M. Rajasekaran et al /J. Pharm. Sci. & Res. Vol.3(9), 2011,1427-1433

ISSN:0975-1459

Journal of Pharmaceutical Sciences and Research

www.jpsr.pharmainfo.in

In Vitro Antioxidant Activity of Ethanolic Extract of a Medicinal Mushroom, *Ganoderma Lucidum*

M.Rajasekaran* and C.Kalaimagal

School of Chemical and Biotechnology, SASTRA University, Thanjavur-613 402, Tamil Nadu, INDIA.

Abstract:

Aim: The aim of the present study was to analyze the antioxidant contents and evaluate the antioxidant potential of *Ganoderma lucidum*.**Materials and Methods**:Wild collected *G.lucidum* was powdered and extracted with ethanol using soxehlet apparatus.Total phenolics,flavones and ascorbic levels were estimated following standard procedures.Subsequently,reducing power,DDPH radical,superoxide,hydroxyl radical and nitric oxide radical scavenging effects of the extracts at different concentrations were evaluated following standard methods. **Results**:The extract contained 42.41±2.21mg/g of phenolics,13.57±1.57 mg/g of flavones and the ascorbic acid amount was 1.33±0.12 mg/g .Total reducing power of the extract was 1.38 OD/ml at 240mg/ml concentration.DPPH radical scavenging activity was concentration dependent, showing maximum percent inhibition(72.24%) at 250mg/g.Similarly,hydroxyl,superoxide and nitric oxide scavenging potential also at their maximum at higher concentrations.Hydroxyl scavenging effect was 64.69% at 250 mg/g,superoxide radical inhibition was 73.54% at 300 ug/ml and 65.87% inhibition of nitric oxide was noted at 200 mg/ml. **Conclusion**:Ethanolic extract of *G.lucidum* showed appreciable amount of antioxidant compounds and also good free radical scavenging effects against different free radicals.The study shows that *G.lucidum*compounds can be better antioxidant supplement for nutrients.

Key words: Antioxidants,ethanol extract, *G. lucidum,* free radical scavenging.

Introduction:

Free radicals and related species have attracted a great deal of interests in recent years. Free radicals are chemical species possessing un unpaired electron formed by the loss of a single electron from a normal molecule.[1]About 5% or more of the oxygen inhaled is converted to reactive oxygen species (ROS) such as O_2 , H_2O_2, and **.**OH by univalent reduction of O_2 [2] .When oxygen is partially reduced it becomes activated and readily reacts with a variety of biomolecules by the addition of one , two or four electron to oxygen leading to formation of ROS such as superoxide anions, hydrogen peroxide, and hydroxyl radical, malondehyde and nitric oxide. Thus cells under aerobic condition are always threatened with the insult of ROS, which however are efficiently taken care of by the highly powerful antioxidant systems of the cell without any untoward effect. When the balance between ROS production and antioxidant defenses is lost, 'oxidative stress' results which through a series of events deregulates the cellular functions leading to various pathological conditions including cardiovascular dysfunction, neurodegenerative diseases, gastroduodenal pathogenesis, metabolic dysfunction of almost all the vital organs, cancer and premature aging.[3]

Cells have developed a comprehensive array of antioxidant defenses to prevent free radical formation or limit their damaging effects. These include enzymes to decompose peroxides, proteins to sequester transition metals and a range of compounds to 'scavenge' free radicals. Though cells are equipped with several defense mechanisms, both enzymatic and non enzymatic, to combat the free radical assault, natural antioxidant supplements containing food may be used to help protect from oxidative damage. Restriction in the use of synthetic antioxidants paved the way for the increased interest towards natural antioxidants.[4,5]

Natural antioxidants are widely known for their defense role against oxidative damage and found abundant in vegetables, food and fruits, which include phenolic compounds, carotenoids, tocopherol, ascorbic acid, etc.[6,7] Equal to the herbal medicines, in recent years mushrooms also have attracted attention for their natural medicinal properties. Generally mushrooms are rich in dietary fibres, minerals, vitamins and low in fat calories and also contain various

