Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2022; 11(5): 202-206
Received: 15-08-2022
Accepted: 03-09-2022

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Morpho-molecular and biochemical characterization of the wild edible mushroom *T. giganteum* (TGS-1)

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Abstract
The present investigation was carried out to identify the morphological and molecular characterization of the mushroom *Tricholoma giganteum* (TGS-1). The collected mushroom sample was identified as *Macrocybe* based on the morphological characters. Furrtherly, the Internal Transcribed Spacer (ITS) 1 and 2- PCR was employed to amplifying the rDNA from the pure culture of the mushroom and yielded 590 bp from the PCR. The amplified product was sequenced and identified using BLASTn in the NCBI. The BLASTn results revealed that, *T. giganteum* (TGS-1) strain was 98.82% similar with the strain *Macrocybe giganteum* (EU051917) and 98.65% with *Tricholoma giganteum* (MK024240). This mushroom was deposited as a new strain in NCBI GenBank with accession number MZ061712. The GC-MS analysis revealed that the presence of the biochemical compounds *viz*., 9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-, 9,12-Octadecadienoic acid (Z,Z)-,2-hydroxy-1-(hydroxymethyl) ethyl ester, with highest peak area of 69.34% and high retention time (RT) of 22.81 min. This study revealed the novelty of the mushroom strain and encourage to further investigation on their benefits.

Keywords: *Tricholoma giganteum*, morphology, molecular identification, ITS-PCR, GC-MS

Introduction
Fungi that influenced the humans affair either direct food source, food processing and as well as medicine (Odeyemi *et al*. 2014) [22]. Mushrooms are important component in environmental diversification and it provides healthy and quality foods and involves in decomposition (Nilsson *et al*. 2011; Suman and Sharma 2018) [21, 29]. Recent years many scientists showed their interest to investigate the mushroom's due to its nutritional, therapeutic and ailment effects against many human diseases and illness (Biswas *et al*. 2012 and Chatterjee *et al*. 2011) [5, 7]. Mushrooms are may be edible, inedible or poisonous and they are morphologically classified as puffballs, stinkhorns, brackets and gilled fungi (Bates 2006) [4].
Tricholoma giganteum are the robust wild edible mushroom. It ubiquitously distributed throughout the world (Suman and Sharma 2018; Prakasam *et al*. 2011) [26, 29]. It was large, fleshy, white, cream to greyish ochraceous, concave, umbonate to depressed basidiomata, saprophytic with clamped hyphae. *Macrocybe* has been treated as *Tricholoma* until Pegler *et al*. (1998) [25]. Morphologically *Macrocybe, Tricholoma* and *Calocybe* are almost similar. *Macrocybe* and *Tricholoma* have close relationship in morphologically and molecularly then *Calocybe.* There are difficult to differentiate phenotypically of *Macrocybe* and *Calocybe* but both DNA pattern was irrelevant (Razaq *et al*. 2016) [27].
Previously, the mushroom identifications were based on the habitat, habit, season, size, shape, colour, margin of carpophore, length, diameter and texture of stipe, the spore bearing surface, spore prints, presents of volva, annulus and other unique features on the fruiting body (FAO 2006, Odeyemi and Adeniyi 2015) [23]. Recently molecular identification of the species was emerging rapidly, Ribosomal RNA (rRNA) analyse and comparison from the different fungal isolates are done by PCR coupled RFLP (Miller *et al*. 1999) [17], direct sequence of the rRNA genes (Moncalvo *et al*.1995) and rDNA from the region of Internal Transcribed Spacer Polymerase chain Reaction (ITS- PCR) (Bruns *et al*. 1998) [6].
In the present study, discussed about the ecological, morphological and microscopical and biochemical aspects of the mushroom *Tricholoma giganteum* (TGS-1) and it was molecularly identified by analysing the ITS sequence of rDNA from the basidiocarp.

Materials and methods
A survey was conducted during North-East monsoon (Nov). The wild mushroom strain *Tricholoma giganteum* (TGS-1) was collected from non-cultivable plains in O.

Sowdapuram Village, Namakkal district, Tamil Nadu, exact GPS location was 11.4776218,78.0836454. Collection of the mushroom sample was followed by the standard procedure proposed by Stojchev *et al*. (1998) [31]. The mushroom was collected from decaying woods and soil debris at different location around two acres of plain land. The collected samples were kept in the refrigerator (4℃) until before analysis.

