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Extraction and application of secondary metabolites from *Parmelia Perlata*

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Abstract

Parmelia perlata is a lichen that is a symbiotic association between fungi and algae belonging to the family Parmeliaceae. It is also consumed as an edible source in India.The present study was focussed on extracting and evaluating the application of secondary metabolites from the lichen sample. The sample was extracted using methanol as solvent by cold extraction method and phytochemical analysis was carried out to determine the presence of secondary metabolites. The antibacterial activity of lichen *Parmelia perlata* was tested against 2 gram (+) ve bacteria *Staphylococcus aureus, Bacillus subtilis* and 2 gram (-) ve bacteria *Pseudomonas aeruginosa* and *Escherichia coli.* Anticancer activity was determined against MCF-7 breast cancer cell lines using MTT assay where the sample showed a rapid decrease in the percentage of cell viability. The antioxidant activity was also determined by DPPH assay, TCA assay and H_2O_2 assay.The sample was purified by means of column chromatography and the purified sample was estimated by Gc-Ms analysis to reveal the presence of major secondary compounds. The dye yielding potential of lichen *Parmelia perlata* was also assessed by boiling water method (BWM) and di-methyl sulphoxide extraction method (DEM) .The extracted dyes were tested onto fabrics with and without the use of mordants and the anticancer activity of the dye obtained by BWM method wasalso assessed in order to test its efficacy as a potential food colorant.

Key words : *Parmelia perlata*, Secondary metabolites, Antibacterial activity, Anticancer activity, Antioxidant activity, Column chromatography, Gc-Ms analysis, Dye.

INTRODUCTION

The occurrence of natural physiological and biological process within the living cells are generally associated with the production or generation of free radicals and reactive oxygen species (ROS).The different forms of reactive oxygen species are Hydrogen peroxide, superoxide anion, hydroxyl radical and singlet oxygen [1]. Free radicals could lead to oxidative damage of cellular macromolecules like lipids, proteins and DNA and could also eventually lead to diseases such as cancer,aging,diabetes and other degenerative diseases. Thus antioxidants are considered to be the compounds that can impede oxidation process by reacting with free radicals chelating catalytic metals and scavenging oxygen in biological system[2]. The synthetic antioxidants such as Butylated Hydroxyl Anisole (BHA), Butylated Hydroxyl Toluene (BHT), and Tertiary butylated hydroquinone were found to cause harmful health impacts[3].

The plants are gifted with free radical scavenging molecules such as tannins, terpenoids, flavanoids, steroids, vitamins, coumarins, alkaloids and studies have shown that many of these antioxidant compounds from plants possess anti-tumor,anti-bacterial,anti-atherosclerotic and anti-mutagenic activities[4,5].

Cancer is the major dreadful disease that causes death all over the world. Breast cancer is considered to be the most common type of cancer diagnosed in women. Plant sources are widely been used for cancer treatment and it has become significant that over 60% of anti-cancer drug come from natural sources. Some of the naturally occurring anti cancer drugs from plant sources are: vinca alkaloids, taxanes, podophyllotoxin and its derivative, camptothecin and its derivatives, anthracyclines and others [6].

About 23 drugs derived from natural sources in the year between 2001 and 2005 were used in the treatment of bacterial and fungal infections, cancer, diabetes and other genetic disorders like gauchers disease.Since all the microbes have developed resistance against allopathic antibiotics, recently attention is paid towards biologically active compounds that are isolated from plant species used in herbal medicine[7]. Metabolites derived from plants and microorganisms are considered to be important sources of drugs and used in therapeutic applications. Among them, 50-60% is produced by plants and 5% have a microbial origin[8].

Parmelia perlata (Huds.) Ach. (Family: Parmeliaceae) is a lichen (i.e., symbiotic association between fungi and algae) and commonly referred to as “Stone Flower"and “Charila"[9]. Other names include: Dagad (North India), Pattharkephool (Hindi), Kallupachi (Telugu) and Kalpasi (Tamil).It is lichen growing in rosettes and spreads irregularly over the substratum giving the appearance of a flower. The thallus is dirty white or greyish brown and has bitter or saline taste [10].

It is generally used as a spice in order to enhance the taste and flavor of foods[11].They also possess antiemetic, analgesic and astringent activities. Its smoke relieves headache, increase flow of menses, heals wound and relieves pain of liver, uterus and stomach[12]. It shows antibacterial activity and is fairly effective against protozoans.It is also used as anti-fungal and anti-viral agent. It helps against baldness, worms and lices.

