Russula senecis: An unexplored wild mushroom for your healthy lunch basket

Somanjana Khatua, Arun Kumar Dutta and Krishnendu Acharya*

Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, Kolkata, West Bengal - 700019, India.

*Corresponding author e-mail: krish_paper@yahoo.com

ABSTRACT

Russula senecis, a worldwide distributed mushroom, is exclusively popular among the tribal communities of West Bengal for food purposes. The present study focuses on its reliable taxonomic identification through macro-, micro-morphological features and DNA barcoding, confirmation of its systematic placement by phylogenetic analyses, myco-chemicals and functional activities. For the first time, complete Internal Transcribed Spacer region of *R. senecis* has been sequenced and its taxonomic position within subsection *Foetentinae* under series *Ingratae* of the subgen. *Ingratula* is confirmed through phylogenetic analysis. For exploration of its medicinal properties, dried basidiocarps were subjected for preparation of a heat stable phenol rich extract (RusePre) using water and ethanol as solvent system. The antioxidant activity was evaluated through hydroxyl radical scavenging (EC_{50} 5 µg/ml), chelating ability of ferrous ion (EC_{50} 0.158 mg/ml), DPPH radical scavenging (EC_{50} 1.34 mg/ml), reducing power (EC_{50} 2.495 mg/ml) and total antioxidant activity methods (13.44 µg ascorbic acid equivalent/mg of extract). RusePre exhibited antimicrobial potentiality against *Listeria monocytogenes*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Furthermore, different parameters were tested to investigate its chemical composition which revealed the presence of appreciable quantity of phenolic compounds along with carotenoids and ascorbic acid. HPLC-UV fingerprint indicated existence of at least 13 phenolics of which 10 were identified (pyrogallol> kaempferol> quercetin> chlorogenic acid> ferulic acid, cinnamic acid> vanillic acid> salicylic acid> *p*-coumaric acid> gallic acid). Result from the present work suggests that the fraction, RusePre, may open novel prospection as a functional ingredient in antioxidant supplements and in drugs to treat infectious disease.

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INTRODUCTION

A recent estimation implies the existence of around 3 million fungi (Hawksworth, 2012) of which approximately 140,000 species pass the criteria as set by Chang & Miles (1992) to be considered as 'Mushroom' (Rajaratnam & Thiagarajan, 2012). Standing into the era of 21st century, our present knowledge on the described mushroom species by far accounts to be only 10% of total estimated mushroom diversity (Chang & Miles, 2004). Out of these 14,000 identified macrofungal species, about 650 have been recognized to possess medicinal properties (Thatoi & Singdevsachan, 2014). Thus, there is a recent trend among mycologists to document therapeutic value of mushrooms all around the globe and the present study is not an exception of that.

To meet the aim, West Bengal (21°38′-27°10′ N latitude and 85°50′-89°50′ E longitude) has been selected as study area due to its unique phyto-geographical feature. It is the only state in India, which shares its topographical extension from Himalayas in the northern side to the Bay of Bengal in the southern with regions such as plateau and Ganges delta prevailing in between. These wide ranges of topographical feature, types of soils and substrata make the state to be ideal for hosting and flourishing rich diversity of mushrooms (Dutta & Acharya, 2014).

Literature review implies that, afterwards the year of 2000 (Berkeley, 1850, 1851a, 1851b, 1852, 1854, 1876; Bose, 1919, 1920; Bose & Bose, 1940; Roy & De, 1996), there is no exhaustive investigation on macrofungal diversity from the state. Since last 10 years, extensive field work by our research team inventoried large no of wild mushrooms from different corner of state (Pradhan et al., 2012; Dutta et al., 2013). Morphological and molecular investigation revealed that many of them are new to science (Acharya, Dutta & Pradhan, 2012; Dutta et al., 2014), new record for India (Dutta et al., 2011, 2012a) and addition to the macrofungal flora of West Bengal (Dutta et al., 2012b; Acharya et al., 2014).

During foray, we have collected a number of wild edible mushrooms with active help from the ethnic and tribal mushroom hunters of the state and among them some revealed to be still not documented as edible mushroom. In this context, an undocumented mushroom from our collection was taxonomically investigated, systematic position was supported by the phylogenetic analysis and medicinal prospect was evaluated.

MATERIALS & METHODS

Mushroom sampling

During field survey (2008-2012), several edible mushrooms were collected from the forest floor of West Bengal, India accompanying with tribal mushroom hunters of the regions. Among the basket of mushrooms which they usually gather for their regular dishes, a unique mushroom, commonly called "JHAL PATRA" (JHAL = because of its acrid taste; PATRA = Mushroom) were chosen and brought to the laboratory for thorough taxonomic investigation. Detailed microscopic works were performed using the protocol of Buyck & Adamik (2011) and were identified as *Russula senecis* S. Imai using standard literatures (Imai, 1938; Zhishu, Guoyang & Taihui, 1993; Das, 2009). Colour codes and terms (mostly) follow Royal Botanic Gardens Edinburgh colour chart (Henderson, Orton & Watling, 1969). Scanning Electron Microscope (SEM) illustrations of basidiospores were carried out with Zeiss EVO-MA10 electron microscope at the Centre for Research in Nanoscience and Nanotechnology,

University of Calcutta, Kolkata, India. After thorough microscopic work, specimen voucher had been deposited in Calcutta University Herbarium (CUH).

Phylogenetic protocols

DNA extraction, Polymerase Chain Reaction and sequencing

Genomic DNA was extracted from dried herbarium specimens (10–50 mg) using the 'Fungal gDNA Mini Kit' (Xcelris Genomics, Ahmedabad, India). ITS region 1 and 2, and the 5.8S rDNA, were amplified using primers pair ITS1 and ITS4 (White et al., 1990). The DNA fragments were amplified on Applied Biosystems® 2720 automated thermal cycler following the protocol as described by Abd-Elsalam et al. (2003) with little modifications. PCR products were purified using QIAquick® Gel Extraction Kit (QIAGEN, Germany) and was subjected to automated DNA sequencing on ABI3730xl DNA Analyzer (Applied Biosystems, USA) using primers identical with amplification for ITS rDNA region. The newly generated sequences were then deposited in GenBank (www.ncbi.nlm.nih.gov).

Taxon sampling

Twenty eight Internal Transcribed Spacer (ITS) nrDNA sequences representing nineteen species were used in the analyses, of which two sequences of *Russula senecis* S. Imai were generated as part of this study. The sequences represent sixteen species of *Russula* distributed over five subgenus viz. *Compacta* (Fr.) Bon (*Russula delica* Fr.), *Heterophyllidia* Romagn. (*Russula cyanoxantha* (Schaeff.) Fr. and *Russula virescens* (Schaeff.) Fr.), *Amoenula* Sarnari (*Russula amoenicolor* Romagn.), *Ingratula* Romagn. (*Russula* cf. *laurocerasi*, *Russula* cf. *subfoetens*, *Russula fellea* (Fr.) Fr., *Russula foetens* Pers., *Russula insignis* Quél., *Russula grata* Britzelm. (in the present study represented as *Russula laurocerasi* Melzer), *Russula ochroleuca* Fr., *Russula pulverulenta* Peck and *Russula senecis* S. Imai), *Russula* emend. Sarnari (*Russula emetica* (Schaeff.) Pers.) and *Incrustatula* Romagn. emend. (*Russula rosea* Pers.). *Stereum hirsutum* (Willd.) Pers., *Amylostereum laevigatum* (Fr.) Boidin, and *Bondarzewia mesenterica* (Schaeff.) Kreisel (here represented as *Bondarzewia Montana* (Quél.) Singer) were selected as outgroup taxa for rooting purpose following Buyck et al. (2008). The accession numbers of newly generated two ITS sequences of *R. senecis* and those pulled from GenBank for the purpose of conducting phylogenetic analysis for this study are cited in Fig. 3.

