IN VITRO ANTIOXIDANT ACTIVITIES OF SALAZINIC ACID AND ITS DERIVATIVE HEXAACETYL SALAZINIC ACID

G. Selvaraj[1], A. Tinabaye[2], R. Ananthi[3]

[1]*Research scholar,Department of Chemistry, Kanchi Mamunivar Centre for Post Graduate Studies, Lawspet, Puducherry, India.*

[2]*Associate professor,Department of Chemistry, Kanchi Mamunivar Centre for Post Graduate Studies, Lawspet, Puducherry, India.*

[3]*Research scholar,Department of Chemistry, Kanchi Mamunivar Centre for Post Graduate Studies, Lawspet, Puducherry, India.*

Abstract

The stress generated upon oxidation can lead to the production of free radicals which may cause many degenerative diseases. In order to eliminate the free radicals antioxidants have been used. The present study was to estimate the antioxidant activity of salazinic acid and its derivative hexaacetyl salazinic acid. The antioxidant property were studied under the categories DPPH, FRAP, Metal chelating activity, Hydroxyl scavenging activity, Lipid peroxidation activity, Phosphomolybdenum activity, Superoxide dismutase activity. We have isolated salazinic acid from the lichen Parmotrema reticulatum collected from Kodaikanal hills. The Derivative hexaacetyl salazinic acid was also prepared. Both the compounds were subjected to determine the in vitro antioxidant activity and their IC_{50} value are also calculated. The Lichen metabolite and its derivative have shown better antioxidant efficiency.

***Keywords**: Oxidative stress, Salazinic acid, Hexaacetyl salazinic acid, Tannic acid.*

--***--

1. INTRODUCTION

Lichens are Unique symbiotic association of algae and fungi can produce a bioactive compounds such as depsides, depsidones, dibenzofurans having antifungal, anticancerous, antinflammatory activities as an immunomodulator[1]. The body metabolic processes can triggers the production of several free radicals such as OH^-, O^{2-}, H_2O_2 radicals which can cause damage to the cell growth, can able to arise several health disorders such as cancer, neurological ailments, arthritis and so on. The antioxidants are very essential to eliminate the effective action of free radicals and protect us from various degenerative diseases[2,3]. Recently, more importance was given for the natural antioxidants. In this we have studied the antioxidant potential of Salazinic acid and Hexaacetyl Salazinic acid.

2. MATERIAL AND METHODS

2.1 Collection and Identification of Lichen Material

The lichen *Parmotrema reticulatum* (Taylor) M. Choisy was collected from Kodaikanal hills, 7000ft, Dindigul district in Tamil Nadu (South India). It was botanically identified by Dr. K. P. Singh, Botanical Survey of India, Allahabad.

2.2 Botanical Information

Phylum : Lichens
Class : Lecanoromycetes
Order : Lecanorales
Family : Parmeliacea
Genus : Parmotrema
Species : Parmotrema reticulatum
Habit : Foliose type

2.3 Preparation of Lichens Extracts

2.3.1 Acetone Extraction:

The air-dried lichen (125g) was extracted with hot acetone (1L, each time) till the last extract was almost colourless (four extraction). The combined acetone extract was concentrated under vacuum using a rotatory evaporator which yields a pale yellow pasty mass (5g). This was found to be heterogeneous by TLC examinations. The pasty mass was subjected to separation by column chromatography with silica gel (Merck) built in benzene. The fraction benzene-ethyl acetate (40:60) yield colourless solid.

2.3.2 Identification of Lichen Acids:

To identify the lichen metabolite via TLC. The compound were then spotted on thin layer chromatography (TLC) plates (silica gel, Merck) and run in solvent system (170:30 toulene/glacial acetic acid). Subsequently, each TLC plate was then sprayed with 10% sulfuric acid and heated at 110^0C for 10 minutes to visualize the lichen compound.

