# Determination of anti-oxidative, anti-microbial activity and heavy metal contents of *Leucoagaricus leucothites*

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**Abstract**: Mushrooms, a treasure of diverse bioactive scaffolds, have been widely admired due to their nutritional and medicinal significance all over the world. The current study intended to evaluate the therapeutic potentiality of an edible mushroom, *Leucoagaricus leucothites* (Vittad.) Wasser. Thus, anti-oxidant potential of *L. leucothites* was determined using DPPH assay and for the determination of anti-microbial potential agar dilution procedure was followed. TOS (total oxidant status), TAS (total anti-oxidant status), and OSI (oxidative stress index) values were evaluated utilizing Rel Assay Kits. For the assessment of heavy metal contents, wet decomposition approach with atomic absorption spectrophotometry was adopted. Screening of phytochemicals present in ethanolic extract of *L. leucothites* were determined by HPLC. TAS, TOS and OSI values were found to be 8.291mmol/L, 10.797µmol/L and 0.130 respectively. Our results declared that heavy metal contents are generally in the safe range. Phytochemical analysis of *L. leucothites* has affirmed the presence of important phenolics such as gallic acid, catechin, and hesperidin. Investigations on anti-oxidant and anti-microbial potential of *L. leucothites* has uncovered the fact that this naturally occurring, biologically active, and therapeutically effective mushroom specie has natural borne anti-oxidant and anti-microbial potential and it would be worthwhile to use it for nutritional as well as medicinal purpose.

**Keywords**: Mushrooms, *Leucoagaricus leucothites*, Anti-oxidative, Anti-microbial, Heavy metals

## INTRODUCTION

Medicinal and nutritional values of mushrooms have been subject of speculation for human therapeutics since ancient times (Houshdar Tehrani *et al.*, 2012; Rathee *et al.*, 2012; Phan *et al.*, 2015). Mushrooms being enriched with diverse bioactive compounds are known to possess human health promoting effects (Cohen *et al.*, 2014; Elsayed *et al.*, 2014; Ivanova *et al.*, 2014). Experimentations on mushrooms from Turkey, Finland, Spain, Italy, Mexico, India, Portugal and Korea have reported them as rich source of nutritive nutraceuticals such as essential proteins, polyphenols, terpenoids, flavonoids, ergosterols, keto acids, minerals, anti-oxidative vitamins along with lower fat contents being acceptable for low calorie diets (Reis *et al.*, 2011; Ren *et al.*, 2012; Wasser, 2014; Nguyen *et al.*, 2016; Sevindik 2018; Colak *et al.*, 2018). Foregoing studies have demonstrated varied pharmacological properties of mushrooms such as anti-viral, anti-microbial, anti-oxidant, anti-tumor, anti-allergic, anti-coagulant and anti-inflammatory (Chang & Wasser, 2012). Mushrooms are enriched with variety of secondary metabolites, inclusive of several phenolic compounds, terpenes, polyketides and steroids, which are known to serve as excellent anti-oxidants in biological systems (Soares *et al.*, 2013). The capability of phenolic compounds, especially that of catechin, gallic acid, and caffeic acid as anti-oxidants has been well documented (Brewer, 2011; Woldegiorgis *et al.*, 2014). Polyphenols are known as multifunctional anti-oxidants because they act as hydrogen donating agents, reducing compounds, and singlet oxygen quenchers (Woldegiorgis *et al.*, 2014). Therefore, it would be worthwhile to investigate the anti-oxidant potentials of such phenolic rich nature's gifts.

The breakneck emergence of multiple resistances against currently used broad spectrum of antibiotics arouses the ultimate need to find out safe and effective alternatives. Natural products that have the capability to prohibit microbial strains could provide such alternatives. Mushrooms have been reported to possess anti-microbial potential against various microbes (Cai *et al.*, 2015; Glamoclija *et al.*, 2015). Investigations on anti-microbial potential of mushrooms will pave a way for researchers towards establishment of mushrooms into promising anti-microbial agents in the years to come.

In addition to their medicinal significance, mushrooms are best known as biomonitors of natural pollution. During

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the decomposition process of organic matter, mushrooms accumulate heavy metals to their bodies at high extent (Rauter, 1975). Thus, determinations of heavy metal contents will help in the assessment of toxic metal pollution in the reference site (Rieder *et al.*, 2011). Moreover, it will also provide clues of potential health risk factors caused by the consumption of mushrooms.

