ANTIOXIDANT STUDY OF USNIC ACID AND ITS DERIVATIVE USNIC ACID DIACETATE

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Abstract

Currently interest towards the study of antioxidant efficiency of the lichen metabolites was given prior attention in the field of research. As the free radical accumulation in human cells causes several chronic diseases which can be eliminated with the help of antioxidants. In our present study we have isolated the lichen secondary metabolite Usnic acid from Usnea luridorufa and also prepared the acetyl derivative of Usnic acid. The in vitro antioxidant activity of Usnic acid and Usnic acid diacetate under DPPH free radical scavenging, FRAP, Superoxide dismutase activity, Metal chelating activity, Phosphomolybdenum activity, Hydroxyl scavenging activity, Lipid peroxidation inhibiting activity were studied. The antioxidant potential of the Usnic acid and its derivative Usnic acid diacetate were compared. The IC_{50} value are also determined. Both the test compounds possesses significant antioxidant activity under the studies.

Keywords: *Antioxidant efficiency, Usnic acid, diacetate, Tannic acid.*

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1. INTRODUCTION

Lichens are in mutual relationship with algae and fungi both of them benefits each other by their association, here the fungal partner was responsible for the production of lichen secondary metabolites such as depsides, dibenzofurans, depsidones etc. The lichen secondary metabolites have been used as medicine traditionally, as they possesses antimicrobial, antitumor, antioxidant, anti-inflammatory, wound healing, antiviral, analgesic properties[1]. The frequent generation of free radicals and Reactive Oxygen Species(ROS) in body metabolism causes several degenerative diseases which includes tumor cell growth, inflammation, defects in cardiovascular system, enhances the aging process and triggers the neurological disorders[2,3]. In order to get rid of those ROS the necessity of antioxidants has to be considered but there is insufficiency in the body metabolism to acquire the antioxidants which leads to the search of external antioxidant resource. Many synthetic antioxidants have been used as supplementary to defend against ROS but are associated with several side effects. Searching of natural antioxidant resources have been very essential for the therapeutic supplements. In our present study, the antioxidant efficiency of usnic acid and its diacetyl derivative usnic acid diacetate were analyzed.

2. EXPERIMENTAL SECTION

2.1 Collection and Identification of Lichen Material

The lichen species *Usnea luridorufa* stirt. was collected from kodaikanal hills, Dindigul district, Tamil Nadu (altitude: 2268m). This lichen species was authenticated from Botanical Survey of India, Allahabad.

2.2 Isolation of Usnic Acid from Usnea Luridorufa

The fresh lichen material was air dried and powdered. A coarsely powdered lichen material (75g) was extracted with acetone (2l) under reflux condition for about 6hr. The extract was concentrated under vacuum using a rotatory evaporator which yields (8g), a brown residue.

The concentrated extract (8g) was fractioned on column chromatography. The benzene fractions yields 100mg of an yellowish crystalline solid which was found to be single in TLC (solvent system: Toluene : Acetic acid, 170:30). The compound was recrystallized from benzene, an yellow prismatic rods which melts at 202◦C. It gave a reddish brown colour with neutral ferric chloride and with conc. Sulphuric acid gave a deep yellow solution turning orange red on standing. It did not give any colour with bleaching power. The compound obtained was confirmed as Usnic acid (Fig: 1) with the help of Co-TLC with the authentic sample and spectral data.

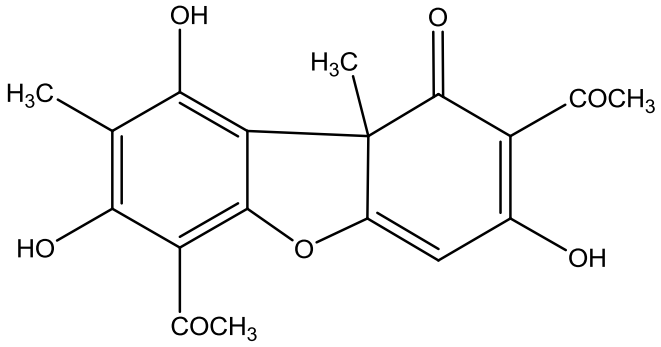

Fig: 1. Usnic acid

2.3 Preparation of Diacetate Derivative for Usnic Acid

The compound usnic acid (0.2g) was suspended in acetic anhydride (2ml) and a drop perchloric acid (60%) was added when the solid went into solution. It was kept undisturbed at room temperature for 2hr and then poured over crushed ice. The oily mass solidified a pale yellow solid. After keeping overnight, it was filtered and recrystallized from methyl alcohol. The pale yellow needles melts at 201◦C. The compound Usnic acid diacetate (Fig: 2) was confirmed with spectral data.