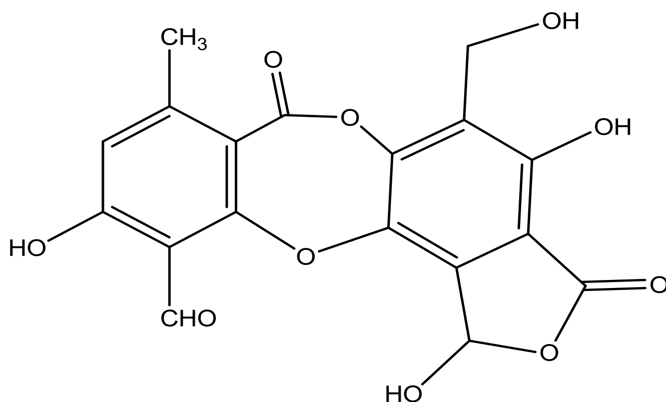

Fig:1. Salazinic acid

Salazinic acid (Fig.1) obtained as colourless needles, m.pt. 260-268^{0}C (with decomposition) with change in colour to brown at 240^0. It forms red colour solution with $FeCl_3$. It gives red crystals of potassium salt when added to hot potassium carbonate. It gives red colour with KOH. The molecular formula $C_{18}H_{12}O_{10}$. The structure was confirmed with spectral data.

2.3.3 Acetylation of Salazinic Acid:

A compound (0.1g) was suspended in acetic anhydride (1ml) and perchloric acid (60% 1drop) were added. The mixture was kept at room temperature for 2 hours and then poured into ice-water. The acetate soon solidified to a colorless crystalline solid. It was filtered off and crystallized from ethyl alcohol when it separated as long colorless prismatic rods. On heating it lost the solvent of crystallization at 150^{0}C and then melted at 177-178^{0}C. The compound hexaacetyl salazinic acid(Fig.2) was confirmed with spectral data. The molecular formula of hexaacetyl salazinic acid is $C_{30}H_{26}O_{17}$.

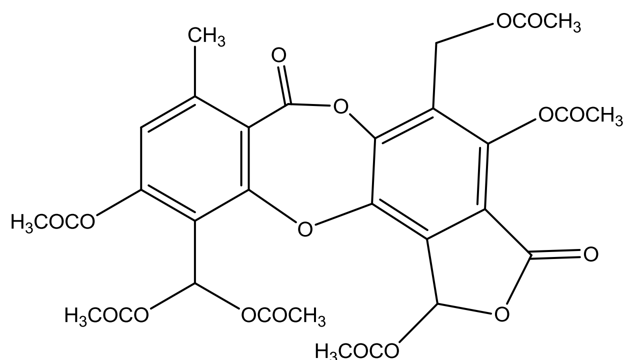

Fig:2 Hexaacetyl salazinic acid

3. MATERIALS AND METHODS

3.1 DPPH Radical Scavenging Assay

The antioxidant ability of a compound is depends upon its hydrogen donating or radical scavenging ability by using the stable radical DPPH. According to the method of Blois (1958)[4]. In this method, the volume was adjusted to 100µl with methanol and sample at various concentration (100-500µg). The standard (0.1mM concentration methanolic solution of DPPH was allowed to stand for 20min at 27^{0}C. The sample absorbance were measured at 517nm. The % of radical scavenging activity of the sample can be determined by using:

% DPPH radical scavenging activity = (Control OD-Sample OD/ control OD)x100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

3.2 Hydroxyl Radical Scavenging Activity:

The method of Hydroxyl radical scavenging activity of the sample was put-forward by Klein *et al.* (1991)[5]. In this method, sample taken at different concentrations (100-500µg) were added with 1mL of Fe-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5ml of EDTA solution (0.018%), and 1mL of dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4).The reaction was initiated by adding 0.5mL of ascorbic acid (0.22%) and incubated at 80-90^{0}C for 15min in a water bath. After the period of incubation by the addition of 1mL of ice cold TCA. The reaction was terminated. 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 left at room temperature for 15min. the intensity of the colour should be measured at 412nm against reagent blank. The % radical scavenging activity of the sample were calculated as follows:

% Hydroxyl radical scavenging activity = (Control OD-Sample OD/ Control OD) x 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration

3.3 Superoxide Radical Scavenging Activity:

The superoxide radical scavenging activity of the sample was determined in terms of Beauchamp and Fridovich 1971[6] to inhibit formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3mL reaction mixture contained 50mM sodium phosphate buffer (pH 7.6), 20mg riboflavin, 12mM EDTA, 0.1mg NBT and various concentrations (100-500µg) of sample. The absorbance was measured at 590nm. Immediately after illumination of reaction mixture with sample extract 90 seconds. The entire reaction should be carried out in box timed with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated as:

% Superoxide radical scavenging activity = (Control OD-Sample OD/ Control OD) x 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

3.4 Ferric Reducing Antioxidant Power (FRAP) Assay:

The reducing capacity of the sample was estimated by using FRAP assay 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 freshly prepared and warmed at 37^oC. 900µL FRAP reagent was mixed with 90µl water and 10µl of the sample. The reaction mixture was incubated at 37^0C for 30 minutes and the absorbance was measured at 593nm.

