
| *Arthrinium* sp. | 87 | *Elaeocarpus serratus* (E) | 15.34 | >100 | | |
| *Auerobasidium pullulans* | 223 | *Spinifex littoreus* (E) | 14.74 | 71 | | |
| *Botrytis* sp. | 69 | *Erythroxylon monogynum* (E) | 38.74 | 100 | | |
| *Chaetomium* sp. | 154 | *Phoebe lanceolata* (E) | 18.38 | 22.5 | | |
| *Chaetomium* sp. | 672 | *Phoebe lanceolata* (E*) | 19.4 | 3.5 | 80 | 22.9 |
| *Cladosporium* sp. | 670 | *Bridelia retusa* (E) | 5.74 | >100 | | |
| *Cladosporium* sp. | 675 | *Elaeocarpus serratus* (E) | 8.17 | >100 | | |
| *Curvularia lunata* | 93 | *Ilex wightiana* (E) | 5.43 | >100 | | |
| *Curvularia* sp. | 100 | *Macaranga peltata* (E) | 24.21 | 31 | | |

| Fungus | Extract ID | Strain isolated from # | Recovery (mg/400 mg XAD) | *Pf3D7* $IC_{50}$ µg $ml^{-1}$ | HeLa $TC_{50}$ µg $ml^{-1}$ | Selectivity Index $IC_{50}/TC_{50}$ |
|---|---|---|---|---|---|---|
| *Curvularia* sp. | 104 | *Rhizophora mucronata* (E) | 4.42 | 12.5 | | |
| *Curvularia* sp. | 456 | *Acacia leucophloea* (E*) | 27.21 | 2.5 | 50 | 20 |
| *Curvularia eragrostidis* | 669 | *Litsea floribunda* (E) | 14.25 | 78 | | |
| *Colletotrichum* sp. | 85 | *Catharanthus roseus* (E) | 12.02 | 97 | | |
| *Colletotrichum gloeosporioides* | 90 | *Cinnamomum wightii* (E) | 14.84 | 14 | | |
| *Corynespora* sp. | 668 | *Kydia calycina* (E*) | 8.11 | 5 | >100 | >20 |
| *Corynespora* sp. | 673 | *Murraya paniculata* (E) | 4.76 | >100 | | |
| *Drechslera* sp. | 83 | *Catharanthus roseus* (E) | 18.64 | 20 | | |
| *Fusarium* sp. | 78 | *Azadirachta indica* (E) | 13.6 | 75 | | |
| *Fusarium* sp. | 204 | *Euphorbia tithymaloides* (E) | 5.02 | 28 | | |
| *Fusarium* sp. | 217 | *Vitex altissima* (E*) | 3.79 | 1.56 | 30 | |
| *Fusarium* sp. | 467 | *Semecarpus anacardium* (E) | 3.97 | 45 | | |
| *Fusarium* sp. | 674 | *Murraya paniculata* (E) | 4.91 | >100 | | |
| *Lasiodiplodia theobromae* | 454 | *Bridelia retusa* (E) | 7.49 | 49 | | |
| *Lasiodiplodia theobromae* | 671 | *Randia dumetorum* (E) | 9.37 | 80 | | |
| *Nigrospora oryzae* | 76 | *Cryptocarya bourdillonii* (E) | 3.68 | >100 | | |
| *Nigrospora oryzae* | 97 | *Daphniphyllum neilgherrense* (E) | 6.09 | >100 | | |
| *Nigrospora oryzae* | 151 | *Macaranga peltata* (E*) | 21.97 | 3 | >100 | >33 |
| *Nodulisporium* sp. | 443 | *Syzygium densiflorum* (E) | 5.52 | 77 | | |
| *Nodulisporium* sp. | 95 | *Glochidion* sp. (E) | 12.03 | 75 | | |
| *Penicillium* sp. | 141 | *Phyllanthus emblica* (E) | 12.44 | >100 | | |
| *Pestalotiopsis* sp. | 117 | *Litsea stocksii* (E) | 4.75 | >100 | | |
| *Pestalotiopsis* sp. | 455 | *Bridelia retusa* (E) | 16.06 | 75 | | |
| *Phomopsis* sp. | 676 | *Murraya paniculata* (E) | 12.78 | 11 | 52 | 4.8 |
| *Phomopsis* sp. | 677 | *Lagestroemia microcarpa* (E*) | 10.67 | 8 | 100 | 12.5 |
| *Pithomyces* sp. | 152 | *Eurya nitida* (E) | 11.3 | >100 | | |
| *Phyllosticta capitalensis* | 666 | *Maytenus emarginata* (E) | 7.82 | >100 | | |
| *Sordaria* sp. | 65 | *Eurya nitida* (E) | 13.86 | >100 | | |
| *Xylaria* sp. | 123 | *Anogeissus latifolia* (E) | 12.32 | >100 | | |
| *Xylaria* sp. | 184 | *Randia dumetorum* (E) | 13.4 | >100 | | |
| *Xylaria* sp. | 195 | *Ziziphus jujuba* (E) | 44.26 | 81 | | |
| *Xylaria* sp. | 315 | *Butea monosperma* (E) | 8.85 | >100 | | |
| *Xylaria* sp. | 667 | *Lasianthus venulosus* (E) | 23.74 | 3.8 | <50 | <13 |
| *Aphanocladium* sp. | 508 | *Thalassia* sp. (Sg) | 9.41 | 97 | | |
| *Auerobasidium pullulans* | 585 | *Thalassia* sp. (Sg) | 20.16 | 93 | | |
| *Curvularia tuberculata* | 516 | *Syringodium* sp. (Sg) | 15 | 33 | | |
| *Gonatophragmium mori* | 622 | *Thalassia* sp. (Sg) | 8.13 | 37 | | |
| *Memnoniella* sp. | 647 | *Cymodocea serrulata* (Sg) | 30.45 | 67 | | |

# A = marine algal endophytes, E = tree endophytes, Sg = seagrass endophytes, * indicates strains selected for sequencing of fungal ITS region

were not observed in the extracts studied by us. Sorbitol-synchronized ring stage parasites (haematocrit: 2%, parasitaemia: 1%, 100 µl) under normal culture conditions were incubated in the presence or absence of increasing concentrations of the extracts. It is important to note that use of synchronized cultures over mixed stage cultures is necessary for (a) consistency in results across laboratories, (b) use of ring stage synchronized cultures can enable the test molecules to interact with all the three stages (ring, trophozoite and schizont) of the 48 hrs long life cycle of *P. falciparum* in culture, (c) starting the experiment with synchronized ring stage culture provides the distinct advantage of observing growth inhibitory effects without a rise in parasitemia during the ring-trophozoite-schizont transitions and (d) the choice of ring stage synchronized cultures is also governed by the procedural ease (5% Sorbitol treatment which destabilizes both trophozoites and schizonts

without affecting ring stage cells) with which pure rings can be obtained against the elaborate Percoll gradient based centrifugal procedures required to obtain either the trophozoites or the schizonts.

