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BIOLOGICAL EVALUATION, CHARACTERIZATION AND DISTRIBUTION OF SOME LICHENS OF HIMALAYAN REGION, PAKISTAN

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ABSTRACT

Present study investigates the distribution, antibacterial, antioxidant, antitumor and cytotoxicity of the acetone and methanol extracts of ten lichen genera of *Dermetacarpon*, *Peltigera*, *Flavopunctelia*, *Flavoparmelia*, *Lecanora*, *Cladonia*, *Caloplaca*,*Collema*, *Rhizocarpon*, *Usnea* of Himalayan region (Mansehra) Pakistan. The bacterial strain used in this study was *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus bacillus*, *Pseudomonas aeroginosa*, *Escherichia coli* and *Enterococcus faecalis*. The antibacterial activity of the lichen extract in both solvent was studied by Disc diffusion assay. The zone of inhibition of these lichen extracts were in range 7 to 12.7 mm in diameter. This study showed that *S. bacillu*s is the most resistance and *S. epidermidis* is the most sensitive bacteria to the extract of all lichen genera. The antioxidant activity was investigated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging. The acetone extract in concentration of 1000ug/ml of *Collema* showed strongest DPPH radical scavenging activity while other acetoneextracts also showed positive results. However, *Rhizocarpon* showed the least DPPH radical scavenging activity.Cytotoxic activity of different concentration of lichen extract was tested against Brine shrimp, *Artemianauplii*. Result of cytotoxicityindicated that both methanol and acetone extract of *Flavopunctelia* were potent against brine shrimp. In potato disc assay, most of the acetone and methanol extracts of lichens showed significant (inhibition≥20%) antitumor activity against *Agrobacterium tumefacien.* Similarly, both acetone and methanol extracts of*Dermetocarpon* showed maximum (40%) tumor inhibition while *Collema* showed minimum tumor inhibition (16.66%).

Keywords: Antibacterial activity, Antioxidant activity, Lichens, Himalayan region, Pakistan.

INTRODUCTION

Lichen is a symbiotic association between algae and fungus. On the globe, they are represented by around 20,000 species (Shyam*et al.,* 2010), growing in terrestrial habitat on a variety of substrates i.e. bark, wood, leaves, rocks, soil and other fixture (Shah, 2011). They are important source of food for animals including man and are also used in alcohols, paints, perfume and pharmaceutical industries. Many natives of American, Indian and European tribes use it as their traditional medicine for treatments of many human and animal diseases (Karthikaidevi *et al.,* 2009). Lichens also produce various types of secondary metabolites i.e. phenolic compound, usnic acid, quinins, depsidones, deposones and pulvinic acid derivatives etc. which are unique with respect to higher plants (Veranja *et al.,* 2005) having remarkable antibacterial, antiviral, antifungal, antioxidant and antitumor activities (Tatjana *et al.,* 2011). Concentrations usually 0.1-1% of dry thallus weight; sometimes up to 30% of these metabolites vary with the species and area in varying amounts. A number of studies are available on biochemical assays of these secondary metabolites of lichen (Santiago*et al.,* 2010:Marijana and Rankovic, 2010: Krystle *et al.,* 2010) but lichens of Manshera or Pakistan are not studied yet.

MATERIALS AND METHODS

Collection of Lichen specimens: Mansehra (Hazara Division) is North Eastern District (34°14´- 35° 11´ NL; 72° 49´- 74° 08´ E) of Khyber Pakhtoonkhawa, Pakistan covering an area of 4,579 km^2 (Ali, 2005). Lichen specimens were collected from Dader and Manroor valley, Lolosar and Behisal area of District Mansehra during 2013. The samples collected were*Dermetacarpon*, *Peltigera*, *Flavopunctelia*, *Flavoparmelia*, *Lecanora*, *Cladonia*, *Caloplaca*, *Collema*, *Rhizocarpon*and*Usnea.* All collected samples were sun/air dried by changing the papers at suitable intervals, mounted on a cardboard paper and covered with thick brown packet, numbered and labeled. Each specimen was then identified based upon morphology of the thallus and micro-chemical color tests (Smith, 1918: Chopta, 1934: Culberson and Kristinsson, 1970: Culberson, 1972:Shyam*et al.,* 2010:Aptroot and Iqbal, 2012).

