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PLECTOLOGICAL AND MOLECULAR IDENTIFICATION OF ECONOMICALLY IMPORTANT WILD RUSSULALES MUSHROOMS FROM PAKISTAN AND THEIR ANTIFUNGAL POTENTIAL AGAINST FOOD PATHOGENIC FUNGUS *ASPERGILLUS NIGER*

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Abstract

Present study deals with the plectological and molecular analysis as well as use of economically important wild Russuloid mushrooms against food pathogenic fungus *Aspergillus niger*. Three different species of mushrooms *viz.*, *Russla laeta, R. nobilis,* and *R. nigricans* were collected and identified from Himalayan range of Pakistan and are found as new records for this country. Major objective of this study was to highlight the importance of these wild creatures as antifungal agents against *A. niger*. For this purpose methanolic extract of selected mushrooms of different concentration levels *viz.*, 1, 1.5, 2 and 3% were used. This activity is also first time reported from Pakistan by using this group of mushrooms. Results showed that all tested mushrooms exhibit growth inhibition of *A. niger* and can be used as biocontrol agents. *R. nigricans* showed maximum inhibition of fungus growth that is 62% at 3% concentrations while minimum inhibition was observed in *R. nobilis* at same concentration that is 43.6%.

Introduction

Many people in Pakistan depend on agriculture but various crops are contaminated by phytopathogenic fungi (*i.e.*, *Aspergillus*, *Fusarium*, *Penicillium*) during pre and post-harvesting processes. These pathogenic fungi produce certain chemicals that change the quality of food. Due to their toxic effects, resistance to the environment and adulteration in foods and feeds these fungi prove hazardous to the health of all organisms on land (Barung *et al.*, 2003). There are many methods to control these fungi but use of mushrooms against these is a cheap and environment friendly technique because mushrooms exhibit antifungal activity. The Himalayan range in Pakistan is considered as diversity rich hotspot for such wild mushrooms (Jabeen *et al.*, 2014). Mushrooms (macrofungi) are most abundant in this area, but unfortunately people in these areas are mostly illiterate and have less knowledge about use of these economically important mushrooms. These fungi are also important component of ecosystem as edible, medicinal, ectomycorrhizal and as decomposers.

For this work, mushrooms belonging to *Russula* genus were selected due to their high diversity in Pakistan (Ahmad *et al.*, 1997; Niazi *et al.*, 2006; Razaq *et al.*, 2014; Jabeen *et al.*, 2016) to check their growth inhibition potential against pathogenic fungus. These mushrooms were collected from coniferous forests of Pakistan. The main objective of this study was to identify the mushrooms up to species level by morphological and molecular analysis and to find

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the potency of wild mushrooms as biocontrol agent and to analyze the potential of their methanolic extracts for checking their latent antifungal potential against food pathogenic fungal species of *A. niger*.

Materials and Methods

Sampling and plectological analysis

The Basidioma were collected from coniferous forests of Pakistan during rainy season in July– August 2016. They were photographed in the field. Following morphological characters of selected specimens were documented from sporocarps: Pileus shape, diameter, colour, texture, ornamentation, bruising reaction of the flesh, margin shape and color, hymenium colour, lamellae tube, size of gills and bruising reactions of gills. Stipe dimensions (width and length), color, shape, ornamentation and texture, bruising reaction of the context, stipe attachment with pileus, presence/absence of annulus on stipe were also taken in to consideration. After being dried on a forced air dryer, samples were sealed in plastic bags with unique ID number for further analyses. Dried sporocarps were used for microscopic examinations. Samples were rehydrated using 10% KOH for microscopic studies. For microscopic analysis, slides were prepared and observed under microscope (light and Scanning Electron Microscope). Measurements were determined for basidiospores, basidia, and other tissues under a light microscope. Average measurements are reported based on 30 spores and 10 basidia. Spore measurements are presented as length/width (l × w) ratios and extreme values are given in parentheses. The range contains a minimum of 90% of the values.

Molecular analyses

DNA was extracted from dried sporocarps following a modified CTAB method (Gardes and Bruns, 1996). Primer pairs ITS1F/ITS4 (White *et al.*, 1990) for the ITS region were used for PCR and Sanger sequencing. All PCR products were evaluated for successful amplification using SYBR Green and 1.5% agarose gels with TAE buffer for gel electrophoresis. Amplicons were prepared for sequencing via enzymatic purification using Exonuclease I and Shrimp Alkaline Phosphatase enzymes (Werle *et al.*, 1994). Purified products were sequenced through MacroGen Company, Korea. Sequence chromatograms were trimmed, edited, and assembled using Sequencher 4.1 (GeneCodes, Ann Arbor, MI). DNA sequences generated for this study were deposited in GenBank.

Phylogenetic analyses

Consensus sequences were analyzed using BLAST searches at NCBI (http://www.ncbi.nlm.nih.gov/). The most similar sequences for ITS region were retrieved from GenBank. These ITS sequences were then aligned using Muscle Alignment Tool to generate alignments (Edgar, 2004). MEGA5 software was used for phylogenetic analysis with maximum likelihood criterion by following algorithm and Jukes and Cantor (1969) model of sequences evolution (Tamura *et al.*, 2011). One thousand bootstrap iterations were performed with rapid bootstrapping. Significant support was considered to be ≥70%. All phylogenetic analyses were performed on the CIPRES Portal v. 3.1. (Miller *et al.*, 2010).