CQ was used as positive control and 0.4% DMSO (v/v), which was found to be non-toxic to the parasite, was used as vehicle control. After 48 h of incubation, 100 µl of SYBR Green I buffer [0.2 µl of 10,000 × SYBR Green I (Invitrogen) per ml of lysis buffer {Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%; wt/vol), and Triton X-100 (0.08%; vol/vol)}] was added to each well, mixed twice gently with multi-channel pipette and incubated in the dark at 37°C for 1 h. Fluorescence was measured using a Victor fluorescence multi-well plate reader (Perkin Elmer) with excitation and emission at 485 and 530 nm, respectively. Fluorescence counts for CQ (0.1 µM for *Pf*3D7) were deducted from counts in each well. A dose–response curve was constructed by plotting fluorescence counts against the drug concentration and $IC_{50}$ (dose of a drug required to slow the rate of growth of a cell population by 50%) was determined. In the experiments described by us, no drug control corresponds to 100 % growth while 100 nM Chloroquine (sufficient to cause total arrest of growth) corresponds to 0 % growth. Between these two boundaries of 100% and 0% growth lie the points where at different concentrations of test samples, the parasite shows different extents of growth. The graph depicting growth inhibition as a function of concentration of test sample is used to obtain $IC_{50}$. Giemsa-stained smears of extract-treated parasite cultures were visualized microscopically to validate the results from fluorescence-based assay.

**Cytotoxic activity of extracts on HeLa cells using MTT assay**

The cytotoxic effects of active extracts on mammalian cells was determined by a functional assay (Mosmann 1983) using HeLa/L929 cells cultured in complete medium containing 16.2 g/l RPMI 1640, 10% fetal bovine serum, 0.21% sodium bicarbonate (w/v) (Sigma) and 50 µg/mL gentamycin. Cells ($10^4$ cells/200 µl/well) were seeded into 96-well flat-bottom tissue culture plates in complete medium. After 24 h of seeding, test solutions were added and cells incubated for 48 h in a humidified atmosphere at 37°C and 5% $CO_2$. DMSO (as positive inhibitor) was added at 10% v/v. Twenty microlitres of a stock solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg/mL in 1X phosphate buffered saline) was added to each well, gently mixed and incubated for another 4 hr. After centrifugation at 300 g for 5 min, the supernatant was removed and 100 µl DMSO (quench agent) was added to the cell pellet. Formazan formation was read using a microtiter plate reader (VersaMax tunable multiwell plate reader) at 570 nm. The 50% cytotoxic concentration ($TC_{50}$) of drug was determined by analysis of dose–response curves. Selectivity index ($TC_{50}/IC_{50}$) was also calculated for the different extracts studied.

**Bioassay Guided Fractionation of Potent Extracts**

Following the screening of 84 different fungal XAD extracts, the extracts of isolate numbers 151 and 580 which showed promising antiplasmodial potency ( $IC_{50} \leq 3$ µg/ml ) and selectivity indices (>30) were chosen for antiplasmodial activity guided reversed- phase HPLC fractionation. Waters C18, Deltapak (19 × 300 mm, 15µ) column was used for fractionation using methanol water gradient (1%/min) at a flow of 10 ml/min on Gilson prep HPLC system. Dual wavelength detection at 214 nm (σ-σ* transitions shown by several aliphatic molecules) and 254 nm (π-π* transition shown by aromatic molecules) is helpful in assessing the chemical nature of molecules eluting from the column. Fractions were collected, dried, weighed and analyzed for antiplasmodial and cytotoxic activity.

**Fungal DNA extraction**

The taxonomic affiliation of seven fungal isolates which showed relatively high antiplasmodial activity was confirmed by sequencing of ITS1-5.8S-ITS2 region. Genomic DNA was extracted from pure cultures of fungal endophytes following the phenol-chloroform extraction method. For this, cultures were grown on potato dextrose agar plates for 7 days and mycelia were collected and transferred to a sterile microfuge tube containing 500 µL of DNA extraction buffer (0.1 M NaCl, 50 mM Tris, 10 mM $Na_2EDTA$, 2% SDS, pH 8.0). The samples were then ground well and to this emulsion, 500 µL of chilled phenol was added and centrifuged at 15,300g for 15 min at 4°C. The upper aqueous phase was transferred to a fresh tube and an equal volume of chilled chloroform: isoamylalcohol (24:1 v/v) was mixed to get a suspension. The tubes were centrifuged at 15,300g for 15 min-at 4°C and the clear aqueous phase was transferred to a fresh microfuge tube. After adding chilled isopropanol (0.6 volume) and 3 M sodium acetate (0.1 volume), the contents were gently mixed and incubated at –80°C for 2 h followed by centrifugation at 15,300g for 15 min at 4°C. The supernatant was discarded and the pellet was washed with 200 µL of 70% ethanol (v/v) followed by centrifugation at 15,300g for 10 min at 4°C. After discarding the supernatant, the pellet was air-dried, dissolved in 50 µL of sterile distilled water and stored at –20°C. The genomic DNA was electrophoresed in 0.8% agarose gel (w/v) and then diluted suitably for amplifying the nuclear ITS region.

