Pharmacological activities of selected wild mushrooms in South Waziristan (FATA), Pakistan

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This study investigates the pharmacological importance of selected wild mushrooms viz., Morchella esculenta (common morel), Calvatia gigantea (Giant puffball) and A. hygrometricus (False earthstar) collected from South Waziristan Agency, Federally Administered Tribal Areas (FATA), Pakistan. The selected mushrooms were collected from Tehsil Makeen, Wana and Birmal of South Waziristan Agency during a sampling survey conducted in April–May 2010. The dry fruiting bodies of mushrooms were methanol extracted and evaluated for total phenolic, protein, antioxidant activity, cytotoxicity and their effects on radish seed growth. The results revealed that methanol crude extracts prepared from fruiting bodies of A. hygrometricus and C. gigantea have higher phenolic content and total antioxidant activity as well as greater brine shrimp cytotoxicity. On the other hand, M. esculenta has a high level of protein content and promoted seedling growth in radish. Antioxidant activity for A. hygrometricus, C. gigantea and M. esculenta at IC50 values were: 9.3 ± 0.3 μg/ml, 22.2 ± 0.3 μg/ml and 18.0 ± 0.1 μg/ml, respectively. Methanolic extract of C. gigantea (10 mg/ml) reduced the seed germination and shoot length of radish by 16%. In contrast, the methanolic extract (10 mg/ml) of M. esculenta and C. gigantea enhanced the shoot length, root length and root/shoot ratio of radish. Higher LC50 value was recorded for methanolic extract of A. hygrometricus (19.0 ± 0.3 μg/ml) followed by M. esculenta (17 ± 0.19 μg/ml), whereas methanolic extract of C. gigantea showed lower LC50 value (16 ± 0.23 μg/ml). It is inferred from the present investigation that mushrooms collected from South Waziristan could be potential source of compounds with beneficial biological activities.

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1. Introduction

The use of wild mushrooms in the diet has increased worldwide, enhancing their marketability and economic contribution by approximately two billion dollars (Wang and Hall, 2004; Pettenella, Kloehn, and 52–68, 2007. The trade and consumption of these wild mushrooms are of vital importance to rural livelihoods in the local communities as food (nutrients) and medicine (ailments treatment) (FAO, 2004). Mushrooms are non-timber forest products that are important for both their nutritional as well as pharmacological effects. They are sources of many biologically active compounds that can help in strengthening the immune system and shielding against carcinogens (Ramesh and Pattar, 2010). Previous studies have shown that mushrooms contain active ingredients, which have numerous therapeutical effects such as antitumor, immune-modulating and amelioration of chronic bronchitis (Seth, Haider, and Mohan, 2014). Several genera of mushrooms are edible providing sources of proteins, carbohydrates, vitamins, minerals and amino acids (Okwuorie and Odunze, 2004). Calvatia gigantea, the largest edible mushroom species, belongs to family Lycoperdaceae (Leffingwell and Alford, 2011). Morchella esculenta (Helvellaceae) is an economically important mushroom species largely collected in the wild. The fruiting body of this mushroom species is edible and is usually used as a flavoring agent in soups and gravies (Prasad et al., 2002). A. hygrometricus (Diplocystaceae) is another wild growing mushroom species in South Waziristan that is used as food and possesses antimicrobial properties (Badshah et al., 2012).

South Waziristan extends over an area of 6,500 sq km, located about 580 km northeast of Islamabad, Pakistan. South Waziristan shares a 300 km border with Afghanistan. It is among the federally administered tribal areas (FATA) of Pakistan, in which poverty is widespread and people source their food and medicine from the wild.

The present study aimed to investigate the content and biological activities: total phenolics, protein content, antioxidant activity and cytotoxicity of selected mushroom species viz., M. esculenta, A. hygrometricus and C. gigantea collected from South Waziristan (FATA), Pakistan, using methanolic extracts on radish seed growth.