Morphological Characters

Primarily, the collected samples were morphologically described (Color, texture, shape, size and odour of the sporosphore) and note the presence or absence of volva, annulus and color changes of the mushroom at different stages. Account the different growth stages of the mushroom. The mushroom comes under which group was identified through compared the morphological characters of the sample with previous authors report and identified.

Isolation

Collected mushroom sample was isolated by standard tissue culture technique proposed by Sud (1985) [32]. Surface of the mushroom was sterilized by using 70 per cent ethanol. The fruiting body of the mushroom was cut across the pileal region with sterile scalpel. Took 1 -2 mm size of the bits of mushroom from the joint of stipe and cap, it was placed a petri plate containing 15 ml of medium after 2 - 3 sterilization. Cover the plates with cling flim for avoiding contamination. The plates were incubated at 28℃. The axenic culture was prepared by sub-culturing from the mother plate and maintained in refrigerator at 4℃. The entire research was done in the Department of Plant Pathology, Annamalai University, Tamil Nadu.

Evaluation of different medium

Mycelial growth of the mushroom was evaluated with seven different medium *viz.,* Potato Dextrose Agar (PDA), Beet Dextrose Agar (BDA), Wheat Bran Agar (WBA), Rice Bran Agar (RBA), Potato Sucrose Agar (PSA), Tapioca Dextrose Agar (TDA) and Cynodon Dextrose Agar (CDA). The medium were prepared by the method was proposed by Ainsworth (1961) [1]. Four sterilized thin mushroom bits were placed on the periphery layer of the plate containing 15 ml of solid medium. The plates were covered with cling flim for avoiding contamination and incubated at 28℃.

Evaluation of different pH and temperature

Five mm size of the 7 – 10 days old mycelial disc of the mushroom *T. giganteum* was inoculated with five different pH *viz.,* 6.0, 6.5, 7.0, 7.5 and 8.0. The pH of the medium was adjusted by using 0.1N HCL or 0.1N NaOH. The plates were incubated under five different temperature *viz.,* 26℃, 28℃, 30℃, 32℃ and 34℃. The mycelial growth was recorded periodically on till the colony covers the full plate. All experiments were maintained three replications.

Molicular Identification

DNA extraction

The mushroom mycelium was grown in 100 ml of PD broth for 7 days at 28±2℃. The genomic DNA was extracted and purified by using the modified CTAB (Cetyl Trimethyl Ammonium Bromide) buffer method (Nicholson *et al*. 1996) [20]. Harvested fungal mycelium was dried and took 2 g of dried mycelium and ground into a fine powder by using liquid nitrogen in mortar and pestle. 10 – 20 mg of powdered mycelium was transferred to 2 ml Eppendorf tubes and 1 ml of CTAB extraction buffer (100 mM tris base (pH: 8.0), 1.4 ml NaCl, 20 mM EDTA and 2 per cent (hexadecyltrimethylammonium bromide) CTAB, 0.2 per cent mercaptoethanol) was added, vortexed and incubated at 65℃ for 45 minutes.

The mixture was transferred to a clean tube and chloroform: isoamyl alcohol (24:1) was added in equal volume. The mixture was centrifuged at 10000 rpm for 10 minutes. Equal volume of 5 M NaCl and ice cold isopropanol was transferred to the supernatant taken in clean tube and mixed well. It was incubated at 65℃ for DNA precipitation or incubated overnight at -20℃. The content was centrifuged at 13000 rpm for 10 minutes and the pellet was collected by discarding the supernatant. The pellet was washed with 70 per cent ethanol twice. 50µl of TE buffer or double sterile b water was used for resuspending the pellet (10 mM Tris, 1 mM EDTA, pH 8.0).

DNA sequencing and phylogenetic analysis

The DNA sample was sent for the sequence analyses to the Eurofins genomics India Pvt Ltd Bangaluru. The sequenced DNA results were involved in phylogenetic analyses. Sequenced 18S rDNA gene was further used to characterize the organisms in order to establish relationships among them. NCBI Genbank nucleotide sequence database was utilized for BLAST (Basic Local Alignment Search Tool) the isolated 18S rDNA sequence (Altshul *et al*. 1997) [3].

