JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4735-4741

Qualitative Phytochemical Screening, Total Phenolic Content, *In vitro* Antioxidant Activity and Antimicrobial Activities in Methanolic Extracts of *Lactarius sanguifluus* (Paulet) Fr.

Monika Thakur and Rukhsaar Sayeed

Amity Institute of Food Technology, Amity University, Noida, UP - 201303, India.

(Received: 30 August 2014; accepted: 31 October 2014)

Lactarius sanguifluus **is a wild edible mushroom belonging to** ***Russulaceae*** **family and commonly known as 'bloody milk cap'. It is one of the most valued mushrooms in the gastronomy sector. The bioactive components present in** ***L. sanguifluus*** **are responsible for the nutraceutical potential of this wild edible mushroom. The present study was carried out to assess the phytochemical screening, total phenolic content, antioxidant potential and antimicrobial activities of methanolic extracts of** ***L. sanguifluus*****. Qualitative phytochemical analysis showed the presence of alkaloids, anthraquonone, glycosides, tannins, saponins, flavonoids, terpenoids, phenolics, carbohydrates as well as proteins and amino acids, but anthocyanins and sterols were found absent. The total phenolic content of methanol extracts of** ***Lactarius sanguifluus*** **was 128.05 (mg Gallic acid equivalents per gram weight). The** ***in-vitro*** **antioxidant potential was analyzed by DPPH and Hydrogen peroxide method. The DPPH scavenging activity was 85.2±0.371 % and Peroxide was 84.1±0.281 % at 500μg/ml concentration, comparable to that of ascorbic acid. The methanolic extracts of** ***L. sanguifluus*** **were found to have significant antimicrobial activity against all the three test microorganisms i.e.** ***Escherchia coli, Pseudomonas aeruginosa*** **and** ***Candida albicans.*** **The present study evaluates the quantitative phytochemicals, total phenolic content antioxidant potential and antimicrobial activity of** ***Lactarius sanguifluus*** **extracts, so that its underexploited nutraceutical potential can further be explored.**

Key words: *Lactarius sanguifluus*, Phytochemical screening, DPPH, Hydrogen peroxide, Antioxidant potential, antimicrobial activity.

Since time immemorial, wild edible mushrooms have been collected and consumed throughout the World. They have been rightly called as, "the plant of immortality" due to their prolonged use in the history of mankind. Mushrooms have been used as food and flavoring agent for bland foods because of their unique and distinct flavour. Wild mushrooms are also underutilized and untapped resources, as wide variety of mushrooms are still underexploited (Lakhanpal, 1994). In last decades, there has been rising worldwide attention on the use of Wild edible fungi in food and medicine (FAO, 2004). They have also continued to generate great interest due to their ability to cure diseases. They also make a sustainable contribution to the diets of people living in developing countries and are considered as a source of income for many rural people. Therefore, wild edible mushrooms contribute towards diet, income and human health. Mushrooms also play an important ecological role, as many species are found living symbiotically with certain tree and their mycorrhizal association helps in sustainable growth of forests. Apart from their

* To whom all correspondence should be addressed.
Tel.: (0120) – 4392474; (M) 9810495426;
E-mail: mthakur1@amity.edu;
monika.harsh05@gmail.com

importance as a source of food for the regional poor and as mycorrhizal partners of host trees, they are excellent sources of bioactive compounds showing medicinal properties (Das, 2010). Wild mushrooms are also important Non-Timber Forest Product (NTFP's) from the forests and are being used as food since time immemorial (Devkota, 2008). From India, many species of edible fungi has been reported to be traditionally and regularly consumed by the local inhabitants without causality and fatality. Wild edible mushrooms having very good commercial value are species of *Morchella, Helvella, Hericium, Sparassis, Hydnum, Trapeinda, Clavaria, Ramaria, Boletus, Albatrellus, Cordyceps, Lactarius* and *Rusulla* (Lakhanpal, 2000). All these wild edible mushrooms are consumed as well as sold fresh, collected and dried for sale (Lakhanpal and Rana, 2005; Lakhanpal *et al*., 2010).