3.5 Metal Chelating Assay

Ability of sample to form chelation with Fe^{+2} ions was estimated by the method Dinis *et al*. (1994)[8]. Briefly, 50µl of 2mM $FeCl_2$ was added to 1 mL of the sample (250µg). The reaction was initiated by the addition of 0.2mL of 5mM ferrozine solution. The mixture was shaken thoroughly and allowed to stand at room temperature for 10min. The absorbance of the solution was measured spectroscopically at 562nm. The analysis was performed in triplicate and the results were expressed as EDTA equivalent.

3.6 Phosphomolybdenum Assay:

For phosphomolybdenum assay a modified procedure by Prieto *et al*.(1999)[9]. The antioxidant activity of the sample can be evaluated. An aliquot of 0.1mL of sample solution was combined in a 4mL vial with 1mL of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The vials were capped and incubated in a water bath at 95^0C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 765nm against a blank. The result reported are mean values expressed as milligrams of ascorbic acid equivalents per gram sample.

3.7 Lipid Peroxidation Inhibiting Assay:

The inhibition ability of the sample against lipid peroxidation was carried out by using modified procedure of Ohkawa *et al*. (1979)[10]. According to the method, the goat liver was washed vigorously in cold phosphate buffer saline (pH 7.4) and homogenized to form 10% homogenate. Then filter the homogenate and centrifuged at 1000rpm for 10 min and the supernatant used to carry out the assay. To 0.5mL of 10% homogenate, 0.5mL of the sample (50-250µg) was added. To this, 0.05 mL of 0.07 M ferrous sulphate was added and incubated at room temperature for 30 min. To the incubated solution, 1.5 mL of 20% acetic acid (pH 3.5) and 1.5mL of 0.8% TCA (in 1% SDS) were added. The tubes were incubated at 100^0C for 1 hour and cooled to room temperature. About 5mL of butanol was added and centrifuged at 3000 rpm for 10min. The upper layer was used to read the absorbance at 532nm. The percentage inhibition was calculated as follows:

% inhibition = [(Control OD-Sample OD)/Control OD] x 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the 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. RESULT AND DISCUSSION

Antioxidant capacity is widely used as a parameter for medicinal bioactive components. Radical scavenging activities are very important due to the deleterious role of free radicals in food and in biological systems. Diverse method are currently used to assess the antioxidant activity of Natural bioactive compounds. In our study, we have analyzed the antioxidant activity of Salazinic acid and hexaaceytyl salazinic acid.

5.1 Free Radical Scavenging by DPPH:

For the assessment of free radical scavenging activity of photomedicine stable DPPH radical has been used. The DPPH radical scavenging activity of the two test compounds salazinic acid and its derivatives hexaacetyl salazinic acid were evaluated at different concentration (100-500µg/mL). The result 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. The % inhibition activity of the test compound were also determined and are compared with the standard Tannic acid. However, the result shows lesser % inhibition than the standard tannic acid. The test compound shows significant DPPH radical scavenging activity because the test compounds are naturally obtained and are free from side effect. It is established that the derivative hexaacetyl salazinic acid shows higher % inhibition and lower IC_{50} value than salazinic acid.