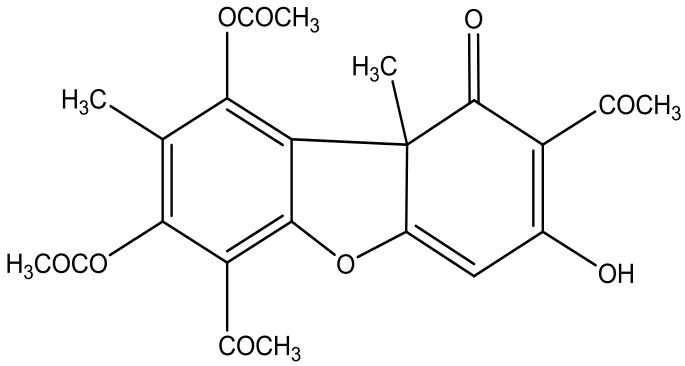

Fig: 2. Usnic acid diacetate

3. MATERIALS AND METHODS

3.1 Free Radical Scavenging Activity by DPPH*

The DPPH radical scavenging ability of Usnic acid and Usnic acid diacetate were determined based on the hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Blois (1958)[4]. The test compounds Usnic acid and Usnic acid diacetate were taken at different concentrations (100-500µg) and their volumes were adjusted to 100µl with methanol. 5mL of 0.1mM methanolic solution of DPPH* was added and allowed to stand for 20 min. at 27◦C. The absorbance of the Usnic acid and Usnic acid diacetate were measured at 517nm. The percentage of DPPH radical scavenging activity of the sample were calculated as follows:

% DPPH radical scavenging activity = (control OD – sample OD/control OD) X 100

The 50% inhibition (IC_{50}) of Usnic acid and Usnic acid diacetate under the DPPH radical scavenging assay condition was calculated from the graph inhibition concentration against sample concentration. The experiment was performed in triplicates.

3.2 Hydroxyl Radical Scavenging Activity

The Hydroxyl radical scavenging activity of Usnic acid and Usnic acid diacetate were measured in reference with Klein et al. (1991)[5]. The Usnic acid and Usnic acid diacetate were taken at different concentrations (100-500µg) 1mL of iron-EDTA solution (0.3% Ferrous ammonium sulfate and 0.26% EDTA solution (0.018%), and 1mL of dimethyl sulfoxide (0.85% V/V in 0.1M phosphate buffer, pH 7.4) are added to it. Using 0.5mL of ascorbic acid (022%) the reaction was initiated and was kept incubated at 80-90◦C for 15min. in a water bath. Later 1mL of ice-cold TCA (175.5% W/V) was added in order to terminate the reaction, then 3mL of Nash reagent (75.0g of ammonium acetate, 3mL of glacial acetic acid, and 2mL of acetyl acetone were mixed and raised to 1L with distilled water) was added and Kept at room temperature for about 15min. The colour intensity was measured spectroscopically at 412nm against the reagent blank. The % hydroxyl scavenging activity was calculated as follows:

% Hydroxyl scavenging activity = (control OD – sample OD/control OD) X 100

The 50% (IC_{50}) of Usnic acid and Usnic acid diacetate under the hydroxyl radical scavenging assay was calculated from the graph of inhibition percentage against sample concentration. The experiment was performed in triplicates.

3.3 Superoxide Radical Scavenging Activity

Superoxide radicals were formed by Beauchamp and Fridovich (1971)[6]. In this assay, the capacity of Usnic acid and Usnic acid diacetate can able to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system was measured. For this reaction a mixture of 50mM of Sodium phosphate buffer (pH 7.6), 20mg of riboflavin, 12mM of EDTA, 0.1mg NBT and Usnic acid and Usnic acid diacetate at different concentrations (100-500µg) were taken in 3mL. The reaction was started upon illumination of the reaction mixture with sample for 90 seconds. The absorbance were measured immediately at 590nm after illumination. The entire reaction assembly were enclosed in a box lined with aluminium foil. The reaction mixture without the sample kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated as follows:
% Superoxide radical scavenging activity = (control OD – sample OD/control OD) X 100

The 50% inhibition (IC_{50}) of Usnic acid and Usnic acid diacetate under Superoxide radical scavenging assay condition was calculated from the graph of inhibition percentage against sample concentration. The experiment was performed in triplicates.

3.4 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was used to estimate the reducing capacity of the Usnic acid and Usnic acid diacetate, by Benzie and Strain, 1996[7]. The FRAP reagent contained 2.5mL of a 10mM TPTZ solution in 40mM HCl, 2.5mL of 20mM $FeCl_3.6H_2O$ and 25mL of 300mM acetate buffer (pH 3.6). It was freashly prepared and warmed at 37◦C. 900µl FRAP reagent was mixed with 90µl water and 10µl of the sample. The reaction mixture was incubated at 37◦C for 30 minutes and the absorbance was measured at 593nm. The experiment was performed in triplicates.

3.5 Metal Chelating Activity

The Chelation of Ferrous ions by Usnic acid and Usnic acid diacetate were estimated by Dinis *et al.* (1994)[8]. 50µl of 1mM $FeCl_2$ was mixed with 1mL of the sample (250µg). 0.2mL of 5mM ferrozine solution was added to initiate the recation. The mixture was shaken vigorously and kept at room temperature for 10 min. The absorbance of the solution was measured at 562nm. The analysis was performed in triplicate and the results were expressed as EDTA equivalent. The experiment was performed in triplicates.