L. sanguifluus is an wild edible mushroom belonging to *Russulaceae* family. It is commonly known as **bloody milk cap** (Plate A). It is one of the most valued mushrooms in the gastronomy sector. *L. sanguifluus is* an ectomycorrhiza species which is a form of symbiotic relationship that occurs between a fungal symbiont and the roots of various plant species. It grows in association with pines. In India, *L. sanguifluus* has been found growing in mixed coniferous forests of Himachal Pradesh, under *Onychium contiguum* (Lakhanpal *et al.*, 1987).

L. sanguifluus is liked by people for its aroma and flavor. The specie is mainly used as a delicacy in Catalonian cuisine (Carles, 2003). At younger stage they are mostly dried by the people for use during harsh and snowy winters. It keeps well without spoiling for a long period of time. Older fruiting bodies usually turn soft and get infested with worms (Paulet, 1811).

Lactarius sanguifluus has been a potent source of nutraceuticals. Very less work has been done on the nutraceutical potential of the mushroom, but Anke *et al*. (1989) studied the synergistic effects of its bioactive compound. Terpenoids (sesquiterpenoids) with anti-microbial and cytotoxic effects have been detected in this mushroom. The compounds tested were a stearic acid ester of a sesquiterpene (I) and a sesquiterpene aldehyde (lactaroviolin, II) and alcohol (deterrol, III). The assays used were for mutagenic activity in the *Salmonella* assay, for antimicrobial activity against bacterial fungi and algae, for cytotoxicity against Ehrlich ascitic tumour cells and L 1210 cells, and for phytotoxic activity against *Lepidium sativum* and *Seteria italica*. All three compounds showed weak mutagenic activity in the Ames assay. Two compounds (II and III) were found to have moderate cytotoxic activity and one (III) exhibited weak antibacterial activity. No compound revealed phytotoxic, algicidal or antifungal activity.

Lactarius sanguifluus also contains a mixture of sterols. The predominant sterol is ergosterol (56.6% of total sterols), with lesser amounts of ergosterol derivatives, including ergost-7-en-3^2-ol, ergosta-7,22-dien-3^2-ol, and ergosta-5,7-dien-3^2-ol (Cerri *et al.*, 1981). Some of these chemicals are thought to undergo enzymatic conversions when the fruit body becomes injured (Sterner *et al.*, 1988). Ferreira *et al*. (2009) studied the antioxidant in wild edible mushrooms.

Kalogeropoulos *et al*. (2013) studied the bioactive micro-constituents and antioxidant properties of wild edible mushrooms from Island of Lesvos, Greece. The extracts obtained from *Lactarius sanguifluus* have been investigated for their antimicrobial activity. Growth inhibition using agar disk diffusion assays was determined against *Candida albicans.* Fruit body extracts have been shown to have some antimicrobial activity against Gram-positive and Gram-negative bacteria (Dulger *et al.*, 2002). Sagar and Thakur (2013) also studied the antibacterial activity of *L. sanguifluus methanolic* extracts.

Therefore, the present study evaluates the quantitative phytochemicals, total phenolic content, *in-vitro* antioxidant potential and antimicrobial activity of *L. sanguifluus* methanolic extracts, so that its underexploited nutraceutical potential can further be explored.

MATERIAL AND METHODS

Chemicals

Ethanol, Methanol, Sodium carbonate, Gallic acid, Folin-Ciocalteu reagent, and DPPH (1,1-diphenyl-2-picrylhydrazyl) were used. All the chemicals and reagents used were of analytical grade.

Microbial cultures

Microorganism slant cultures were obtained from MTCC, IMTECH Chandigarh - 160036, India. The following cultures were used for the study:

- *Escherchia coli* (MTCC1698),
- *Pseudomonas aeroginosa* (MTCC6458),
- & *Candida albicans* (MTCC7315).

Collection of plant material and sample preparation

The wild edible mushroom specie was collected from the Northwest Himalayan region of Shimla, India (31º6'12"N77º10'20"E). The fruiting bodies were thoroughly cleaned of extraneous matter and dried completely and coarsely grounded.

Preparation of extracts

Grounded mushroom was extracted with methanol at room temperature prior to removal of solvent. 10 grams of the ground sample was mixed with six times of 99.6% methanol and kept for 24 hour. This process was repeated thrice and filtrates were collected. The filtrates obtained were concentrated under vacuum on a rotary evaporator (Buchi Rotary Evaporator, Model R-124) and stored at 4°C for further use (Song *et al*., 2010).