Antifungal activity

To check the antifungal potential of selected mushroom samples, standard protocols were followed (Javaid and Samad, 2012).

Selection of Pathogen

Pure cultures of *Aspergillus niger* were prepared from available strain by inoculating on Malt extract (ME) medium. After seven days of inoculation, pure culture of selected fungus pathogen was obtained.

Preparation of mushroom extracts

For this purpose, fresh mushroom samples were dried to lose water content completely. Dried samples were cleaned and weighed accordingly (5 g). For soaking, samples had been finely grind to be dipped in 100% methanol for seven days**.** The extract was filtered and the liquid was fan dried to make up crude extract; further dilutions were made depending on final crude extract mass. To make 60 ml liquid media, 1.6 grams of ME medium was used to make final volume by adding dist. water in it. Sterilized glassware was used in order to avoid contamination. Autoclaved medium was taken in to Laminar Air Flow (LAF) where dilutions of crude extracts were added and simmering processes were performed. Antibacterial capsules (chloramphenicol 250 mg) were used to depress bacterial growth that may directly affect pure culture strain and ultimately the main objective of study. Equal volume for each concentration was made in labelled flasks.

Checking activity with organic solvents extracts

Wrapped flasks with aluminium foil were ready for inoculation next day. Inoculated flasks were checked after seven days of inoculation, fungal mass was filtered by using filter paper and made them dry in drying oven. The dried mass was weighed to recognize potency of mushroom metabolites against pathogenic fungus *A. niger*. Percentage growth inhibition was measured by using formula:

$$\text{Growth Inhibition (\%)} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

Statistical Analysis

The data was analyzed statistically for significance error; least standard deviation (L.S.D.) and Duncan's Multiple Range test were applied by using software package COSTATE version 3.03 and Minilab version 5.17 according to Steel *et al*., (1997). Mean values and standard error were calculated. The data was presented as Mean $\pm$ S.E. (Mean $\pm$ Standard Error).

Results

Identification of mushrooms

Russula laeta Jul. Schäff. **(Fig. 1)**

GenBank accession No. MK389376

Plectological analysis

Pileus 3.4−4 cm in diameter, ovate, firm, parabolic, slightly striate at margins, pileus not dehiscent, colour variable but mostly orange to light brown; Margins smooth, entire, wavy at margins and flesh white, contex change on bruising; Gills attachment crowded, lamellae tube attachment adnate, gill space moderate, gills in series, white in colour; Stipe 5 cm long, 1.3 cm wide, centrally attached with pileus, clavate/club shaped, tapering to the apex with un swollen base, chalky, white in colour, Volva absent, Taste not recorded, Odour indistinctive.

Basidiospores 6.3 − 10.7 µm (avg = 6.3 µm) spiny, globose to ellipsoid, symmetrical, thick walled, light grey in 10% KOH; Basidia 9.9 × 31.8 − 14.1 × 42.4 µm (avw=12.8 µm, avl=37.5 µm) clavate, 2–3 sterigmata, sterigmata long, thin walled with oil granules, without clamp connection, hyaline to light grey in 10% KOH; Cystidia 5.3 × 28.3 − 10.6 × 38.9 µm (avw=7.6 µm, avl=33.8 µm) clavate, thin walled, without clamp connection, abundant with oil granules,

hyaline to light grey in 10% KOH; Pileipellis 3.1 µm, loosely arranged, aseptate, accompanied by number of cystidia and spores, cylindrical with clamp connection; Stipitipellis 4.5 µm, loosely arranged, aseptate, cylindrical with no clamp connection.