Thus in the present study the anti-oxidant,anti-microbial and anti-cancer potential as well as the dye yielding ability of lichen *Parmelia perlata* was evaluated .

MATERIALS AND METHODS

SAMPLE PREPARATION

Dried samples of lichen *Parmelia perlata* was obtained from the supermarket. The sample was cleaned, washed under tap water several times to remove the dirt, dried and powdered using a

mixer.The powdered lichen sample was stored in clean bottles until when required.

PREPARATION OF CRUDE EXTRACT

The crude extracts from the lichen sample was obtained by means of cold extraction method. About 30g of the powdered lichen sample was added to 300ml of methanol separately in a conical flask, covered with aluminium foil and kept on a rotary shaker for 24 hours at room temperature. The solution was filtered with the help of Whatman No.1 filter paper and the filtrate obtained was evaporated. The dried extracts were then dissolved in methanol and utilized for further phytochemical analysis. The yield of respective extracts were calculated as: Percentage yield (%) = (dry weight of extract/dry weight of samples) × 100.

PHYTOCHEMICAL ANALYSIS

The lichen extracts were subjected to phytochemical anlaysis based on the standard phytochemical methods as given by Sibi G (2013)[13]. The coloration developed was used in identifying the major natural chemical groups such as tannins, saponins, flavonoids, phenols, terpenoids, alkaloids, glycosides and steroids .The preliminary phytochemical results are given in table 1.

ANTIOXIDANT ACTIVITY

DPPH assay: Two ml of 6×10^{-5} M methanolic solution of DPPH were added to 50µl of a methanolic solution (10 mg ml-1) of the sample. Absorbance was read at 515 nm in a spectrophotometer and the decrease in absorbance at 515 nm was continuously recorded for 16 min at room temperature. Methanolic solutions of pure compound [quercetin] were tested at 1mg/ml concentration. The scavenging effect was plotted against the time. The percentage of inhibition was calculated as: Inhibition (%) = (A of control A of test) / A of control × 100 [14].

Hydrogen peroxide scavenging assay: Solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4).Concentration of H_2O_2 was determined by absorption at 230 nm in spectrophotometer. About 1ml of the extract was added to 3ml of H_2O_2 solution (0.6ml, 40mM). Solution containing phosphate buffer without hydrogen peroxide was used as control and the absorbance was read at 230nm after 10 minutes against control. The % of inhibition was calculated as Inhibition (%) = (A of control A of test) / A of control × 100 [15].

Total antioxidant capacity assay: About 1ml of the test sample is added to 3 ml of freshly prepared TAC reagent and is incubated for about 15 minutes at room temperature. The sample without TAC reagent was used as control and the absorbance of control and test samples were measured at 650nm in a colorimeter .The Total antioxidant capacity (TAC) can be calculated using the formula: (A of control A of test) / A of control × 100.

ANTIBACTERIAL ACTIVITY

The antibacterial activity of the lichen extracts were screened against 2 gram positive bacteria (*Staphylococcus aureus and Bacillus subtilis*) and 2 gram negative bacteria *(Escherichia coli and Pseudomonas aeruginosa*) and was carried out by agar well diffusion assay.

Agar well diffusion assay: The bacterial strains were precultured in nutrient broth overnight using rotary shaker at 37˚C and the cultures were used for antibacterial studies. Muller Hinton Agar medium was prepared, sterilised and about 25ml of the media was poured into the petriplates and were allowed to solidify. Once solidified the bacterial cultures were swabbed onto the media using a sterile cotton swab. The wells were made with the use of cork borer and about 150µl of the extracts diluted in methanol solvent were added to the well while pure methanol was used as control. Then the plates were incubated at 37˚C in the incubator for 24 hours and after incubation the inhibition zones were measured from the edge of the well to the edge of the zone.

ANTICANCER ACTIVITY

MTT assay: The breast cancer cell line (MCF-7) were seeded in a 96 well microtitre plate and incubated for 24 hours at 37˚C in a humidified 5% CO_2 incubator. The extracts at different concentration (50,100,150 and 200 µl) were added into the well and then incubated for 72 hours in CO_2incubator. After 72 hours of incubation the media was removed from the wells and about 20µl of MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide was added to each well and incubated for 3-4 hours.After incubation with MTT reagent the media was removed from the wells and about 100µl DMSO (Dimethyl sulphoxide) was added to rapidly solubilize the formazan.The absorbance for each well was measured at 650nm in a microtitre plate reader and the percentage inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = (\text{Control}_{A590} - \text{Sample}_{A590}) / \text{Control}_{A590} \times 100.$$

COLUMN CHROMATOGRAPHY

Silica gel (60-120 mesh) was chosen as the stationary phase. The column was filled with the silica gel using hexane and the crude residue from methanol (cold) extract was transformed on to the bed of silica gel. The column was run by using the mobile phase Hexane: Ethyl acetate in the ratio of (70:30).The fractions obtained were collected at an interval of 5ml each and they were monitored by thin layer chromatography. These obtained fractions were evaporated to dryness and the compounds obtained were stored.