Quantitative Phytochemical screening

Methanolic extracts of *L. sanguifluus* were used for qualitative screening of twelve phytochemicals as per standard biochemical procedures. The preliminary tests for methanol extracts were performed to confirm the presence of Alkaloids, Anthraquinones, Anthocyanins, Carbohydrates, Flavonoids, Glycosides, Phenols, Proteins and amino acids, Saponins, Steroids, Tannins and Terpenoids (Tiwari *et al*., 2011).

Estimation of Total Phenolic content

The total phenolic content in methanolic extracts of grounded sample was estimated by Folin-Ciocalteu reagent, as described by Singleton and Rossi (1965). 100 mg of gallic acid was

Table 1. Qualitative phytochemical screening of *Lactarius sanguifluus* extracts

S. no.	Phytochemical	*L. sanguifluus* extract
1	Alkaloids	+ + +
2	Anthraquinone	+ +
3	Anthocyanins	-
4	Proteins and amino acids	+ + +
5	Carbohydrates	+ + +
6	Phenols	+ +
7	Terpenoids	+ + +
8	Sterols	-
9	Saponins	+ + +
10	Glycosides	+ + +
11	Flavonoids	+ +
12	Tannins	+ +

Table 2. Percent scavenging activity in *L. sanguifluus* extracts at different concentrations

S. No.	Sample	Absorbance at 765nm (Mean ± Standard error)	Total phenolic content (mg Gallic acid equivalents per gram weight)
1	*L. sanguifluus*	0.861 ± 0.0012	128.05

Table 3. Absorbance and percentage scavenging activity of the mushroom extract at different concentrations at 765 nm using DPPH method.

Con., (µg/ml)	Absorbance of *L.sanguifluus*	Percent scavenging activity
100	0.494	39.8
200	0.348	57.6
300	0.196	76.1
400	0.171	79.1
500	0.084	89.7

Table 4. Absorbance and percent scavenging activity of the mushroom extract at different concentrations using Hydrogen peroxide at 230 nm.

Con., (µg/ml)	Absorbance of *L.sanguifluus*	Percent scavenging activity
100	0.478	40.3
200	0.381	52.4
300	0.193	75.9
400	0.184	77.0
500	0.086	89.2

dissolved in 100 ml ethanol to prepare Gallic acid stock solution (1000 µg/ml). Various dilutions of standard gallic acid were prepared from this stock solution. 1 ml aliquots of 1.0, 2.5, 5.0, 10, 25, 50 and 100 µg/ml of gallic acid solution were mixed with 5.0 ml of Folin-Ciocalteu reagent (diluted ten-fold) and 4.0 ml of sodium carbonate solution (75 g/l) and calibration curve was plotted. The absorbance was measured after 30 min at 20°C at 765 nm. 1 ml extract was mixed separately with the same reagents and absorbance was measured at 765 nm after 1 hour. The total phenolic compound in the extract was determined using the formula:

$$C = C_1 \times V/m$$

C= Total content of phenolic compounds in mg/g in GAE (Gallic acid equivalent); C_1= The concentration of gallic acid established from the standard curve in mg/ml; V=The volume of extract in ml, M=Weight of extract in grams.

***In vitro* evaluation of antioxidant activity**

In vitro antioxidant activity of the extract of *L. sanguifluus* was determined using two methods: Free radical scavenging activity using DPPH method and Free radical scavenging activity using hydrogen peroxide.

Free radical scavenging activity using DPPH method

The free radical scavenging activities of sample extract was measured by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Kaur *et al.*, 2008). Briefly, 0.1 mM solution of DPPH in ethanol and 1.5 ml of this solution was added to 0.5 ml of extract solution in ethanol at different concentrations (50-300 ¼l/ml). The mixture was shaken and allowed to stand

Plate A: Fruiting body of *Lactarius sanguifluus*

Plate B: Antimicrobial activity of *Lactarius sanguifluus* against *Escherchia coli*