polyphenolic and flavanoid compounds recognized as antioxidants.[8] Several important compounds including polysaccharides(β-glucan), ergosterol, vitamins, *a*-tocopherol and *b*-carotene have been isolated from a variety of mushrooms. Phenolics and flavanoids have been reported in two species of *Pleurotus.*[9] Studies on five different species of *Agaricus* have also showed the presence of rich antioxidant compounds like phenol, flavanoids, ascorbic acid, *ß*-carotene and lycopene and significant antioxidant activity.[10] Despite the increased awareness on the role of antioxidants in preventing disease onset and curing , mushrooms have received only little attention with regards to their antioxidant chemicals and radical scavenging activity. In the present study, free radical scavenging activity of ethanol extracts of a medicinal mushroom *Ganoderma lucidum* has been evaluated. *Ganoderma lucidum* is a large, dark mushroom with a glossy exterior and a woody texture. Among cultivated mushrooms, *Ganoderma* is unique in being consumed for its medicinal,rather than nutritional value [11]. It is widely consumed throughout the world as a health food, being commonly and regularly used by many Asian individuals for the promotion of health and longevity, and as a remedy for illness .[12,13]

Materials and Methods:

Reagents and Chemicals

2, 2-diphenyl-1-picryl-hydrazyl or 1,1-diphenyl-2-picryl-hydrazyl,Nitro Blue Tetrazolium, NADH, curcumin, quercetin, gallic acid and ascorbic acids were purchased from Himedia Laboratories Ltd, Mumbai, India. All other chemicals were analytical grade reagents.

Mushroom Extracts

Fresh *Ganoderma lucidum* specimens were collected from coconut trees in Tamil Nadu Agricultural University, Sugar Cane Research Centre, Siruganur, Trichy, Tamil Nadu. The specimens were cut into small pieces and powdered for extraction. Ethanol extract was obtained using soxehlet apparatus and the ethanol was vacum evaporated .The *Ganoderma lucidum* ethanol (GDE) extract was resuspended in ethanol to make a concentration of 100mg/ml and stored at 4^0c for further use.

Total phenolic content (TPC)

The amount of total phenol content was determined by Folin-Ciocalteau reagent method [14]. 0.5 ml of extract and 0.1 ml of Folin-Ciocalteu reagent (0.5 N) were mixed and incubated at room temperature for 15 min. Then 2.5 ml saturated sodium carbonate was added and further incubated for 30min at room temperature and absorbance measured at 760 nm. Gallic acid was used as a standard, and the results were expressed in terms of gallic acid equivalence (GAE) in µg.

Total flavonoid (TF) content

Flavonoid content in the GDE extract was determined by aluminium chloride method [15]. The reaction mixture (3.0 ml) comprised of 1.0 ml of extract, 0.5 ml of aluminum chloride (1.2%) and 0.5 ml of potassium acetate (120 mM) is incubated at room temperature for 30 min and absorbance measured at 415 nm. Quercetin was used as a positive control. The flavonoid content is expressed in terms of standard equivalent (mgg-1 of extracted compound).

Ascorbic acid determination

For ascorbic acid determination, GDE extract (100 mg) was re-extracted with metaphosphoric acid (1%, 10 ml) for 45 min at room temperature and filtered through Whatman No. 4 filter paper. The filtrate (1 ml) was mixed with 2,6-dichlorophenolindophenol (9 ml) and the absorbance was measured within 30 min at 515nm against a blank. Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid [16].

In vitro free radical scavenging assays

Superoxide anion radical scavenging (SO) assay

The superoxide anion scavenging activity was determined as described below. [17] Briefly, 0.5ml of NBT (0.3mM), 0.5ml of NADH (0.936mM), 1ml of ethanol extract of *G.lucidum* in concentrations ranging from 100-500 ug/ml and 0.5ml Tris-Hcl buffer (16mM, pH 8) were mixed with 0.5ml PMS solution (0.12mM)and the reaction started. The mixture was incubated at 25^0c for 5min.Absorbance was measured at 560nm against a blank. Curcumin was used as control.

Nitric oxide radical scavenging (NO) assay

Nitric oxide radical scavenging was measured in the GDE extract.[18] 3ml of 10mM sodium nitroprusside in phosphate buffer was added to 2ml of the extract and reference compound in different concentrations (40 -200µg/ml). The solutions were then incubated at 25°C for 60min.To 5ml of the incubated sample, 5ml of Griess reagent (1% sulphanilamide, 0.1% naphthy-ethylene diamine dihydrochloride in 2% H_3PO_3) was added and absorbance was measured at 540 nm. Percent inhibition of the nitrite oxide generated is measured by comparing the absorbance values of sample and standard solution preparations. Ascorbic acid was used as a positive control.