Presently, there are 14,000 estimated species of mushrooms. Among these, approximately 50% of species are known to possess varying potency of edibility and about 31 genera with 3000 species are reported as prime comestible mushrooms. Furthermore, around 2000 of them has been accounted for medicinal significance and there are 270 species which are ensured as preventive agents and have established therapeutic characteristics in human health perspective. There are very finite numbers of species which are known to exhibit poisonous attributes, whereas only 30 species are lethal (Sharma & Gautam, 2015).

To date, limited knowledge is available on the nutritional as well as medicinal significance of *Leucoagaricus leucothites* (Vittad.) Wasser. Hence, in the present study we have evaluated the anti-oxidant, anti-microbial, and heavy metal contents of *L. leucothites* to ascertain its value for nutraceutical and food manufacturing industries.

## MATERIALS AND METHODS

***Laboratory studies***

Edible *L. leucothites* samples required for this study were gathered from Yedigoller National Park (Bolu/Turkey). Morphological (shape, color, size) and ecological characteristics of the samples were recorded in the field conditions. The microscopic characteristics of the specimens transported to the laboratory under appropriate conditions were determined by light microscopy using a % 3 KOH solution (Leica DM750). The specimen was identified morphologically using the references of Breitenbach and Kranzlin (1995) and Lange (2012). Collected mushrooms were dried at 40°C using an incubator in laboratory environment. Subsequently, the samples were shattered by mechanical grinding. After that, sample (30g) was weighed and subjected to ethanolic extraction for 6 hours at 50°C with Soxhlet apparatus (BUCHI Extraction System Model B-811). Solvent from the extract were removed under reduced pressure by laboratory rotary evaporator (Heidolph Laborator 4000 Rotary Evaporator). Extract was stored at +4°C for further studies.

***Anti-oxidant activity***

Anti-oxidant potential of mushroom extract was evaluated by DPPH (Shimada *et al.*, 1992). DPPH (1,1 diphenyl-2-picrylhydrazyl) is a dark coloured powder comprised of stable free radicals showing maximum absorbance at 517 nm. It is readily transformed from violet-purple colour into light yellow colour whenever a radical medium contains an anti-oxidant molecule. Stock solutions of extract (1mg/ml) with DMSO (Dimethyl Sulfoxide) were prepared. 160µL of 0.039% DPPH solution was mixed with 50µL of stock solution. The resulting admixture was subjected to incubation in the dark at ambient temperature for about 30 minutes. The absorbance was checked at 517nm. This process was repeated for all the concentrations of solutions. Reference anti-oxidants that were used are caffeic acid and rosmarinic acid. Following formula was used to determine the DPPH free radical scavenging activity (Shimada *et al.*, 1992).

$$\text{Scavenging activity (\%)} = \frac{[(\text{ADPPH} - \text{AExample})}{(\text{ADPPH})]} \times 100$$

***Anti-microbial activity***

To evaluate the anti-microbial potential of mushroom extract, agar dilution method was followed in accordance to the recommendation of Clinical and Laboratory Standards Institute (CLSI). Bacterial strains used in this study are as following: *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213. Strains of fungus that are used to access anti-microbial potential of mushrooms are: *Candida tropicalis* ATCC 13803 and *Candida albicans* ATCC 10231. Pre-culturing of bacterial strains were done in Muller Hinton broth (Merck) medium and for fungal strains RPMI 1640 broth (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used. In order to acquire the standard inoculum, bacterial and fungal turbidity was obtained in line to McFarland scale no. 0.5. 50% diluted DMSO was used to prepare the solutions of all the extracts. The concentrations of tested compounds were 6.25, 12.5, 25, 50, 100, 200, 400 and 800µg/mL. For fluconazole fungi, Ciprofloxacin and Ampicillin were utilized as standard bacterial drug. With the help of sterile plastic ring nose loop (0.01mL), standard bacterial and fungal inoculums (106 CFUs/mL) were seeded on agar petri plates. After 16-20 hours (for bacterial strains), and 48 hours (for fungal strains) of storage, evaluation of seeded plaques were done at 35°C. The minimum concentration of tested compounds that inhibited the proliferation of bacterial and fungal strains was represented as minimal inhibitor concentrations (MIC) (Hindler *et al.*, 1992; CLSI 2002; CLSI 2003).