Biochemical identification

GC-MS Analysis

The GC-MS analysis was carried out using an Equipment TSQ 9000 Triple Quadrupole GCMS/MS, Detector TSQ Quadrupole Mass Spectrometer, and the Column Trace of GOLD™ TG-5MS, 30m x 0.25 mm ID x 1.4 µm of capillary column. The Carrier gas flow was 1ml per min and the sample was injected into Split 10:1 proportion. In software side "Xcaliber" Software was used. One µl of the sample was loaded for the analysis. The initial temperature of the instrument was set to 110℃ and continuously allow for 3.5 minutes. After 3.5 minutes, the temperature is increased at the rate of 10℃ for every one minute without any holding. After the temperature reaching 200℃, the temperature was increased at the rate of 5℃ per minutes. When the temperature reaches 280 ℃, it has to be hold for 20 minutes. The sample injector temperature was 280℃. Total Gas Chromatograph running time was 40.50 minutes.

In Mass Spectrophotometer programme, NIST Version-2011 upgraded version library was used. While processing, the Inlet line temperature was 290° C, temperature of injection port was 250 ° C and flow of helium was 1ml / min. The ionized voltage Electron energy was 70 eV. The mass spectral scan range was set at 50-500 (m/z) amu. The detecting time delay upto 0 – 3.5 minutes. Total MS running time was 40.50 minutes. The GC-MS analysis was carried out by the method proposed by Oni *et al*. (2020) [24] and Franelyne *et al*. (2016) [10].

Result and Discussion

Morphological Characters analysis

Results obtained from the present findings that the pileus of the mushroom *Tricholoma giganteum* (TGS-1) was 8 -15 cm in diameter with white creamy in colour. The pileus, initially concave and turn flattern when matured. Stipe was 4×15 cm long cylindrical, enlarge towards base and inner portions are solid with fleshy and outer surface shows scaly nature. Gills

are 5 – 10 mm broad, milky white in colour, lamellae are crowded with 3 cm length. Basidiospores are ellipsoidal in colour. Hyphae are branched with septations and clamp connections are present, volva annulus are absent. (Table 1)

Almost similar results were already reported by Kuppuraj (2019) [14] he isolated *Tricholoma giganteum* (TGSLM) strains from Yarcaud hills, Tamil Nadu. *T. giganteum* (TGS-1) strain was almost similar to the strain released by Tamil Nadu Agricultural University *Tricholoma giganteum* Co (TG)-3 in 2012 except the size of the gills, pileus and stipe. Suttipun *et al*. (2017) [33] and Nguyen Xuan Hoe *et al*. (2017) [19] reports about *Tricholoma giganteum* (M4) was also supported to the current research.

Effect of different media on the radial growth of *T. giganteum*

Result indicated that PDA and BDA were much superior and found to be on par with each other in enhancing the vegetative growth (90.00 mm) and also biomass production respectively (219.70 and 210.46 mg). Attain maximum radial growth at 14th day. These were followed by WBA (81.09 mm), the least mycelial growth was recorded on CDA (63.68 mm) with lowest biomass (99.43 mg). (Table 2)

The present finding is in confirmation of the finding of PDA and MEA medium enhanced the mycelial growth of the milky mushroom *C. indica* (Sandeep *et al*. 2018) [28]. PDA enriched the vigorous mycelial growth of *T. giganteum* (Netam *et al*. 2018) [18]. PSA followed by PDA medium more suitable for the cultivation of *C. indica* (Kerketta *et al*. 2018) [13].

Effect of different temperature and pH on the radial growth of *T. giganteum*

The present study revealed that the neutral pH (7.0) was the most suitable for the mycelial growth of *T. giganteum* followed by acidic pH (6.5 and 6.0). (Table 3) The least growth was recorded in alkaline pH (7.5 and 8.0). Similar to the present observation a pH of 7.0 (Sharma *et al*. 2006) [29] and pH of 7.2 (Marcelo *et al*. 2016) [15] promote the mycelial growth of *T. giganteum.* The reduction in the mycelial growth of *T. giganteum* could be attributed to the reason that the strong acidic or alkaline pH (below 5.5 and above 7.5) might have corroded the cell wall and impaired the selective permeability function of the cell as observed by Hopkins (1995) [11].