**PCR amplification and sequencing of fungal ITS region**

The nuclear ribosomal internal transcribed spacers (ITS) along with 5.8S region were PCR amplified using primer sets ITS5 and ITS4 or ITS1F and ITS4 (White *et al.* 1990; Gardes and Bruns 1993). A 25 µL amplification reaction consisted of 2.5 µL dNTPs (10 µM), 2.5 µL PCR buffer (10X), 1 µL primers (forward and reverse – 10 µM each), 1 µL Taq DNA Polymerase (1 U), 1 µL DMSO (1%, v/v), 1 µL $MgCl_2$ (25 mM) and 1 µL fun-

gal genomic DNA as template (approx. 25–50 ng). Cycling reactions were performed in a Master Cycler Thermocycler (Eppendorf, USA) with following conditions: 94 ºC for 3 min, followed by 34 cycles of 94 ºC for 30 s, 54 ºC for 30 s, 72 ºC for 1 min; and finally 72 ºC for 10 min. The amplified products were run on 1% agarose gel (w/v) to determine the size and purity of amplicons. The purified PCR products were then sequenced in an automated sequencer (ABI 3130 Genetic Analyzer). The sequences obtained were manually edited before performing a BLAST search using the megablast algorithm to arrive at the nearest match. The results were used to confirm the identity of fungal morphotypes at species level wherever possible. The nucleotide sequence data obtained were deposited in GenBank database (Accession numbers KF135618-KF135624).

### Phylogenetic analysis

The nuclear ITS1-5.8S-ITS2 sequences obtained for different endophytes isolated in the present study were aligned with 250 related sequences available in the GenBank database based on the results obtained from BLAST search. The sequences were then aligned by ClustalW (Thompson *et al.* 1994) using the default settings and manually re-edited to build an initial neighbor-joining analysis. From this dataset, we selected 89 closely related sequences to perform the final analysis. The neighbour joining method of Saitou and Nei (1987) was used to construct the evolutionary tree from the datasets by using MEGA version 5.1 (Tamura *et al.* 2011). The evolutionary distance was calculated using the Maximum Composite Likelihood method and branch support was calculated with average distances from 1,000 bootstrap replicates (Felsenstein 1985). All gaps and missing data in the dataset were eliminated for this analysis.

## Results

### Antiplasmodial potencies, mammalian cell cytotoxicities and selectivity indices of endophytic fungal secretions

Sixteen endophyte isolates belonging to twelve different fungal genera (*Alternaria*, *Aspergillus*, *Chaetomium*, *Curvularia*, *Corynespora*, *Fusarium*, *Nigrospora*, *Paecilomyces*, *Penicillium*, *Phomopsis*, *Trichoderma* and *Xylaria*) which were isolated from marine algae and angiosperm trees, representing about 19% of the 84 fungi screened, showed high ($IC_{50}$<10 µg/ml ) antiplasmodial activity (Table I). The antiplasmodial $IC_{50}$ potency profile of the remaining XAD elutes was 11–25 µg/ml (18% isolates), 26–50 µg/ml (21% isolates), 51–100 µg/ml (19% isolates) (Fig. 1). The growth curves obtained using the SYBR Green assay for two representative XAD extracts viz. 151 ($IC_{50}$ 2.88 µg/ml) and 580 ($IC_{50}$ 1.94 µg/ml) together with Giemsa stained smears for control and treated samples are shown in Fig. 2. The correspondence between % growth as scored in SYBR Green assay and % parasitemia in microscopic images provides validity to the results obtained from the SYBR Green assay. Furthermore, the extracts which showed $IC_{50} \leq 11$ µg/ml were analysed for their selectivity against *Plasmodium* over mammalian cells. Most of these extracts were found to be nontoxic with selectivity index of >10 (Table I).

### Antiplasmodial activity guided RPHPLC fractionation of extracts 151 and 580

The extracts of *Fusarium* sp. (580) endophytic in a marine alga and *Nigrospora* sp. (151) endophytic in a tree species which showed the best combination of the features of high yield

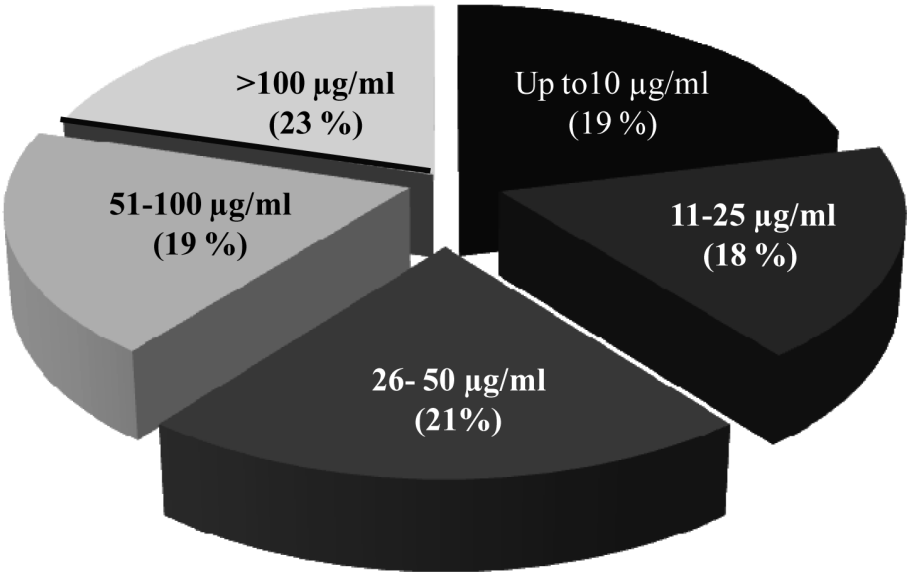



**Fig. 1.** Frequency of fungal endophytes displaying different antiplasmodial potencies as $IC_{50}$ in µg $ml^{-1}$ obtained after screening eighty four different fungal secretions captured on XAD resin. The relative percentage of endophyte strains within each potency range is indicated in parentheses

