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Original Article

ENDOPHYTIC FUNGI FROM *PHYLLANTHUS AMARUS* SCHUM. & THONN. CAPABLE OF PRODUCING PHYLLANTHIN, HYPOPHYLLANTHIN AND/OR RELATED COMPOUNDS

DHANDAYUTHAPANI KANDAVEL[1], SOUNDARAPANDIAN SEKAR[2]

[1]Department of Botany, Periyar EVR College (Autonomous), Tiruchirappalli–620023, India, [2]Department of Industrial Biotechnology, Bharathidasan University, Tiruchirappalli-620024, Tamilnadu
Email: kandavel1976@gmail.com

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ABSTRACT

Objective: To isolate endophytic fungi from various parts of *Phyllanthus amarus* (family Phyllanthaceae) and to probe for production of the bioactive compounds Phyllanthin and Hypophyllanthin.

Methods: The fungi were isolated using Potato Dexotrose Agar (PDA) and a novel *P. amarus* powder amended (2 g/l) PDA (PPDA). The isolates were cultured in liquid medium and after liquid-liquid extraction they were screened for production of Phyllanthin, Hypophyllanthin and/or other related compounds using Shimadzu HPLC system equipped with PDA and UV detector at 230 nm using phosphate buffer (pH 2.8) and acetonitrile in a gradient elution at a flow rate of 1.5 ml/min. Isolates which showed close overlaps were subjected to HPTLC in Pre-coated silica gel $F60_{254}$ on aluminium plates using Linomat V, Camag TLC applicator and Toluene: Ethyl acetate (2: 1) as solvent system. Developed plates were sprayed with 10% v/v sulphuric acid in methanol. Isolates which showed promising results were identified using morphological characters and ITS sequences. The sequences were submitted to GenBank.

Results: Twenty three endophytic fungal isolates embracing eighteen from PDA and five from PPDA were obtained with roots serving as major home. HPLC analyses resulted in shortlisting of four isolates SATR2-A, SATR1-A, KOPS4-A and SKTS2-B and HPTLC analyses exposed further agreement. Bands apprise putative role of these endophytes in bi °Chemical synthesis of phyllanthin and hypophyllanthin. Morphological characters and ITS sequences identified the isolates as *Fusarium oxysporum* (SATR2-A), *Gibberella moniliformis* (Synonym: *Fusarium verticillioides*, SATR1-A), *Alternaria* sp. (KOPS4-A) and *Edenia gomezpompae* (SKTS2-B).

Conclusion: Phyllanthin and Hypophyllanthin, the twin magic bullets from *P. amarus* has widespread clinical importance and especially as hepatoprotectives. The study enlightens the role of endophytic fungi from *P. amarus* which can synthesize these bioactive compounds. This is the first such report from this plant and further research will result in confirming the potential.

Keywords: *Phyllanthus amarus*, Endophytic Fungi, Phyllanthin, Hypopllanthin, HPLC, HPTLC.

INTRODUCTION

Microbes which live within interior tissues of healthy plants without causing disease symptoms are 'endophytes' [1]. Initial reports describing these microbes date back to the turn of the nineteenth and twentieth century [2]. The most often encountered endophytes are representatives of the fungi; however, the existence of many endophytic bacteria including actinomycetes has been d °Cumented as well [3]. Of the nearly 300, 000 plant species inhabiting our planet, each individual one is host to several to hundreds of endophytes [4], creating an enormous biodiversity: a myriad of undescribed species, a rich source of novel natural products therefrom and an unknown genetic background of all the interdependencies thus implied. Functional metabolites of endophytic origin have already demonstrated a considerable potential to impact the pharmaceutical arena [5-8]. Endophytic fungi produce a number of important secondary metabolites, including anti-cancer, anti-fungal, anti-diabetic and immunosuppressant compounds. Some of these compounds are those produced by their respective host plants as well. For example, *Taxomyces andreanae* isolated from the yew plant (*Taxus brevifolia*), produced paclitaxol, the multibillion dollar anti-cancer compound, just as it is produced by the plant [9]. Following this report, a number of endophytic fungal sources of important plant secondary metabolites including camptothecin (CPT) [10, 11], podophyllotoxin [12, 13], vinblastine [14], hypericin [15], azadirachtin [16], rohitukine [17], etc., have been reported.