Phylogenetic analysis

Sequences were edited with the CodonCode Aligner software (CodonCode Corporation, Dedham, Massachusetts). The newly generated two ITS1-5.8S-ITS2 sequences of *R. senecis* and those retrieved from GenBank were aligned with the help of ClustalX (Thompson et al., 1997) using default setting. A final set of 28 sequences were aligned. The appropriate substitution model was determined using Bayesian information criterion (BIC) in MEGA6 (Tamura et al., 2013). The K2+G model (with lowest BIC scores of 4931.469) was selected as the best-fit model.

Phylogenetic analyses was performed in MEGA6 (Tamura et al., 2013) using Maximum Likelihood (ML) method based on the Kimura 2-parameter model. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A

discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5337)).

Beside ML method, phylogenetic analyses were also carried out using Neighbor-Joining (NJ) method (Saitou & Nei, 1987) to determine whether different methods (Maximum Likelihood vs. Neighbor-Joining) alters the resulting phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The sum of branch length of the optimal tree was 1.12069257. In both the cases, all positions containing gaps and missing data were eliminated and a bootstrap test of 1000 replicates was performed to obtain the percentage of replicate trees for clustering the associated taxa.

Preparation of extract

Polyphenol rich fraction was extracted according to the method of Dasgupta et al. (2014). Dried and powdered basidiocarps of *R. senecis* were steeped with ethanol at 25ºC for 2 days to eliminate the alcohol soluble constituents such as coloured material, small organic molecules (steroid, terpenoids etc.) and fat. After filtration, the residue was then re-extracted with ethanol, as described above. The filtrate was air dried and extracted by stirring with distilled water at 100ºC for 7 hrs. Solvent was separated and 4 volume of ethanol was added slowly and kept at 4ºC overnight. Precipitate was discarded by centrifugation and supernatant was reduced in volume using a rotary evaporator (Butchi, Switzerland). This concentrated polyphenol rich extract of *R. senecis* (RusePre) was stored at -20ºC until further analysis.

Antioxidant activity

Hydroxyl radical scavenging assay

The method described by Halliwell et al. (1987) was followed for this study. The reaction mixture (1ml) consisted of potassium phosphate buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), variable concentration (1–10 µg/ml) of RusePre, $FeCl_3$ (100 mM), EDTA (104 µM), ascorbic acid (100 µM) and H_2O_2 (1 mM) and was incubated at 37˚C for 1 h. 2ml Thiobarbituric acid (TBA)- trichloro acetic acid (TCA) solution (0.375% (w /v) TBA, 15% (w /v) TCA and 0.25 N HCl) was added to stop reaction and incubated at boiling water bath for 15 min. After cooling, absorbance was measured at 535 nm against buffer. Identical reaction mixtures were prepared where TBA-TCA solution was added prior incubation to subtract background colour. Butylated hydroxyanisole (BHA) was used as positive control. EC_{50} value expressed the effective concentration at which the scavenging free radical activity was 50%. The degree of scavenging was calculated by following equation:

$$\text{Scavenging effect (\%)} = \{(A_0\text{-}A_1)/ A_0\} \times 100$$

Where A_0 was the absorbance of control and A_1 was absorbance in presence of sample

DPPH radical scavenging assay

Radical scavenging activity in RusePre was evaluated using purple coloured 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radicals based on the method by Shimada et al. (1992). The reaction mixture (2 ml) consisted of methanol solution of DPPH (0.101 mM) and various concentrations of RusePre (0.5–1.5 mg/ml). The mixture was shaken and left to stand for 30

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min in dark. Absorbance was measured at 517 nm against blank. EC_{50} value is the effective concentration at which DPPH radicals were scavenged by 50%. Ascorbic acid was used for comparison. The degree of scavenging was calculated by following equation:

Scavenging effect (%) = $\{(A_0-A_1)/ A_0\} \times 100$

Where A_0 was the absorbance of control and A_1 was absorbance in presence of sample

Chelating ability of ferrous ions

The ability of investigated extract to chelate ferrous ion was determined (Dinis, Mudaira & Alnicida, 1994) with slight modification. Reaction mixture (2 ml) contained different concentration of RusePre (0.05–0.2 mg/ml) mixed with water and 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 min incubation at room temperature, the absorbance was determined at 562 nm against a blank. EDTA was used as positive control. EC_{50} value is the effective concentration at which ferrous ions were chelated by 50%. The percentage of inhibition of ferrozine- Fe^{2+} complex formation is given by this formula:

Scavenging effect (%) = $\{(A_0-A_1)/ A_0\} \times 100$

Where A_0 was the absorbance of control and A_1 was absorbance in presence of sample

Determination of reducing power

A modified method of reducing power described by Oyaizu (1986) was considered. Various concentrations of RusePre (1–3 mg/ml) were mixed with 2.5 ml sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated for 20 min and then 2.5 ml of TCA (10%) was added. 2.5 ml of solution was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%) and incubated for 15 min. The absorbance was measured at 700 nm against buffer. Ascorbic acid was used as positive control. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration.

Determination of total antioxidant capacity by phosphomolybdenum method

The assay was carried out as described by Prieto, Pineda & Aguilar (1999) with little modification (Mitra et al., 2014). The reaction mixture consisted of 0.3 ml sample solution (0.1 mg/ml) and 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium sulphate and 4 mM ammonium molybdate). Blank was prepared by adding 0.3 ml water and 3 ml reagent solution. Tubes were capped and incubated at 95ºC for 90 min. Samples were cooled at room temperature and absorbance was measured at 695 nm against blank. Concentrations of ascorbic acid (1–30 µg/ml) were used to obtain a standard curve. Total antioxidant activity was expressed as the number of equivalents of ascorbic acid.

Antimicrobial activity

Test bacteria

Listeria monocytogenes MTCC Code 657, *Salmonella typhimurium* MTCC Code 98 and *Bacillus subtilis* MTCC Code 736, *Escherichia coli* MTCC Code 68, *Pseudomonas*

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aeruginosa MTCC Code 8158 and *Staphylococcus aureus* MTCC Code 96 were obtained from the culture collection of the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. They were incubated for 24 hours by inoculation into nutrient broth.

Disk diffusion method

The determination of inhibitory effect of RusePre on test bacteria was carried out by agar-disc diffusion method (Bauer et al., 1966). Nutrient agar was poured into each sterilized petridish (90 mm diameter) after injecting cultures (100 µl) of bacteria and medium was distributed homogeneously. Paper discs (5 mm) were loaded with 20 µl of 20 mg/ml concentrated RusePre. The impregnated discs were air dried before placing it on the petri dishes with the test microorganisms. Plates were incubated as per the bacterial requirement. Studies were performed in triplicate and the inhibition zones were compared with those of blank discs.