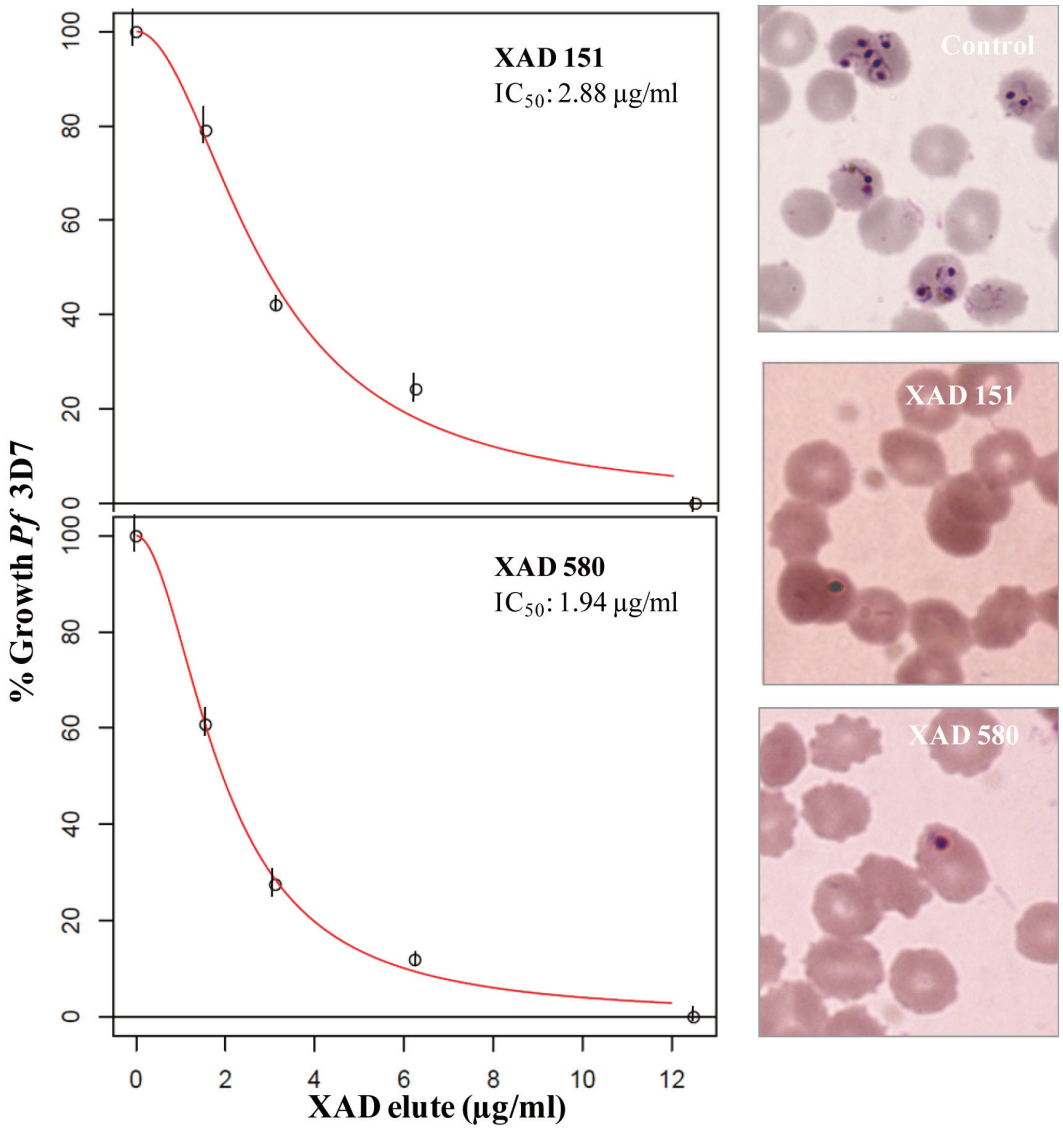
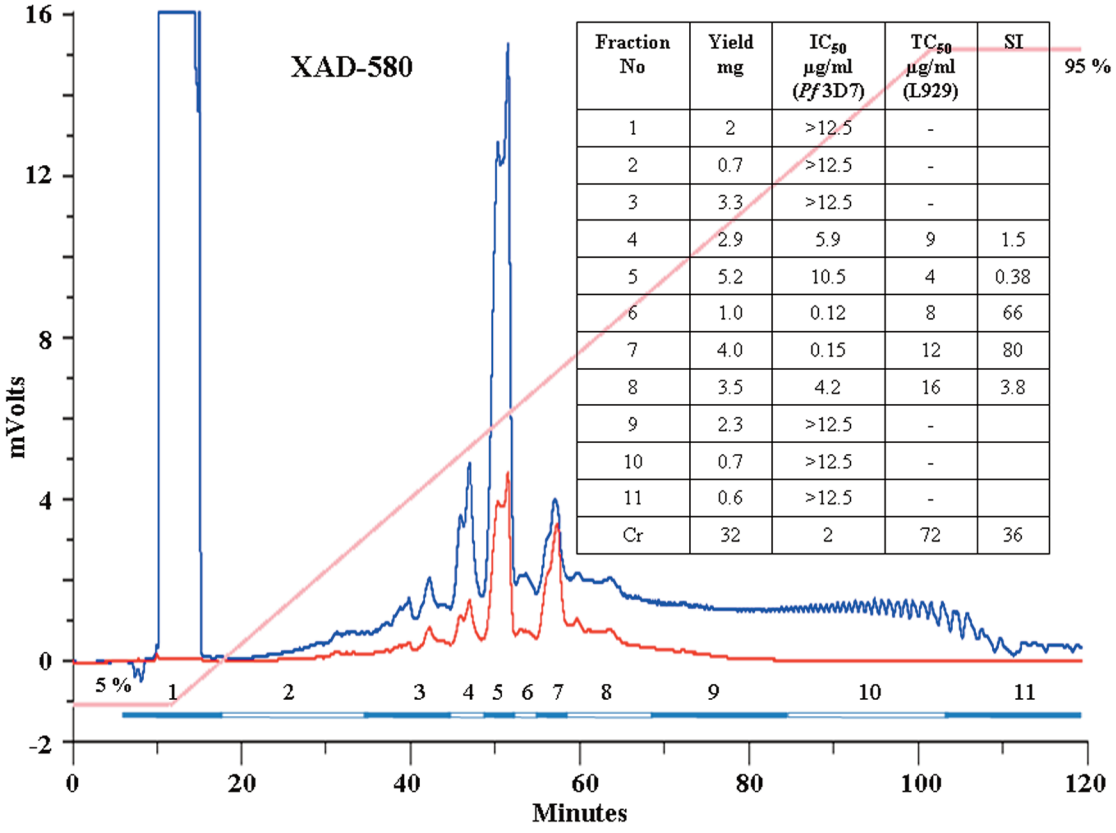