Phyllanthus amarus Schum. & Thonn. belongs to the family Phyllanthaceae and is a small medicinal herb widely used throughout the world. In Indian Ayurvedic system of medicine as well as in traditional remedies the whole plant is used for problems of stomach, genitourinary system, liver, kidney and spleen [18]. It elaborates different classes of organic compounds including alkaloids, flavonoids, hydrolysable tannins, lignans, polyphenols, triterpenes, sterols and volatile oil. Lignans isolated from P. amarus include phyllanthin, hypophyllanthin, niranthin, phyltetralin, nirtetralin, isonirtetralin, hinokinin, lintetralin, isolintetralin, demethylenedioxy-niranthin, 5-demethoxyniranthin, etc [19]. The lignans are one of the major classes of phytoestrogens and are phenolic derivatives formed from the shikimate pathway produced by oxidative dimerization of two phenylpropanoid units [20]. Phyllanthin (a bitter constituent) and Hypophyllanthin (a non-bitter constituent) [21, 22] are the active principles responsible for the hepatoprotective role [23] of this plant and are present in combination only in P. amarus and not in the other related Phyllanthus species [24]. P. amarus with these twin "magic bullets" was chosen for the current study and endopthytic fungi from different parts were isolated and screened for their production *in vitro*.

MATERIALS AND METHODS

Study areas

Tiruchirappalli district is centrally located in the state of Tamilnadu, India with a total geographic extent of 4404.12 sq. Km. It lies between 78010' to 7905' east longitude and 10015' and 1102' north latitude. It lies at an altitude of 78 m above sea level. Three Revenue Villages were chosen as study sites representing the major soil types; red (Koppu North), brown (Sathamangalam) and grey (Sikkathambur).

Isolation of Endophytes

The seedlings from the study areas were uprooted, kept in sterile bags and brought to the laboratory. Root sections of 2-3 cm were excised using a sterile scalpel from ten plants. Root portions were

taken just below the soil line. The samples were surface disinfected with sodium hyp °Chlorite (1.05%) and washed in four changes of 0.02 M phosphate buffer (pH 7.0) solution. Measured quantity of 0.1 ml aliquots from the final buffer wash was removed and transferred in 9.9 ml Potato Dextrose Agar to serve as sterile check. Samples were discarded, if growth was detected in the sterile check within 48 h [25]. Two different media were used for their isolation: Potato Dextrose Agar (PDA) medium; Potato Dextrose Agar medium amended with 2g/l P. amarus whole plant powder (PPDA). Five plants were chosen per study area per medium and after surface sterilization, they were cut and separated as root, stem and leaflet segments. The leaflet segments include rachis, petiole, leaflets and fruits. The cut ends which had emanated mycelium were considered and representatives of colony morphology were transferred to fresh Trypticase Soy agar plates and pure cultures were raised. The cultures were then stored at 4 °C in Sabouraud Dextrose Agar (SDA). The medium was supplemented with penicillin G 100 units/ml and streptomycin 100 µg/ml concentrations.

Liquid culture and extraction

The isolates were grown in 500 ml Erlenmeyer flasks containing 250 ml of PDbroth medium and incubated for 21 days (Photoperiod 12L: 12D; Light intensity 3.70 e1 µMoles/µEinsteins; Growth temperature 27±2 °C). After 3 weeks of still culture, the culture with culture fluid was passed through four layers of cheese cloth to remove solids. From the filtrate, 50 ml was transferred into 250 ml separating flask and 50 ml of chloroform was added. It was vigorously shaken for 5 minutes and kept for separation. The Organic layer was transferred to a beaker. The same pr °Cess was repeated two more times and all the organic layers were combined and passed through anhydrous sodium sulphate. The organic layer was evaporated under reduced pressure at 35 °C using a rotary vacuum evaporator to dryness and the residue was redissolved in 2 ml of methanol.