GAS CHROMATOGRAPHY-MASS SPECTROMETRY (Gc-Ms)

An Agilent 6890 gas chromatograph was equipped with a straight deactivated 2 mm direct injector liner and a 15mm Alltech EC-5 column (250µI.D., 0.25µ film thickness).A split injection was used for sample introduction and the split ratio was set to 10:1.The oven temperature program was programmed to start at 35˚C, hold for 2 minutes then ramp at 20˚c per minute to 300˚C and hold for 5 minutes. The helium carrier gas was set to 2ml/minute flow rate (constant flow mode).A JEOL Gcmate II benchtop double focussing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-2000 software was used for all analyses.Low resolution mass spectra were acquired at a resolving power of 1000 (20%height definition) and scanning from m/z 25 to m/z 700 at 0.3 seconds per scan with a 0.2 second inter-scan delay.High resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 750 at 1 second per scan.

EXTRACTION OF DYES

The natural dyes can be extracted from the lichen *Parmelia perlata* by means of two methods: Boiling water method (BWM) and Di-methyl sulphoxide extraction method (DEM).

Boiling water method (BWM): About 10g of powdered lichen sample was added to 100ml of distilled water and is heated

till boiling and was maintained at simmer for 1 hour. The content was filtered into a clean conical flask. It is cooled and presoaked threads and fabric were added to the dye bath and was kept for 2 weeks at room temperature. Then the thread as well as the fabric were removed from the dye bath, rinsed in water and dried.

Di-methyl sulphoxide extraction method (DEM): About 10g of powdered lichen sample was added to 100ml of crude Di-methyl Sulphoxide solution in conicalflask. The content was stirred vigorously, covered with aluminium foil and left for 1 week at room temperature for the color to develop. Then it was filtered using filter paper and the filtrate was taken in a conical flask. Presoaked threads and fabric were added into the dye bath and was left for 2 weeks at room temperature. Then the thread as well as the fabric were removed from the dye bath, rinsed in water and dried[16].

Mordants: The fabrics (Cotton and Synthetic) were first immersed in the mordant for about 1 hour. (Mordant Ratio = 5g of alum in 50ml distilled water).Then the fabric fixed with the mordant were kept immersed in the dye bath and was left for about 2-3 hours. The fabric is then removed from the dye bath, rinsed in water, dried and were compared with those fabric that were dyed without using mordants

RESULT

The Percentage yield of lichen extract was found to be 2.56% and it has been reported to be the total yield for about 30 grams of the dry weight of the sample.The phytochemical analysis of lichen extracts were found to show positive results for components likealkaloids, terpenoids, glycosides, carbohydrates, phenols and negative results for saponins, resin, aminoacid, flavanoids and proteins (Table 1).

The antioxidant activity of the lichen extract was determined by DPPH assay, TAC assay and H_2O_2 assay. By DPPH assay the % scavenging of lichen extract was found to be 41%. By H_2O_2 assay the % scavenging of lichen was found to be 42% and the total antioxidant capacity of lichen extract was found to be 39%.(Table 2).

The antibacterial activity of lichen extract was tested against both gram (+) ve and gram (-) ve bacteria and the zone of inhibition was determined.The lichen extract is found to inhibit both gram (+) veas well as gram (-) ve bacteria. The maximum zone of inhibition was observed against *Staphylococcus aureus* with a zone of about 10mm in diameter followed by *Escherichia coli* and *Bacillus subtilis* with 6mm and *Pseudomonas aeruginosa* with 5mm (Table 3).

The anticancer activity of the lichen extract was determined by means of MTT assay. The lichen extract has been shown to greatly reduce the percentage of cell viability with increasing concentration wherein the % of cell viability at the highest concentration of 200µg/ml was found to be 41% (Table 4).

GC-MS is an analytical method used for identifying different compounds within a test sample The analysis of the lichen ***(Parmelia perlata)*** extract was carried out and were found to show large number of secondary compounds like Dodecane, tetradecane, heneicosane, Eicosane and so on (Table 5).