3.6 Phosphomolybdenum Reduction assay

The antioxidant activity of the Usnic acid and Usnic acid diacetate were evaluated by the phosphomolybdenum in reference with Prieto et al. (1999)[9]. In a 4mL vial, 1mL regent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) and an aliquiot of 0.1mL of sample were taken. The vials were capped and incubated in a water bath at 95◦C for 90 minutes. After cooling them to room temperature, the absorbance of the mixture were measured at 765nm against a blank. The results were expressed as milligrams of ascorbic acid equivalents per gram sample. The experiment was performed in triplicates.

3.7 Lipid Peroxidation Inhibiting Activity

The lipid peroxidation inhibition ability of the Usnic acid and Usnic acid diacetate were carried out using a procedure of Ohkawa et al. (1979)[10]. Goat lliver was washed thoroughly in cold phosphate buffer saline (pH 7.4) and homogenized to give a 10% homogenate. The homogenate was filtered and centrifuged at 10000 rpm for about 10 min. and the supernatant used to carry out the assay. To the 0.5mL of 10% homogenate, 0.5mL of the sample (50-250µg) was added followed by the addition of 0.05mL of 0.07M ferrous sulphate and the mixture was incubated at room temperature for 30 min. Then add 1.5mL of 20% acetic acid (pH 3.5) and 1.5mL of 0.8% TCA (in 1% SDS) to the incubated solution. The tubes were incubated at 100◦C for1hr and cooled to room temperature. To this 5mL of butanol was added and centrifuged at 3000 rpm for 10 min. The absorbance for the upper layer was measured at 532nm. The percentage inhibition was calculated as follows:

% Lipid peroxidation inhibition = (control OD – Sample OD/control OD) X 100

The 50% inhibition (IC_{50}) of Usnic acid and Usnic acid diacetate under Lipid peroxidation assay condition was calculated from the graph of inhibition percentage against sample concentration.

4. STATISTICAL ANALYSIS

All assays were carried out in triplicates and result are expressed as mean ± SD. Data were analyzed in Microsoft EXCEL-2010 by taking triplicates and thus mean and Standard Deviation (SD) obtained.

5. RESULTS AND DISCUSSION

Antioxidants can offer resistance against oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation, thus it provides prevention from many other diseases. In our present study, the antioxidant activity of the two test samples 1.Usnic acid and Usnic acid diacetate were analyzed.

5.1 DPPH Free Radical Scavenging Activity

1,1-Diphenyl-2-picrylhydrazl(DPPH), is one of the stable organic free radical which can facilitate the free radical scavenging assay of bioactive phyto-compounds. The DPPH free radical scavenging activity of the two test compounds Usnic acid and Usnic acid diacetate were determined at different concentration (100µl, 200µl, 300µl, 400µl and 500µl). The IC_{50} values of the test compounds were also determined. The results were given in the Table:1. The dose dependent activity of the two test compounds and the standard were expressed in Chart: 1 and Chart: 2. From the results, it has been found that both the test compounds shows higher % DPPH free radical scavenging activity as the concentration increases and also the Diacetate derivative of Usnic acid shows better scavenging activity than Usnic acid. Eventhough the two test compounds shows lesser activity as that of the standard tannic acid.

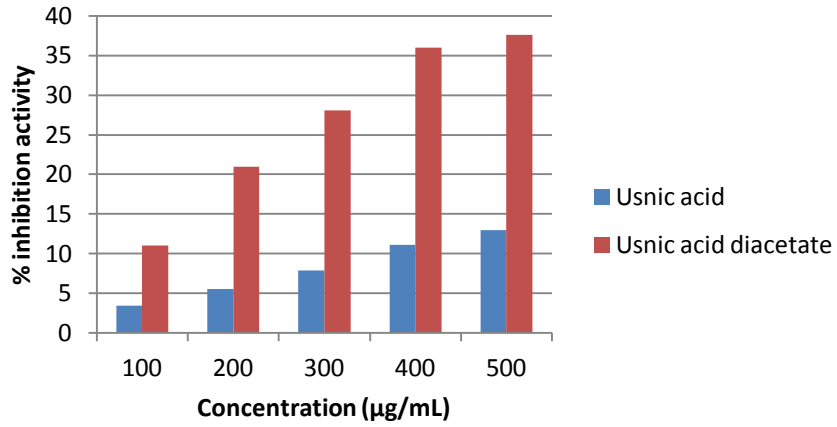

Chart: 1-DPPH Free Radical Scavenging Activity of Usnic acid and Usnic acid diacetate