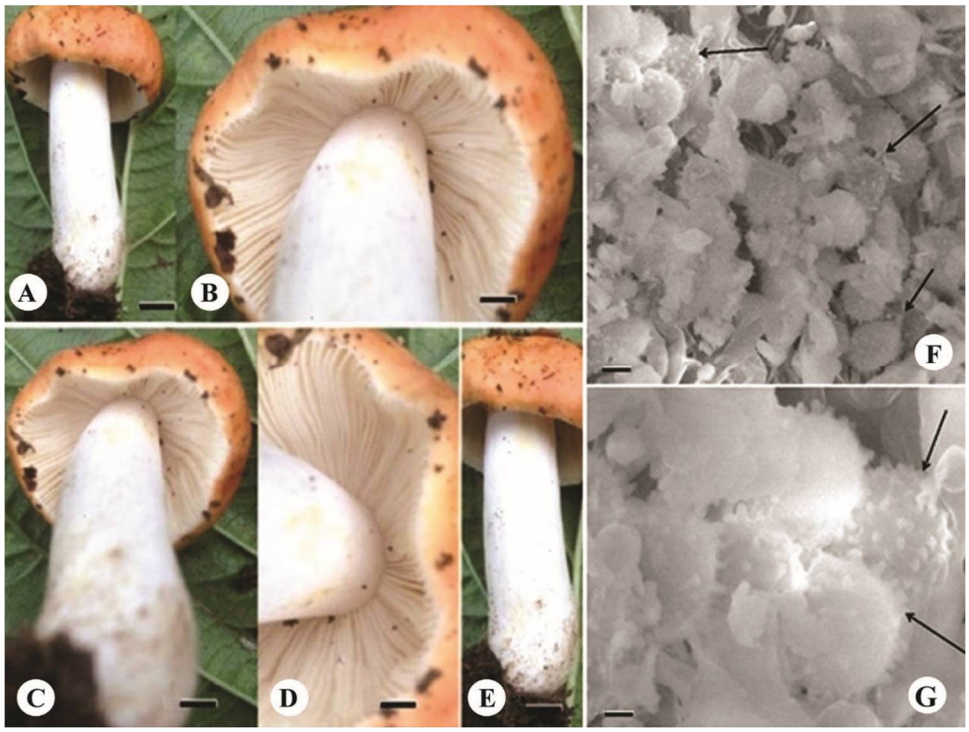

Fig. 1. Macroscopic characters of *Russula laeta* showing Pileus (A), Gills (B–D), Stipe (E), Basidiospores SEM (F–G)

Material examined: Pakistan Khyber pakhtunkhwa, Swat Miandam, 25 July 2016, under *Abies pindrow* Royle, at 1800 meter above sea level (m.a.s.l.), MH032272016.

Phylogenetic analysis **(Fig. 4)**

Molecular description of *R. laeta* was carried out by amplifying ITS regions of nrDNA. After BLAST analysis, this species showed 100% similarity with *R. laeta* with accession no. MG679812. Among 1042 characters, 315 were conserved, 542 were variable and 189 were parsimony informative sites. In phylogenetic tree, *R. laeta* sequence formed a clade with *R. laeta.*

Russula nobilis Velen. (**Fig. 2)**

Plectological analysis

Pileus is 9.2 cm in diameter, shape plane with uniform depth, plicate (folded), colour variable but mostly dark red; surface smooth and shiny; Margins wavy, splitting at maturity, margin curved to inner side when young, gradually straight with maturity, flesh creamy off white, unchanging on bruising or on exposure; Gills attachment curate, gill spacing moderate, attachment with pileus at base, white in colour when young and turns to creamy shade when old; stipe 9.7 long and 2 cm wide, equal in shape, attached centrally with pileus and gradually tapered at base;

white to creamy in colour, surface smooth, colour change on bruising, volva absent, Annulus absent, Taste not recorded, Odour indistinctive.

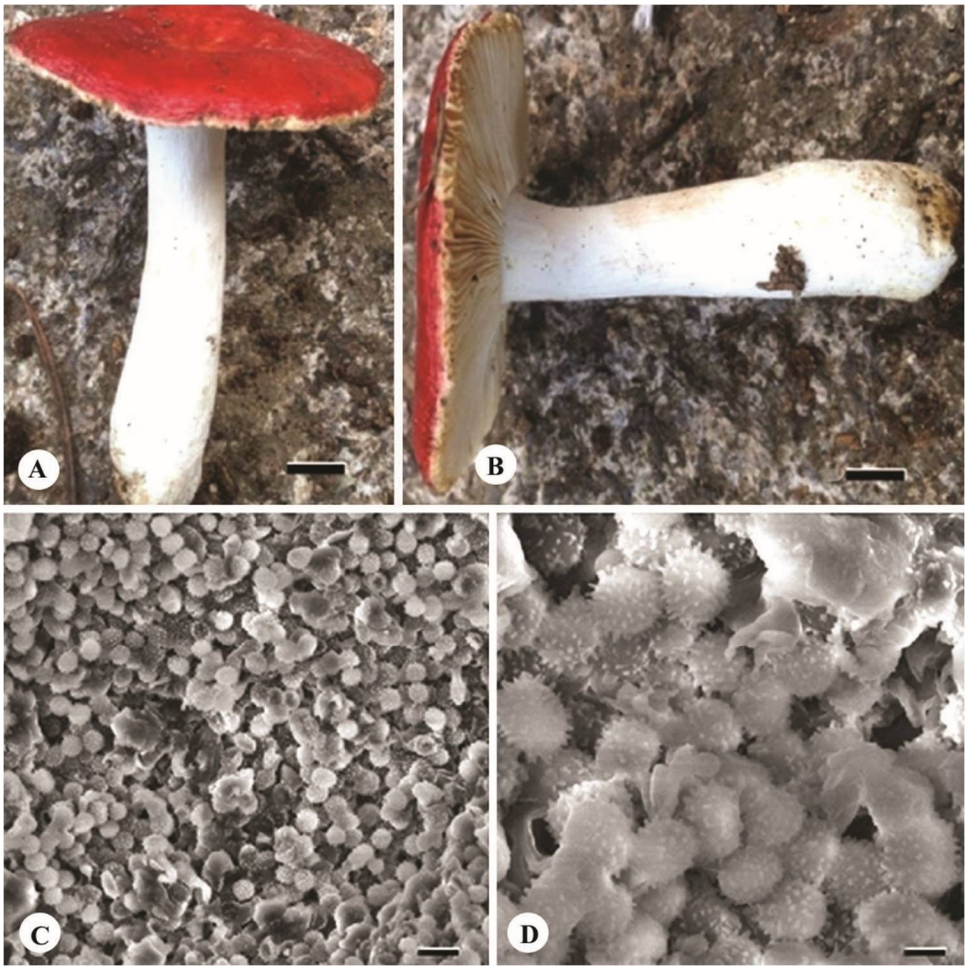

Fig. 2. Macroscopic Characters of *Russula nobilis* showing Pileus, Stipe and Gills (A–B), Basidiospores SEM (C–D)