Likewise, 30 and 32℃ was found to be the most favorable temperature. The mycelial growth and biomass production showed a harsh reduction above 34℃ and below 28℃ respectively. Several earlier workers have also reported that a temperature range of 30℃ in optimum for the mycelial growth of *T. giganteum* (Suttipun *et al*. 2017; Shukla *et al*. 2014). Recently, Akhtar *et al*. (2019) [33, 30, 2] observed 32℃ as more favorable for the mycelial growth of *T. giganteum.*

DNA Sequencing analysis

The isolated genomic DNA from the pure culture of the mushroom *T. giganteum* (TGS-1) was amplified with ITS region 1 and 2 through PCR and obtained 590 bps. The obtained sequences from this study, was deposited in the GenBank database with accession number MZ061712.

AGTGAATGAA CTTGGTCAAG TTGTTGCTGG CCCTTTCAGA GCATGTGCAC
GCTTGGCTAT TGTTTCTTAA ACCACTTGTG CACCTTTTGT AGACTTTGGG
TAAAGTTTTG AGTCGAGAGT GATCTTGGCC CTTATACTCC AAAGTCTATG
TCTTTTCATA TCATTTACTC TATGTATAAG AATGTTTCTA AGGCATTTCT
TTGATGCCTT TAAATCATAT TACAACTTTC AACAACGGAT CTCTTGGCTC
TCGCATCGAT GAAGAACGCA GCGAATGCGA TAAGTAATGT GAATTGCAGA
ATTCAGTGAA TCATCGAATC TTTGAACGCA CCTTGCGCTC CTTGGTATTC
CGAGGAGCAT GCCTGTTTGA GTGTCATGAA ATTCTCAACC TGTTGTTACT
TTAGTTTGTT TCAAAGTGTT TGGATGTGGA GTTGCTGGCT TATTTGTGTT
CAAAGAGTCA GCTCTTCTGA AATACATTAG TGGGACCCAT CGTTGATTTA
GCCCCTGGTG TGATAGTTAT CTACGCCGTG GCTTAGCACG ATATTGTGTG
GTTCAGCTTT CTAACAGGAC AATTAACTGT CTTTGATCCA

Fig 1: ITS sequence data of *Tricholoma giganteum* showed 590 bp.

After BLAST, 590 bp of gene sequence was deposited as new strain of edible milky mushroom *Tricholoma giganteum* (TGS-1) or *Macrocybe giganteum* (TGS-1) in NCBI. Results of BLAST showed the closely related sequences of the strain. ITS sequence of the pure culture of *T. giganteum* showed 98.82%, 98.65%, 98.48% and 98.29% similarity with *Macrocybe giganteum* (EU051917) was isolated by Tang *et al*. (2007), *Macrocybe giganteum* (MK024240) was isolated by Brode *et al*. (2018), *Macrocybe giganteum* (JX041888) was isolated by Mo *et al*. (2012), *Macrocybe giganteum* (JN006792) was isolated by Pushpa *et al*. (2011) respectively.

Characterization (GC- MS) of antimicrobial compounds from fruiting body of *T. giganteum*

The dry powdered sample of the *T. giganteum* sent to IIFPT, Thanjavur for GC-MS analyses. (Table 4) The results showed the presence of compounds namely 9,12,15-Octadecatrienoic acid, 6,9,12,15-Docosatetraenoic acid, Androstane-11,17-dione, 1-Hexadecanol, 2-Dodecanol, 4-Trifluoroacetoxytetradecane and 1-Tetradecene were detected through GC-MS analysis. Among the compounds detected, 9,12,15-Octadecatrienoic acid and 6,9,12,15-Docosatetraenoic acid showed highest peak with the probability of 69.34 at the retention time of 22.81 respectively. The biomolecules present in the mushroom *T. giganteum* is presented in the table 4. It is agreed that GC-MS with high specificity, high sensitivity, stability and small amount of sample characteristics, are unanimously accepted as the method for the analysis of volatile constituents (Jiang and Sliva 2010) [12].

Table 1: Morphological Characteristics of *T. giganteum*

Characters	*T. giganteum* (TGS-1)	*T.giganteum* (TGSLM) Kuppuraj (2019) [14]	*T. giganteum* (M4) Nguyen Xuan Hoa *et al.* (2017) [19]
Pileus	8-15 cm in diameter, White creamy colour, initially convex later on expanded & flattened, irregular margin	8-12 cm in diameter, White creamy colour, first convex later on expanded & flattened, smooth, regular margin	14- 16 cm in diameter, White creamy colour, initially convex, later on expanded & flattened, smooth
Stipe	4 × 15 cm long cylindrical, enlarge toward base with solid, scaly, fleshy surface.	3 × 15 cm long cylindrical, swelled base with solid, fibrillose, fleshy surface	2.5 × 15 cm long cylindrical, swelled base with solid, fleshy surface
Gills	5-10 mm broad, white, crowded with gills of three cm length.	4-9 mm broad, crowded, off white, with many tiers, unequal gills	6-10 mm broad, off white, crowded with gills of three cm length
Spore shape	Ellipsoid	Ellipsoid	Ellipsoid
Hypal system	Branches with septate; presents of clamp connection	Thin walled parallel hyphae, branched, septate with clamp connections	Branches with septate; presents of clamp connection
Annulus & volva	Absent	Absent	Absent
Spore Print	White	White	White