Chemical composition

Mycochemical Analyses

The content of total phenolic compounds in RusePre was estimated using Folin-ciocalteu reagent and gallic acid as standard (Singleton & Rossi, 1965). The results were expressed as µg of gallic acid equivalents per mg of dry extract. Total flavonoid content was determined using aluminium nitrate and potassium acetate. Quercetin (5–20 µg/ml) was used to calculate the standard curve (Park et al., 1997). The results were expressed as µg of quercetin equivalents per mg of dry extract. β-carotene and lycopene were estimated by measuring absorbance at 453, 505 and 663 nm (Nagata & Yamashita, 1992). Ascorbic acid was determined by titration against 2, 6-dichlorophenol indophenol dye (Rekha et al., 2012).

Determination of phenolic profile by HPLC

For quantitative analysis of phenolic compounds, a 3-level calibration curve was obtained by injection of known concentrations (10–50 µg/ml) of eleven standard compounds: gallic acid ($y = 34.773x - 9.2238$; $R^2 = 0.9991$), chlorogenic acid ($y = 13.776x – 2.9025$; $R^2 = 0.9993$), vanillic acid ($y = 19.225x + 0.2588$; $R^2 = 0.9994$), *p*-coumaric acid ($y = 49.773x - 10.541$; $R^2 = 0.9994$), ferulic acid ($y = 30.425x - 2.8188$; $R^2 = 0.9995$), myricetin ($y = 5.0676x - 6.0375$; $R^2 = 0.9937$), salicylic acid ($y = 4.4974x - 0.4763$; $R^2 = 0.9994$), quercetin ($y = 5.2478x - 5.9763$; $R^2 = 0.9954$), cinnamic acid ($y = 108.07x - 111.55$; $R^2 = 0.9979$), pyrogallol ($y = 10.8x + 0.3333$; $R^2 = 0.9999$) and kaempferol ($y = 18.667x – 80.875$; $R^2 = 0.9997$). The results were expressed as µg/mg of dry extract.

0.5 mg RusePre was dissolved in1 ml of methanol and water (1:1 v/v) and filtered through 0.2 µm filter paper. 20 µl filtrate was loaded on the HPLC system (Agilent, USA). Separation was achieved on an Agilent Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 µm) using a flow rate of 0.8 ml/min at 25°C. The mobile phase consisted of eluent A (acetonitrile) and eluent B (aqueous phosphoric acid solution, 0.1% v/v). A gradient program was used for elution: 0-2 min, 5% A; 2-5 min, 15% A; 5-10 min, 40% A; 10-15 min, 60% A; 15-18 min, 90% A. The absorbance of standard and sample solution was measured at 280 nm. Sample compounds were identified on the basis of retention times and absorption spectra of standard materials. Components were quantified by comparing their peak areas with those of standard curves.

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RESULTS & DISCUSSION

Taxonomy

Russula senecis S. Imai

Pileus 5.5–7(–13) cm broad, convex when young, becoming plano-convex to applanate at old, usually with broad central depression, glabrous, slightly viscid when wet, hygrophanous, bay, pale ochraceous buff to ochraceous-tawny towards centre, pallid to ochraceous buff towards margin, surface turns translucent rust to rusty-tawny with KOH; margin decurved, tuberculate striate; cuticle not easily separable from the context, cracking up into patches near margin; context up to 3.5 mm thick, creamy buff, unchanging color when exposed (Fig. 1A-B). **Lamellae** 4.5–6 mm broad, adnexed, regular, bifurcate near the attachment of stipe, rarely one tiered, creamy buff, entire, even, edge discolorous, with fine brown to sienna buff margin. **Stipe** 5.5–7.5(–14) ×1.1–1.3(–2.4) cm towards top × 1.2–2.5 cm towards base, tapered towards the base, central to slightly eccentric, fleshy, slightly curved, cylindrical, becoming compressed, multi chambered at maturity; surface smooth, moist, slight shiny, creamy buff to dull yellow, often with fine dark brown warts, becoming clay buff on bruising, turns rusty-tawny to bay with KOH (Fig. 2A). **Odor** strong. **Taste** very acrid. **Spore print** creamy white.

Basidiospores (7.5–)8.2–8.6–8.9(–9.7) × 7.8–8.3–8.6 µm, Q= 0.95–1.04–1.18, globose to subglobose, ornamentation amyloid, up to 2.1–3.2 µm high, composed of large wings and isolated warts, never forming reticulum (Fig. 1C-D; 2B). **Basidium** 32–38 × 10–10.7 µm, clavate, 4–spored (Fig. 2C). **Hymenial cystidia** (61–)64–68(–82) × 8.6–9.7(–10.7) µm, lanceolate to fusoid or elongated fusoid, with mucronate to moniliform apex, thin-walled, mostly with heteromorphous contents (Fig. 2D). **Lamellar trama** ca. 143–150 µm broad towards middle, 96 µm broad towards edge, mainly composed of sphaerocytes. **Subhymenium** pseudoparenchymatous. **Pileipellis** orthochromatic in cresyl blue, sharply delimited from underlying sphaerocytes of the context, distinctly divided into a dense, gelatinized, ca. 143–157(–161) µm deep subpellis composed of horizontally oriented hyphae, 3.2–3.6(–4.3) µm wide, mostly scattered with oleiferous fragments, (5.7–)6.4–7.2(–8.6) µm wide, and a less gelatinized, 36–72(–89) µm deep suprapellis of erect or repent hyphal ends. Incrustations absent. Pileocystidia up to 4.3–7.2 µm broad, mostly lanceolate, apex cylindrical to often with a minute rounded capitulum, thin-walled, recognizable by their distinct heteromorphous contents (Fig. 2E). Underlying sphaerocytes globose to sub-globose, ca. 12.5–13.9(–14.3) × 13.6–14.3 µm, hyaline. **Stipitipellis** up to 107–143 µm thick, composed of 3.6–3.9 µm broad hyphae, frequently with interspersed oleiferous hyphae, measuring 5.7–8.9 µm broad. **Caulocystidia** absent. **Stipe trama** composed of nested subglobose sphaerocytes, measuring 21–36(–44) µm diam.

Habit and habitat: common, ectomycorrhizal with *Shorea robusta* C.F.Gaertn. and *Castanopsis* sp.

Specimen examined: INDIA: West Bengal, Burdwan district, Malandighi, 11 July 2008, Prakash Pradhan, CUH AM103; Burdwan district, Malandighi, 25 August 2008, Prakash Pradhan, CUH AM104; Bankura district, Bishnupur, 10 August 2009, Prakash Pradhan, CUH AM105; Bankura district, Manjhulia, 15 July 2010, Prakash Pradhan, CUH AM106; Birbhum district, Gonpur, 08 July 2011, Arun Kumar Dutta and Prakash Pradhan, CUH AM107; East Midnapur district, Ramnagar, Kasaphaltalya, 24 July 2011, Arun Kumar

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Dutta and Prakash Pradhan, CUH AM108; Darjeeling district, Jawbari, 28 June 2012, Prakash Pradhan, CUH AM102; Darjeeling district, 7th mile Jungle, near Gurdum, 1 July 2012, Prakash Pradhan, CUH AM081.