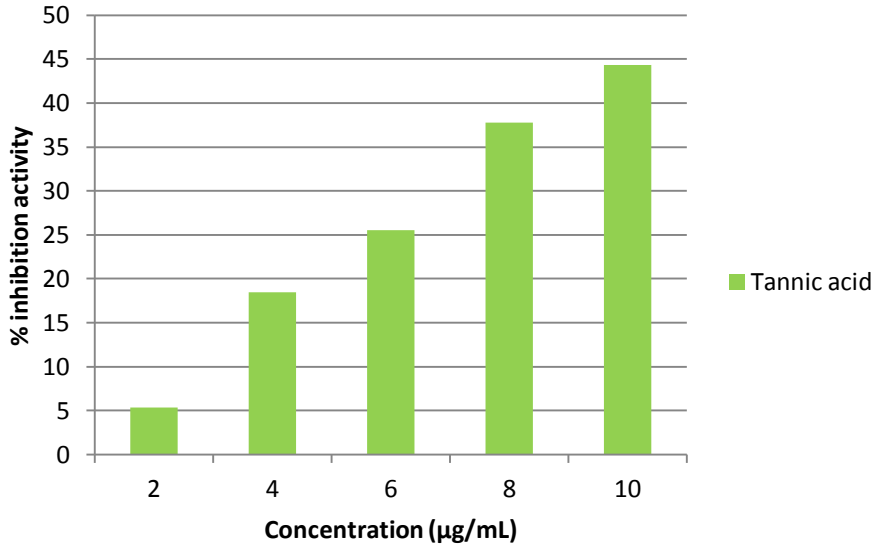

Chart: 2-DPPH Free Radical Scavenging activity of Standard Tannic acid

Table: 1-DPPH Free Radical Scavenging Assay

Test compound	**Concentration (µg)**	**% Activity**	**IC_{50} (µg/ml)**
Usnic acid	100 200 300 400 500	3.43±0.26 5.56±0.50 7.92±0.15 11.09±0.71 12.99±0.45	691.36±31.10
Usnic acid diacetate	100 200 300 400 500	11.02±1.08 20.94±0.94 28.07±2.76 35.98±1.92 37.62±2.33	215±14.12
Tannic acid	2 4 6 8 10	5.31±0.54 18.45±0.17 25.50±0.38 37.78±0.38 44.31±0.22	4.31±0.02

5.2 Hydroxyl Radical Scavenging Activity

Hydroxyl radical was formed from the various biological mechanism can damage protein, facilitates lipid peroxidation, affects the DNA sequences. The Hydroxyl radical scavenging activity of the two test compounds Usnic acid and Usnic acid diacetate were determined at different concentration (100µl, 200µl, 300µl, 400µl and 500µl). The IC_{50} values of the test compounds were also determined. The results were given in the Table: 2. The dose dependent activity of the two test compounds and the standard were expressed in Chart: 3 and Chart: 4. From the result, it has been revealed that the % inhibition activity of two test compounds shows higher activity as increase in their concentration and also the usnic acid has better efficiency than Usnic acid diacetate in scavenging the Hydroxyl radicals. But, the two test compounds shows only lesser activity when compared to the standard Tannic acid.

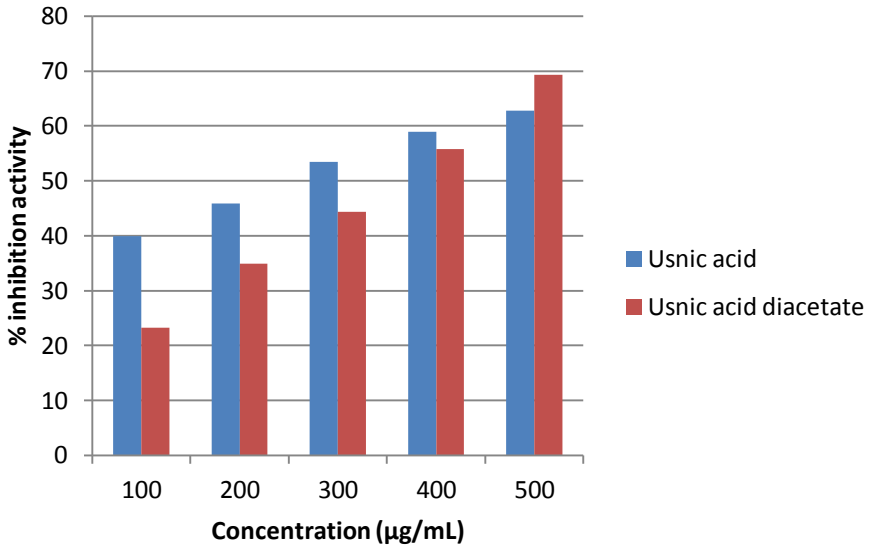

Chart: 3-Hydroxyl Radical Scavenging Activity of the Usnic acid and Usnic acid diacetate