High pressure liquid chromatography (HPLC)

The extracts of the isolates (10 µl) were subjected to gradient HPLC separately. The Mobile phase as given in table 1 has 0.1M Phosphate Buffer as Solvent A and Acetonitrile as Solvent B. High Performance Liquid Chromatographic system Shimadzu, Japan equipped with LC10A pump with SPD-M 10A*vp* Photodiode Array Detector & UV detector (230 nm) in combination with Class-VP software and LC 2010HT integrated system equipped with Quaternary gradient, auto injector in combination with Lab solution software was used at a flow rate of 1.5 ml/min. Overlay of all chromatograms with Phyllanthin and Hypophyllanthin standard mix was done and samples suspected to synthesize Phyllanthin, Hypophyllanthin and/or their related compounds were shortlisted based on observation of peaks in retention times close to standards and subjected to High Performance Thin Layer Chromatography (HPTLC).

Table 1: The ratio of mobile phase solvents in the HPLC gradient elution

Time (min)	Buffer (Solvent A)	Acetonitrile (Solvent B)
0.01	95	5
5.00	95	5
10.00	90	10
13.00	65	35
15.00	50	50
30.00	50	50
35.00	65	35
38.00	90	10
40.00	95	5
45.00	95	5

HPTLC

Each shortlisted extract preparation (20 µl) was coated independently and then in combination with standard mix preparation of 1.6 µg/ml to Pre-coated silica gel $F60_{254}$ on aluminium plates separately using the TLC applicator (Linomat V, Camag). The Solvent System was Toluene: Ethyl acetate (2:1). The plate was developed in twin trough developing saturated chamber (Camag) to a distance of 8-9 cm and sprayed with 10% *v/v* sulphuric acid in methanol.

Identification of fungi based on classical method and Internal transcribed spacer (ITS) sequences

Colony morphology of the chosen isolates were observed and then Lactophenol Cotton Blue based slides were prepared to see mycelia and spore characters using Labomed CXR3, LaboAmerica, Inc., US., microscope. The fungi were identified using standard identification manuals [26, 27]. DNA extraction was performed using 10–15 mg (wet weight) of freshly subcultured fungi in Potato Dextrose broth [28]. Amplification of genomic DNA was done with a reaction mix (100 µl): DNA 1 µl (100 ng); Forward Primer 1 µl (400 ng); Reverse Primer 1 µl (400 ng); dNTPs (2.5 mM each) 4 µl; 10x Taq DNA Polymerase Assay Buffer 10 µl; Taq DNA Polymerase Enzyme (3U/ µλ) 1 µl; MilliQ Water 82 µl. ITS1 and ITS4 primers were used and the amplifications were carried out using a DNA engine therm °Cycler (Eppendorf Pro-S gradient cyler) and the PCR conditions were Initial denaturation 94 °C/5 min followed by Denauration 94 °C/30 sec; Annealing 55 °C/30 sec; Extension 72 °C/1 min (35 cycles) and Final extension 72 °C/15 min with Mgcl2 1.5 mM final conc. The amplified PCR products were purified and sequenced using Big Dye Terminator version 3.1 with ABI 3130 Genetic Analyzer (Hitachi). The sequence similarity of each amplicons was identified using BLASTn tool and phylogenetic tree was constructed based on similarities using Neighbour Joining method with Mega 6.0 software. Sequences were submitted to Gen Bank and accession numbers were procured.

RESULTS

Isolation and HPLC profile

Twenty three endophytic fungal isolates comprising eighteen from PDA and five from PPDA was obtained. Roots of *P. amarus* contain maximum number of sixteen followed by stem with four, leaflets with two and finally rachis one.