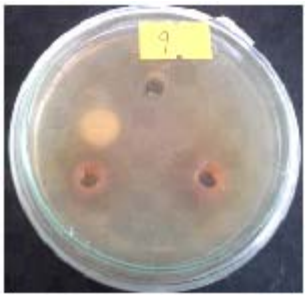

Plate C: Antimicrobial activity of *Lactarius sanguifluus* against *Pseudomonas aeruginosa*

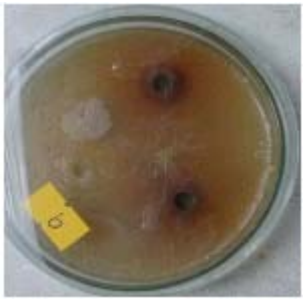

Plate D: Antimicrobial activity of *Lactarius sanguifluus* against *Candida albicans*

Table 5. Antimicrobial activity against *E. coli*, *P. aeruginosa* and *C. albicans*

S. No.	Sample Extracts	Zone of inhibition (mm) *E. coli*	*P. aeruginosa*	*C. albicans*
1	*L. sanguifluus*	22 ± 0.286 (C)	10.5 ± 1.002 (C)	20 ± 0.186 (C) 17 ± 1.22 (D)

at room temperature for 30 minutes. The absorbance was then measured at 517 nm using a spectrophotometer. A blank without DPPH was used to remove the influence of the color of the extracts and an ethanolic solution of DPPH was used as a negative control. Ascorbic acid was used as a reference. All of the measures were carried out in triplicates. The DPPH radical scavenging activity was calculated using the following equation:

$$\% \text{ scavenging effect} = \frac{A_0 - A_s}{A_0} \times 100$$

Where,

A_o = Absorbance of negative control

A_s = Absorbance of sample

Free radical scavenging activity using hydrogen peroxide

The free radical scavenging activity of sample extract was determined by using hydrogen peroxide (Mohamad *et al*., 2010). An aliquot of 0.6 ml of hydrogen peroxide (43m) and 1.0 ml of various concentrations of the extract prepared using phosphate buffer (200-400 micro gm/ml) were mixed followed by 2.4 ml of 0.1 M phosphate buffer (pH 7.4). The resulting solution was kept for 10 minutes and the absorbance was recorded at 230 nm. All measures were repeated triplicate. For each concentration, mixture without sample was taken as a control and a mixture without hydrogen peroxide was taken as a blank. Ascorbic acid was used as a standard compound. The percentage scavenging activity of hydrogen peroxide was calculated as:

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_s}{A_s} \times 100$$

Where,

A_o = Absorbance of negative control

A_s = Absorbance of sample

***In - vitro* Anti-microbial Activity**

The *in- vitro* antimicrobial activity was carried out by Disc diffusion method. The microbial suspension of different dilutions (10^0, 10^{-1}, 10^{-2} and 10^{-3}) was prepared in 0.85% saline and 100 µl of 10^0 and 10^{-3} dilutions were spread evenly on separate Nutrient Agar (NA) Plates. The extracts both diluted (D) and concentrated (C) were used. The dilution of the extracts was done with DMSO (Di-methyl sulfoxide). Then the blank sterile discs containing 20 µl of the sample extract were placed on these NA plates. The experiment was carried out in triplicates. The plates were then incubated in an incubator at 37°C for about 24 hours and the plates were observed for zone of inhibition, and the diameter of the zone of inhibition was calculated for all the plates. Antimicrobial activity was evaluated by measuring the diameter of the zone of inhibition around the extract (Duyilemi & Lawal, 2009). The assay was repeated three replicates and the results were recorded as mean + SD.

RESULTS AND DISCUSSION

Qualitative phytochemical analysis of *L. sanguifluus* extracts showed the presence of alkaloids, anthraquonone, glycosides, tannins, saponins, flavonoids, terpenoids, phenolics, carbohydrates as well as proteins and amino acids (Table 1). Anthocyanins and sterols were not found. The results revealed that *L. sanguifluus* may be used as potential sources of phytochemicals and thus can be used for designing drugs that can prove to be of keen interest in the treatment and prevention of diseases like cancer, tumor, heart diseases, etc

The amount of total phenols was determined with Folin- Ciocalteu reagent. Gallic acid was used as standard compound. The standard curve of Gallic acid concentrations and absorbance is shown in Fig 1. The absorbance for various dilutions of Gallic acid with Folin- Ciocalteu reagent and sodium carbonate were found.