Distribution: Samples of different lichen genera were collected from different localities of Manshera, Pakistan.*Dermetacarpon, Flavopuntelia, Collema* were collected from Dader, *Flavoparmelia, Cladonia*and *Peltigera* from Manoor Valley, *Lecanora* from Lalosar and *Caloplaca* from Behisal. The altitude of each location are given in Table 1.

Preparation of Lichen extract: The plant extracts of 10 lichen genera samples i.e. *Dermetacarpon, Peltigera, Flavopunctelia, Flavoparmelia, Lecanora, Cladonia, Caloplaca, Collema, Rhizocarpon*and*Usnea*were prepared using acetone and methanol. The weighed quantity one gram of each lichen was homogenized with 50 ml of respective solvents. Crude preparations were left overnight in orbital shaker at room temperature. It was the filtered and evaporated at 60°C. The extract was then weighed and then dissolved in distilled water to obtain final concentrations for each sample (Claudia *et al.,* 2014).

Bacterial Strains: Six bacterial strains (ATCC bacterial strains) (Gram +ve and –ve) were used in antibacterial assay i.e.*E.coli, Enterococcus faecalis, Staphylococcus bacillus, Staphylococcus aureus, Pseudomonas aeroginosa*and *Staphylococcus epidermidis.* These bacterial strains were cultured at 37 °C in 40ml of Liquid broth (LB) media and were maintained at 4 °C.

Antibacterial Assay: An antibacterial activity of each lichen extract wastested by measuring inhibition zone by disc diffusion assay (Sharma *et al.,* 2011). For this purpose Muller Hinton agar (bacteria) were seeded with appropriate inoculums. Paper discs (6 mm diameter) were impregnated with different concentrations of lichen extract and placed on organism seeded plates. Blank disc was impregnated with dimethyl sulphoxide (DMSO) and used as negative control while roxithromycin was used as positive control. The plates were incubated and the zones of inhibition around each disc were measured (Sati and Joshi, 2011)

Cytotoxicity Assay: Cytotoxicity of Lichen extracts was tested by Brine shrimp cytotoxicity assay, most widely used tool for detection of bioactive compound. A container filled with Brine solution was used for hatching of Brine shrimps eggs. Brine solution was prepared by dissolving 32 g of sea salt and 1.5% agar in distilled water. Ten shrimps were transferred to each tube containing brine solution 4.5ml/tube and different concentration of Lichen extract Acetone and Methanol (0.05 mg/ml, 0.01mg/ml and 0.001mg/ml) were added in each dilution for each sample to check its cytotoxicity. After 24 hours of incubation the number of surviving shrimps was counted by magnifying glass(Olowa andMuneza,2013)

Antitumor activity: Antitumor activity was evaluated through Potato disc bioassay (Coker *et al.,* 2003). Potatoes taken from local market were surface sterilized with 20% bleach solution. The experiments were performed in two groups, extract of four lichen species and positive control. Stock solution of 0.05 mg/mL in DMSO was further diluted with sterilized water to achieve 0.01mg/mL and 0.001mg/mL concentration. Same concentration of positive control (Vincrystien) was also prepared. Potato disc were made by cork borer and placed on 2% agar plates (6 disc/ plates). *Agrobacterium tumefacien* culture of 48 hours was transferred on the surface of each plate(50µl). Same concentration (0.05mg/mL, 0.01mg/mL and 0.001mg/mL) of the lichen extract and positive control were applied to each plats. The disc were then stain with lugols solutions (10% KI, 50% I) after 21 days of incubation at 28 °C. Number of tumor were counted under dissecting microscope and magnifying glass. Percentage inhibition was counted by following formula:

Percentage inhibition: 100 – No of tumor per sample/Number tumor per control X100.