Notes: *Russula senecis* was originally described from Japan (Imai, 1938), and reported to frequently grow in association with *Vateria indica* plant among the dipterocarp forests of Western Ghats (Natarajan et al., 2005), and in mixed forests under *Lithocarpus* and *Castenopsis* plant from Sikkim Himalaya, India (Das, 2009; Das, Van de Putte & Buyck, 2010). This well known widely distributed species can be easily recognized by the combination of an ochraceous-tawny pileus which turns rust to rusty-tawny with KOH, ochraceous buff tuberculate striate margin; creamy buff lamellae which often bifurcate near the attachment of stipe, discolorous lamellae with fine brown to sienna buff edges; creamy buff to dull yellow coloured, multi chambered stipe; acrid taste; strong odor; cream spore print; globose to sub-globose basidiospores (7.5–9.7 × 7.8–8.6 µm) with large wings and isolated warts, often with ridges (up to 2.1–3.2 µm high), but never form reticulum, absence of amyloid suprahilar spot; lanceolate to fusoid or elongated fusoid hymenial cystidia with mostly mucronate to moniliform apex; and lanceolate pileocystidia. The presence of these morphological features, categorize *Russula senecis* within the subgen. *Ingratula* Romagn., series *Ingratae* (Quél.) Maire and subsect. *Foerentinae* (Melzer & Zvára) Singer (Sarnari, 1998).

Being a member of series *Ingratae* (of subgenus *Ingratula*), *R. senecis* closely resembles with *Russula laurocerasi* and *Russula foetens*. However, *R. laurocerasi* differs from the present species by a light yellow to brilliant yellow or orange yellow coloured pileus with viscid to sticky surface, yellowish white lamellae, presence of lamellulae, pale yellow coloured spore-print, up to 5 µm broad pileocystidia and *R. foetens* differs by having characters like brilliant to dark or deep orange yellow or soft yellowish brown pileus, yellowish white coloured lamellae with lamellulae of two series, a stipe with veined surface, pale yellow spore-print, partially amyloid and mostly conic to acute tipped isolated warts basidiospores, fusoid shaped hymenial and pileocystidia. A recently described species from India, *Russula dubdiana* K. Das, Atri & Buyck, differs from *R. senecis* by having a white coloured lamellae which turns sienna after bruising, white stipe when young, becoming faintly greying in places at maturity or hazel which turns fulvous to cinnamon towards base on bruising, smaller (5.2–7 × 4.2–5.5 µm) broadly ellipsoid to ellipsoid basidiospores with mostly of cylindrical warts and very few ridges and fertile lamellae edge (Das, Atri & Buyck, 2013).

Molecular phylogeny

Phylogenetic analyses were performed on an ITS dataset of 28 sequences of which 25 sequences were *Russula* species, and the remaining three viz. *S. hirsutum*, *A. laevigatum*, and *B. Montana* were used as an outgroup for rooting purposes. Sequencing products of the collected samples from different places in subsequent years ranged from 578 to 632 nucleotides. All sequences were aligned and the ends trimmed to create a dataset of 560 nucleotides that included 336 positions in the final dataset.

The resulting phylogram with the highest log likelihood value (–2215.8014) is considered to represent in the present manuscript. The phylogram obtained using Neighbor-Joining method displayed same topology with the phylogram obtained using ML analyses.

Data obtained from ML analyses and NJ analyses (Bootstrap percentage) has been indicated in Fig. 3.

Twenty five sequences of in-group *Russula* species distributed over five subgenus (Sarnari, 1998), resulted two distinct clades (I and II) with moderate bootstrap support (BS). Morphologically, all members of Clade-I are being well characterized by having basidiospores without an amyloid spot where as members under clade-II possess basidiospores with distinct amyloid spot. Clade-I is further subdivided into two subclades (viz. subclade-A and subclade-B) with 56% BS (NJ) and 64% BS (ML) respectively.

Within subclade-A, *Russula senecis* clusters with the members of the subsect. *Foetentinae* (viz. *R. laurocerasi*, *R.* cf. *laurocerasi*, *R. foetens* and *R.* cf. *subfoetens*) with high bootstrap support (99% BS and 94% BS) and clearly separate from that of the sect. *Subvelatae*. Distinct differentiation of the subsect. *Foetentinae* from that of sect. *Subvelatae* based on the molecular data (ITS sequence) is also supported by the morphological characters like no reddening reaction with KOH and absence of arachnoid veil (Sarnari, 1998). *R. farinipedes* of the subsect. *Farinipedes* (subgen. *Ingratula*), clusters with that of *R. cyanoxantha* (subsect. *Cyanoxanthinae*; subgen. *Heterophyllidia*) with relatively low bootstrap support (50% BS in NJ analysis). Morphologically both the species shows white coloured spore-print, whereas species belonging to sect. *Subvelatae* and subsect. *Foetentinae* shows cream coloured spore-print. A similar result was also observed by Eberhardt (2002), where subsect. *Cyanoxanthinae* comes basal to the subsect. *Foetentinae* with bootstrap values ≥ 50 %. In the present study, incorporation of species belonging to the section *Subvelate*, results a single clade with high bootstrap support values and subsect. *Cyanoxanthinae* (represented here by *R. cyanoxantha*) along with subsect. *Farinipedes* comes basal to the clade which contains members of subsect. *Foetentinae* and sect. *Subvelatae* (subgenus. *Ingratula*) with bootstrap values ≤ 50 %.

R. amoenicolor and *R. virescens* cluster together and form subclade-B with 75% BS (NJ) and 76% BS (ML) respectively (morphologically, in both species number of lamellulae is rare) and clearly separates from that of *R. delica* (subgen. *Compacta*), generally known to posse's abundant number of lamellulae (Sarnari, 1998), with 54% BS (NJ) and 62% BS (ML).

Clade-II consists of four species, distributed within three subgenus viz. *Incrustatula*; sect. *Felleinae* of subgen. *Ingratula*; and subgen. *Russula*. Member representing the subgenus *Incrustatula* (*R. rosea*) forms subclade-C and distinctly separates from that of subclade-D with moderate bootstrap supports (72% BS and 65% BS respectively). The separation of these two subclades within clade-II is also supported by the morphological characters like presence (members belonging to subclade-D) or absence (species clusters within subclade-D) of pileocystidia.

Although sect. *Felleinae* is within the subgenus *Ingratula*, the present study reveals that sect. *Felleinae* (represented here by *R. fellea*) is more closely related to subgenus *Russula* than that of the remaining section *Subvelatae* and series *Ingratae* (subsect. *Foetentinae* and *Farinipedes*) of subgen. *Ingratula*. The discrete morphological difference of the members belonging to section *Felleinae*, is the presence of basidiospores with amyloid spot which is completely absent among the remaining sections of the subgen. *Ingratula* (Sarnari, 1998).