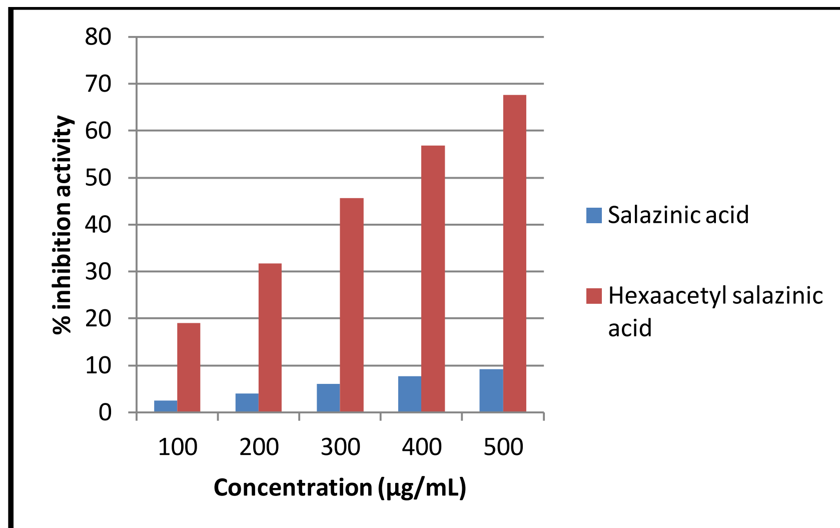

Chart: 1-DPPH Free Radical Seavenging Activity of Salazinic acid and Hexaacetyl salazinc acid

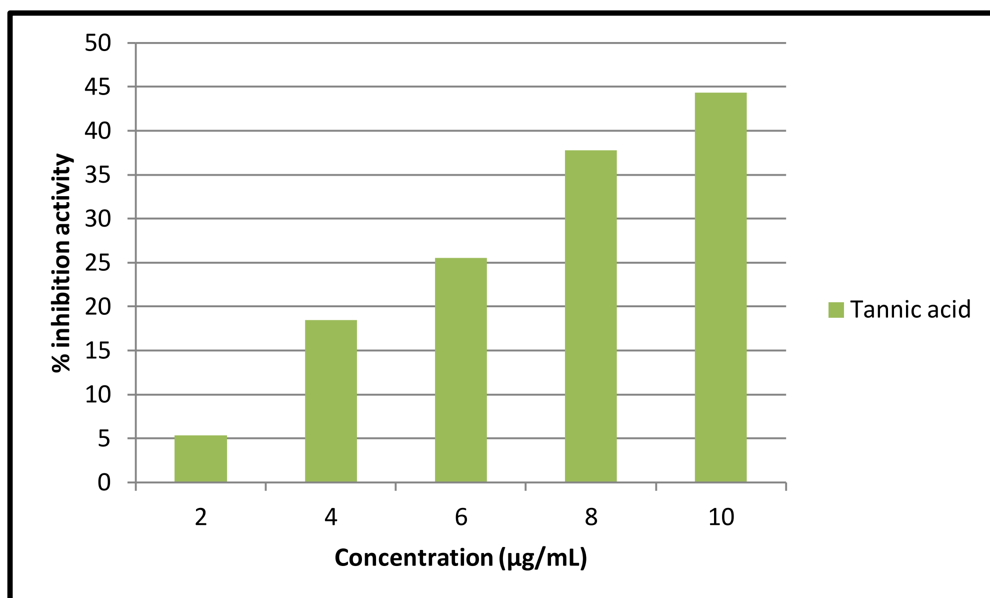

Chart: 2-DPPH Free Radical Scavenging Activity of Tannic acid

Table: 1-DPPH Free Radical Scavenging Activity

Test compound	Concentratio n(µg/mL)	% Activity	IC_{50} (µg/ml)
Salazinic acid	100 200 300 400 500	2.45±0.20 3.99±0.20 6.08±0.10 7.72±0.20 9.22±0.20	974.66±30.69
Hexaacetyl salazinic acid	100 200 300 400 500	19.04±1.13 31.73±2.17 45.70±1.31 56.89±1.03 67.58±0.69	127.78±2.76
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:

Among all the reactive oxygen species, the hydroxyl radical was the major active oxygen species which causes lipid peroxidation and several biological disorders. The Hydroxyl radical scavenging activity of the two test compounds salazinic acid and hexaacetyl salazinic acid were determined at different concentration (100-500μg/mL). 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 results, it has been found that both the test compounds shows nearly similar hydroxyl radical scavenging activity and also possess lesser activity than the standard Tannic acid.