The dyes were extracted from lichen (*Parmelia perlata*) by means of two methods: Boiling water method and Di-methyl sulphoxide extraction method and the dyes obtained were found to be in the shades of light orange and light brown and were tested onto the fabric.

Table 1: Phytochemical analysis of *Parmelia perlata.*

S.NO	PHYTOCHEMICAL TEST	RESULT
1	Carbohydrate (Molisch Test)	Positive (+)
2	Proteins (Biuret test)	Negative (-)
3	Amino acid (Ninhydrin test)	Negative (-)
4	Glycosides (Keller killani test)	Positive (+)
5	Tannins (Ferric chloride test)	Positive (+)
6	Alkaloids (Hagers test)	Positive (+)
7	Flavanoids	Negative (-)
8	Phenol (Ferric chloride test)	Positive (+)
9	Terpenoids (Salkowski test)	Positive (+)
10	Saponins	Negative (-)
11	Resin (Acetone water test)	Negative (-)

Table 2: Determination of scavenging % of lichen extract by DPPH assay and H2O2 assay and total antioxidant % by TAC assay.

S.NO	ASSAY	% ACTIVITY
1	DPPH assay	41%
2	Hydrogen peroxide scavenging assay	42%
3	Total antioxidant capacity assay	39%

Table 3: Antibacterial activity of lichen extract against 2 gram (+) ve bacteria *Staphylococcus aureus, Bacillus subtilis* and 2 gram (-) ve bacteria *Escherichia coli, Pseudomonas aeruginosa.*

ORGANISM	ZONE OF INHIBITION (diameter in mm)	
	CONTROL	LICHEN
Staphylococcus aureus	No Zone	10mm
Escherichia coli	No Zone	6mm
Bacillus subtilis	No Zone	6mm
Pseudomonas aeruginosa	No Zone	5mm

Table 4: Determination of cell viability % of lichen extract against breast cancer cell lines at different concentrations.

S.NO	SAMPLE	CONCENTRATION (µG/ml)	SAMPLE OD VALUE	CELL VIABILITY (%)
1	LICHEN ***(Parmelia perlata)***	Control	0.69	100
		50	0.15	78
		100	0.26	62
		150	0.32	54
		200	0.41	41

Table 5: Determination of compounds in lichen purified extracts by GC-MS.

PEAK NO	RT (MIN)	COMPOUND	PEAK AREA	PEAK AREA (%)
1	18.94	1,2-15,16-Diepoxyhexadecane	4040688	10.88
2	19.65	Dodecane,5,8-diethyl-	2096144	5.64
3	20.55	Tetradecane,2,6,10-trimethyl-	3534000	9.52
4	21.44	Tetracosane	8305776	22.37
5	22.45	Heneicosane	10115632	27.24
6	23.68	Cis-1-Chloro-9-octadecene	9041136	24.35
		Total	37134176	100.00