In accordance with the morphological features, phylogenetic analysis based on ITS1-5.8S-ITS2 sequence data revealed that, *R. senecis* clusters within the same clade (clade-A)

together with that of *R. laurocerasi* and *R. foetens*, confirming its position within the same subsection *Foetentinae* under the series *Ingratae* of the subgen. *Ingratula*.

Antioxidant activity

Hydroxyl radicals are formed by an electron transfer from transition metals to H_2O_2 and can easily cross cell membranes. It has ability to interact with biomolecules (carbohydrate, protein, lipid, DNA) immediately after formation and cause tissue damage or cell death. The radical itself and its subsequent radicals are considered to be the most toxic among all reactive oxygen species (ROS) (Valko et al., 2007). Therefore, removing hydroxyl radicals is important for the protection of living systems. In order to determine radical scavenging potentiality of RusePre the method described by Halliwell et al. (1987) was followed. RusePre showed excellent scavenging activity which rose gradually with the increase of doses (Fig. 4A). At 1, 5 and 10 µg/ml concentrations RusePre scavenged radicals at the rate of 4.34%, 49.32% and 67.14% respectively. The action was highly comparable with standard, BHA. At the same concentrations BHA exhibited radical scavenging at the rate of 17.74%, 40.08% and 70.23% respectively. EC_{50} value of RusePre was found to be 5 ± 0.2 µg/ml, whereas that of BHA was 6.9 ± 0.3 µg/ml which by far suggested extremely high activity of the extract. Paloi & Acharya (2013) have reported antioxidant activity of phenol rich fraction from *Amanita vaginata* (Bull.) Lam., AvaPre, using various methods. Based on the comparison of EC_{50} values, RusePre possessed higher activity than AvaPre (EC_{50} 10 µg/ml concentration).

The ability of extracts to quench reactive species by hydrogen donation was measured through the DPPH radical scavenging activity test. DPPH radical is considered to be long lived commercially available organic nitrogen radical and can directly react with antioxidants in a simple, rapid and sensitive procedure. Hence, model of scavenging stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of samples. Antioxidants may react with purple coloured DPPH radical and convert it into a yellow coloured hydrazine (Amić et al., 2003). As shown in Figure 4B, RusePre exhibited radical scavenging activity at the rate of 19.42%, 40.38%, 58.8% at 0.5, 1 and 1.5 mg/ml concentrations respectively, while standard ascorbic acid scavenged 98-99% DPPH radical at these concentrations. EC_{50} value of RusePre was found to be at 1.34 ± 0.07 mg/ml which was much higher than that of ascorbic acid (EC_{50} of 4.3 ± 0.3 µg/ml). Similar kind of result was found with phenol rich extract of *Amanita vaginata* (AvaPre) (EC_{50} 1.45 mg/ml) (Paloi & Acharya, 2013). Although phenolic extract of *Russula albonigra* (Krombh.) Fr., RalPre, exhibited higher activity than RusePre as evidence by its low EC_{50} value i.e. 0.47 mg/ml (Dasgupta et al., 2014).

Transition metals e.g. ferrous ion (Fe^{+2}) are known as catalysts in radical formation. Fe^{+2} can move single electron and stimulate hydroxyl radical formation via Fenton reaction as well as also take part in production of peroxyl and alkoxyl radicals. The main strategy to avoid ROS generation involves chelating of metal ions (Ebrahimzadeh, Pourmorad & Bekhradnia, 2008). Thus determination of chelating potentiality of natural compounds would be beneficial with respect to reduction damages by ROS. Chelating ability of RusePre was determined according to Dinis et al. (1994) and result reveals high potentiality of RusePre as chelators of ferrous ion. At 0.05 mg/ml and 0.1 mg/ml concentrations RusePre chelated 13.77% and 38.51% ferrous ions respectively which increased to 58.55% at 0.2 mg/ml concentration (Fig. 4C). The standard, EDTA, chelated >90% at these same concentrations. EC_{50} value of RusePre was very low i.e. 0.158 ± 0.01 mg/ml. Hasnat et al. (2014) have extracted water and ethanol fraction from *Russula virescens* (Schaeff.) Fr. and have subjected them for determination of

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chelating ability. Both the extracts had high EC_{50} values (3.63 and 6.82 mg/ml respectively) suggesting strong capacity of RusePre.

The reducing power of any secondary metabolites such as phenolic compounds is directly related with electron donation capacity. Antioxidant activity is also based on their ability to donate hydrogen atoms or electrons. So reducing power assay indicates total antioxidant potentiality of investigated extract. Principle of the method was based on ability of antioxidants to reduce ferric ion to ferrous ion (Reis et al., 2012). Investigation revealed that RusePre has high reducing ability which increased in a dose dependent manner. At 1 and 2 mg/ml concentrations it displayed reducing power of 0.231 and 0.408 respectively which increased to 0.596 at 3 mg/ml concentration (Fig. 4D). EC_{50} value of RusePre was 2.495 ± 0.015 mg/ml which was much higher than standard, ascorbic acid (EC_{50} 0.015 ± 0.001 mg/ml). Gursoy et al. (2010) have conducted reducing power determination assay of methanol extract of *Russula delica* Fr. at different concentrations ranging from 4 to 20 mg/ml and EC_{50} value was >12 mg/ml. Thus it can be assumed that RusePre possessed much stronger reducing power than the fraction of *R. delica*.

Total antioxidant capacity can also be determined by phosphomolybdenum method. The assay is based on reduction of Mo (VI) to Mo (V) by antioxidant compound and formation of green phosphate/Mo (V) complex at acidic pH. Increase in absorbance of the reaction mixture indicates increase in reducing power (Prieto, Pineda & Aguilar, 1999). Total antioxidant capacity of RusePre was investigated and compared against ascorbic acid. Result indicated that, 1 mg of RusePre acted equivalent to 13.44 ± 0.67 µg of ascorbic acid.

Antimicrobial activity

The antimicrobial effect of RusePre was tested against six species of pathogenic bacteria. Moderate inhibitory effect was found against *L. monocytogenes*, *B. subtilis*, *P. aeruginosa* and *S. aureus* and the inhibition zones were between 5 mm to 7 mm. However RusePre was ineffective against *E. coli* and *S. typhimurium* as the inhibition zones were < 5 mm. It may be expected that the antimicrobial activity of fraction would be related to its phenolic compounds. Our finding was somewhat similar with ethanolic fraction of *Russula delica* as it was effective against *Bacillus cereus*, *L. monocytogenes* and *S. aureus*. On the other hand, *E. coli*, *P. aeruginosa* and *Salmonella enteritidis* were inhibited very weakly (Yaltirak et al., 2009).

Chemical composition

To investigate chemical nature of RusePre different parameters such as phenol, flavonoid, β-carotene, lycopene and ascorbic acid were tested. Results showed that phenol was the major naturally occurring antioxidant component and value was 14.142 ± 1.05 µg gallic acid equivalent/mg of extract. RusePre also contained flavonoid as 4.427 ± 1.123 µg quercetin equivalent/mg of extract. Very negligible amount of β-carotene and lycopene were found such as 0.633 ± 0.01 µg /mg and 0.59 ± 0.01 µg /mg of the extract respectively. Ascorbic acid was also present in small quantities and the obtained value was 1.22 ± 0.17 µg/mg of dry extract. Puttaraju et al. (2006) have reported phenolic content of water and methanol extract of *Russula brevipes* and the recorded values were 5.5 and 0.7 µg gallic acid equivalent/mg of sample. The total phenolic and flavonoid contents of methanolic extract of *R. delica* were 2.09 µg gallic acid equivalent/mg of extract and 0.16 µg quercetin equivalent/mg of extract

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(Gursoy et al., 2010). Thus it can be assumed that our extraction procedure was appropriate to produce a fraction with adequate phytochemicals.