**Fig. 2.** *P. falciparum* 3D7 was *ex vivo* cultured for 48 hrs in human red blood cells in absence and presence of increasing concentrations of XAD extracts of 151 and 580 respectively. Graphs show % growth measured by SYBR Green fluorescence assay as a function of increasing concentrations of extracts. Each data point represents the mean +/- standard deviation of three independent observations. ICEstimator-version 1.2 (http://www.antimalarial-icestimator.net/MethodIntro.htm) was used for plotting the graph and obtaining the $IC_{50}$ values. Microscopic images of Giemsa stained smears of untreated vs XAD extract (taken at $IC_{100}$) treated cultures are shown. The high ring stage parasitemia in control vs the low moribund trophozoite stage parasitemia in extract treated cultures provides validity to the SYBR Green results

(weight of metabolites obtained on elution from XAD beads), antiplasmodial potency and selectivity index were subjected to RPHPLC based activity guided fractionation. As shown in Fig. 3 and Fig. 4, the fractionation of crude extracts led to identification of fractions with high activity. A marked increase in potencies of crude ($IC_{50}$: 2 µg $ml^{-1}$) vs fractions 6 & 7 ($IC_{50}$: 0.12 µg $ml^{-1}$ and 0.15 µg $ml^{-1}$) was observed in the case of 580 (Fig. 4).

**Phylogenetic study**

The seven representative fungal isolates which showed low $IC_{50}$ values ($IC_{50}$<4.8 µg $ml^{-1}$) were selected and studied using molecular techniques to infer their phylogeny. The ITS region was amplified and sequenced using fungal specific primers and the sequences obtained were compared with GenBank (using megablast algorithm) and CBS databases to identify the closest matching sequences (Table II). The search results showed that the seven isolates belonged to six different genera namely, *Alternaria*, *Chaetomium*, *Curvularia*, *Fusarium*, *Nigrospora* and *Trichoderma*. However, we found that the top match with reference to species names from two different databases varied for three strains (strains 456, 151, 590). Isolate 580 (marine algal endophyte) showed 100% match with *Fusarium oxysporum* f. sp. *aechmeae* and isolate **151** (tree endophyte) showed a 100% match with *Nigrospora sphaerica* (Table II). The discrep-



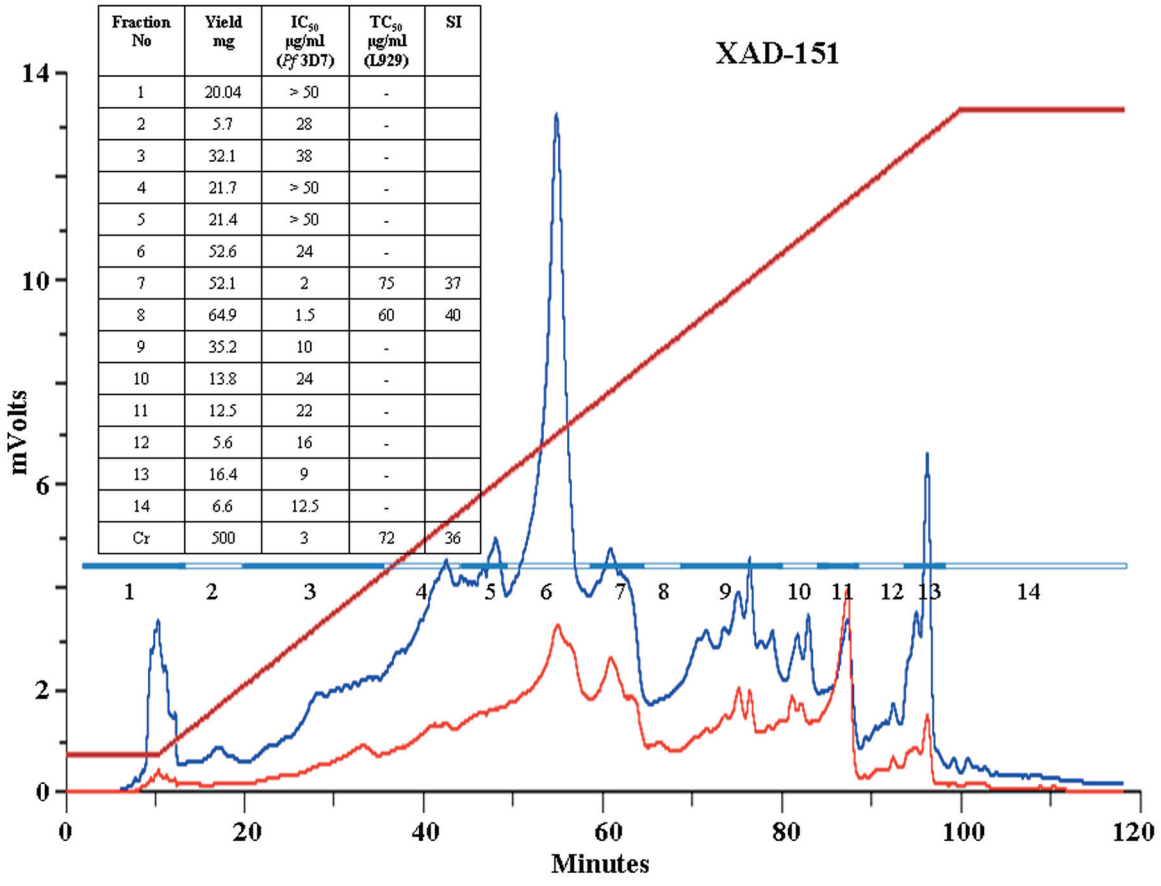

| Fraction No | Yield mg | $IC_{50}$ µg/ml (*Pf* 3D7) | $TC_{50}$ µg/ml (L929) | SI |
|---|---|---|---|---|
| 1 | 2 | >12.5 | - | |
| 2 | 0.7 | >12.5 | - | |
| 3 | 3.3 | >12.5 | - | |
| 4 | 2.9 | 5.9 | 9 | 1.5 |
| 5 | 5.2 | 10.5 | 4 | 0.38 |
| 6 | 1.0 | 0.12 | 8 | 66 |
| 7 | 4.0 | 0.15 | 12 | 80 |
| 8 | 3.5 | 4.2 | 16 | 3.8 |
| 9 | 2.3 | >12.5 | - | |
| 10 | 0.7 | >12.5 | - | |
| 11 | 0.6 | >12.5 | - | |
| Cr | 32 | 2 | 72 | 36 |