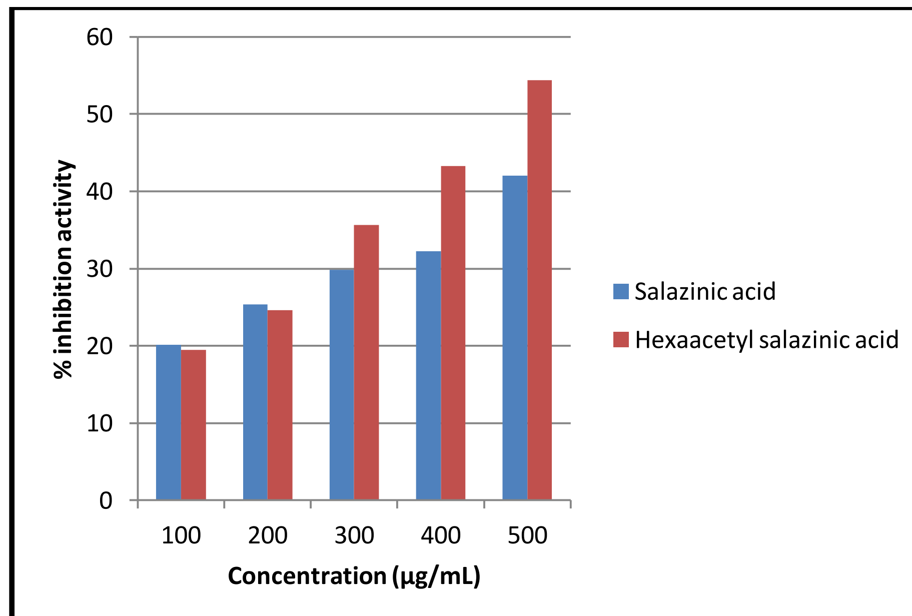

Chart: 3-Hydroxyl Radical Scavenging Activity of Salazinic acid and Hexaacetyl salazinic acid

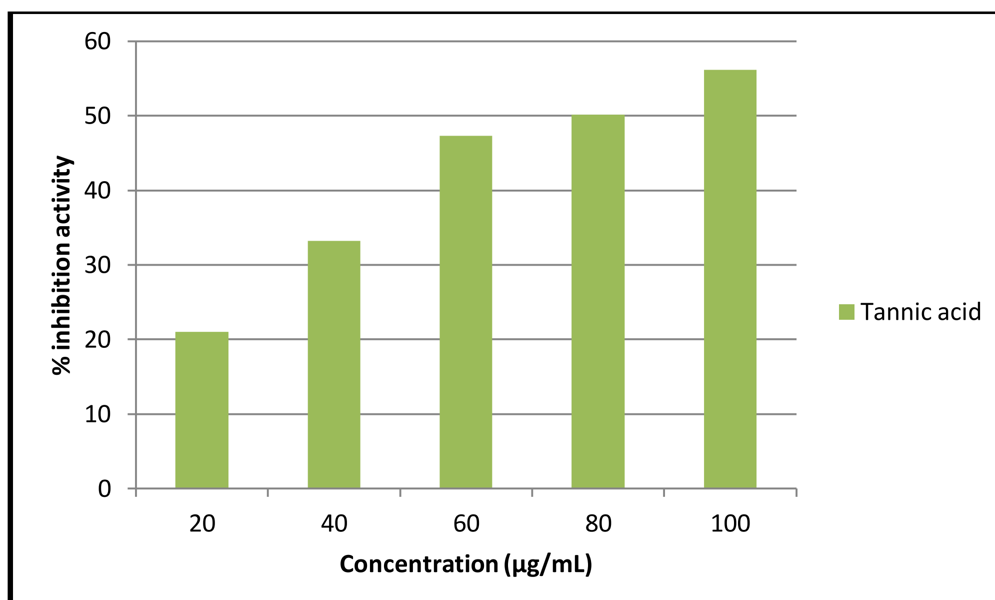

Chart: 4-Hydroxyl Radical Scavenging of Tannic acid

Table: 2-Hydroxyl Radical Scavenging Activity

Test compound	Concentration (μg/mL)	% Activity	IC_{50} (μg/ml)
Salazinic acid	100 200 300 400 500	20.15±0.07 25.38±0.30 29.83±0.39 37.27±0.52 42.02±0.65	193.36±0.12
Hexaacetyl salazinic acid	100 200 300 400 500	19.46±0.47 24.60±0.61 35.62±1.10 43.28±0.15 54.39±0.40	161.87±1.67
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 Radical Scavenging Activity