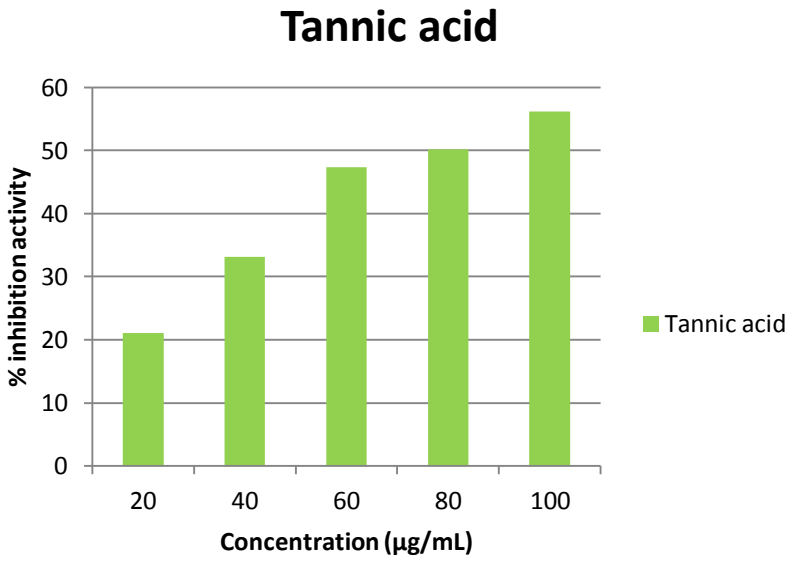

Chart: 4-Hydroxyl Radical Scavenging Activity of the standard Tannic acid

Table: 2-Hydroxyl Radical Scavenging Assay

Test compound	Concentration(µg)	% Activity	IC_{50} (µg/ml)
Usnic acid	100 200 300 400 500	39.95±0.52 45.87±0.46 53.44±0.47 58.97±0.47 62.73±0.54	119.10±0.45
Usnic acid diacetate	100 200 300 400 500	23.22±0.57 34.89±0.52 44.31±0.20 55.77±0.79 69.30±0.49	125.98±1.01
Tannic acid	20 40 60 80 100	21.06±0.52 33.18±0.00 47.35±0.52 50.15±0.92 56.14±0.79	21.51±0.19

5.3 Superoxide Dismutase Activity

The superoxide anion radical is a most potent reactive oxygen species which causes harmful effects on the biological defence mechanism. The Superoxide radical scavenging activity of the two test compounds Usnic acid and Usnic acid diacetate were determined at different concentration (100µl, 200µl, 300µl, 400µl and 500µl). The IC_{50} values of the test compounds were also determined. The results were given in the Table: 3. The dose dependent activity of the two test compounds and the standard were expressed in Chart: 5 and Chart: 6. From the result, it was found that Usnic acid shows higher % inhibition activity as the concentration increases. But the Usnic acid diacetate shows very lesser activity in scavenging the superoxide radicals. Also in comparison with the standard Tannic acid, both the test compounds shows lesser activity.

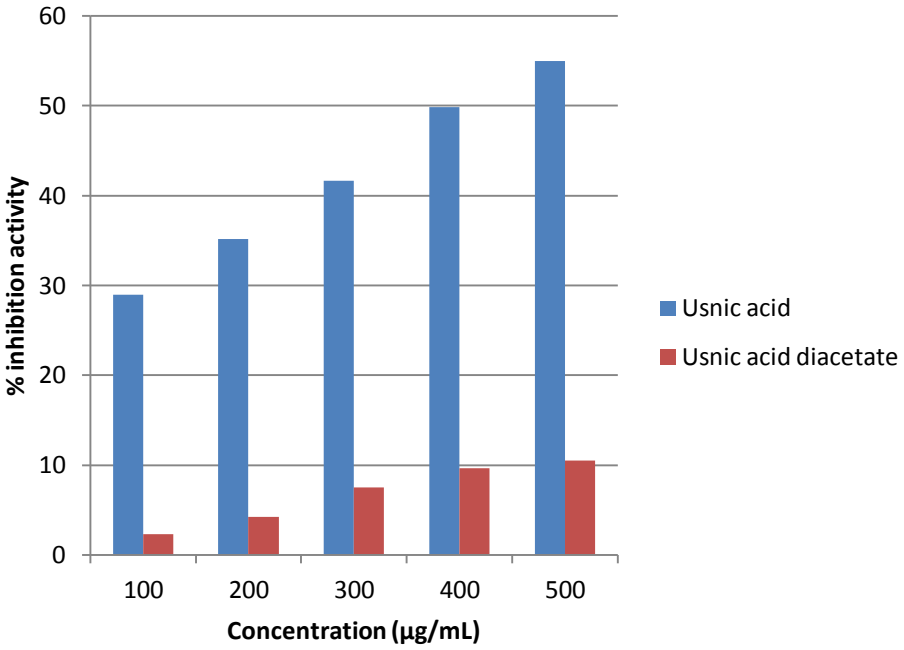

Chart: 5-Superoxide Radical Scavenging Activity of the Usnic acid and Usnic acid diacetate