**Fig. 3.** Antiplasmodial activity guided Semiprep Reversed Phase HPLC Chromatogram of *Fusarium* sp. (580) XAD extract. Supernatant (32 mg/ ml DMSO) was injected to Deltapak (C18, 19 × 300 mm, 15µ) column and fractionated using Methanol – water (10 ml/min, 1%/min) gradient (slanting line). Absorbance at 254 nm (lower chromatogram) and 214 nm (upper chromatogram) are shown. Fractions (Frc, 1–11 indicated by filled and empty bars) were collected, dried, weighed and analyzed for antiplasmodial and HeLa cells cytotoxic activity. Inset shows amount (mg) recovered, antiplasmodial $IC_{50}$, cytotoxic activity and selectivity index (SI). Note the significant enhancement in antiplasmodial potency and selectivity indices of fractions 6 and 7 in comparison with the activities for the crude extract (Cr)

ancy in species level identification for the three strains can be attributed to the fact that more than 10% of sequences available in public sequence databases may have incorrect species annotation (Nilsson *et al.* 2006; Ko *et al.* 2011). In our study, we have carried out morphological characterization of these strains to further validate the results obtained from molecular characterization. Furthermore, we selected sequences related to these seven strains from the GenBank database (Table III) based on the results obtained from BLAST search to construct a neighbour joining tree. The sum of branch length of optimal tree constructed using the maximum composite likelihood approach was found to be 0.667. The phylogram showed that the endophyte isolates and the related sequences were grouped into six (A-F) major clades (Fig. 5) with all clades receiving very high branch support. Clades (A-F) represented the isolates belonging to species of *Fusarium*, *Nigrospora*, *Trichoderma*, *Chaetomium*, *Alternaria*, and *Curvularia* respectively. Interestingly, however, a few of the *Alternaria* species grouped together with species belonging to *Curvularia*. Two of the isolates (542 and 548) could be grouped together with *Chaetomium* species in clade D.

## Discussion

Many recent studies have shown that endophytes produce an extraordinary array of functional metabolites (Schueffler and Anke 2011; Tejesvi and Pirttila 2011). This need for such a diversity of metabolites may be triggered by the fact that endophytes which survive in living plant tissues may need them for their constant interaction with the defence chemicals produced by the plant host (Schulz *et al.* 1999). It is quite likely that survival in an alien life form may have resulted in the evolution of heightened synthetic ability of endophytes (Suryanarayanan *et al.* 2009). The hypothesis that horizontal gene transfer events from the host plant to its endophytes being instrumental in the production of some of these metabolites has evoked considerable interest as well as great controversy (Heinig *et al.* 2013; Sachin *et al.* 2013). Pharmaceutically important secondary metabolites-producing endophytes, compatible with *in vitro* cultures offer special advantages that include cost- effective large scale production of the metabolites in industrial laboratory settings. In this regard, endophytes from marine habitats appear to be unique since many of them produce different novel




| Fraction No | Yield mg | $IC_{50}$ µg/ml (*Pf* 3D7) | $TC_{50}$ µg/ml (L929) | SI |
|---|---|---|---|---|
| 1 | 20.04 | > 50 | - | |
| 2 | 5.7 | 28 | - | |
| 3 | 32.1 | 38 | - | |
| 4 | 21.7 | > 50 | - | |
| 5 | 21.4 | > 50 | - | |
| 6 | 52.6 | 24 | - | |
| 7 | 52.1 | 2 | 75 | 37 |
| 8 | 64.9 | 1.5 | 60 | 40 |
| 9 | 35.2 | 10 | - | |
| 10 | 13.8 | 24 | - | |
| 11 | 12.5 | 22 | - | |
| 12 | 5.6 | 16 | - | |
| 13 | 16.4 | 9 | - | |
| 14 | 6.6 | 12.5 | - | |
| Cr | 500 | 3 | 72 | 36 |

**Fig. 4.** Antiplasmodial activity guided Semiprep Reversed Phase HPLC Chromatogram of *Nigrospora* sp. (151) XAD extract. Supernatant (500 mg/2 ml methanol) was injected to Deltapak (C18, 19 × 300 mm, 15µ) column and fractionated using Methanol – water (10 ml/min, 1%/min) gradient (slanting line). Absorbance at 254 nm (lower chromatogram) and 214 nm (upper chromatogram) are shown. Fractions (Frc, 1–14 indicated by filled and empty bars) were collected, dried, weighed and analyzed for antiplasmodial and HeLa cells cytotoxic activity. Inset shows amount (mg) recovered, antiplasmodial $IC^{50}$, cytotoxic activity ($TC_{50}$) and selectivity index (SI), crude extract (Cr)

**Table II.** Fungal cultures with their closest matches from GenBank and CBS databases based on ITS sequence similarity. Numbers in parenthesis indicate the accession number of the closest matched sequences

| Culture Code | Identified as | Top match in GenBank | Similarity | Top match in CBS | Similarity |
|---|---|---|---|---|---|
| 542 | *Chaetomium* sp. | *Chaetomium spirochaete* (JN209921) | 100% | *Chaetomium spirochaete* (CBS730.84) | 100% |
| 456 | *Curvularia* sp. | *Cochliobolus nisikadoi* (JN943428) | 98% | *Curvularia ischaemi* (CBS630.82) | 97% |
| 151 | *Nigrospora* sp. | *Nigrospora* sp. (HQ631070) | 99% | *Nigrospora sphaerica* (CBS167.26) | 100% |
| 590 | *Alternaria* sp. | *Alternaria brassicae* (JX984695) | 99% | *Alternaria rhizophorae* (CBS118816) | 99% |
| 580 | *Fusarium* sp. | *Fusarium oxysporum* (KC254033) | 100% | *Fusarium oxysporum* f. sp. *aechmeae* (CBS244.61) | 100% |
| 568 | *Trichoderma* sp. | *Trichoderma harzianum* (KC330218) | 100% | *Trichoderma harzianum* (CBS354.33) | 100% |
| 548 | *Chaetomium* sp. | *Chaetomium globosum* (JF826006) | 100% | *Chaetomium globosum* (CBS 105.40) | 100% |