Superoxide radical plays a pivotal role in the pathogenesis of diseases such as atherosclerosis, neurodegenerative disease, rheumatoid arthritis, age-related degeneration and cancer initiation. These effects of free radicals are through their action on proteins, lipids and DNA. Cells have the innate antioxidant defense system which protects against the dreadful action of free radicals. The superoxide radical scavenging activity of the two test compounds salazinic acid and hexaacetyl salazinic acid were determined at different concentration (100-500μg/mL). The IC_{50} values of the test compounds were also determined. The result 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 has been found that the SOD activity of the test samples were dose dependent as the concentration increases the % inhibition activity also increases and their IC_{50} values evaluated. IC_{50} values for the derivative hexaacetyl salazinic acid was less than that of salazinic acid, therefore the derivative of salazinic acid shows higher % inhibition activity than salazinic acid.

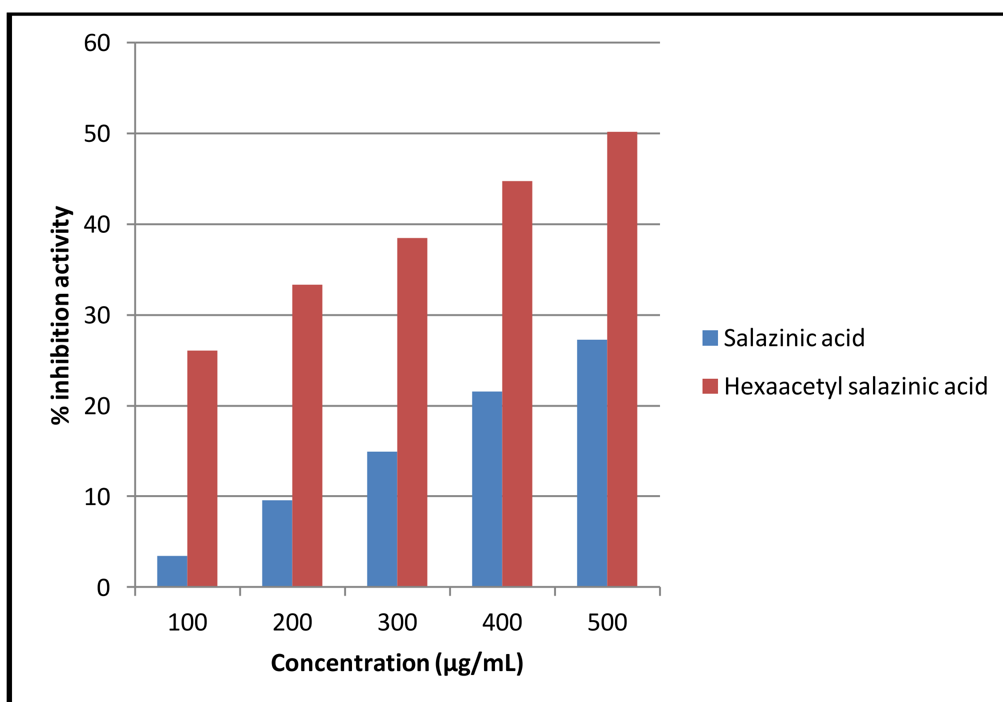

Chart: 5-Superoxide dismutase Scavenging Activity of Salazinic acid and Hexaacetyl salazinic acid