Found standard curve equation was;

$Y = 0.0106 x + 0.041$

$R^2 = 0.996$

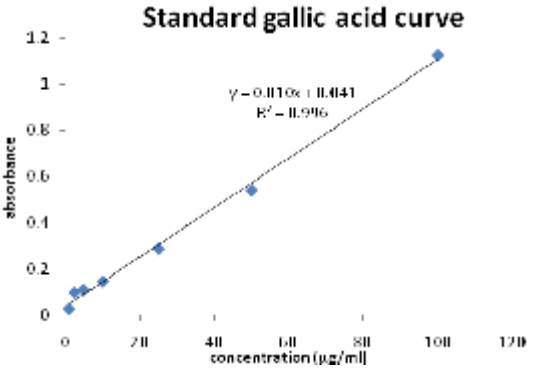

Fig. 1. Standard Gallic acid curve

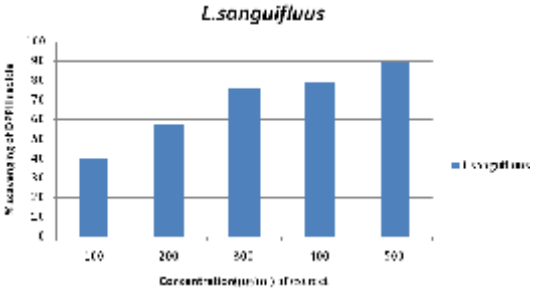

Fig. 2. Percentage Scavenging of DPPH Radical of *L. sanguifluus*

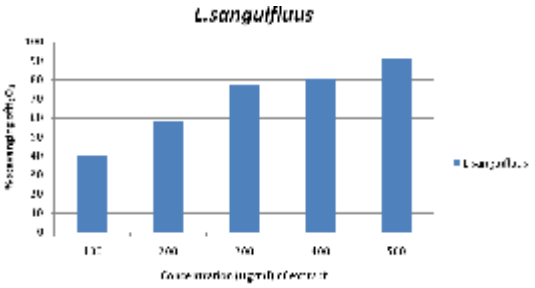

Fig. 3. Percentage Scavenging of Hydrogen peroxide Radical of *L. sanguifluus*

The total phenolic content of methanol extracts of *L. sanguifluus* was 128.05 (mg Gallic acid equivalents per gram weight) (Table 2). Data expressed as mean ± standard error of three samples analyzed separately.

Antioxidant activity of sample extract at different concentrations was determined using two different techniques viz. DPPH method and H_2O_2 method. The DPPH is a stable organic free radical with an absorption maximum band around 515-528 nm (Stankovic, 2011) and is widely used for evaluation of antioxidant potential of compounds. The methanolic extracts of *L. sanguifluus* showed good antioxidant activity as evaluated by both the methods. Results are presented in Table 3 and Table 4. The results obtained from both the methods revealed that *L. sanguifluus* exhibits high antioxidant activity. At the concentration of 500 µg/ml, the extracts showed 89.7% and 89.2% scavenging activity by DPPH and hydrogen peroxide method respectively. The results are represented by Figure 2 and 3.

The methanolic extracts of *L. sanguifluus* were found to have significant antimicrobial activity against all the test micro-organisms microorganisms i.e. *Escherchia coli, Pseudomonas aeroginosa and Candida albicans.* The zones of inhibition shown by concentrated (C) and diluted (D) methanolic extracts of the mushroom are presented in Table 5. Plates B, C and D, show the antimicrobial activities against *E. coli, P. aeruginosa and C. albicans* respectively.