Antioxidant activity: The antioxidant activity of investigated lichen genera were reported through DPPH (1, 1- diphenyl-2 picrylhydrazyl) assay. Lichen extract of concentration 1000ug/ml, 500ug/ml, 250ug/ml and 125ug/ml prepared in methanol and acetone were used in this assay. Ascorbic acid was used as standard. (Aliyu *et al.*,2012, Sharma and Kalikotay, 2012)

RESULTS

The area of Manshera was covered in the current study and sample of *Dermetacarpon*, *Flavopuntelia, Collema* were collected from Dader, *Flavoparmelia, Cladonia*and *Peltigera* from Manoor Valley, *Lecanora* from Lalosar and *Caloplaca* from Behisal. The altitude of each location is given in Table 1. The antibacterial activities of the lichen extract were measured by Disc diffusion assay. The extract of these ten lichen genera showed variable range of zone of inhibitions against tested bacteria (Table 2). The maximum rate of antibacterial activity was observed in acetone extract of *Flavoparmelia* against *E.coli* (12.66±0.577) while methanol and acetone extract of *Peltigera* have no activity against *E.coli, S.bacillus* and *S.aureus*. Similar result was also recorded for *Flavopuntelia*and *Collema* against *S. bacillus*. The result of antioxidant activity of methanol and acetone extract and control is show in Table 3 and 4.For methanol extract of concentration 1000ug/ml, the strongest (66%) DPPH radical scavenging activity was shown by the lichen genera *Cladonia*, followed by *Rhizocarpon* (63%) while in acetone maximum activity was *Rhizocarpon* (57%) and *Peltigera*(56%). Brine shrimp cytotoxicity is the easiest

and simple method for detection of bioactive compound. In the above study Brine shrimps lethality of the ten lichen extract were shown in (table 4). In acetone extract highest rate of mortality was observed in *Flavopuntelia* (60%) followed by *Flavoparmelia* (40%) and *Peltigera* (30%). For methanol extract highest mortality were recorded for Flavoparmelia (53%) followed by Flavopuntelia (50%) and Dermetacarpon, Peltigera, Usnea (30%). The result of antitumor activity of different concentration is shown in table (5). In acetone extract of concentration 0.05mg/ml, maximum (40%) antitumor activity was recorded for lichen genera *Flavoparmelia* followed by *Rhizocarpon* (53%), while in methanol extract maximum antitumor activity 47% and 45% for same genera respectively (table 6). Figure 1-4 represent the percentage mortality and tumor inhibition resulting from methanol extract and acetone extract,

Table 1: Distribution of Different Genera of Lichens collected from Mansehra District Khyber PakhtoonKhawa, Pakistan.

Sr. No.	Lichen genera	Altitude (m)	Localities
1	*Dermetacarpon*	1055	Dader
2	*Peltigera*	2580	Manoor valley
3	*Flavopuntelia*	1050	Dader
4	*Collema*	1030	Dader
5	*Flavoparmelia*	2540	Manoor valley
6	*Lecanora*	3334	Lalosar
7	*Caloplaca*	3283	Behisal
8	*Cladonia*	2560	Manoor valley
9	*Rhizocarpon*	3550	Lalosar
10	*Usnea*	2580	Manoor valley