***Determination of TAS, TOS and OSI Values***

For the evaluation of TAS values of mushroom samples, Trolox (as a calibrator) and Rel Assay brand commercial kit (Rel Assay Kit Diagnostics, Turkey) was used. The results obtained are represented in mmol Trolox equiv./L (Erel, 2004). To determine TOS values, hydrogen peroxide (as a calibrator) and Rel Assay brand commercial kits were used. The results obtained are represented in µmol $H_2O_2$ equiv./L. For the calculation of OSI, following formula was applied (Erel, 2005).

**Table 1**: Antioxidant activity of *L. leucothites* (% inhibition)

| | 100 (%) | 75 (%) | 50 (%) | 25 (%) |
|---|---|---|---|---|
| Caffeic acid | 54.47±0.05 | 38.39±0.66 | 21.34±0.66 | 8.62±0.91 |
| Rosmarinic acid | 61.92±0.15 | 35.09±7.96 | 7.00±0.41 | 6.03±0.15 |
| Ascorbic Acid | 95.96±1.96 | 93.72±1.39 | 91.40±0.70 | 89.45±1.44 |
| *L. leucothites* | 63.74±3.60 | 52.08±0.76 | 46.40±1.78 | 28.20±0.71 |

Values are presented as mean±S.D.; n=6 (Experiments were made as 3 parallel)

**Table 2**: TAS, TOS and OSI Values of *L. Leucothites*

| | TAS | TOS | OSI |
|---|---|---|---|
| *L. leucothites* | 8.291 ±0.043 | 10.797 ±0.117 | 0.130 ±0.001 |

Values are presented as mean±S.D.; n=6 (Experiments were made as 5 parallel)

**Table 3**: Antimicrobial activity of *L. Leucothites*

| | *S. aureus* (µg/mL) | *E. faecalis* (µg/mL) | *E. coli* (µg/mL) | *P. aeruginosa* (µg/mL) | *C. albicans* (µg/mL) | *C. tropicalis* (µg/mL) |
|---|---|---|---|---|---|---|
| *L. leucothites* | 200 | 200 | 100 | 400 | 50 | 50 |
| Flukonazole | - | - | - | - | 1.56 | 3.12 |
| Ampicillin | 3.12 | 1.56 | 3.12 | - | - | - |
| Ciprofloxacin | 0.78 | 0.78 | 1.56 | 3.12 | - | - |

* 400, 200, 100 and 50 (µg / mL) indicate concentrations of extracts affecting microorganisms.

**Table 4**: Heavy metal contents of *L. Leucothites*

| | Fe | Zn | Cu | Pb | Ni | Mn | Co | Cd | Cr |
|---|---|---|---|---|---|---|---|---|---|
| *L. leucothites* | 94.38±6.49 | 7.63±1.27 | 2.93±0.34 | 1.48±0.27 | none | 5.25±0.11 | 0.99±0.06 | 0.16±0.04 | 3.34±0.28 |

Values are presented as mean±S.D.; n=3 (Experiments were made as 3 parallel)

**Table 5**: Phenolic contents of *L. leucothites*

| | Gallic acid | Catechin | Hesperidin |
|---|---|---|---|
| *L. leucothites* | 1.84 ppm | 333.26 ppm | 49.26 ppm |

$$OSI = \frac{TOS, \mu mol\ H_2O_2 equiv./L}{TAS, mmol\ Trolox\ equiv./L} \times 10$$

***Determination of heavy metal content***

Collected mushrooms were dried using an incubator in laboratory environment at 40°C. Subsequently, the samples were shattered by mechanical grinding. Three samples (each 1g) were weighed and put into glass beaker (50ml). After the addition of 10ml of $HNO_3$, these glass beakers were placed at room temperature for 24-48 hours. Then, the beakers were heated until the resulting solutions become transparent. Then, 10ml of HCl (concentrated) were added to each sample with the repetition of same burning step. After that, 20 ml of HCl (diluted) was added followed by filtration step (Akgul *et al.*, 2016). Now, the solution was ready for analysis. Perkin Elmer (Analyst 400) instrument was used for the determination of element concentrations of final solution.

***Phenolic content***

For phytochemical analysis of *L. leucothites* extract, protocol introduced by Caponio et al (Caponio *et al.*, 1999), was followed some modifications, utilizing a DAD detector and SHIMADZU HPLC apparatus. Injection volume was maintained to 20µL. As mobile phase A, 3% acetic acid and as mobile phase B, methanol was used. Flow rates were maintained to 0.8mL per 60 seconds. Chromatographic separation was done at 30°C with Agilent Eclipse XDBC18 column (250x4.6 mm id 5µM).