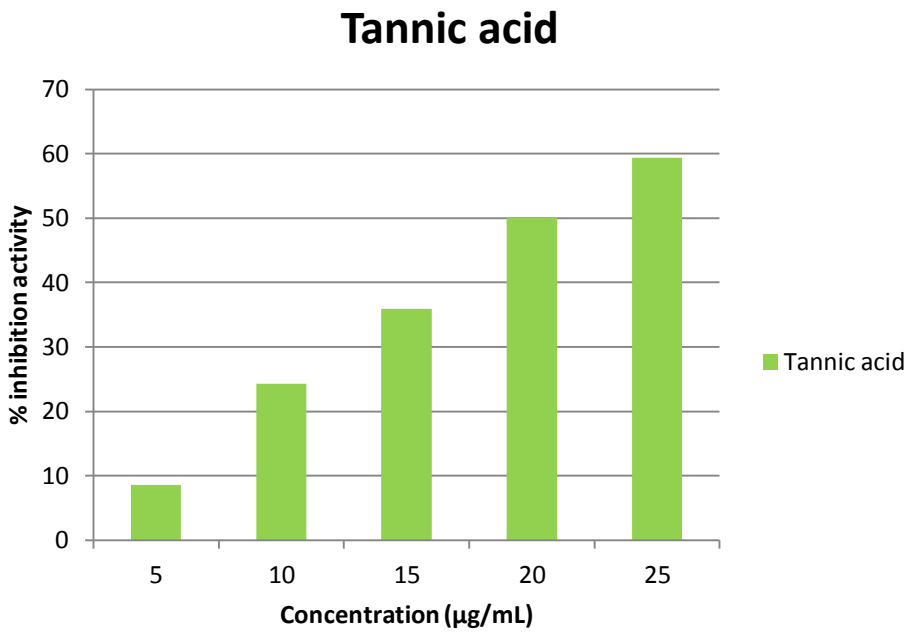

Chart: 6-Superoxide Radical Scavenging Activity of the standard Tannic acid

Table: 3-Superoxide Radical Scavenging Activity

Test compound	**Concentration(µg)**	**% Activity**	**IC_{50} (µg/ml)**
Usnic acid	50 100 150 200 250	6.21±0.81 9.75±1.11 17.02±1.06 25.18±0.18 32.45±0.53	44.57±1.06
Usnic acid diacetate	50 100 200 150 250	7.45±0.92 12.41±1.34 18.26±0.81 23.23±1.11 29.26±1.06	46.58±1.03
Tannic acid	50 100 150 200 250	46.77±0.78 57.14±1.35 71.43±2.23 87.41±2.06 94.56±1.06	13.37±0.21

5.4 Lipid Peroxidation Inhibiting Activity

Lipid peroxidation was toxicological process, responsible for the excessive production of variety of reactive oxygen species, which in turn causes modification of lipoprotein, DNA sequences, proteins. The Lipid peroxidation inhibiting activity of the two test compounds Usnic acid and Usnic acid diacetate were determined at different concentration (100µl, 200µl, 300µl, 400µl and 500µl). The IC_{50} values of the test compounds were also determined. The results were given in the Table: 4. The dose dependent activity of the two test compounds and the standard were expressed in Chart: 7. From the result, it has been identified that the lipid peroxidation inhibiting activity of the two test compounds have shown significant activity at higher concentration but shown lesser activity than the standard Tannic acid.

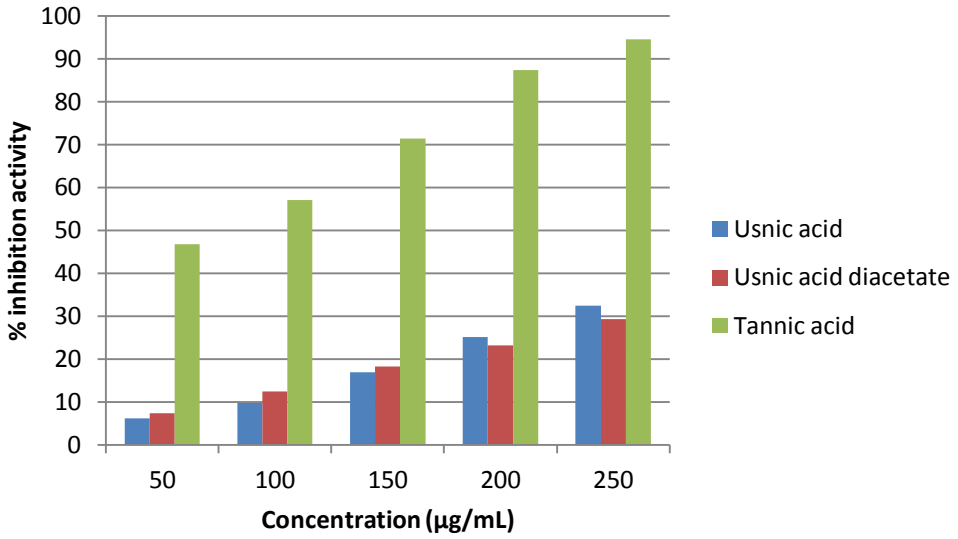

Chart: 7-Lipid Peroxidation Inhibiting Activity of Usnic acid, Usnic acid diacetate and Tannic acid

Table: 4-Lipid Peroxidation Inhibiting Activity

Test compound	**Concentration (µg)**	**% Activity**	**IC_{50} (µg/ml)**
Usnic acid	100 200 300 400 500	28.94±0.46 35.13±0.73 41.63±0.73 49.85±0.90 54.94±0.66	121.46±0.56
Usnic acid diacetate	100 200 300 400 500	2.33±0.33 4.26±0.62 7.55±0.65 9.66±0.20 10.50±0.20	689.17±17.55
Tannic acid	5 10 15 20 25	8.54±0.36 24.24±0.23 35.90±0.23 50.04±0.49 59.36±0.59	6.40±0.05