**Table III.** Sequences of fungal species used for constructing the phylogram

| Species | GenBank Accession Number | Species | GenBank Accession Number |
|---|---|---|---|
| *Alternaria* aff. *longipes* | DQ156337 | *Cochliobolus australiensis* | JN943408 |
| *Alternaria alternata* | GQ916545 | *Cochliobolus cymbopogonis* | JQ783057 |
| *Alternaria arborescens* | JQ676197 | *Cochliobolus geniculatus* | AB245085 |
| *Alternaria brassicae* | JX857165 | *Cochliobolus lunatus* | HQ607991 |
| *Alternaria californica* | JQ693645 | *Cochliobolus nisikadoi* | JN943428 |
| *Alternaria dianthicola* | JQ693640 | *Curvularia affinis* | GU073105 |
| *Alternaria frumenti* | JQ693654 | *Curvularia clavata* | GQ179976 |
| *Alternaria hordeicola* | JQ693642 | *Curvularia coicicola* | AB453880 |
| *Alternaria humuli* | JQ693652 | *Curvularia fallax* | JQ360963 |
| *Alternaria incomplexa* | JQ693658 | *Curvularia geniculata* | GU073454 |
| *Alternaria intercepta* | JQ693656 | *Curvularia inaequalis* | HM101095 |
| *Alternaria merytae* | JQ693651 | *Curvularia intermedia* | GU073103 |
| *Alternaria metachromatica* | JQ693660 | *Curvularia pseudorobusta* | AB453879 |
| *Alternaria novae-zelandiae* | JQ693655 | *Curvularia sichuanensis* | AB453881 |
| *Alternaria panax* | JQ693662 | *Curvularia trifolii* | GQ241277 |
| *Alternaria porri* | HM204456 | *Fusarium* cf. *solani* | JX270188 |
| *Alternaria rosae* | JQ693639 | *Fusarium equiseti* | HQ649908 |
| *Alternaria solani* | JX469421 | *Fusarium incarnatum* | JX885463 |
| *Alternaria* sp. | KC010550 | *Fusarium oxysporum* | KC254033 |
| *Alternaria tenuissima* | JX860514 | *Fusarium oxysporum* | JX885462 |
| *Chaetomium arcuatum* | AB746177 | *Fusarium oxysporum* f. sp. *cyclaminis* | JQ676177 |
| *Chaetomium atrobrunneum* | JN034195 | *Fusarium oxysporum* f. sp. *melonis* | GU934523 |
| *Chaetomium brasiliense* | FR718872 | *Fusarium oxysporum* f. sp. *ranunculi* | JQ340086 |
| *Chaetomium cancroideum* | HM449046 | *Fusarium pseudoanthophilum* | HF548703 |
| *Chaetomium carinthiacum* | HF548694 | *Fusarium* sp. | HQ130709 |
| *Chaetomium coarctatum* | HM365260 | *Gibberella moniliformis* | JQ277275 |
| *Chaetomium cruentum* | HM365266 | *Hypocrea lixii* | JX436467 |
| *Chaetomium dolichotrichum* | HM449049 | *Hypocrea nigricans* | JN943372 |
| *Chaetomium elatum* | JN209874 | *Hypocrea rufa* | AY380908 |
| *Chaetomium erectum* | HM449044 | *Hypocrea stilbohypoxyli* | AY380916 |
| *Chaetomium funicola* | AB746176 | *Hypocrea virens* | JX969615 |
| *Chaetomium globosum* | JF826006 | *Nigrospora* cf. *sphaerica* | JQ676183 |
| *Chaetomium gracile* | HF548698 | *Nigrospora oryzae* | EU821485 |
| *Chaetomium grande* | HM365253 | *Nigrospora* sp. | HQ631070 |
| *Chaetomium murorum* | GQ376100 | *Nigrospora* sp. | JF694932 |
| *Chaetomium nigricolor* | AB746178 | *Nigrospora sphaerica* | GQ258792 |
| *Chaetomium nigricolor* | AJ458185 | *Trichoderma aureoviride* | FJ487919 |
| *Chaetomium ochraceum* | JN093258 | *Trichoderma* cf. *harzianum* | KC176363 |
| *Chaetomium piluliferum* | HE649377 | *Trichoderma harzianum* | KC330218 |
| *Chaetomium reflexum* | HM449051 | *Trichoderma ovalisporum* | AY380896 |
| *Chaetomium sphaerale* | AB625588 | *Trichoderma piluliferum* | JQ517493 |
| *Chaetomium spirochaete* | JN209921 | *Trichoderma pleuroticola* | HM142362 |
| *Chaetomium subaffine* | HM365247 | *Trichoderma pleurotum* | HM142363 |
| *Chaetomium undulatulum* | HM365250 | *Trichoderma* sp. | FJ645728 |
| | | *Trichoderma tomentosum* | FJ487916 |