Hydroxyl radical scavenging (HO) assay

The scavenging ability for hydroxyl radicals was measured as described below. [19] The reaction mixture (1ml) consisted of 100 µl of 2-deoxy-D-ribose (28 mM in 20 mM KH_2PO_4 -KOH buffer, pH 7.4), 500 µl of the extract, 200 µl EDTA (1.04 mM) and 200 µM $FeCl_3$ (1:1 v/v), 100 µl of H_2O_2 (1mM) and 100µl of ascorbic acid (1mM) which was incubated at 37ºC for 1 h.1ml of thiobarbituric acid (1%) and 1ml of trichloroacetic acid (2.8%) were added and incubated at 100ºC for 20 min. After cooling, absorbance was measured at 532 nm, against a blank sample. Ascorbic acid and quercetin were used as positive controls.

Reducing power (RP)

The reducing power of the extract was determined as follows. [20] Briely, 1ml extract was mixed with 2.5ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30mM) and incubated at 50° C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600mM) was added to the reaction mixture and centrifuged for 10min at 3000 rpm. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6mM) and absorbance was measured at 700nm. Ascorbic acid was used as positive control.

α, α-Diphenyl-β-picryl-hydrazyl radical scavenging (DPPH) Assay.

The free radical scavenging activity was measured by using 2, 2-diphenyl-1-picryl-hydrazyl or 1,1-diphenyl-2-picryl-hydrazyl.[21] The reaction mixture (3.0 ml) consisted of 1ml of DPPH in methanol (0.3 mM), 1ml of the extract and 1ml of methanol. It was incubated for 10 min in dark, then the absorbance was measured at 517 nm. In this assay, the positive controls were gallic acid and quercetin.

Statistical Analysis:All the analysis were done in triplicate. Statisical analysis of the data was done using Graphpad prism 5 software.

Results and Discussion:

Natural antioxidants have been proved to be effective protectors of body from the adverse effects of free radicals caused oxidative stress.Mushrooms are found to be rich source of these antioxidants with immense antiradical activity [22,23] .*Ganoderma lucidum* with wider medicinal values also contains rich antioxidants. In the present study, ethanol extract of the GDE contained 42.41±2.21mg/g total phenolics,

13.57±1.15 mg/ g of flavones and 1.33±0.12mg/g ascorbic acid. Several mushrooms have been reported to contain high levels of these compounds. In a study with several mushrooms 63 mg/g of phenolics from hot water extracts of *G.lucidum* was reported.[24] Low level of phenolics noticed in the present study might be due the solvent .It is reported that the polarity of the extraction solvent affect the level of phenolics [25,26].Higher extraction yields of phenolics were noted with increased polarity.Phenolic acids were the major phenolic compounds reported in mushrooms.Evidence show that gallic acid, tannic acid,protocatochuic acid and gentistic acids were some of the important phenolics acids isolated from edible in mushrooms in India.Extracts from fruiting bodies and mycelia of *G. lucidum* from southern parts of India have shown antioxidants properties [25].

One of the important indices to know the antioxidant activity of mushrooms is evaluating the reducing power.Usually the ability of mushroom to donate electron and reducing Fe^3 to Fe^2 is assessed. The reducing power was measured as the absorbance. In the present study, GDE extract showed concentration dependent increase of reducing power as given in Fig:1.

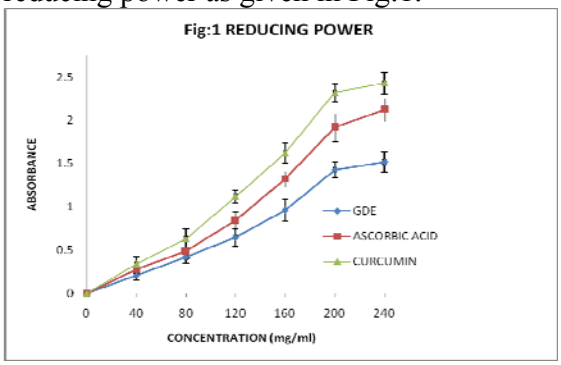

Fig. 1: Reducing power of GDE,Ascorbic acid and Curcumin at various concentrations.Values are Mean±S.E of three parallel observations.