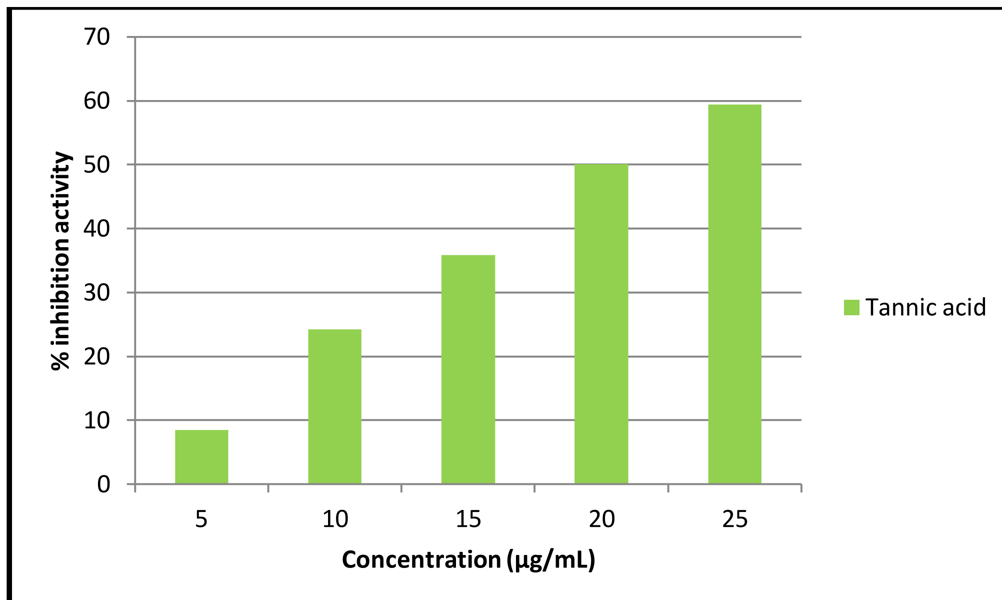

Chart: 6-Superoxide dismutase Scavenging Activity of Tannic acid

Table: 3-Superoxide Dismutase Activity

Test compound	Concentration (µg/mL)	% Activity	IC_{50} (µg/ml)
Salazinic acid	100 200 300 400 500	3.47±0.27 9.57±0.81 14.93±2.14 21.52±0.33 27.27±0.13	294.07±6.57
Hexaacetyl Salazinic acid	100 200 300 400 500	26.09±1.91 33.33±0.60 38.43±0.20 44.71±0.66 50.15±0.42	133.40±0.67
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

5.4 Lipid Peroxidation

ROS are responsible for the lipid peroxidation in the biological systems. It may be initiate by reactive free radicals by abstracting an allylic hydrogen atom from a methylene group of poly unsaturated fatty acid side chains. Followed by rearrangement of bond. This leads to the stabilization by diene conjugate formation. The Lipid peroxidation inhibiting activity of the two test compounds Salazinic acid and hexaacetyl salazinic acid were determined at different concentration (100-500µg/mL). The IC_{50} values of the test compounds were also determined. The result were given in the Table: 4. The dose dependent activity of the two test compounds and the standard were expressed in Chart: 7. The lipid peroxidation inhibition activity of the test compounds shows higher inhibition activity as the concentration increases. When comparing the two test compounds the lipid peroxidation % inhibition activity of salazinic acid was better than hexaacetyl salazinic acid. But both the test compound are less active than the standard tannic acid.

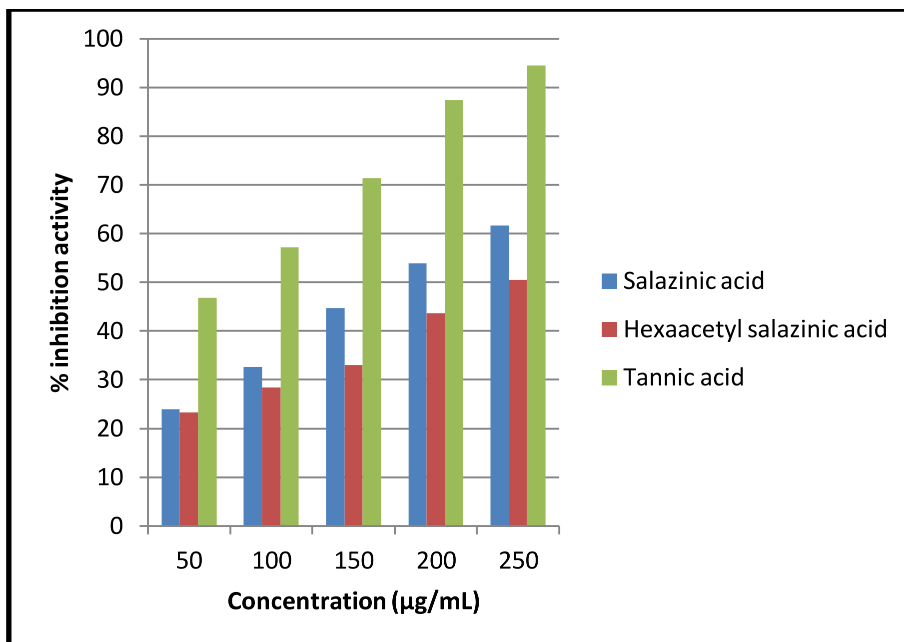

Chart: 7-Lipid Peroxidation Inhibiting Activity of Salazinic acid and Hexaacetylsalazinic acid