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2. Materials and methods

The mushroom samples were collected from Tehsil Makeen (lat, 32.62° and long, 69.84°), Wana (lat, 32.30° and long 69.57°) and Birmal (lat, 32.51° and long, 69.29°) of South Waziristan Agency during April-May 2010 and identified at Pakistan Museum of Natural History (PMNH), Islamabad. The fresh mushroom samples were washed with sterile distilled water, cut into slices and shade dried. The dried material was ground finely with an electric grinder and stored at 4 °C.

A total of 20 g of each mushroom sample, ground and powdered, was soaked in a flask in 200 ml of methanol for 3 days with occasional shaking (Mau, Chang, Huang, and Chen, 2004). The mixture was filtered through Whatman filter paper No. 1 in a Buchner funnel using suction pump. To the residue left in the flask, the same amount of the solvent was added and the process was repeated. The extracts were concentrated to dryness using a rotary evaporator at 40 °C under reduced pressure and was further stored at 4 °C in a refrigerator.

2.1. Antioxidant activity (DPPH free radical scavenging activity)

The free radical scavenging activity of methanolic extract of *M. esculenta*, *A. hygrometricus* and *C. gigantea* was measured in terms of hydrogen donating or radical scavenging ability using the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH), prepared by dissolving 10 μg/ml (1:10) folin-ciocalteu reagent. The mixture was added to a glass vial followed by the addition of 0.2 ml of test sample solution, in methanol, leading to the final concentration of 1 μg/ml, 5 μg/ml, 10 μg/ml, 25 μg/ml, 50 μg/ml and 100 μg/ml (Khan, Khan, Shaheen, and Ahmad, 2012). These solution mixtures were kept in the dark for 30 min (incubation period) at room temperature and absorbance was measured at 517 nm. A lower absorbance value of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid was used as standard in 1 – 100 μg/ml solution. All the tests were carried out in triplicate (Williams, Ongskul, Proudfoot, Croft, and Bellin, 1995).

The radical scavenging activity was calculated as percentage of DPPH discoloration using the following equation:

\[
\text{% scavenging DPPH free radical} = \frac{AE}{AD} \times 100
\]

AE is the absorbance of the solution when extract was added, and AD is the absorbance of the DPPH solution with no addition (blank without extract). Thus, the IC50 value was calculated as the concentration of sample required to inhibit 50% of the DPPH free radical using GraphPad Prism v.5.0 (GraphPad software Inc.) (Khan et al., 2012).

2.2. Estimation of total phenolic contents

Total phenolic contents (TPC) were estimated using the method of Oyetayo, Nieto- Camacho, Ramirez-Apana, Baldomero, and Jimenez (2013). The 200 μl (1–5 mg/ml in respective solvent) of each fraction was added to 10 ml of 1:10 folin-ciocalteu reagent. The mixture was mixed and incubated for 5 min before the addition of 7 ml of 0.115 mg/ml Na2CO3. The solution was incubated for 2 h before absorbance readings were taken at 765 nm. Gallic acid (50, 75, 100, 125, 150, 175 and 200 μg/ml) was used for the calibration curve. Results were expressed as milligram gallic acid equivalent (GAE) per gram of dried fraction.

2.3. Brine shrimp cytotoxicity assay

The brine shrimp cytotoxicity assay was performed according to Meyer-Alber, Hartmann, Sumpel, and Creutzfeldt (1992). Samples were prepared by dissolving 10 mg of extract in methanol to form a stock solution that was used to prepare further dilutions. Brine shrimps were incubated in a two-compartment rectangular tray containing sea salt saline. The sea saline was prepared by dissolving 38 g sea salt in 1 L of deionized H2O with continuous stirring for 2 h. Eggs were sprinkled in a dark compartment of the tray and after 24 h the hatched larvae were collected by pipette from the lighted side.