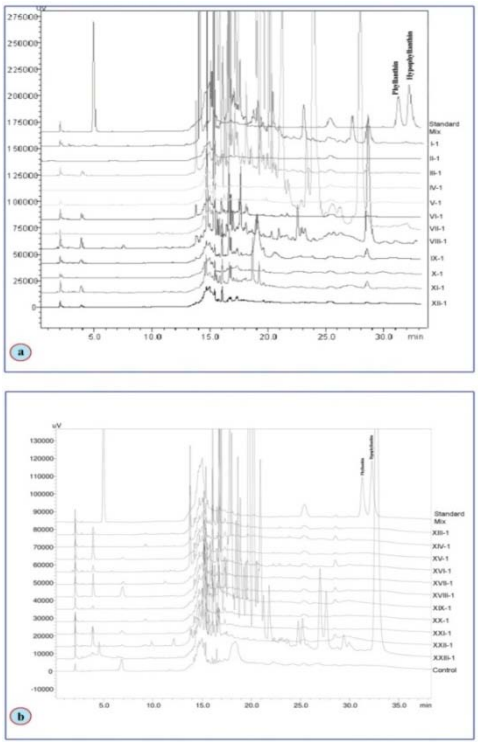

Fig. 1: Overlay of HPLC chromatograms obtained for different fungal isolates

HPLC analyses of control spectrum was detected from basal Potato Dextrose Broth and the remaining spectra from twenty three endophytic isolates (Coded I to XXIII). The chromatograms were then overlaid. The overlay indicated similar peaks of standard mix at many retention times as seen from fig. 1.

Standard mix and fungal samples I to XII, b. Standard mix and fungal samples XIII to XXIII

Endophytic fungal isolate one had similar twin peaks before phyllanthin and hypophyllanthin peaks; isolates seven and eight had large single peaks in the same region and isolate twenty three had large peak in hypophyllanthin retention time.

They were then shortlisted for spiking analysis. Of the shortlisted four, two isolates were from roots (SATR2-A & SATR1-A) and two were from stems (KOPS4-A & SKTS2-B).

HPTLC Profile of the shortlisted isolates

HPTLC analysis of the extracts from the four isolates revealed through fig. 2 show parallel bands for phyllanthin and hypophyllanthin in all. When the extracts were mixed with a known quantity of mixed

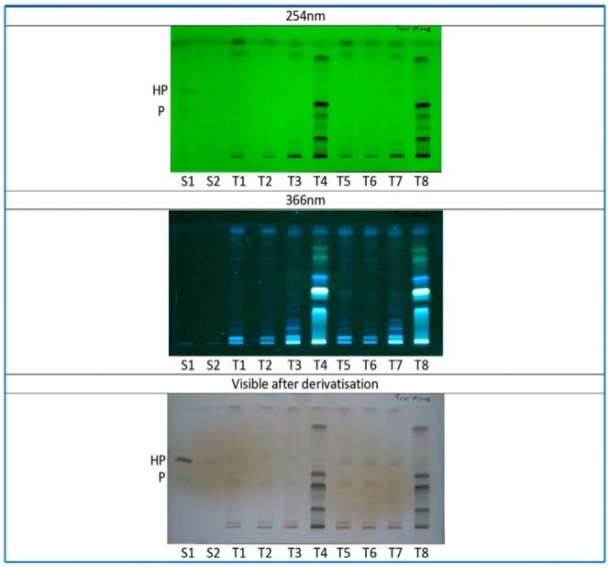

Fig. 2: HPTLC plate showing spiking analysis using phyllanthin and hypophyllanthin standard and viewed under different light regimes

S1: Phyllanthin and Hyphophyllanthin standard (16 µg/ml each), S2: Phyllanthin and Hyphophyllanthin standard (1.6 µg/ml each), T1: Extracellular extract of fungal sample I, T2: Extracellular extract of fungal sample VII, T3: Extracellular extract of fungal sample VIII, T4: Extracellular extract of fungal sample XXIII, T5: Extracellular extract of fungal sample I spiked with Phyllanthin and Hyphophyllanthin standards (1.6 µg/ml each), T6: Extracellular extract of fungal sample VII spiked with Phyllanthin and Hyphophyllanthin standards (1.6 µg/ml each), T7: Extracellular extract of fungal sample VIII spiked with Phyllanthin and Hyphophyllanthin standards (1.6 µg/ml each), T8: Extracellular extract of fungal sample XXIII spiked with Phyllanthin and Hyphophyllanthin standards (1.6 µg/ml each). standards of phyllanthin and hypophyllanthin and eluted slightly overlapped twin bands were observed for hypophyllanthin in all the four samples and clear single bands for phyllanthin. This confirms the putative role of these endophytes to synthesis phyllanthin and hypophyllanthin.

Identification of the chosen isolates and Gen Bank submission

Based on the morphological characters of colonies, observations on spores and mycelia characters they were identified. The isolate SATR2-A was identified as *Fusarium oxysporum*; isolate KOPS4-A was identified as *Alternaria* sp.; isolate SATR1-A was identified as *Gibberella moniliformis* (Synonym: *Fusarium verticillioides*) and isolate SKTS2-B was identified as *Edenia gomezpompae* and displayed in fig. 3 and fig. 4. It is for the first time these endophytes are reported from *P. amarus*.