## RESULTS

***Anti-oxidant activity***

Anti-oxidant activity of ethanolic extract of *L. leucothites* was evaluated using DPPH assay. Obtained results are provided in table 1. Our findings represent that free radical (DPPH) scavenging potential of ethanolic mushroom extract increases as the concentration of mushroom extract was increased. A comparison of anti-oxidant activity of *L. leucothites* with standard anti-oxidants such as caffeic acid and rosmarinic acid declared that *L. leucothites* have highest free radical scavenging

***TAS, TOS and OSI values***

TAS, TOS and OSI values, represented in mmol/L, and µmol/L respectively, was evaluated by Rel Assay Kits. The TAS value for *L. leucothites*, gathered from Yedigoller National Park was 8.291mmol/L, while the

TOS value of mushroom extract was 10.797µmol/L. OSI value represents concentration of oxidant molecules produced by anti-oxidant compounds during *in vivo* investigations, which was 0.130. TAS, TOS and OSI vales for *L. leucothites* are provided in table 2.

***Anti-microbial activity***

For the evaluation of anti-microbial potential of mushroom extract, agar dilution method was followed in accordance to the recommendation of Clinical and Laboratory Standards Institute (CLSI). Results obtained from these evaluations are provided in table 3. As shown in table 3, the provided results declared that *L. leucothites* ethanolic extract was most effective against *E. coli* with MIC 100µg/mL. While it was also active against *S. aureus* and *E. faecalis* with MICs 200µg/mL. In the current study *L. leucothites* extrat was found to be most affective against *C. albicans* and *C. tropicalis* having MIC 50 µg/mL.

***Heavy metal content***

The Fe, Cu, Zn, Pb and Ni heavy metal contents concentrated in *L. sulphureus* were evaluated in $mg.kg^{-1}$. The heavy metal contents determined in the current study are provided in table 4 as mean ± S.D. Mushrooms are best known as biomonitors of natural pollution. During the decomposition process of organic matter, mushrooms accumulate heavy metals to their bodies at high extent (Garcia *et al.*, 1998). So, it is worthwhile to investigate about heavy metal contents to provide an image of soil pollution or toxicological aspects. Analysis of heavy metal contents of *L. leucothites* has uncovered the facts that there were no Ni contents in *L. leucothites.* While Fe and Zn was found to be most abunant heavy metal. Obtained results have declared the contents of Fe as 94.38±6.49, contents of Zn as 7.63±1.27, contents of Cu as 2.93±0.34, contents of Pb as 1.48±0.27, contents of Mn as 5.25±0.11, contents of Co as 0.99±0.0, contents of Cd as 0.16±0.04 and contents of Cr as 3.34±0.28 in *L. leucothites*.

***Phenolic contents***

Screening of *L. leucothites* methanolic extract for the determination of phenolic contents was done with HPLC. Results are represented in table 5. Investigations on phenolic contents have revealed the presence of phenolics including gallic acid, catechin and hesperidin. Catechin was found to be most abundant phenolic compound in mushroom extract, while gallic acid found to be in less amount. Thus, it could be argued that *L. leucothites* anti-oxidant potential reported in this study is due to its polyphenolic contents such as hesperidin, gallic acid and especially catechin.