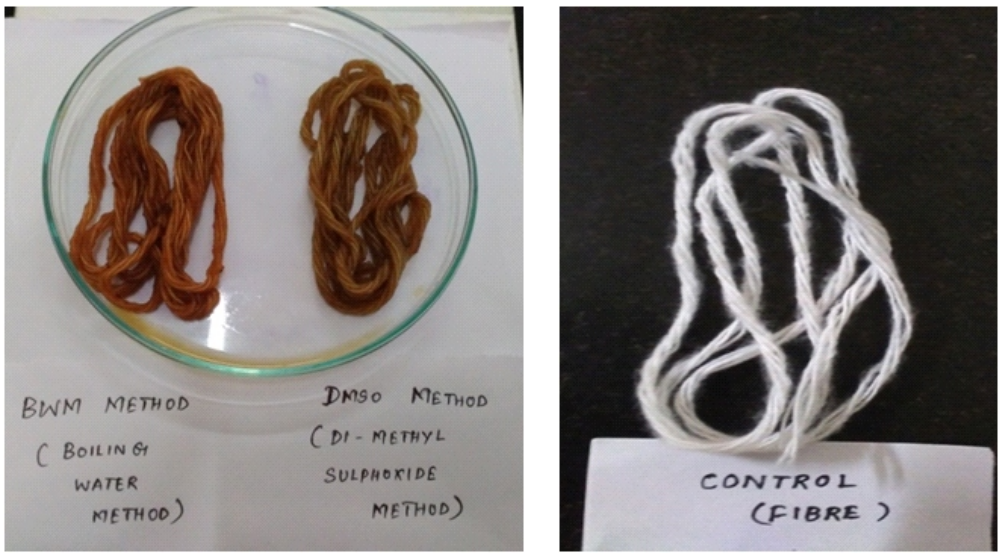
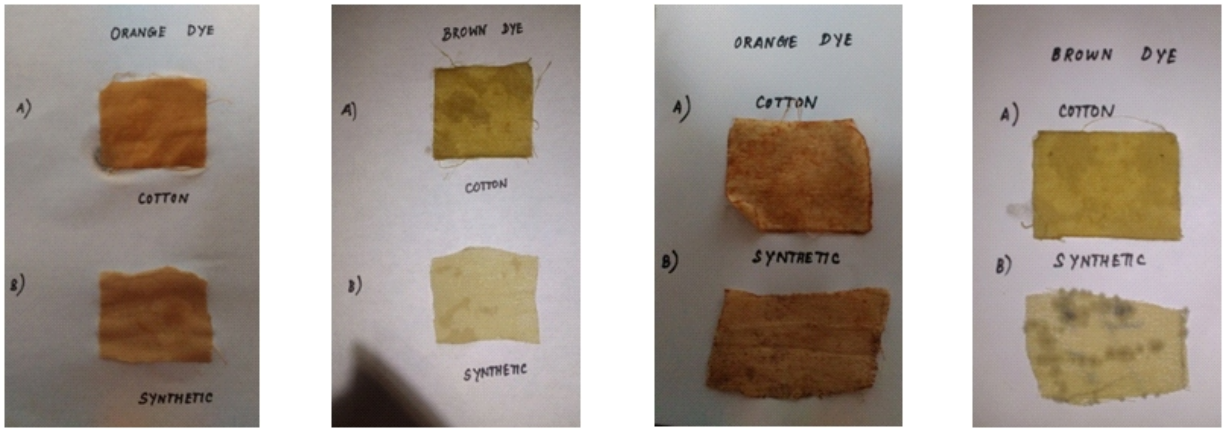


Fig 1: Comparison of the control fibre (undyed) with that of the dyed fibre

Table 6: Percentage of cell viability at different concentration of dye from lichen.

S.NO	SAMPLE	CONCENTRATION (µg/ml)	SAMPLE OD	CELL VIABILITY (%)
1	Dye from aqueous extract	Control 100 200	0.69 0.59 0.63	100 13 8



WITHOUT MORDANT **WITH MORDANT**

Fig 2: Comparison of the fabrics that were dyed with and without mordant.

In order to test the effectiveness of mordant the fabrics dyed and fixed with mordants were compared to those fabrics without mordants. The fabrics dyed without the use of mordant appears to be natural and smooth while those fabric fixed with mordant were found to be slightly rough.So it is inferred that fabric without use of mordant seems to be better than those with the use of mordant (Fig 1,2).

The anti cancer potential of the dyes obtained from aqueous extract of lichen *Parmelia perlata* were tested by means of MTT assay. The dye obtained from the aqueous extract of the lichen are also tested against breast cancer cell line at two different concentrations (100 and 200 µl) and were found to effectively reduce the percentage of cell viability hence they can also be used as natural food colorant and can also be used for therapeutic applications (Table 6).

DISCUSSION

The antioxidant activity of lichen extract was determined in which the % of DPPH inhibition as reported earlier by other authors in different lichen species fall under the same range between 25-45 % [17]. In case of antibacterial activity *Escherichia coli* showed an inhibition zone of about 6mm while study reported earlier showed an inhibition zone of about 19mm[18]. Earlier study carried out on lichen extracts against colon cancer cell lines were found to show 50 % cell viability at a concentration of 150µg/ml[19].

In case of column chromatography the studies carried out earlier has been reported to have used solvents like petroleum ether: ethyl acetate in the ratio of 9:1 as mobile phase for column chromatography[18] while in the present study Hexane: Ethyl acetate in the ratio of (70:30) was used because the solvent combination is found to be good for difficult seperations.The GC-MS analysis of the purified lichen extract was reported to have compounds like alkanes and other fatty acids and was also reported to possess numerous therapeutic properties[20]. The dye yielding potential of lichen *Parmelia perlata* was determined and similar experiments were also found to be carried out on different lichen species[16].