The amplicon generated after ITS amplification was 552 bases long for the isolate SATR2-A, 544 bases long for the isolate KOPS4-A, 515 bases long for the isolate SATR1-A and 514 bases long for the isolate SKTS2-B. The Blastn homology sequence search confirmed their nomenclatural assignments done earlier and Neighbour Joining phylogenetic tree based on the sequences were constructed and given in fig. 5.

All the four accessions were submitted to Genbank in National Centre for Biotechnology Information, Bethesda and accession numbers were procured after performing required screening and processing (SATR2-A–KF560408; SATR1-A–KF560409; KOPS4-A–KF560406; SKTS2-B–KF560407).

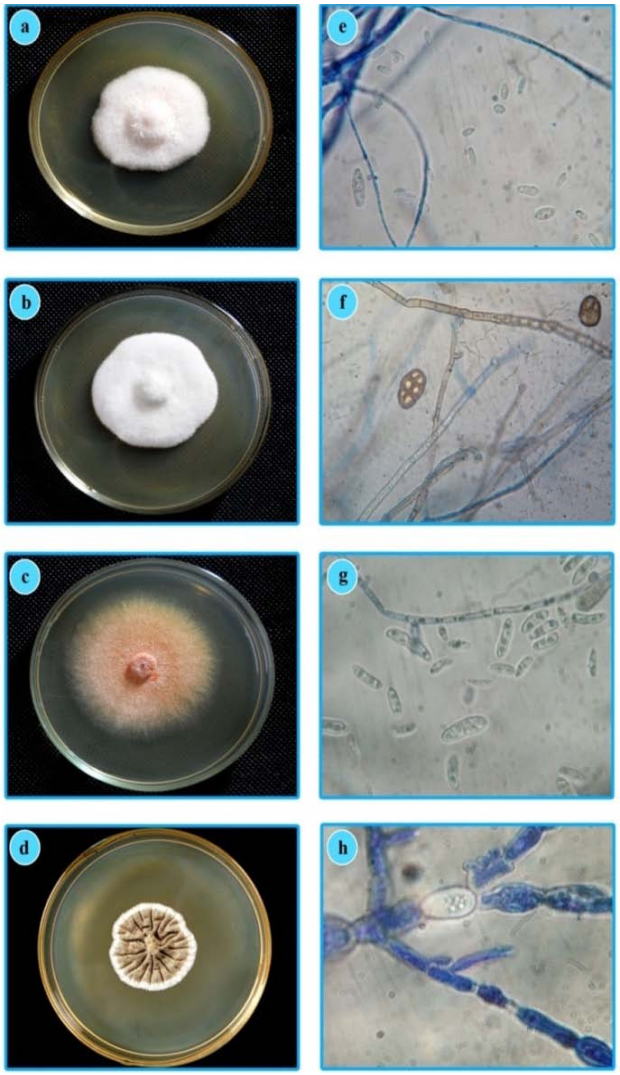

Fig. 3: Colony morphology and microscopic characteristics of chosen endophytic fungal isolates

a and e: *Fusarium oxysporum* (SATR2-A, Isolate I), b and f: *Alternaria sp.* (KOPS4-A, Isolate VII), c and g: *Gibberella moniliformis* [Syn. *Fusarium verticillioides*] (SATR1-A, Isolate VIII), d and h: *Edenia gomezpompae* (SKTS2-B, Isolate XXIII), a to d: Colony morphology; e to h: microscopic characters.