For shrimp treatment, 0.5 ml of each concentration (1000 μg/ml, 100 μg/ml, or 10 μg/ml, 1 μg/ml) was placed in vials and the solvents were evaporated. Residues were re-dissolved in 2 ml saline. Ten shrimps were transferred to each vial and the volume was made up to 5 ml by incubating the vials at 25–28 °C. The same assay was performed for the standard Ampicillin trihydrate. After 24 h of incubation, the survivors were counted and recorded for calculating the LC50 values using GraphPad Prism v.5.0 (GraphPad software Inc.).

2.4. Assay for determining methanolic extract effects on radish growth

Following the method described by Rehman (1991) and Khan, Tariq, and A Khan (2011), the methanolic extract (500 mg) was dissolved in 50 ml methanol to make a stock solution of 10 mg/ml, i.e., 10,000 mg/L or 10,000 ppm concentration. The stock solution was further diluted to 1000 ppm with methanol. Autoclaved distilled water and pure methanol were used as control and vehicle control, respectively. An aliquot (0.5 ml) of each sterilized concentration was placed onto sterilized filter paper (Whatman No. 1) in a 10 cm Petri dish. Methanol was vacuum evaporated separately, and then 5 ml autoclaved distilled water was added to each Petri plate. Three replicates were prepared for each concentration. For negative control, 5 ml methanol was added to the plate, it was vacuum evaporated and then 5 ml autoclaved distilled water was added to each plate. Three replicates were prepared for each control. Ten sterilized radish seeds were placed at sufficient distances with a sterilized forceps in each plate. For the sterilization of radish seeds, 0.1% of mercuric chloride (HgCl2) solution was prepared in a beaker, where radish seeds were put in for 3 min. Furthermore, it was rinsed with autoclaved distilled water followed by drying on sterilized blotting paper. Petri plates were incubated in a light of 350 μmol m−2 s−1 at 25 °C (Miri et al., 2013). Seed germination percentage was calculated after 7 days. Root length was measured after 7 days, and % inhibition of the root length was calculated as follows:

\[
\text{% inhibition of root length} = \frac{\text{root length in test sample} - \text{root length in control}}{\text{root length in control}} \times 100
\]

Dry weights of shoots and roots were determined after drying the shoot and root separately at 72 °C for 3 days in an oven.

2.5. Determination of total protein contents

The mushrooms were dried at 100 °C for 24 h and analyzed for their protein contents using the micro Kjeldahl method (AOAC, 1990).

2.6. Statistical analysis

The data for the effect of methanolic extracts on radish growth were analyzed by analysis of variance (ANOVA) according to Steel and Torrie (1980), and comparison among treatment means was made by Duncan’s multiple range test (DMRT) using MSTAT-C version 1.4.2.

Table 1

| Antioxidant activity measured as DPPH radical scavenging activity tests. |
|----------------|-----------------|
| Mushrooms | IC50 (μg/ml) |
| A. hygrometricus | 9.3 ± 0.3 |
| C. gigantea | 22.2 ± 0.3 |
| M. esculenta | 18.0 ± 0.1 |
| Ascorbic acid | 7.5 ± 0.2 |

± represent standard error.
3. Results and discussion

Antioxidant activity of selected mushrooms measured as DPPH radical scavenging activity tests has been summarized in Table 1. Among the methanolic crude extracts prepared and standard tested for in vitro antioxidant activity using the DPPH method, the crude methanolic extracts of A. hygrometricus, C. gigantea and M. esculenta showed antioxidant activity with IC50 values of 9.3 ± 0.32 μg/ml, 22.2 ± 0.3 2 μg/ml and 18.0 ± 0.12 μg/ml, respectively. The IC50 value recorded for ascorbic acid was 7.5 ± 0.2 μg/ml. The results showed that IC50 value of A. hygrometricus is close to ascorbic acid. These results are in agreement with those of Fui, Shieh, and Ho (2002), who found that various cultivated and wild mushrooms possess significant antioxidant and free radical scavenging activities. Huangs (2000) also found excellent scavenging effects (96.3–99.1 and 97.1%) with methanolic extracts from Antrodia camphorata and Brazilian mushroom (Agaricus blazei) at 2.5 mg/ml, respectively.