Basidiospores 9.9 − 13.8 µm (avg=11.3 µm) spiny, thick walled, epiculated, abundant, light green in 10% KOH; Basidia 10.6 × 24.7 − 17.7 × 37.1 µm (avw=12.9 µm, avl=30.6 µm) clavate, 3–4 sterigmata, sterigmata long, thick walled, with oil granules, without clamp connection, light green in 10% KOH; Cystidia 10.6 × 31.8 − 17.7 × 38.9 µm (avw=14.8 µm, avl=34.8 µm) clavate , thick walled, with oil granules, without clamp connection, light green in 10% KOH; Pileipellis 3.2 µm, loosely arranged, narrow, aseptate, cylindrical with no clamp connection; Stipitipellis 3 µm, loosely arranged, narrow, aseptate, cylindrical with no clamp connection.

Material examined: Pakistan Khyber Pakhtunkhwa, Swat Miandam, 25 July 2016, under *Abies pindrow* Royle, at 1800 m.a.s.l., MH132272016.

Russula nigricans Bull Fr. **(Fig. 3)**

GenBank accession No. MK389375

Plectological analysis

Pileus 6.75 cm in diameter, convex (outwardly rounded) to umbeliform, smooth entire sulcate, colour shade range from creamy to dark brown, no bruising and no colour change on cut; Margins plane and rounded, tuberculately striated, margins incurved when young and remain intact at maturity; Gills attachment crowded, lamellae tube free to slightly adnate (gills widely attached to stem), gills in series, subdistant at maturity, split near the pileus margins, first off white and then turning to creamy with age,; Stipe 6.8 cm long and 2.75 cm wide, club shaped and broadened at base, white in colour and discolouring to brown, no reticulation, no bruising and no colour change on cut, chalky, contex solid to stuffed in stipe; Volva absent; Taste not recorded; Odour indistinctive.

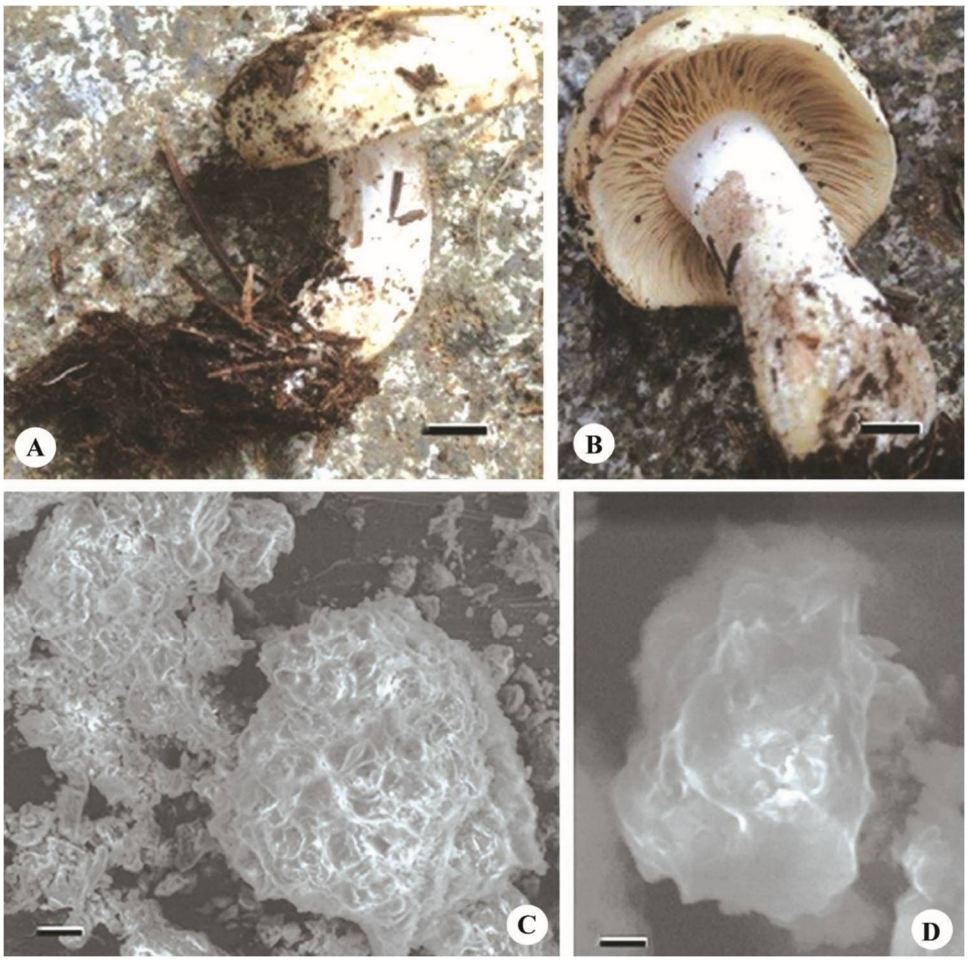

Fig. 3. Macroscopic Characters of *Russula nigricans* showing Pileus, Stipe and Gills (A–B), Basidiospores SEM (C–D)