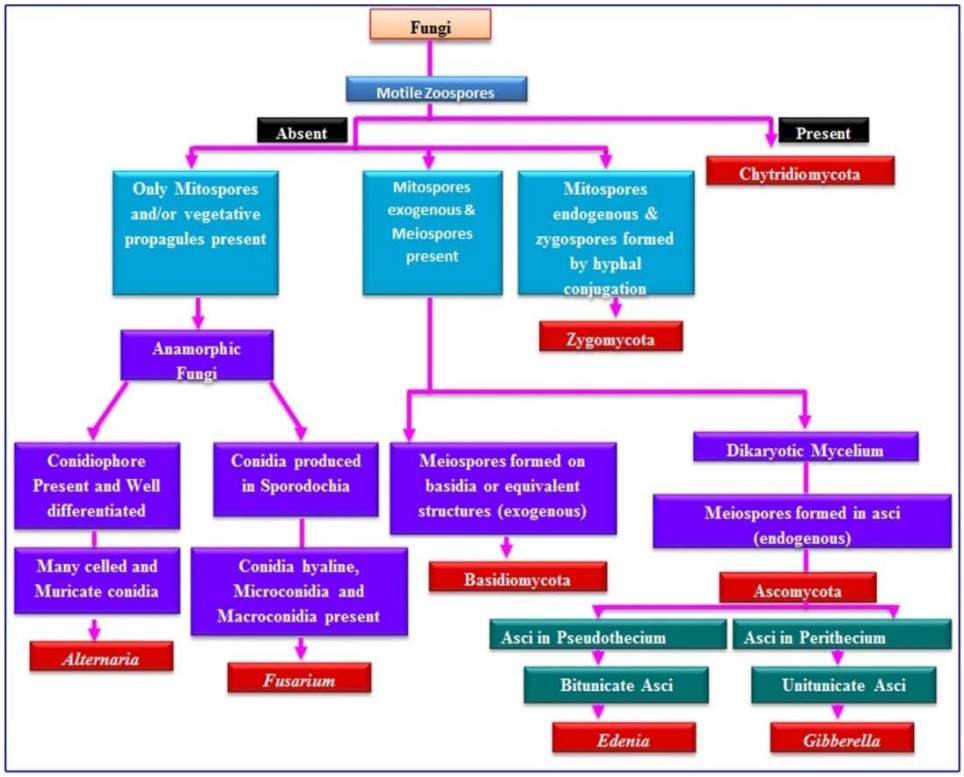

Fig. 4: Key framework evolved on the basis of microscopic characters for the identification of chosen endophytic fungal isolates

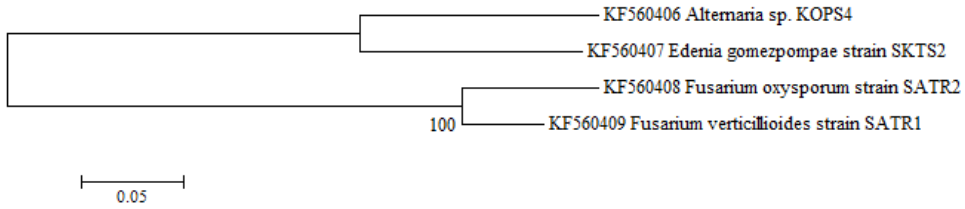

Fig. 5: Phylogenetic tree of endophytic fungal species using Neighbour Joining method with Mega 6.0 software. Corresponding Gen Bank accession number is given before each isolate

DISCUSSION

This study revealed the endophytic diversity of fungi in *P. amarus* and possibly their ability to synthesize phyllanthin and hypophyllanthin in the host plant. It is established that new endophytes can be isolated using the host plant powder/extract in the culture medium. Up to 51% of biologically active substances isolated from endophytic fungi have been reported to be new [29] and as the results indicate other intense peaks in HPLC results can be fractionated and evaluated in further studies to ascertain their chemical and clinical nature. Since, our inspections were at *in vitro* levels of isolated endophytic fungi, their biosynthetic capacity inside the host plant with multitude of interactions among themselves and also between the host plants may have a strong influence [30]. Co-culture approaches trying to imitate real *in vivo* scenario happens to be the need of the hour to understand these complicated and cryptic interactions [31]. Changing the culture conditions may have an impact on the range of secondary metabolites produced [32] and hence optimization of culture conditions through further studies is a broad scope.

CONCLUSION

The clinical acumen of medicinal plants is largely influenced by collateral silent partners residing deep inside their cells, as apparent from the case study results obtained here. This study has opened new doors into the possibility of synthesizing Phyllanthin and Hypophyllanthin, without the requirement of the host plant and employing nature's own machinery. Understanding plant metabolic routes and transformation is a huge enchantment awaiting the researchers to flood with new revelations.

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CONFLICT OF INTERESTS

We declare that we have no conflict of interest

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