The brine shrimp lethality test was performed to assess the cytotoxic effects of methanolic extract prepared from fruiting bodies of A. hygrometricus, C. gigantea and M. esculenta as presented in Table 2. A higher LC50 value was recorded for methanolic extract of A. hygrometricus (19.0 ± 0.32 μg/ml) followed by M. esculenta (17.0 ± 0.2 μg/ml). However, the methanolic extract of C. gigantea exhibited relatively lower LC50 (16.0 ± 0.22 μg/ml), which was comparable to LC50 of antibiotic Ampicillin trihydrate.

Cytotoxicity-based screening for identification of compounds has been previously shown to be successful in the discovery of many clinically useful anticancer natural products (Koneman, Allen, Janda, Schreckenberger, and Winn, 1997). Previous studies showed that C. gigantea produced an antitumor compound calvacin (Babich and Borenfreund, 1992). Although during the present investigations emphasis was not given to anticancer activity of selected mushrooms collected from South Waziristan, these native mushroom species indicated their cytotoxic potential against brine shrimp. Several studies have shown that brine shrimp assay has been an excellent method to screen cytotoxic activity in plant and mushroom species and also for the isolation of biologically active compounds (Faridur, Rezaul, Farhadul, Rowshshanul, and Tofazzal, 2010). The method provides a preliminary toxicity screening basis for further experiments on mammalian models (Wu, 2014). The lethal effect of wild mushroom methanolic extracts on brine shrimp depicts the presence of potential cytotoxic compounds which warrants further investigation as anticancer agents.

Table 2
LC50 for brine shrimp cytotoxicity test.

| Mushrooms        | LC50 (μg/ml) |
|------------------|--------------|
| Calvatia gigantea| 16.0 ± 0.2   |
| Astraeus hygrometricus | 19.0 ± 0.3   |
| Morchella esculenta | 17.0 ± 0.2   |
| Ampicillin trihydrate | 15.1 ± 0.2   |

± represent standard error.

Table 3
Effects of methanolic extract (1 mg/ml) of mushrooms on radish seeds. The data represent mean of three replicates.

| Mushrooms Species | Seed germination (%) | Shoot length (cm) | Root length (cm) | Root/Shoot |
|-------------------|----------------------|-------------------|------------------|------------|
| Control           | 100.0                | 6.3 a             | 4.1 b            | 1.3 b      |
| Astraeus hygrometricus | 100.0              | 6.0 a             | 4.84 ab          | 0.7 b      |
| Calvatia gigantea | 100.0                | 5.33 a            | 5.33 a           | 1.0 a      |
| Morchella esculenta | 100.0              | 7.0 a             | 3.0 c            | 0.7 b      |
| F value           | 0.3, ns              | 14, ns            | 12.0*            | 14.2*      |
| LSD               | 2.4                  | 2.3               | 0.9              | 0.9        |

All mean values that share a common English letter are similar; otherwise, these values are differ significantly at P < 0.05. * Significant value.

The extracts obtained from natural products with LC50 value less than 100 μg/ml, as observed in the brine shrimp lethality assays, are considered toxic (Nondo, et al., 2011). However, the mushroom species used in this study are edible and used by tribal population as a food since ages. This toxicity revealed may not be seen to human possibly because the toxins are heat labile and must be detoxified during cooking and toxins may be inactivated by gastric juices and proteolytic enzymes in the gastrointestinal tract (Ambali, et al., 2008).