## DISCUSSION

In this study, we have analyzed the anti-oxidative, anti-microbial activity and heavy metal contents of of ethanolic extract of *L. leucothites*. A comparison of anti-oxidant activity of *L. leucothites* with standard anti-oxidants such as caffeic acid and rosmarinic acid declared that *L. leucothites* have highest free radical scavenging activity Anti-oxidant activity of *L. leucothites* was also reported earlier (Aslim & Ozturk, 2011). Consistent with these findings, it is speculated that ethanolic extract of *L. leucothites* can serve as potential anti-oxidant agent due to its potent free radical scavenging ability. Previous investigations on edible mushroom *T. terreum* collected from Mugla provincial center have provided TAS value as 0.38 (Akgül *et al.*, 2016b). Moreover, TAS values for *Auricularia polytricha*, *Coprinus micaceu*, *Pleurotus eryngii*, *Auricularia auricular*, *Trametes versicolor* were reported as 0.93, 0.46, 1.93, 1.010, 0.820 respectively Thus, evidences from aforementioned studies have declared that TAS value for *L. leucothites* tends to be significantly higher when compared to *A. polytricha*, *T. terreum*, *C. micaceus*, *P. eryngii*, *A. auricular*, *T. versicolor* mushrooms. So, it can be speculated that *L. leucothites* are good enogh at producing anti-oxidants. Moreover previous investigations have declared the TOS values for *T.* terreum, *C. micaceus*, *A. auricula*, *and T. versicolor* as 16.76, 16.87, 23.910, and 17.760. Similarly, OSI values for *T. terreum*, *C. micaceus*, *A. auricula*, *T. versicolor* were reported as 4.41, 3.67, 2.367 and 2.166 (Yildirim et al., 2012; Akgül et al., 2016a; Avcı et al., 2016; Akgul et al., 2017). When compared with above-mentioned studies, TOS and OSI values of *L. leucothites* found to be very much lesser than that of *T. terreum*, *C. micaceus*, *A. auricula*, *and T. versicolor*. These findings uncover the fact that collection site of mushrooms is satisfactory and good enough for the growth of edible *L. leucothites*. It is conclued that *L. leucothites* could be utilized as natural anti-oxidant agents due to its significal anti-oxidant levels. These naturally occurring, biologically active, and therapeutically effective chemical entities have turned up as novel paradigm for the prevention of multiple pathological conditions. *L. leucothites* is reported to exhibit notable anti-microbial potential (Aslim & Ozturk, 2011). *L. leucothites* reported to be affective against gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella enteritidis*, *Yersinia enterocolitica*, *Shigella sonnei*) and gram positive (*Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*) bacterial strain (Aslim & Ozturk, 2011). However, anti-fungal potential of *L. leucothites* has not been reported yet. As shown in Table 3, the provided results declared that *L. leucothites* ethanolic extract was most effective against *E. coli* with MIC 100 µg/mL. While it was also active against *S. aureus* and *E. faecalis* with MICs 200 µg/mL. In the current study, *L. leucothites* extrat was found to be most affective against *C. albicans* and *C. tropicalis* having MIC 50 µg/mL. Highest and lowest range of heavy metals reported in literature is as follow: for Fe 14.6-835, for Zn 29.8-306, for Cu 64.8-290, for Pb 0.04-6.88, for Ni 1.18-5.14, for

Mn 18.1-103.0 and for Cd 2.71-7.50 mg.kg-1 (Mallikarjuna et al., 2013; Liu et al., 2015; Lalotra et al., 2016). Based upon theses findings, it could be concluded that Fe, Zn, and Pb contents are within range of the reported values of literature. While the contents of Cu, Mn, and Cd found to be much lower than the reported values in literature. Comparative analysis of obtained results with literature have declared that heavy metal contamination in the reference site is acceptable. In phytochemicals, phenolic compounds are best known as free radical scavengers (Alafiatayo et al., 2014). Catechin, a nutaruuly occuring triphenolic compound, has been reported to possess verstile pharmacological features such as anti-bacterial, anti-oxidant, and anti-arteriosclerotic (Koo & Cho, 2004). Hesperidin, a flavanone glycoside, is known to exhibit anti-bacterial, anti-fungal properties. Hesperidin has been reported to be abunantly found in various mushroom species such as *Ganoderma lucidum* and *Cordyceps sinensis*. Gallic acid exhibits significant free radical scavenging properties along with anti-cancer potential (Suzuki et al., 2014; Choubey et al., 2015). Catechin was found to be most abundant polyphenol in *L. leucothites* extract. Thus, it could be argued that *L. leucothites* anti-oxidant potential reported in this study is due to its polyphenolic contents such as hesperidin, gallic acid, and especially catechin.

## CONCLUSION

It could be speculated that that ethanolic extracts of *L. leucothites* have most potent inhibitory activity against *C. albicans*, *C. tropicalis* and *E. coli*. So, *L. leucothites* might find applications as natural antibiotic against microorganisms. Furthermore, antioxidant activity was found to be lesser than caffeic acid and rosmarinic acid and higher than the ascorbic acid that are used as standards, recommending its usage as an alternative anti-oxidant source. TAS, TOS, and OSI values were found to be 8.291mmol/L, 10.797µmol/L and 0.130 respectively. Our results declared that heavy metal contents are generally in the safe range. Phytochemical analysis of *L. leucothites* has affirmed the presence of important phenolics such as gallic acid, catechin, and hesperidin. As concluding remarks, *L. leucothites* possess good biological activity and have a natural borne potential to be used as anti-microbial and anti-oxidant agent, but isolation and characterization of bioactive compounds from extract and further experimentation are yet mandatory to elucidate full spectrum of its pharmacological activities.

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