GDE showed 1.38±0.21 at 200 mg/ml concentration.However, ascorbic showed 1.89±0.13 at the same concentration. Curcumin exhibited a maximum of 2.28±.12.Similar findings with *G.lucidum and G.tsugae* have been reported [27].The reducing power of the medicinal mushroom might be due to hydrogen donating ability.The medicinal mushrooms containing more reductones might react with the peroxides and certain precursors to halt the peroxide formation and termination of radical chain reactions [24].In addition, the phenolic compounds present in the mushroom can chelate pro- oxidant metal ions, thus preventing radical formation.

The radical scavenging activity of the GDE was assessed by DPPH scavenging assay. The antiradical activity is defined as the amount of antioxidant necessary for decreasing the initial DPPH concentration.Scavenging effect of GDE on DPPH radical was found to increase with increasing concentration(50-250 mg/ml), showing 72.24% inhibition at the highest concentration, whereas gallic acid and quercetin showed 86.87 and 82.32 % inhibition, respectively (Fig:2).

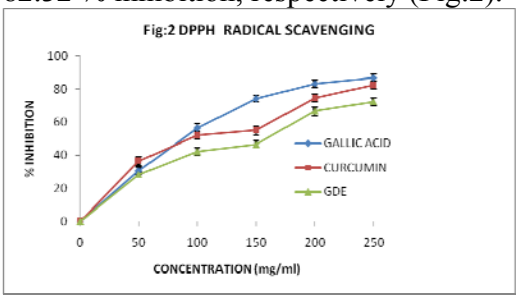

Fig. 2: DPPH scavenging activity of GDE,curcumin and gallic acid at various concentrations.Values are Mean±S.E of three parallel observations.

The IC_{50} for GDE was 172.52 g/ml.However, earlier studies with G. *lucidum and G.tsugae* hot water extracts showed 5.28mg/ml and 0.40mg/ml IC_{50}, respectively [24,26].The variations may be attributed to differences in the concentrations of the antioxidant compounds because of the solvent used for the extraction.Hot water extraction

with more phenolic contents exhibited higher scavenging activity [26].

Hydroxyl radicals are the major active oxygen species that cause lipid oxidation and enormous biological damage.The percentage of hydroxyl radical scavenging activity of the GDE increased with increasing concentration as given in Fig:3.

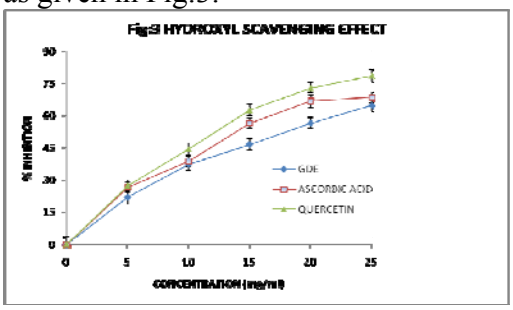

Fig. 3: Hydroxyl free radical scavenging effect of GDE,ascorbic acid and quercetin.

Values are Mean±S.E of three parallel observations.

At 25 mg/ml concentration the activity was over to 64.69% as against 68.45 and 78.45% inhibition exhibited by ascorbic acid and quercetin controls, respectively at this concentration.The scavenging effects of methanolic extracts from *G.lucidum* and its antlers showed 51.2 and 52.6% at 16 mg/ml,respectively.*G.tsugae* however, showed only 44.7% [27],indicating variations among different solvent extracts and parts of mushrooms in hydroxyl scavenging activity.

Superoxide radical is a well known precursor for formation of more harmful reactive substances in the body.The major risk of the superoxide generation is related to its interaction with the nitric oxide to form peroxynitrite, a potent oxidant that causes nitrosative stress. Decrease in the absorbance of the superoxide anion with GDE is noted in the present study, indicating effective scavenging potential comparable to the curcumin control. Fig:4 shows the inhibition of superoxide ions by GDE and curcumin at concentrations ranging from 100 ug/ml to 300ug/ml.Maximum inhibition of 73.54% was noted with GDE whereas curcumin showed 85.35%.Similar findings were reported for *Boletus edulis and B.auranticus* [28].

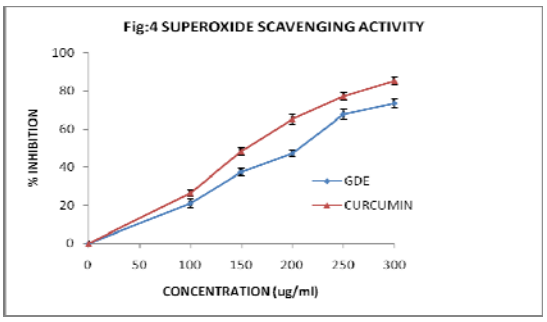

Fig. 4: Superoxide scavenging activity of GDE extract and curcumin control at various concentrations.Values are Mean±S.E of three parallel observations.