Basidiospores 8.7 × 10.7 µm (avg=9.8 µm) globular, epiculated, thick layered, not abundant, hyaline to light green in 10% KOH; Basidia 8.5 × 21.2 − 10.6 × 30.0 µm (avw = 8.5 µm, avl = 24.2 µm) cylindrical, 3–4 sterigmata, sterigmata long, thin walled, without clamp connection, hyaline in 10% KOH; Cystidia 7 × 31.8 − 10.9 × 49.5 µm (avw= 9.3 µm, avl=40.3 µm) cylindrical, thin walled, without clamp connection, hyaline in 10% KOH; Pileipellis 7µm, loosely arranged, aseptate, cylindrical, abundant cystidia, with no clamp connection; Stipitipellis 9 µm, loosely arranged, aseptate, cylindrical with no clamp connection.

Material examined: Pakistan: Khyber Pakhtunkhwa, Swat Miandam, 25 July 2016, under *Abies pindrow* Royle, at 1800 m.a.s.l., MH212272016.

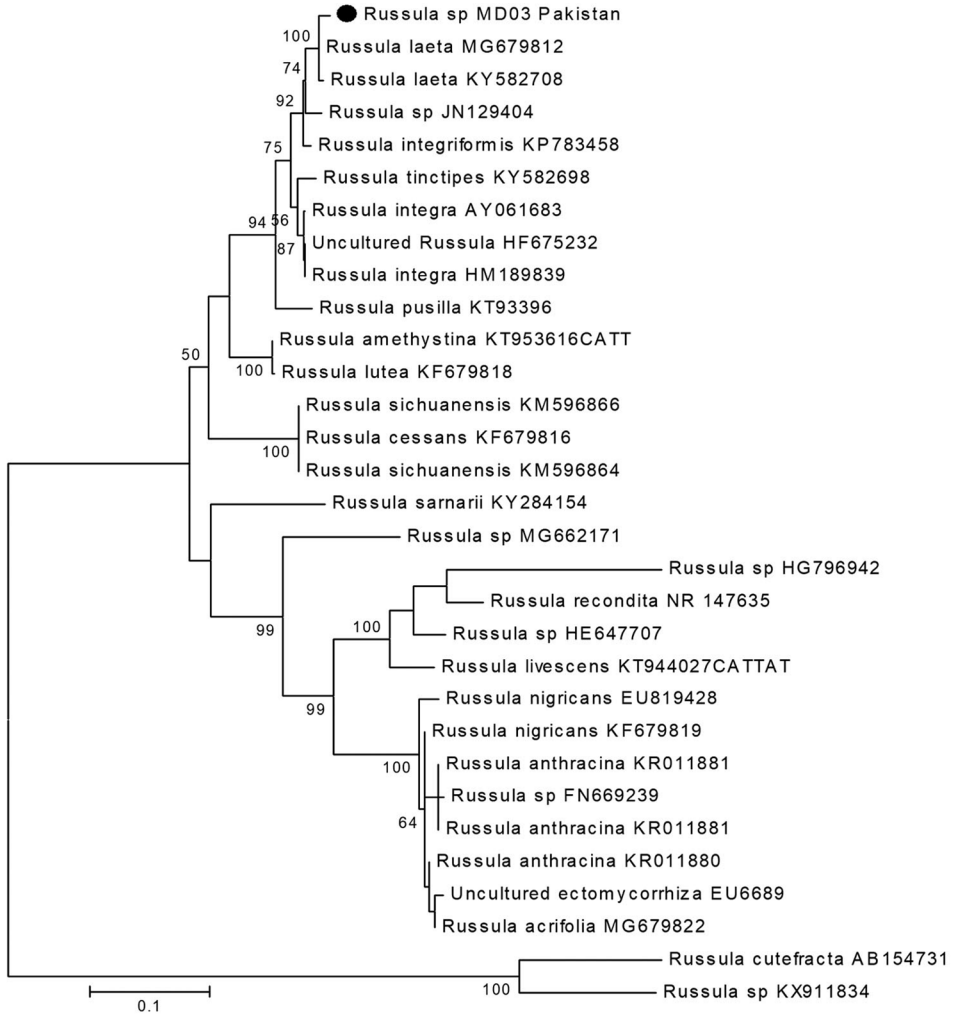

Fig. 4. Phylogenetic position of *Russula latea* from Pakistan with respect to other *Russula* spp. Tree inferred by maximum likelihood analysis based on rDNA sequences, including *ITS* region. The numbers against branches indicate the percentage (>50%) at which a given branch was supported in 1000 bootstrap replications. GenBank accession number is given at the end of species names. ● indicate species reported from Pakistan.