Table 2: Antibacterial activity of different Lichen extracts

			Zone of inhibitions					
	Lichen Genera	**Extract**	***E.coli***	***S.epidermidis***	***S.bacillus***	***E. faecalis***	***P.aeroginosa***	***S. aureus***
1	***Dermetacarpon***	Acetone	10.33±o.577	8	8.00±1.00	8.33±0.577	8.33±0.577	8.33±0.577
		Methanol	0	7.00±0.23	0	9.00±0.577	7	7.66±0.577
2	***Peltigera***	Acetone	0	0	0	8.66±0.577	0	0
		Methanol	0	7	0	0	7	0
3	***Flavopuntelia***	Acetone	0	9	0	8.00±1.00	0	9.00±1.00
		Methanol	8.00±0.47	7.00±0.23	0	8	8.00±0.47	8.66±0.577
4	***Collema***	Acetone	0	0	0	0	8.66±0.577	8.66±o.577
		Methanol	0	7.00±0.23	0	10.66±0577	7.±0.23	8.00±0.47
5	***Flavoparmelia***	Acetone	12.66±0.577	12.33±1.15	9.66±0.577	10.33±0.577	11.33±0.577	11.33±0.577
		Methanol	12.33±0.577	7.00±0.23	10.33±0.577	10	12.33±0.577	9.66±0.577
6	***Lecanora***	Acetone	10	12.33±0.577	10.66±0.577	10	7	12.66±0.577
		Methanol	8.33±0.577	7.00±0.23	9.66±0.577	10.66±0.577	10	9
7	***Caloplaca***	Acetone	8.66±0.577	10	0	10.33±0.577	10.66±0.577	10
		Methanol	10.33±0.577	7.00±0.23	8.33±0.577	10.66±0.577	8	8.33±0.577
8	***Cladonia***	Acetone	9.66±0.577	8.33±0.577	8	12.66±0.577	9.66±0.577	8.33±0.577
		Methanol	0	7.00±0.23	0	10	0	0
9	***Rhizocarpon***	Acetone	7.66±0.577	11.33±0.577	10.66±0.577	9.33±0.577	7.33±0.577	8.66±0.577
		Methanol	12.33±0.577	7.00±0.23	10	0	10	7
10	***Usnea***	Acetone	8.33±0.577	7.66±0.577	8.33±0.577	7.66±0.577	8.33±0.577	8
		Methanol	8	7	9	10	10.66±0.577	7

Table 3: Antioxidant activity of Methanol extract of different Lichens extract

Methanol Extracts		**Percent radical scavenging activity**			
S. No	**Lichen species**	**1000 µg/ml**	**500 µg/ml**	**250 µg/ml**	**125 µg/ml**
1	*Dermetacarpon*	52	47	43	36
2	*Peltigera*	58	56	51	41
3	*Flavopuntelia*	29	26	22	20
4	*Collema*	37	31	30	25
5	*Flavoparmelia*	46	39	33	26
6	*Lecanora*	40	37	29	26
7	*Caloplaca*	17	12	0	0
8	*Cladonia*	66	54	44	40
9	*Rhizocarpon*	63	60	56	49
10	*Usnea*	33	31	26	24
	Ascorbic acid	79	72	65	61

Table 4: Antioxidant activity of Acetone extracts of different Lichens extract

Acetone extracts		Percent free radical scavenging activity			
S. No	**Lichen species**	**1000 µg/ml**	**500 µg/ml**	**250 µg/ml**	**125 µg/ml**
1	*Dermetacarpon*	48	46	37	33
2	*Peltigera*	56	55	51	38
3	*Flavopuntelia*	31	29	23	21
4	*Collema*	26	19	13	11
5	*Flavoparmelia*	51	48	43	41
6	*Lecanora*	37	36	30	28
7	*Caloplaca*	29	20	13	0
8	*Cladonia*	49	47	41	33
9	*Rhizocarpon*	57	55	46	44
10	*Usnea*	26	23	16	8
	Ascorbic acid	79	72	65	61

Table 5: Showing Cytotoxic activity acetone and methanol extract

		Acetone Extract concentration			Methanol extract concentration		
S.No	**Lichen genera**	**0.05**	**0.01**	**0.001**	**0.05**	**0.01**	**0.001**
1	*Dermetacarpon*	10%	10%	0%	30%	30%	20%
2	*Peltigera*	30%	20%	20%	30%	20%	20%
3	*Flavopuntelia*	60%	40%	30%	50%	40%	30%
4	*Collema*	0%	0%	0%	0%	0%	0%
5	*Flavoparmelia*	40%	30%	26%	53%	27%	13%
6	*Lecanora*	30%	10%	0%	10%	7%	0%
7	*Caloplaca*	4%	0%	0%	10%	0%	0%
8	*Cladonia*	7%	4%	0%	10%	7%	0%
9	*Rhizocarpon*	24%	13%	4%	27%	17%	7%
10	*Usnea*	17%	7%	0%	20%	3%	0%

Table 6: Showing Percentage of Tumor inhibition.