Table: 5-IC_{50} value of the test compounds and the standard

Method of antioxidant assay	**Usnic acid IC_{50} Value (µg/mL)**	**Usnic acid diacetate IC_{50} Value (µg/mL)**	**Standard Tannic acid IC_{50} Value (µg/mL)**
DPPH Radical Scavenging	691.36±31.10	215.36±14.12	4.31±0.02
Superoxide Dismutase activity	121.46±0.56	689.17±17.55	6.40±0.05
Hydroxyl radical Scavenging	119.10±0.45	125.98±1.01	21. 51±0.91
Lipid Peroxidation	44.57±1.06	46.58±1.03	13.37±0.21

5.5 Phosphomolydenum Assay

The Phosphomolybdenum assay of the two test compounds and standard were given in the Table: 6. The total antioxidant activities of the test compounds and the standard were shown in the Chart: 8. From the Result, it has been established that the Usnic acid diacetate shows higher total antioxidant activity than the standard Tannic acid. But Usnic acid shows comparatively lesser total antioxidant activity than Usnic acid and Tannic acid.

Table:6-Phosphomolybdenum assay

Sample	**Phosphomolydenum (mg ascorbic acid eq./g sample)**
Usnic acid	59.36±3.96
Usnic acid diacetate	383.38±1.92
Tannic acid	187.21±80.27

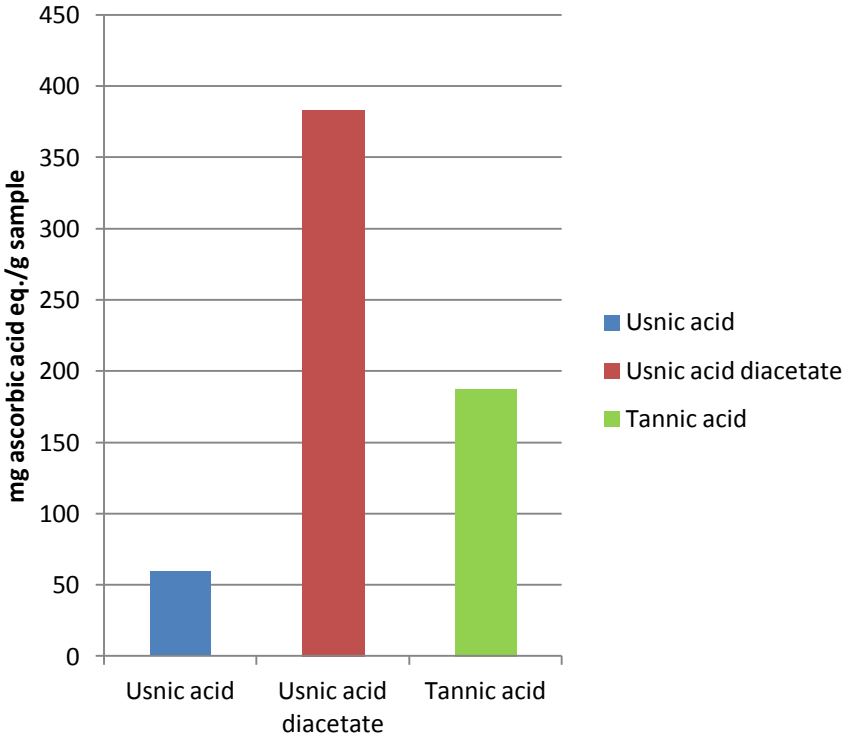

Chart: 8-Phophomolybdenum Assay of Usnic acid, Usnic acid diacetate and Tannic acid

5.6 FRAP Assay

The FRAP assay of the two test compounds and standard were given in the Table: 7. The Ferric reducing antioxidant activities of the test compounds and the standard were shown in the Chart: 9. From the results, Usnic acid diacetate shows higher activity than Usnic acid. But both the test compounds are less active than Tannic acid in reducing Ferric ions.