Furthermore, the molecular phenolic profile of RusePre was determined using HPLC-UV, an efficient tool for quantitative analysis. Figure 5A depicts a typical HPLC chromatogram of eleven phenolic compounds each at 0.05 mg/ml concentration and Fig. 5B represents HPLC chromatogram of RusePre at 0.5 mg/ml concentration. The results showed a qualitative profile of RusePre which is composed of all standard phenolic compounds except myricetin and two unrecognized phenolic substances (λmax in inset). Quantitatively pyrogallol was present in the highest amount (Table 1). Overall, flavonols (166.01 µg/mg of dry extract) along with cinnamic acid and its derivatives (106.15 µg/mg of dry extract) were the main contributors in phenolic profile than hydroxybenzoic acid derivatives (73.59 µg/mg of dry extract). Thus it can be assumed that RusePre might be enriched with flavonols and hydroxycinnamic acid derivatives.

Till now, only a few studies were recorded concerning the molecular profiling of phenolic compounds in edible species of *Russula*. Puttaraju et al. (2006) have reported phenolic composition of water and methanol fraction from *Russula brevipes* Peck which is made up of tannic acid, protocatechuic acid, gallic acid, gentisic acid, vanillic acid, *p*-coumaric acid and syringic acid. Ribeiro et al. (2006) informed *Russula cyanoxantha* (Schaeff.) Fr. consisted of *p*-hydroxybenzoic acid whereas quercetin was undetected. Gallic acid, caffeic acid and rutin have been detected in *Russula delica* Fr. whereas cinnamic acid is reported in two *Russula* species namely *Russula caerulea* Fr. and *Russula sardonia* Fr. (Alves et al., 2013). Gallic acid, vanillic acid, *p*-coumaric acid, cinnamic acids were also detected in RusePre supposing these compounds are common in phenolic fingerprint of *Russula* species.

CONCLUSION

DNA barcoding and therapeutic value of *Russula senecis*, a wild mushroom exclusively consumed by ethnic people of West Bengal, was unexplored to the scientific world until this work. First time complete ITS region of *R. senecis* has been sequenced and its taxonomic position within the subsection *Foetentinae* under the series *Ingratae* of the subgen. *Ingratula* has been supported with molecular phylogenetic analysis. To determine medicinal properties, a heat stable phenol rich extract (RusePre) has been prepared using water and ethanol as solvent system. Results clearly indicated that RusePre have antioxidant activity against various *in vitro* systems, even after heat treatment. The fraction showed extreme potentiality in scavenging hydroxyl radical and chelating ability of ferrous ion than DPPH radical scavenging, reducing power and total antioxidant method. Furthermore, administration of RusePre inhibited several pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The pronounced activity was possibly due to its high phenol and flavonoid content in addition with carotenoids and ascorbic acid which were presented in minor amounts. Molecular phenolic profiling of RusePre by HPLC-UV indicated existence of at least 13 phenolics of which 10 were identified such as pyrogallol, flavonols (Kaempferol, quercetin), benzoic acid derivative (vanillic acid> salicylic acid> gallic acid), cinnamic acid and its derivatives (chlorogenic acid> ferulic acid, cinnamic acid> *p*-coumaric acid). Thus, the studied mushroom may have great potential for food and nutraceutical industries as a source of bioactive molecules such as phenolic components for dietary supplements and functional food.

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661

662 Figure 1. Fresh basidiomata and basidiospore ornamentation of *Russula senecis*. A-B.
663 Basidiomata. C-D. SEM microphotograph of basidiospores. Bars A-B: 10 mm; C-D: 2 µm.