		Percentage of Tumor inhibition					
		Acetone extract concentration			Methanol extract concentration		
S. NO	**Lichen species**	**0.05**	**0.01**	**0.001**	**0.05**	**0.01**	**0.001**
1	*Dermetacarpon*	38%	33%	26%	28%	19%	10%
2	*Peltigera*	34%	23%	20%	30%	16%	8%
3	*Flavopuntelia*	36%	23%	20%	38%	30%	27%
4	*Collema*	16.66%	13%	12%	17%	10%	0%
5	*Flavoparmelia*	40%	38%	34%	37%	31%	15%
6	*Lecanora*	18%	13%	0%	0%	0%	0%
7	*Caloplaca*	0%	0%	0%	0%	0%	0%
8	*Cladonia*	17%	8%	0%	18%	10%	0%
9	*Rhizocarpon*	39%	33%	29%	37%	25%	13%
10	*Usnea*	29%	20%	11%	28%	20%	9%

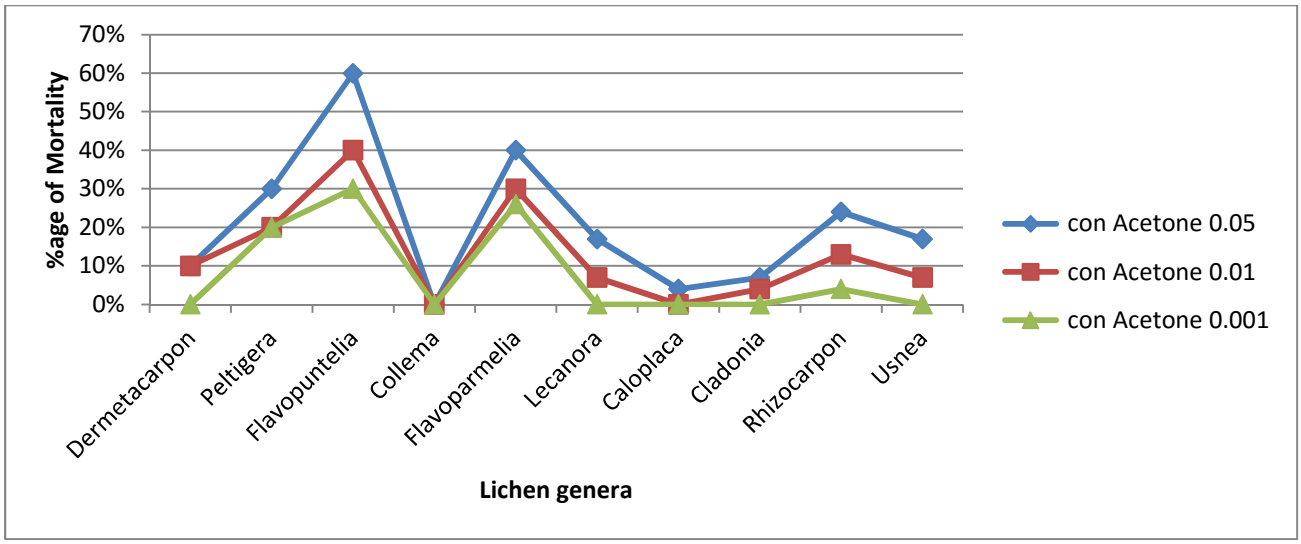

Fig: 1. Percentage mortality rate of Acetone extract

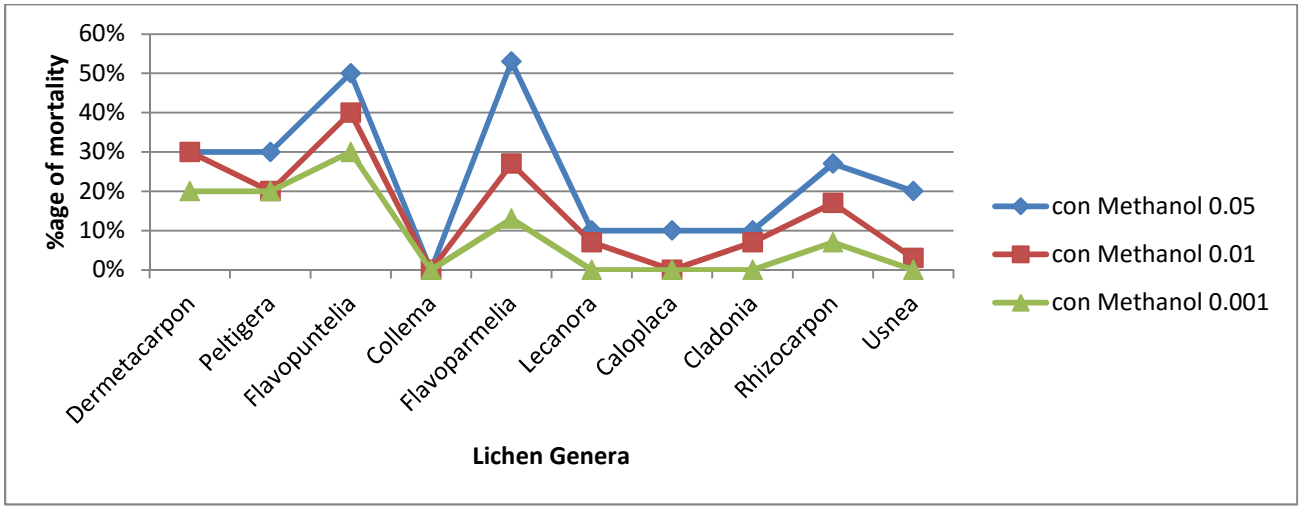

Fig: 2. Percentage of mortality rate of Methanol extract

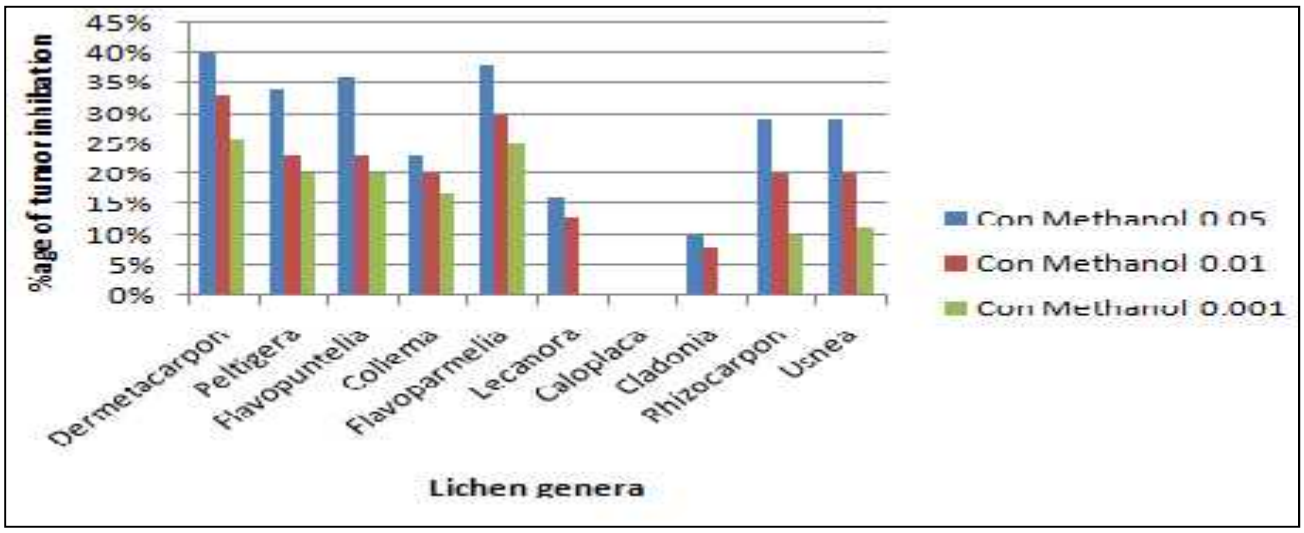

Fig: 3. Percentage of Tumor inhibition of methanol extract