Phylogenetic analysis **(Fig. 5)**

Molecular description of *Russula* sp. was carried out by amplifying ITS regions of nrDNA. During BLAST analysis, this species matched 100% with *R. nigricans* with accession no. KF679819. From Genbank, closely related sequence of related species of genus *Russula* was retrieved. Among 720 characters, 368 were conserved, 314 were variable and 195 were parsimony informative sites. Phylogenetic analysis of *Russula nigricans* showed maximum likelihood with other members from 25 sequences. In phylogenetic tree, *Russula nigricans* sequence formed a clade with *R. nigricans.*

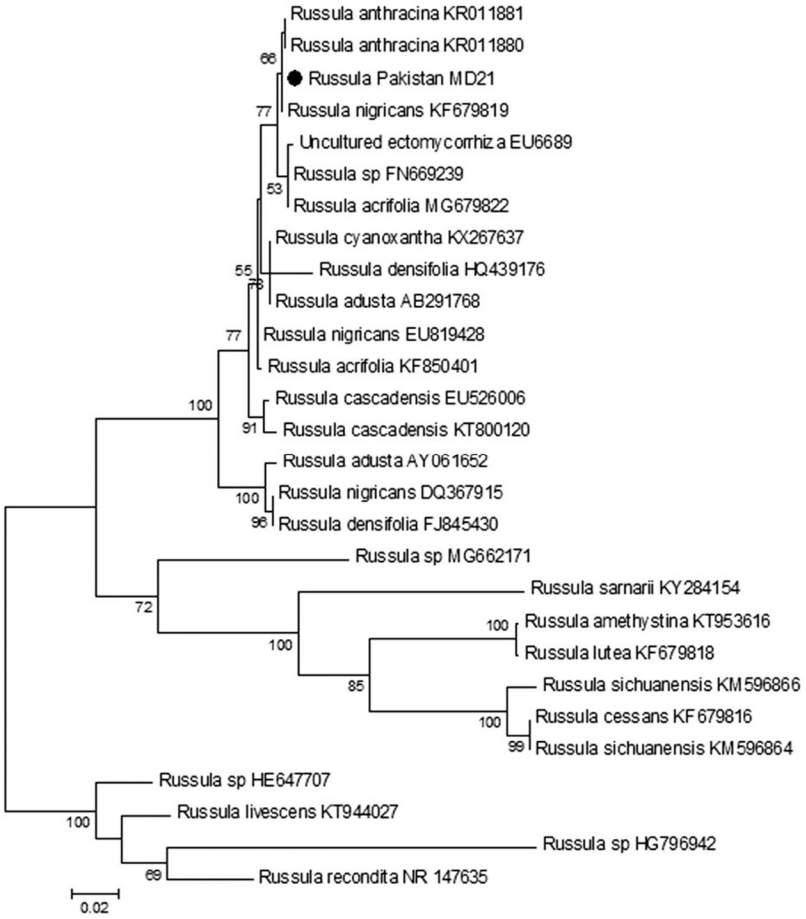

Fig. 5. Phylogenetic position of *Russula nigricans* from Pakistan with respect to other *Russula* spp. Tree inferred by maximum likelihood analysis based on rDNA sequences, including *ITS* region. The numbers against branches indicate the percentage (>50%) at which a given branch was supported in 1000 bootstrap replications. GenBank accession number is given at the end of species names. ● indicate species reported from Pakistan.

Antifungal activity **(Fig. 6)**

Effect of methanolic extract of R. laeta against A. niger

The effect of different concentrations of crude extract of *R. laeta* inspected against food pathogenic fungi *A. niger* in Malt Extract (ME) liquid medium. Extract of mushrooms were

prepared in methanol with different concentrations *i.e.*, 1%, 1.5%, 2% and 3%. The triplicates of different concentrations of methanolic mushroom extracts were observed. Fungal biomass calculated and compared with control *i.e.*, without methanolic mushroom extract concentrations. Four tested methanolic extract concentrations showed 36 %, 43.8%, 46.2% and 60.6% inhibition of pathogenic fungus growth respectively. Maximum inhibition was recorded with highest concentration of mushroom extract.

Effect of methanolic extract of R. nobilis against A. niger

The effect of different concentrations of crude extract of *R. nobilis* inspected against plant pathogenic fungi in Malt Extract (ME) liquid medium. Extract of mushroom were prepared in methanol with different concentrations *i.e.*, 1%, 1.5%, 2% and 3%. The triplicates of different concentrations of methanolic mushroom extract were observed. Fungal biomass was calculated and compared with control *i.e.*, without methanolic mushroom extract concentrations. Four tested methanolic extract concentrations showed 27.8%, 35%, 40% and 43.6% inhibition of pathogenic fungus growth respectively. Maximum inhibition was recorded with highest concentration of mushroom extract.