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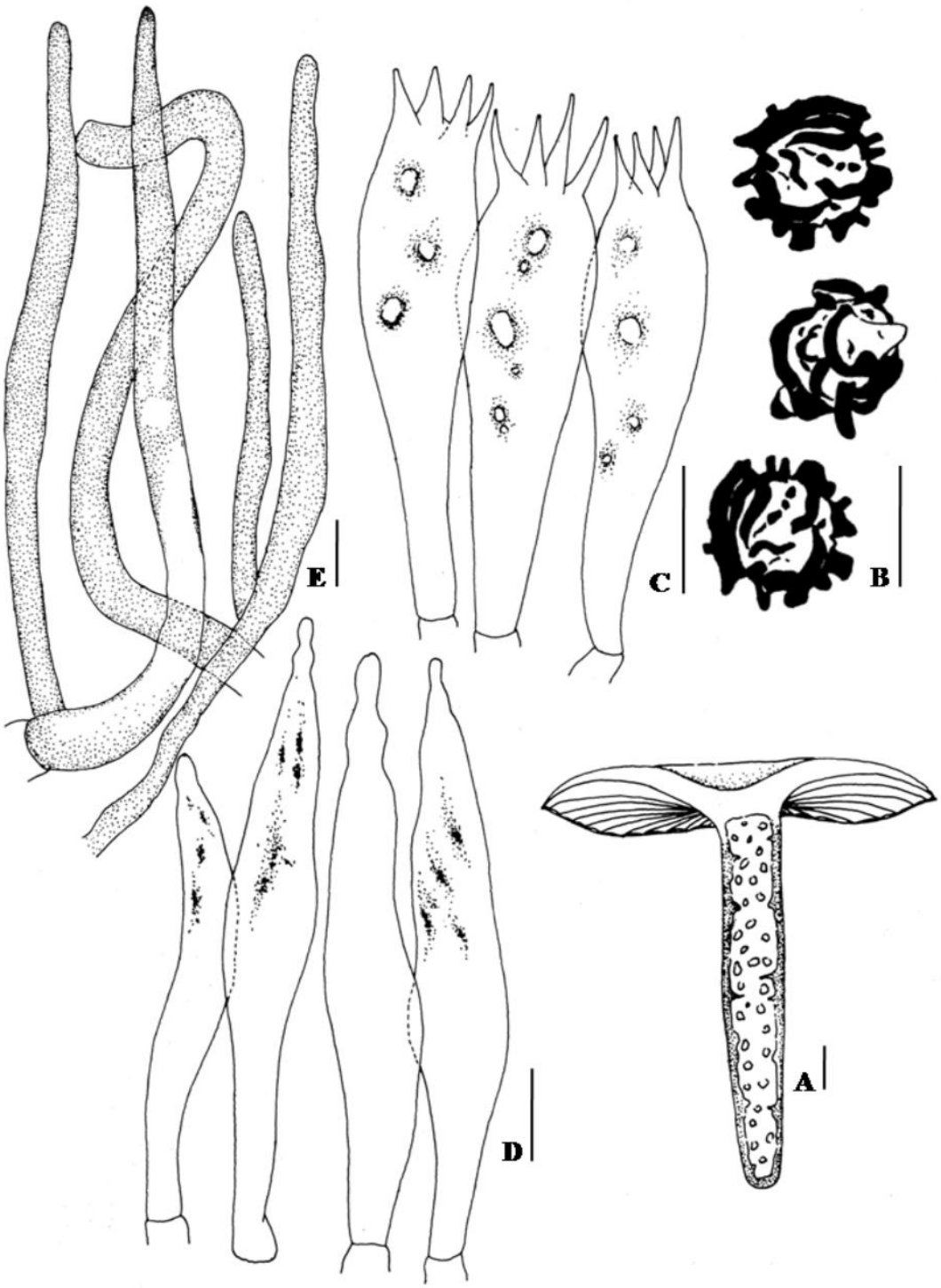
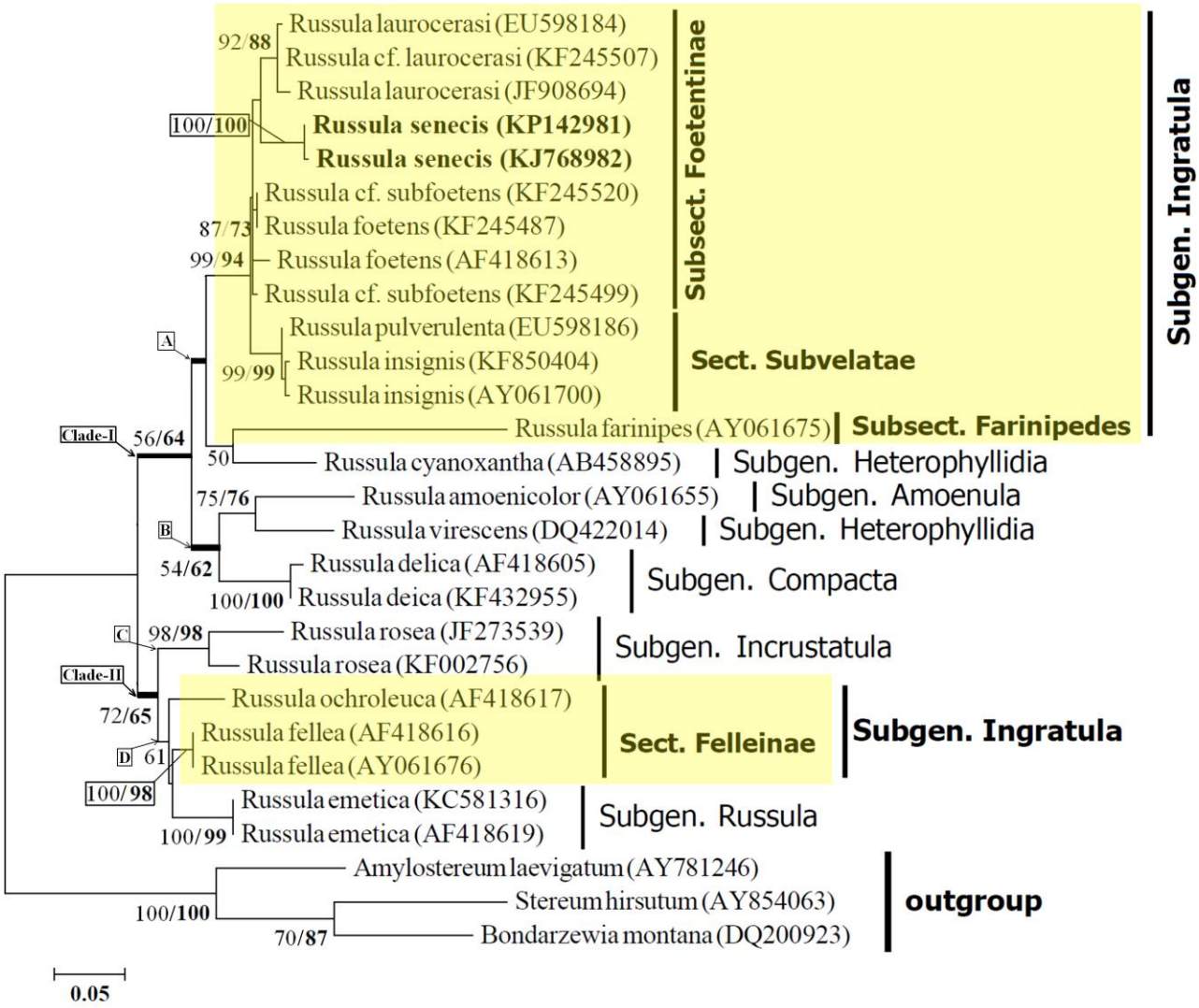


664

665 Figure 2. Hand drawing of macro- and microscopic characters of *Russula seneceis*. A. Fresh
666 basidiomata showing stipe context. B. Basidiospores. C. Basidium. D. Hymenial cystidia. E.
667 Pileocystidia. Bars A: 1 mm; B-E: 10 µm.

668



669

670 Figure 3. Maximum likelihood tree with the highest log likelihood (-2215.8014) generated
671 using K2+G model. The percentage of trees in which the associated taxa clustered together is
672 shown next to the branches. The tree is drawn to scale, with branch lengths measured in the
673 number of substitutions per site. Values to the left of / are Neighbour Joining bootstrap (BS)
674 support, and those to the right indicate the ML bootstrap support of that clade. BS values ≥
675 50% are shown. *Russula senecis* is placed in bold font to highlight its phylogenetic position in
676 the tree.