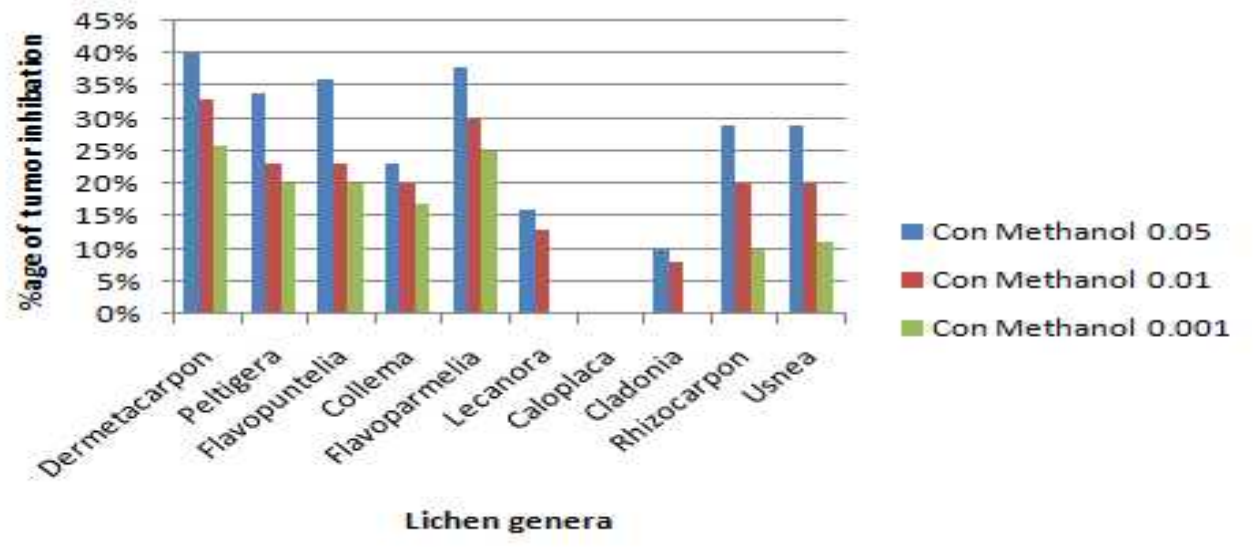

Fig: 4. Percentage of tumor inhibition of Acetone extract

DISCUSSION

Our study concluded that all ten genera of lichen had antibacterial activity against most of tested bacterial strains. *S.bacillus* was the most resistant and *S. epidermidis* was the most sensitive bacteria to most of the investigated lichen. According to Aydin and Kinalioglu (2013), lichen *Cetraria aculeate* showed the antimicrobial activity with extracts of acetone, diethyl ether and ethanol. All extracts were found active against bacterial strains of *E.coli, Staphylococcus aureus, Aeromonashydrophila, Proteus vulgaris, Streptococcus faecalis, Bacillus cereus, Bacillus subtilis, Pseudomonas aeruginosa, and Listeriamonocytogenes.*

In the present study, cytotoxcity assay indicated that both methanol and acetone. *Flavopuntelia* were potent against brine shrimp and there mortality rate were60% and 50% respectively. Babita *et al.*(2012) reported that methanolic extract of lichen *Heterodermia* sp. and *Ramalina*sp. show comparable toxic effect.

The present study also reported that the methanol extract of *Rhizocarpo*n have highest free radical scavenging activity in all of the investigated genera. Sharma and Kalikotay 2012 investigated the antioxidant activity of lichen *Parmotremareticulatum*and Usneasp. They observed 10-35% DPPH free radical scavenging for methanol extract of both lichen species.

In potato disc assay, most of the lichen extract show significant antitumor (inhibition ≥ 20%) inhibition activity against *Agrobacterium tumefacien.* Extract of *Flavoparmelia* show maximum (40%) tumor inhibition while *Collema*show minimum tumor imbibition.

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