CONCLUSION

The present study deals with various therapeutic applications of the natural sources wherein the antimicrobial, antioxidant and anticancer potential of lichen (*Parmelia perlata*) was determined. The lichen sample showed positive result for the presence of various phytochemicals such as alkaloids, flavanoids, terpenoids, phenols and tannins which were found to exhibit a wide range of medicinal properties. A number of lichen compounds were also screened for their antibacterial potential and the study has shown that lichen had the ability to inhibit both the gram +ve and gram ve bacteria and shows that lichen extracts can be widely used in developing therapeutically important drugs. Similarly the anticancer , antioxidant and dye yielding potential of lichen Parmelia perlata was also assessed.

Hence the study proves that the use of drugs derived from natural sources could help in treating a number of diseases due to the accumulation of several bioactive compounds.

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REFERENCES

1. Halliwell B (1995) How to characterize an antioxidant: an update. BiochSocSym 61: 8591

2. Halliwell B, Gutteridge JMC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219: 14 [PubMed].

3. W.M. Zhang, B. Li, L. Han, H.D. Zhang, HD,(2009) "Antioxidant activities of extracts from areca (Areca catectu L.) flower, husk and seed," Afr J Biotechnol., vol. 8: 3887-3892.

4. Rice-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M. and Pridham, J.B .(1995). The relative activities of Plant-derived polyphenolic flavonoid. Free Radical Research, 22:375-383.

5. Sala, A., Recio, M.D., Giner, R.M., Manez, S., Tournier, H., Schinella, G., Rios, J.L. (2002). Anti-inflammatory and antioxidant properties of Helichrysumitalicum. Journal of Pharmacology, 54(3):365-371.

6. Cragg GM, Kingston DGI, Newman DJ.(2005) Anticancer agents from natural products. Taylor & Francis Group, Boca Raton, FL. Brunner-Routledge Psychology Press

7. Essawi T and Srour M (2000). Screening of some palestinian medicinal plants for antibacterial activity, Journal of Ethno pharmacology. 70(3): 343-349

8. Demain AL and Sanchez SJ (2009). Microbial drug discovery: 80 years of progress.Antibiot., 62: 5-16.

9. Kiritikar KR and Basu BD (1987). Indian Medicinal plants. Ed. 3. Pub. Lalit Mohan Basu MB. Allahabad; 1996 , pp 2757

10. K.R. Kiritikar, B.D. Basu,(1956) Indian Medicinal plants. 2nd Ed. M.B. Lalit Mohan Basu, Allahabad, vol. 3.

11. Bhattarai, Tribikram; Subba, Dilip; Subba, Rama (1999).Nutritional value of some edible lichens of east Nepal, 73(½).

12. Lindley J (1981). The Flora Medica**.** Pub. Ajay Book Service. New Delhi

13. Sibi G, Apsara V, K.Dhananjaya, K.R.Ravikumar and H.Mallesha (2013) Phytochemical and antibacterial properties of spices against food borne bacteria with special reference to Parmelia perlata G.J.B.B.,VOL.2(2):145-149.

14. Von Gadow A, Joubert E, Hansmann CF (1997). Comparison of antioxidant activity of aspalathin with that of other plant phenols of Rooibosed tea (Aspalathonlinearis), a-tocopherol, BHT and BHA**.** J. Agric. Food Chem., 45: 632-638.

15. Ruch, R.J., Cheng, S.J., and Klaunig, J.E. (1989) Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis, 10, pp. 1003-1008.

16. PreetiShukla, DK Upreti, S Nayaka and PritiTiwari (2014) Natural dyes from Himalayan lichens. Indian Journal of Traditional Knowledge.Vol 13 (1),pp.19-201.

17. A.Mastan, B.Sreedevi, J.PramodaKumari (2014) Evaluation of the in vitro antioxidant and antibacterial activities of secondary metabolites produced from lichens.Asian Journal of Pharmaceutical and Clinical Research, Vol 7, Issue 1.

18. ThippeswamyB ,Sushma N.R. and Naveenkumar K.J.(2013) Evaluation of antimicrobial property of lichen Parmeliaperlata.AJPP Vol.7(20),pp.1242-1250.

19. HafizurRahman, VijayaB, SoumyaGhosh, Geetika Pant, Sibi G (2014) In vitro studies on antioxidant,hypolipidemic and cytotoxic potential of Parmelia perlata.American Journal of Life Sciences;2(6-1):7-10.

20. P.T.V Lakshmi and Pa Rajalakshmi (2011**)** Identification of phytocomponents and its biological activites of Aloe vera through the Gas chromatography Mass spectrometry.IRJP 2(5) 247-249.