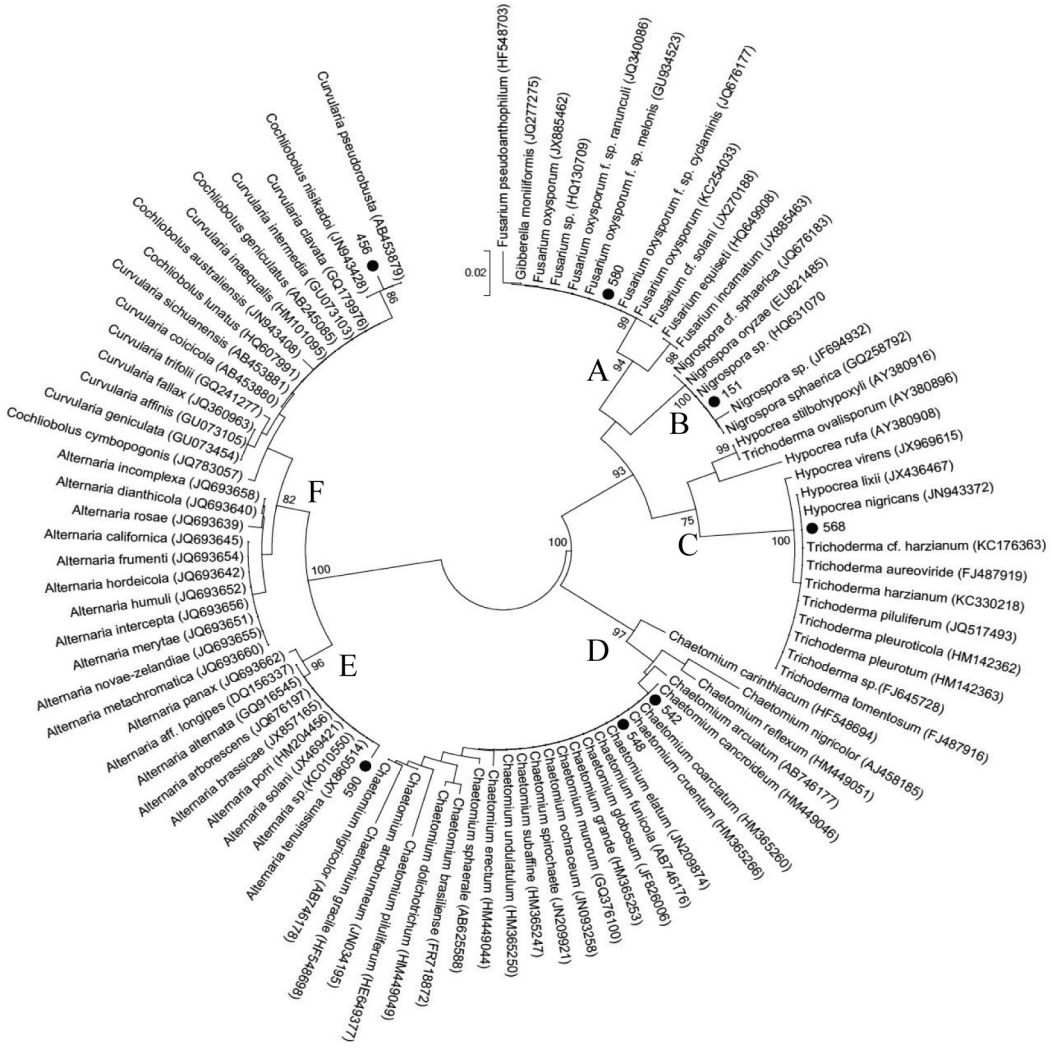



**Fig. 5.** Neighbour joining tree for sequences obtained from the present study (marked as dark circles) along with 89 other related sequences obtained from GenBank database. The evolutionary distance was computed following the Maximum Composite Likelihood method. Bootstrap values above 75% are shown as numbers above nodes of branches. Sequences used in the tree construction are listed in Table III

metabolites (Bugni and Ireland 2004; Jones *et al.* 2008; Raghukumar 2008; Schulz *et al.* 2008; Kjer *et al.* 2010; Suryanarayanan *et al.* 2011; Flewelling *et al.* 2013). Nearly 30% of the novel metabolites produced by marine-derived fungi are from fungi associated with marine algae (Bugni and Ireland 2004); some of these fungi produce metabolites having hitherto unknown carbon frameworks (Kjer *et al.* 2010) . This underscores the importance of our study with the secretions from *in vitro* grown endophytes of not only angiosperms but also seagrasses and seaweeds that exhibit potent anti *P. falciparum* activities.

Although only a limited number of endophyte isolates were screened in the present study, many of them produced antiplasmodial compounds; the finding that as high as 19% and 18% of the screened fungal isolates showed $IC_{50}$<10 µg/ml and $IC_{50}$ of 11–25 µg/ml, respectively, suggests that deeper exploration of endophytic fungi may reveal a rich repertoire of antiplasmodial molecules. Our findings have indicated that endophytic fungi belonging to the genera *Alternaria*, *Aspergillus*, *Chaetomium*, *Curvularia*, *Corynespora*, *Fusarium*, *Nigrospora*, *Paecilomyces*, *Penicillium*, *Phomopsis*, *Trichoderma* and *Xylaria* should be studied intensely for novel antiplasmodial compounds. With reference to the identification of the fungal isolates, we could confirm only 4 of our seven endophyte isolates to the species level based on the similarity search from two different databases. This could be attributed to the fact that nearly 50% of the ITS sequences in the databases are without a species name (Benson *et al.* 2010) and nearly 10% of them have incorrect names (Nilsson *et al.* 2006). We used ITS sequences for characterising the fungi taxonomically as this region has been used widely to infer phylogeny and species identification in fungi (Begerow *et al.* 2010). ITS sequences have been found to be a better marker for fungi than even the genes for ribosomal large and small subunits, since this region has good species resolution capabilities (Schoch *et al.* 2012).

Although a few terrestrial plant endophytes have been shown to have antiplasmodial activity (Kongsaeree *et al.* 2003; Cao *et al.* 2011), our results show that endophytes colonizing seaweeds are equally a potential source to provide new leads against malaria. Further, our data suggest that antiplasmodial potential is more widespread across diverse fungal genera. Our attempts at antiplasmodial activity-guided RPHPLC purification of secretions from isolates 151 and 580 have led to the segregation of fractions with low and high activity. Indeed, in the case of 580 ($IC_{50}$ crude 1.94 μg/ml), we were able to obtain fractions with $IC_{50}$ down to 120 ng/ml. We are optimizing the production of such potent antiplasmodial metabolites from the identified endophytes to be followed by their isolation and identification for the exploration of their potential as novel drugs against malaria. Additionally, we are also examining the qualitative and quantitative changes in the antiplasmodial metabolites spectrum of isolates 151 and 580 as influenced by culture conditions since culture conditions are known to induce production of several new metabolites by fungi (Bode *et al.* 2002; OBrian *et al.* 2007).

**Acknowledgements.** NKK thanks the Indian Council of Medical Research for its Senior Research Fellowship; TSM thanks Manipal University and TIFAC-CORE in Pharmacogenomics for providing the required facilities; TSS thanks the Department of Biotechnology, Govt. of India, for funding the Indo-German project BT/IN/German/11/TSS/2010. We thank Thaddeus Ezeji, Biotechnology and Fermentation Group, Department of Animal Sciences, Ohio State University for reading a draft of the manuscript and for his suggestions. We thank the anonymous reviewers whose critical and incisive comments have enabled us to provide a new spirit to our manuscript.

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**Received:** February 12, 2014
**Revised:** July 11, 2014
**Accepted for publication:** August 7, 2014