Nitric oxide is another free radical generated in human cells. Though associated with many regulatory functions, excess production would be detrimental to the body system as it readily reacts with oxygen to produce stable products of nitrate and nitrites.Nitic oxide inhibition by the GDE has been shown in FIG:5.

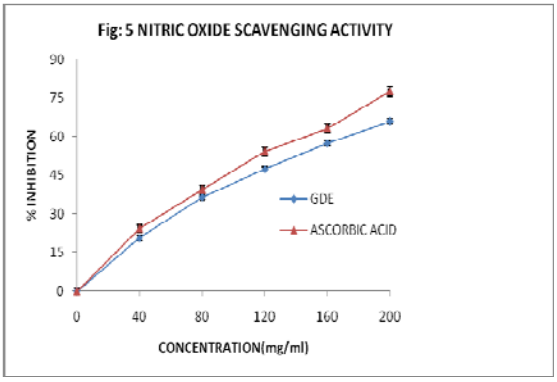

Fig. 5: Nitric oxide scavenging effect of GDE and ascorbic acid at various concentrations.Values are Mean±S.E of three parallel observations.

The study shows a maximum inhibition of 65.87% at 200mg/ml concentration when compared to ascorbic acid control(77.52%).Similar observation with *Pleurotus squarrolus* has been reported [29]. Correlations of total antioxidant components , tocopherols and total phenolics with antioxidant activity, reducing power, scavenging

ability on various radicals were well established [30].Considerable variations among different species of mushrooms in their antioxidant compounds concentration as influenced by the extraction solvent drastically alter the antioxidant potential. Also the level of observed antioxidant activity depend on the bio-ecological differences of tested strains (geographical origination, type of wood substrate, mycelial growth rate, and morphology), as well as the experimental conditions[31] .

Conclusion:

G. lucidum is a popular medicinal mushroom effective against a variety of human diseases.Ethanolic extract of this mushroom showed appreciable amount of phenolics,flavones and ascorbic acid with potent free radical scavenging activity .Though not edible,their broad medicinal properties make this mushroom unique. Development of a better fermentation technology to augment the compound production and extraction protocol will make this mushroom available for everyone. Their rich antioxidant contents make the mushroom ideal nutritional supplement with good medicinal properties.

Acknowlegement:

The authors wish to thank the Vice – Chancellor and the Dean,School of Chemical and Biotechnology,SASTRA University for the facility and encouragement to carry out the present research work. Thanks are also due to the Associate Dean and the Dean, Research for the support.

References:

[1] Ray G, Husain SA. Oxidants,antioxidants and carcinogenesis.*Indian.Jl..Exp.Biol* 2002;40(11):1213-32.

[2] Harman D. Free radical involvement in aging.Pathophysiology and the therapeutic implications.*Drugs Aging1993*,3(1):60-80.

[3] Halliwell, B., in *Oxygen Radicals and Disease Process* (Eds), Thomas, C. E. and Kalyanaraman, B.), Hardwood Academic Publishers, The Netherlands 1997, pp. 1–14.

[4] Ames BM. Dietary carcinogens and anticarcinogens: Oxygen radical and egenerative disease. *Science* 1983, 221: 1256–1263.

[5] Branen AL. Toxicology and biochemistry of butylated hydroxy anisole and butylated hydroxy toluene. *J Am Oil Chem Soc* 1975; 52:59–63.

[6] Cazzi R, Ricardy R, Aglitti T, Gatta V, Petricone, P, DeSalvia, R. Ascorbic acid and b-carotene as modulators of oxidative damage.*Carcinogenesis* 1997; 18: 223–228.

[7] Elmastasa M, Isildaka O, Turkekulb I, Temura N. Determination ofantioxidant activity and antioxidant compounds in wild edible mushrooms. *Journal of Food Composition and Analysis* 2007; 20: 337–345.

[8] Cheung, LM,Cheung PC, Ooi V E. Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chem* 2003;81: 249-255.

[9] Mohamed MI, Raja MMM , BasithJA ,Asarudeen A.Determination of total phenol,flavonoid and antioxidant activity of mushrooms *Pleurotus florida and Pleurotus eous.International Food Research Journal* 2011;18:574-577.