Table 2: Selection of suitable media for the mycelial growth of *T. giganteum*

Tr. No.	Medium	Average Radial Growth (mm)			Mycelial Biomass (mg)	Mycelial character
		7th day	11th day	14th day		
1	Beet Dextrose Agar (BDA)	46.51^{a}	70.39^{a}	90.00^{a}	210.46^{b}	Milky white, Fluffy, Thick mat
2	Potato Dextrose Agar (PDA)	44.83^{b}	69.27^{b}	90.00^{a}	219.70^{a}	Milky white, Fluffy, Thick mat
3	Wheat bran Agar (WBA)	41.38^{c}	61.07^{c}	81.09^{b}	197.58^{c}	White, Thick mat, Strandy margin
4	Rice Bran Agar (RBA)	40.18^{d}	58.97^{d}	78.18^{c}	179.14^{d}	Milky white, woolly growth, slow growth
5	Potato Sucrose Agar (PSA)	38.18^{e}	56.44^{e}	73.22^{d}	143.90^{e}	White, Thin mat
6	Tapioca Dextrose Agar (TDA)	37.98^{e}	52.26^{f}	69.33^{e}	115.64^{f}	White, Thin mat, slow growth
7	Cyanodan Dextrose Agar (CDA)	33.42^{f}	49.80^{g}	63.68^{f}	99.43^{g}	White, Very thin mat, Slow growth

Values not sharing a common superscript differ significantly at $P< 0.05$ (DMRT)

Table 3: Effect of different temperature and pH on the mycelial growth of *T. giganteum*

Tr. No.	Temperature (°C)	Mycelial growth (14th Day)	Mycelial dry weight (mg)	pH	Mycelial growth (14th Day)	Dry Mycelial weight (mg)
1	26	76.84^{d}	143.57^{d}	6.0	79.93^{b}	200.73^{c}
2	28	81.40^{c}	194.62^{c}	6.5	90.00^{a}	217.64^{b}
3	30	90.00^{a}	228.05^{a}	7.0	90.00^{a}	225.97^{a}
4	32	90.00^{a}	226.97^{a}	7.5	75.63^{c}	182.70^{d}
5	34	86.56^{b}	204.93^{b}	8.0	36.08^{d}	67.16^{e}

Values not sharing a common superscript differ significantly at $P< 0.05$ (DMRT)

Table 4: Analysis of Volatile Compounds present in the Mushroom *T. giganteum* by GC-MS

S. No.	Compound	Retention Time	Molecular Formula	Molecular Weight	Peak Area (%)
1.	1-Tetradecene	8.53	C14H28	196	3.68
2.	4-Trifluoroacetoxytetradecane	8.53	C16H29F3O2	310	3.68
3.	1-Hexadecanol	11.29	C16H34O	242	4.02
4.	2-Dodecanol	11.29	C12H26O	186	4.02
5.	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	22.81	C21H36O4	352	69.34
6.	6,9,12,15-Docosatetraenoic acid, methyl ester	22.81	C23H38O2	346	69.34
7.	Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]-, 17-[O-(phenylmethyl)oxime], (3à,5à)-	23.22	C29H43NO3Si	481	6.33

Conclusion

Results of the current research showed that the mycelium of the wild edible mushroom *T. giganteum* was grown well in PDA medium with neutral pH at 30-32°C temperature. The molecular sequencing techniques are performs better for the identification and differentiation of the morphologically similar mushrooms. GC-MS analysis of the mushrooms helps to identify the pharmaceutical benefits.

Acknowledgement

I would like to thank my Research guide Dr. R. Sudha Raja Kumar, Asst. professor, Dept. of Plant Pathology, Annamalai University for his support and guidelines. My parents, friends and who all are supported me to complete my research.

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