REFRENCES

1. Anke, H., Bergendorff, O. Sterner O. Assay of the biological activities of guidance sesquiterpenoides isolated from the fruit bodies of edible *Lactarius* species. *Food Chem. Toxicol.* 1989; **27**:393-398.
2. Carles, I.J. La Cuina tradicional dels bolets. Cossetània Edicions. 2003; pp. 54.
3. Cerri, R. , DeSimone, F. Senatore, F. Sterols from three *Lactarius* species. *Biochem. Syst. Ecol.* 1981; **9** (4): 247–8.
4. Das, K. Diversity and conservation of wild mushrooms in Sikkim with special reference to Barsey *Rhododendron* Sanctuary. *NeBIO*. 2010; **1**(2): 1-13.
5. Devkota, S. Distribution and status of highland mushrooms: a study from Dolpa, Nepal. *J. Nat. History Mush.* 2008; **23**: 51-59.
6. Dulger, B., Yilmaz, F. Gucin, F. Antimicrobial activity of some *Lactarius* species. *Pharma. Biology.* 2002; **40**(4): 304 – 306.
7. Duyilemi, O. P Lawal, I. O. Antibacterial activity and phytochemical screening of *Chrysophyllum albidum* leaves. *Asian J. Food Agro-Indus*. 2009; S75-S79.
8. FAO. Non wood forest products, wild edible fungi: A global overview of their use and importance. (ed. Boa, E). FAO Publication, Rome. 2004; pp 17-147.
9. Ferreira, I.C., Barros, L. Abreu, R. M. Antioxidants in wild mushrooms. *Curr Med Chem.* 2009; **16**(12):1543-60.
10. Kalogeropoulos, N., Yanni, A. E., Koutrotsios, G. Aloupi, M. Bioactive micro-constituents and antioxidant properties of wild edible mushrooms from the island of Lesvos, Greece. *Food Chem. Toxicol.* 2013; **55**:378–385.
11. Kaur, P., Singh, B., Kumar, S. Kaur, S. *In vitro* evaluation of free radical scavenging activity of *Rubia cordifolia* L. *J. Chinese Clinical Med.* 2008; **3**(5): 278-284.
12. Lakhanpal, T.N., Bhatt, R.P., Kaisth, K. *Lactarius sanguifluus* Fr. – an edible mushroom new to India. Current Sci. 1987; **56** (3): 148–149.

13. Lakhanpal, T.N. Rana, M. Medicinal and Nutraceutical genetic resources of mushrooms. Plant Genetic Resources – *Characteriz. Utilizat*. 2005; 1-17.
14. Lakhanpal, T.N. Prospects of Mushrooms from the Wild. Advances in Mushroom Biotechnology (eds. M.C., Nair, G. Gokulapalan and L. Das) Scientific Publishers, Jodhpur. 1994; pp 15-22.
15. Lakhanpal, T.N. Wild and cultivated mushrooms. Natural Resources and Development in Himalaya. (ed. L.R. Verma). Malhotra Publishing House, New Delhi. 2000; pp 402-417.
16. Lakhanpal, T.N., Shad, O. Rana, M. Biology of Indian Morels. I K International Publishing House, 2010; pp 266.
17. Mohamad, A., Seyed, M., Seyed, F., Fatehmeh, B. Ahmad, R. Antioxidant and free radical scavenging activity of *H. officinais* H, var. *Angustifolia*, *V. odorata*; *B. hyrcana*; *Speciosu*m. *Pak. J.Pharma. Sci*. 2010; **23**(1):29-34.
18. Paulet, J.J. Traité des champignons **2** (9 ed.). 1981; p. plate 81, fig 3-5.
19. Sagar, A. Thakur, K. Study on Ultrastructure and Antibacterial Activity of *Lactarius sanguifluus* (Paulet) Fr. *J. Pure App. Microbiol*. 2013; **7** (4), 2873-2877.
20. Singleton, V.L. Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic*. 1965; **6**: 144-158
21. Song, W.Y., Ku, K.H. Choi, J.H. Effect of ethanol extracts from red pepper seeds on antioxidative defense system and oxidative stress in rats fed high-fat, high-cholesterol diet. *Nutr res Pract.* 2010; **4**(1): 11-15.
22. Stankovic, M.S. Total phenolic content, flavonoid concentration and antioxidant activity of *Marrubium peregrinum* L. extracts. *Kragujevac J. Svi*. 2011; **33**: 63-72.
23. Sterner, O., Bergendorff, O., Bocchio, F. The isolation of a guaiane sesquiterpene from fruit bodies of *Lactarius sanguifluus*. *Phytochem*. 1988; **28** (9): 2501–2.
24. Tiwari, P., Kumar, B, Kaur, M., Kaur, G. Kaur, H. Phytochemical screening and extraction: a review. *Intern. Pharmaceu. Sci*. 2011; **1**(1):98-106.