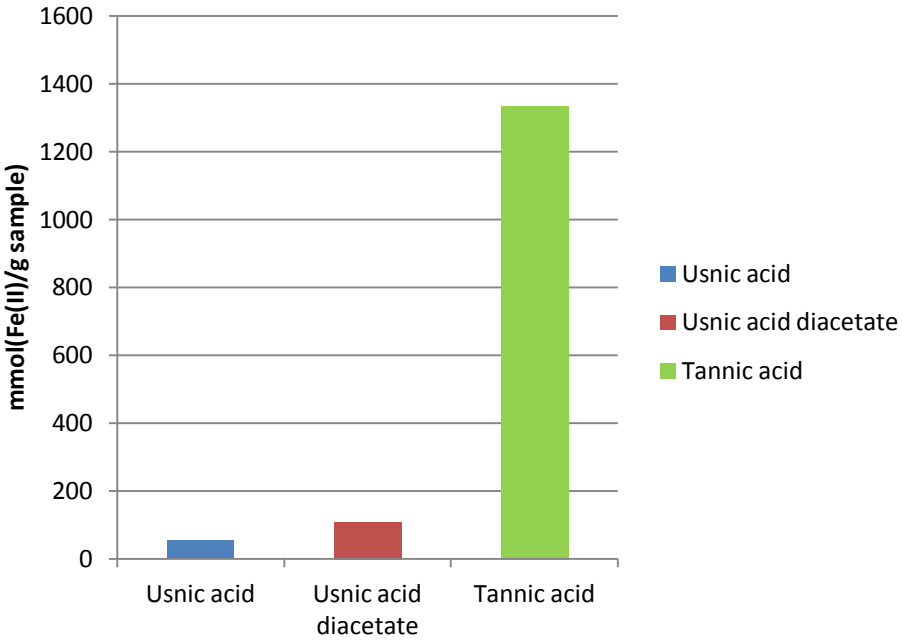

Chart: 9-FRAP assay of Usnic acid, Usnic acid diacetate and Tannic acid

Table: 7-FRAP Assay

Sample	**FRAP mmol(Fe(II)/g) sample**
Usnic acid	54.89±5.07
Usnic acid diacetate	108.42±6.94
Tannic acid	1335.00±160.09

5.7 Metal Chelating Activity

Fe^{2+} ions have the tendency to trigger free radical generation to some extent, it would be beneficial to chelate such Fe^{2+} ions. The Metal chelating activity of the two test compounds and standard were given in the Table: 8. The metal chelating activities of the test compounds and the standard were shown in the Chart: 10. From the results, it has been found that both Usnic acid and Usnic acid diacetate shows nearly similar metal chelating activity and also very less active than the standard Tannic acid.

Table: 8-Metal Chelating Activity

Sample	**Metal chelating activity (mg EDTA eq./g sample)**
Usnic acid	12.35±0.08
Usnic acid diacetate	10.46±0.13
Tannic acid	126.85±4.04

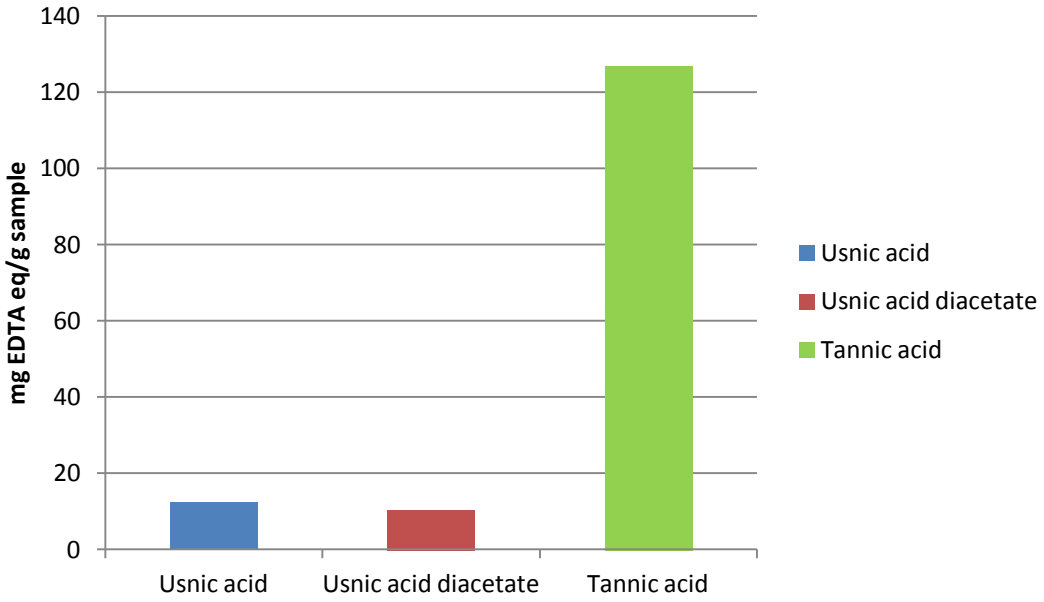

Chart: 10-Metal Chelating Activity of Usnic acid, Usnic acid diacetate and Tannic acid

6. CONCLUSION

In the present study, it is concluded that the lichen secondary metabolite Usnic acid and its derivative Usnic acid diacetate have possesses efficient antioxidant properties and are reported that they are dose dependent. But among the two test compounds Usnic acid gives lower IC_{50} value for Hydroxyl scavenging activity, Superoxide dismutase scavenging activity and shows better activity than Usnic acid diacetate, for DPPH radical scavenging while Usnic acid diacetate gives lower IC_{50} value and possesses better activity than Usnic acid for lipid peroxidation inhibiting activity both the test compounds shows lesser activity. It was known that lower the IC_{50} value higher the inhibition activity. Also Usnic acid diacetate possesses higher total antioxidant activity via Phosphomolydenum assay than Usnic acid and Tannic acid. In FRAP assay Usnic acid dicetate shows higher activity than Usnic acid. On the other hand metal chelating activity of both Usnic acid and Usnic acid diacetate was found to be very less. From the result it was found that both the two test compounds shows lesser activity than the standard tannic acid for DPPH, Hydroxyl scavenging activity, Superoxide dismutase scavenging activity , lipid peroxidation inhibiting activity, Metal chelating activity and FRAP asaay. Since, our test compounds are obtained naturally and are free from side effects. Thus, both the test compounds can found to be a good primary antioxidant.

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