Effect of methanolic extract of R. nigricans against A. niger

The effect of different concentrations of crude extract of *Russula nigricans* inspected against *A. niger* in Malt Extract (ME) liquid medium. Extract of mushroom was prepared in methanol with different concentrations *i.e.*, 1%, 1.5%, 2% and 3%. The triplicates of different concentrations of methanolic mushroom extracts were observed. Fungal biomass was calculated and compared with control *i.e.*, without methanolic mushroom extract. Four tested methanolic extract concentrations showed 36%, 46%, 58% and 62% inhibition of pathogenic fungus growth respectively. Maximum inhibition was recorded with highest concentration of mushroom extract.

Assessment of antifungal activity

Results showed that *R. nigricans* showed maximum inhibition of fungus growth as compare to others selected mushrooms and minimum inhibition was observed in *R. nobilis*. This result revealed that each mushroom shows different potential against *A. niger* growth inhibition and also depends upon the concentration used.

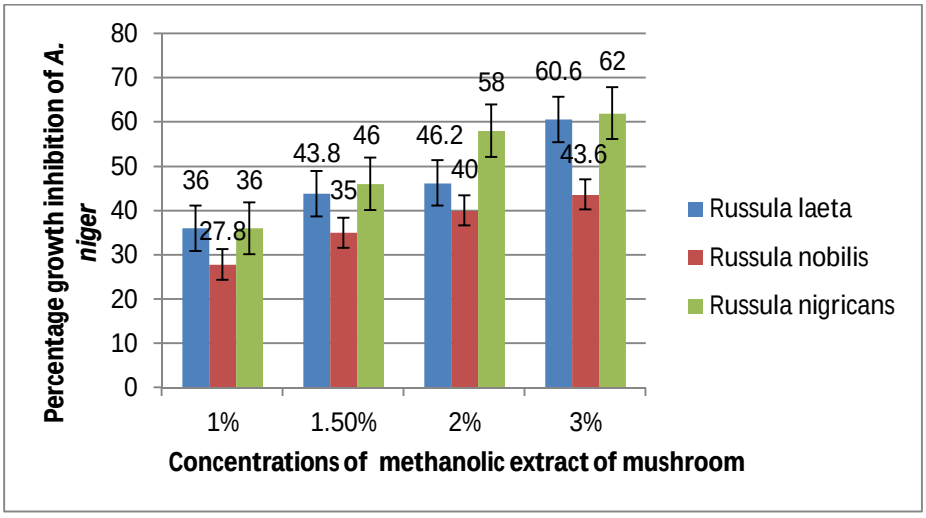

Fig. 6. Comparison of antifungal activity of selected mushrooms against test fungus.

Discussion

The present study was conducted to carry out the morphological and molecular identification of mushrooms *viz., R. laeta, R. nobilis* and *R. nigricans* belonging to order Russulales along with their antifungal activity against food pathogenic fungus. Phylogenetic analysis was carried out by amplifying ITS nrDNA using primers (ITSI-5.8 and ITS4) and identified by using literature (KolmaKov, 2015; Morozova *et al.,* 2012; Tschen and Tschen, 2005; Sarnari, 1998; Romagnesi, 1985).

Present research work also showed that different mushrooms have different potential against tested fungus to inhibit its growth. During this research, crude extracts of *Russula* spp. were examined against the test fungus *Aspergillus niger*. Different concentrations of mushrooms extract (1%, 1.5%, 2% and 3%) were applied *in vitro* against the test fungus. These mushrooms showed pronounced antifungal activity and selected concentrations of the extracts were effective in inhibiting the biomass of target fungus growth up to 62%. Results were evaluated by weighing dry mass of fungus growth against extracts of mushrooms with different concentrations. It was found that *R. nigricans* showed maximum inhibition (62%) of target fungus growth at 3% concentration. The other two mushrooms showed inhibition at same 3% concentration as 60.6% and 43.6% respectively. Study conducted by Emilija *et al.* (2005) on wild mushroom extracts *viz., Russula cyanoxantha, Suillus fluryi* and *Tricholoma acerbatum* analyzed against selected microorganisms *viz., Saccharomyces cerevisiae* and *A. niger* and their antifungal activity were screened. The highest antifungal activity was showed by *R. cyanoxantha* against *S. cerevisiae* and *A. niger*.

Presently, fungal diseases are causing huge problem to yield healthier food. Antifungal agents will be useful to stop spreading of disease in future (Feng and Zheng, 2007). Mushrooms contain compounds (*i.e.,* phenols, flavonoids, ascorbic acid, etc.) that have growth inhibitory potential against fungi, bacteria and other microbes and can be a good alternative source against these pathogens. These features enable their use in combating fungi that cause food damage such as *Alternaria solani* (Atti-Serafini *et al.,* 2002). The methanolic mushroom extracts which are used in present study showed a significant level of antifungal activities depending on concentration used. The determined activities showed that these mushrooms can be used as biocontrol against different pathogenic fungi. This could be a good alternative method to control microbes (Abdulrahman and AlKhail, 2005). In addition, first time antifungal activity was checked by using mushrooms extracts belonging to order Russulales with reference to Pakistan. Due to high economic value of genus *Russula* along with antifungal and antioxidant value this requires further research to check antifungal activities of these mushrooms against different pathogenic fungi because a large number of species of *Russula* is available and most of them are unidentified or somewhat misidentified due to lack of advance technology by molecular markers and phylogenetic analysis

Conflict of interest

The author(s) declare(s) no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country(-ies) where they were performed.