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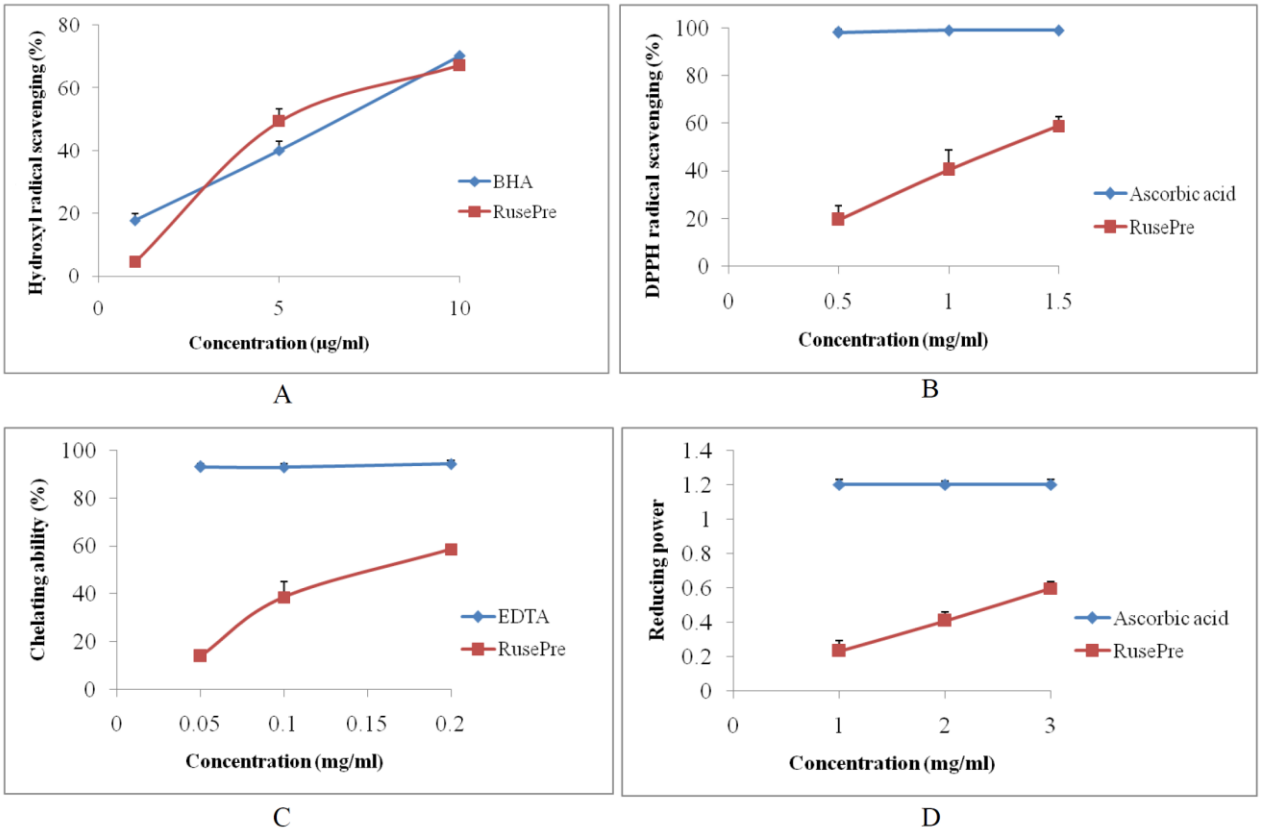

Figure 4. Antioxidant activity of phenol rich fraction from *Russula senecis* (RusePre). Results are presented as the mean ± SD of three separate experiments, each in triplicate. A: Hydroxyl radical scavenging activity B: DPPH radical scavenging activity C: Chelating ability of ferrous ion D: Reducing power.

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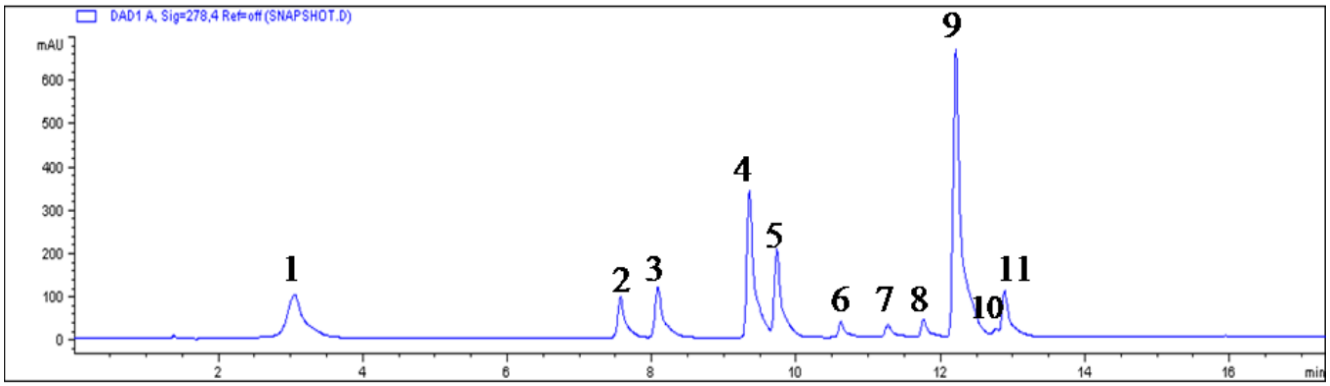
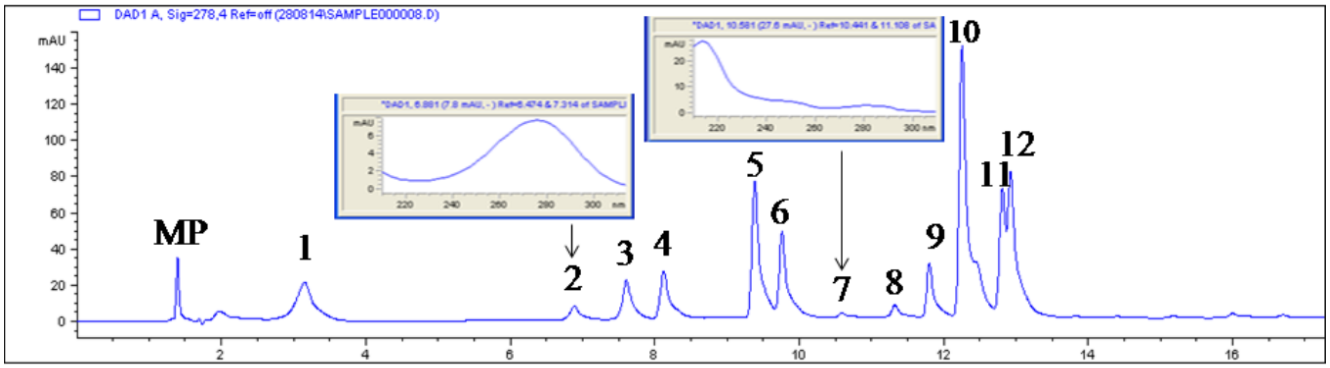


A



B

Figure 5. HPLC chromatogram of standards and phenol rich extract of *R. senecis* (RusePre). A. Standards each in 50 µg/ml concentration (peaks: 1: gallic acid; 2: chlorogenic acid; 3: vanillic acid; 4: p-coumaric acid; 5: ferulic acid; 6: myricetin; 7: salicylic acid; 8: quercetin; 9: cinnamic acid; 10: pyrogallol; 11: kaempferol) B. Phenolic profile of RusePre with UV spectra of two unidentified peaks (inset) (MP: mobile phase; 1: gallic acid; 3: chlorogenic acid; 4: vanillic acid; 5: p-coumaric acid; 6: ferulic acid; 8: salicylic acid; 9: quercetin; 10: cinnamic acid; 11: pyrogallol; 12: kaempferol).

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691 Table 1. Phenolic profile of phenol rich extract of *Russula senecis* (RusePre). ND: Not
692 determined.

Peak no.	RT (min)	λmax (nm)	Area	Concentration (µg/mg)	Compound
1	3.146	272	378.6	22.31	Gallic acid
2	6.879	276	77.1	ND	ND
3	7.599	273	201.2	29.63	Chlorogenic acid
4	8.117	260, 295	247.5	25.72	Vanillic acid
5	9.376	310	572.5	23.43	*p*-coumaric acid
6	9.753	290, 325	401	26.55	Ferulic acid
7	10.581	284	24.1	ND	ND
8	10.627	250, 373	ND	ND	Myricetin
9	11.314	303	57	25.56	Salicylic acid
10	11.792	255, 372	203.5	79.83	Quercetin
11	12.244	277	1322.6	26.54	Cinnamic acid
12	12.8	276	403.3	133.9	Pyrogallol
13	12.918	265, 365	723.5	86.18	Kaempferol

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