The effect of methanolic extracts (1 mg/ml) and (10 mg/ml) of selected mushrooms on radish (Raphanus sativus L.) seeds have been presented in Tables 3 and 4, respectively. Our results revealed that methanolic extracts of A. hygrometricus, C. gigantea and M. esculenta at 1 mg/ml did not show any significant effect on seed germination and shoot length of radish. However, root length and root/shoot ratio was significantly increased by C. gigantea as compared to control. The methanol extract of C. gigantea at 10 mg/ml decreased the seed germination and shoot length of radish by 16%. In contrast, a methanolic extract (10 mg/ml) of M. esculenta significantly increased the shoot length as compared to control. The effect of methanolic extract of A. hygrometricus on seed germination and shoot length was not significant. Similarly, the root length and root/shoot was not significantly affected by methanolic extract (10 mg/ml) of all three mushroom as compared to that of control (Table 4).

The application of higher concentration (10 mg/ml) of M. esculenta methanol extract showed stimulatory effects on shoot length of radish. In contrast, C. gigantea extract (10 mg/ml) inhibited seed germination and shoot length of radish. Allelopathy is a biological phenomenon by which an organism produces one or more biochemicals that influences the growth, survival, and reproduction of other organisms (Nancy, 2003). Biochemical is known as allelochemical and can have beneficial (positive Allelopathy) or detrimental (negative Allelopathy) effects on the target organisms. Allelochemicals are a subset of secondary metabolites which are not required for metabolism (i.e. growth, development and reproduction). Yet these may help plants improve their growth and may also help reduce the risk of pathogen attacks on crop plants.

Table 4
Effects of methanolic extract (10 mg/ml) of mushrooms on radish seeds. The data represent mean of three replicates.

| Mushrooms Species | Seed germination (%) | Shoot length (cm) | Root length (cm) | Root/Shoot |
|-------------------|----------------------|-------------------|------------------|------------|
| Control           | 100.0 a              | 5.4 bc            | 3.8 a            | 0.7 a      |
| Astraeus hygrometricus | 99.7 a             | 6.3 ab            | 4.3 a            | 0.7 a      |
| Calvatia gigantea | 95.0 b              | 4.5 c             | 4.1 a            | 1.0 a      |
| Morchella esculenta | 99.0 a             | 6.8 a             | 4.8 a            | 1.4 a      |
| F value           | 12.1                 | 7.0*              | 2.4, ns          | 1.0, ns    |
| LSD               | 2.3                  | 1.2               | 1.4              | 1.9        |

All mean values that share a common English letter are similar; otherwise, these values differ significantly at P < 0.05. * Significant value.
Maximum phenolic content was recorded in the methanolic extract of A. hygrometricus followed by M. esculenta (Fig. 1). Our results are congruent with the findings of Seng, Chy, Kheng, and Wai (2009), who studied several wild mushrooms for their phenolic contents. Similarly, Kim et al. (2008) reported 28 phenolic compounds in mushrooms. According to this study, the average concentration of phenolic compounds was 326 μg/g; for edible mushrooms 174 μg/g and 477 μg/g for medicinal mushrooms. Phenolic compounds have significant biological and pharmacological properties and some also demonstrate remarkable ability to alter sulfate conjugation. The bioactivity of phenolic compounds may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (Decker, 1997). The mushroom species growing naturally in South Waziristan are a valuable source of natural antioxidants, phenolics and proteins. In rural communities, there is no adoption of mushroom cultivation or plays a significant role in the rural livelihood. It is sold in the local market in huge quantities and the production is mainly from natural stands. In rural communities, there is no adoption of mushroom cultivation or conservation resulting in its decline from these localities.

4. Conclusion

The mushroom species growing naturally in South Waziristan are a source of natural antioxidants, phenolics and proteins. M. esculenta has been a rich source of protein and contributes towards the food requirement of people of remote tribal belt of Pakistan. Similarly, A. hygrometricus and C. gigantea also carry higher pharmacological importance in treating various ailments. However, there is a dire need for documenting and conserving these economically important mushroom species.

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