[10] Barros L, Falcão S, Baptista P , Freire C, Vilas-Boas M , Ferreira I CFR. Antioxidant activity of *Agaricus* sp. mushrooms by chemical, biochemical and electrochemical assays. *Food Chemistry* 2008;111:61-66

[11] Chang ST, Buswell JA. *Ganoderma lucidum* (Curt.:Fr.) P. Karst. (Aphyllophoromycetideae) – a mushrooming medicinal mushroom. *Int J Med Mushr* 1999;1: 139–146.

[12] Jong SC, Birmingham JM. Medicinal benefits of the mushroom *Ganoderma. Adv Appl Microbiol* 1992; 37: 101–134.

[13] Rajarathnam S, Shashirekha MN, Bano Z. Biodegradative and biosynthetic capacities of mushrooms: present and future strategies. *Crit Rev Biotechnol* 1998; 18: 91–236.

[14] McDonald S, Prenzler PD, Antolovich M, Robards K. Phenolic content and antioxidant activity of olive extracts. *Food Chem* 2001; 73: 73-84.

[15] Chang C, Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J.Food Drug Anal* 2002; 10: 178-182.

[16] Kumari D, Reddy MS, Upadhyay RC. Antioxidant activity of three species of wild mushroom genus *Cantherellus* collected

from north-western Himalaya, India. *Int.J.Agric.Biol* 2011;13:415-418.

[17] Robak J, Gryglewski RJ. Flavonoids are scavengers of superoxide anions. *Biochem. Pharmacol* 1988; 37: 837-841.

[18] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS,Tannenbaum SR. Analysis of nitrate, nitrite and 15N nitrate in biological fluids. *Anal. Biochem* 1982; 126: 131-138.

[19] Kunchandy E, Rao MNA. Oxygen radical scavenging activity of curcumin. *Int. J. Pharm* 1990; 58: 237-240.

[20] Athukorala Y, Kim KN, Jeon YJ. Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga *Ecklonia cava*. *Food Chem. Toxicol* 2006; 44: 1065-1074.

[21] McCune LM, Johns T. Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the indigenous peoples of the North American boreal forest. *J.Ethnopharmacol* 2002; 82: 197-205.

[22] Barros, L., M.J. Ferreira, B. Queiros, I.C.F.R. Ferreira and Baptista P. Total phenols, ascorbic acid, b-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. *Food Chem* 2007*;*103: 413–419.

[23] Valentão P, Lopes G, Valente M, Barbosa P, Andrade PB, Silva BM, *et al.* Quantification of nine organic acids in wild mushrooms. *J. Agric. Food Chem* 2005;53: 3626–3630.

[24] Abdulla N, Ismail SM, Aminudin N, Shuib AS, Lan BF.Evaluation of selected culinary-medicinal mushrooms for antioxidants and ACE inhibitory activities.*Evidence Based Compl Alter Medicine 2011;*2012:

[25] Puttaraju NG, Venkateshaiah SU, Dharmesh SM, Urs SMN, Somasundaram R. Antioxidant activity of indigenous edible mushrooms. *J. Agric. Food Chem2006;* 54(26): 9764–9772.

[26] Cheung LM, Cheung PCK, Ooi VEC. Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry 2003;* 81(2): 249–255.

[27] Mau JL , Lin HC, Chen CC. Antioxidant Properties of Several Medicinal Mushrooms. *J. Agric. Food Chem* 2002; *50:* 6072□-6077.

[28] Vidović SS, Mujić IO, Zeković ZP, Lepojević ZD, Tumbas VT, Mujić AI. Antioxidant Properties of Selected Boletus Mushrooms. *Food Biophysics* 2010; 5:49–58

[29] Pal J,Ganguly S,Tahsin KS, Acharya K.*In vitro* free radical scavenging activity of wild edible mushroom ,*Pleurotus squarrolus*(Mont.) Singer.*Ind.Jl.of Exp.Biol* 2010;47:1210-1218.

[30] Lee Y, Jian S, Lian P, Mau J. Antioxidant properties of extracts from a white mutant of the mushroom *Hypsizygus marmoreu. J Food Compos Analysis* 2008; 21:116-124.

[31] Badalyan, SM . Edible and medicinal higher Basidiomycetes mushrooms as a source of natural antioxidants. *Int J Med Mushr 2003; 5:153-162.*