References

Abdulrahman, A. and AlKhail, A. 2005. Antifungal activity of some extracts against some plant pathogenic fungi. Pakistan J. Biol. Sci. **8**: 413–417.

Ahmad, S., Iqbal, S.H. and Khalid, A.N. 1997. Fungi of Pakistan. Sultan Ahmad Mycological Society, Pakistan.

Atti-Serafini, L., Pansera, M.R., Atti-Santos, A.C., Rossato, M., Pauletti, G.F., Rota, L.D., Paroul, N. and Moyna, P. 2002. Variation in essential oil yield and composition of *Lippia alba* (Mill.) N. E. Br. grown in southern Brazil. The Revista Brasileira de Plantas Medicinais. **4**: 72–74.

Barung, D., Egmond, H., Garcia, L.R., Osenbruggen, V.T. and Visconti, A. 2003. Meeting the mycotoxins menace, Wageningen, The Netherlands. Wageningen Academic Publishers. **4**: 11–15.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic. Acids. Res. **32**: 1792–1797.

Emilija, M.M., Smilja, K., Milan, V., Drago, S., Mladen, B. and Verica, V.B. 2005. *Candida* infections of diabetic foot ulcers. Diabeto. Croat. **34**: 29–35.

Feng, W. and Zheng, X. 2007. Essential oils to control *Alternaria alternata in vitro* and *in vivo*. Food Control. **18**: 1126–1130.

Gardes, M. and Bruns, T.D. 1996. ITS primers with enhanced specificity for basidiomycetes, application to the identification of mycorrhizae and rusts. Mol. Ecol. **2**: 113–118.

Jabeen, S., Niazi, A.R. and Khalid, A.N. 2016. First record of *Russula anthracina* and its ectomycorrhiza associated with Himalayan cedar from South Asia. Mycotaxon. **131**: 31–44.

Jabeen, S., Sarwar, S., Niazi, A.R. and Khalid, A.N. 2014. Checklist of Ectomycorrhizae from Pakistan. Annals of Applied Bio-Sciences. **1**: 10–20.

Javaid, A. and Samad, S. 2012. Screening of allelopathic trees for their antifungal potential against *Alternaria alternata* strains isolated from dying–back *Eucalyptus* spp. Nat. Prod. Res. **26**(18): 1697–1702.

Jukes, T.H. and Cantor, C.R. 1969. *Evolution of Protein Molecules*. New York: Academic Press. pp. 21–132.

Kolmakov, P. 2015. Checklist of fungi of the genus *Russula* from Belarusian-Valdai Lake District [Baltarusijos ir Valdajaus ežeryno regiono *Russula* Pers. genties grybų sąrašas]. Bot. Lith. **21**(1): 22–33

Miller, M.A., Pfeiffer, W. and Schwartz, T. 2010. Creating the CIPRES Science Gateway for Inference of Large Phylogenetic Trees. In Proceedings of the Gateway Computing Environments Workshop (GCE). (New Orleans, November 14, 2010).

Morozova, O.V., Popov, E.S. and Kovalenko, A.E. 2012. Studies on mycobiota of Vietnam. I. Genus *Entoloma*: new records and new species. Mycology and Phytopathology **46**(3): 182–200.

Niazi, A.R., Iqbal, S.H. and Khalid, A.N. 2006. Biodiversity of Mushrooms Ectomycorrhiza. *Russula brevipes* Peck. its ectomycorrhiza, a new record from Himalayan Moist Temperate Forests of Pakistan. Pak. J. Bot. **38** (4): 1271–1277.

Razaq, A., Ilyas, S., Khalid, A.N. and Niazi, A.R. 2014. *Russula foetentoides* sp. nov. (Russulales, Russulaceae) a new species from Pakistan. Sydowia. **66**: 289–298.

Romagnesi, H. 1985. Les Russules d' Europe et d' Afrique du Nord. Reprint with supplement. J. Cramer, Lehre.

Sarnari, M. 1998. Monografia illustrate del genere Russula in Europa. Italy: Tromo Primo.

Steel, R.G.D., Torrie, J.H. and Dicky, D.A. 1997. Principles and Procedures of Statistics, A Biometrical Approach. 3rd Edition, McGraw Hill, Inc. Book Co., New York, 352-358.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28**: 2731–2739.

Tschen, E.F.T. and Tschen, J.S.M. 2005. Three species of *Russula* new to Taiwan. Fungal Science. **20**(1&2): 47–52.

Werle, E., Schneider, C., Renner, M., Volker, M. and Fiehn, W. 1994. Convenient single–step, one tube purification of PCR products for direct sequencing. Nucleic. Acids. Res. **22**: 4354–4355.

White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (eds) PCR protocols. A guide to methods and applications. Academic Press, San Diego. pp. 315–322.

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