Table:4-Lipid Peroxidation Inhibiting Activity

Test compound	Concentration (µg/mL)	% Activity	IC_{50} (µg/ml)
Salazinic acid	50 100 150 200 250	23.94±1.41 32.62±1.71 44.68±1.41 53.90±2.15 61.70±1.41	20.25±0.04
Hexaaacetyl Salazinic acid	50 100 150 200 250	23.23±1.11 28.37±1.34 32.98±0.92 43.62±1.60 50.53±0.53	24.98±0.50
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

5.6 Metal Chelating Activity

Chelating agents can prevent radical generations by chelating the transition metals, because the transition metal ions was responsible for the generation of free radicals. The Metal chelating activity of the two test compounds and standard were given in the Table: 5. The metal chelating activities of the test compounds and the standard were shown in the Chart: 8. However, the two test sample shows lesser metal chelating activity than the standard tannic acid. Among the test samples derivatives hexaacetyl salazinic acid shown higher activity than the salazinic acid.

Table: 5-Metal Chelating Activity

Sample	Metal chelating activity (mg EDTA eq./g sample)
Salazinic acid	6.79±0.63
Hexaacetyl Salazinic acid	18.78±0.26
Tannic acid	126.85±4.04

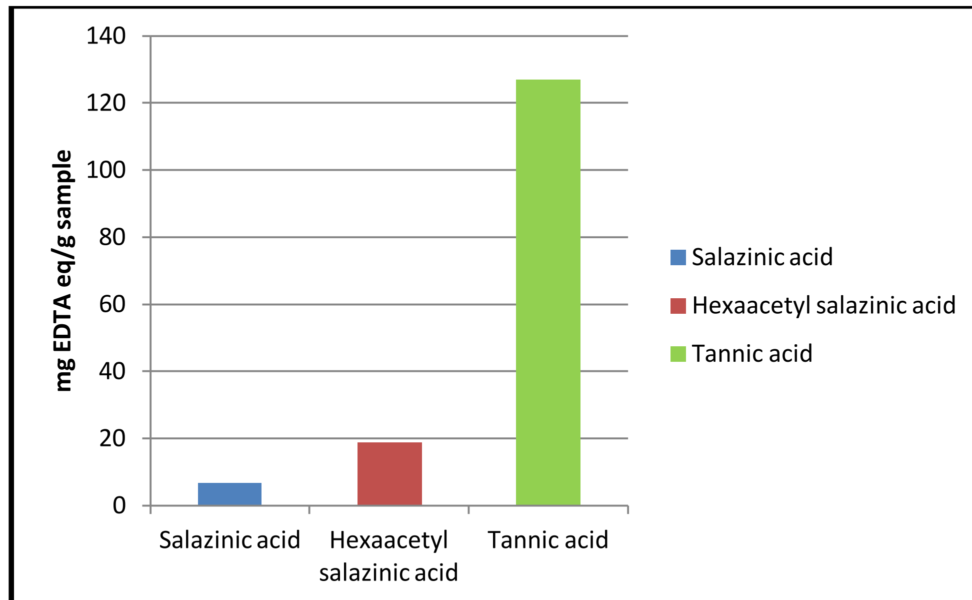

Chart: 8-Metal Chelating Activity of Salazinic acid and Hexaacetyl salazinic acid

5.7 Phosphomolybdenum Assay

The Phosphomolybdenum method has been used to investigate the total antioxidant capacity of the pure compound. The total antioxidant capacity of the Salazinic acid and hexaacetyl salazinic acid were assayed by Phosphomolybdenum 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: 9. The result shows that the salazinic acid and hexaacetyl salazinic acid both shows lesser total antioxidant activity than the standard Tannic acid. But salazinic acid shows higher total antioxidant activity than that of hexaacetyl salazinic acid.

Table: 6-Phosphomolybdenum assay

Sample	Phosphomolydenum (mg ascorbic acid eq./g sample)
Salazinic acid	117.08±0.84
Hexaacetyl Salazinic acid	85.84±6.61
Tannic acid	187.21±80.27

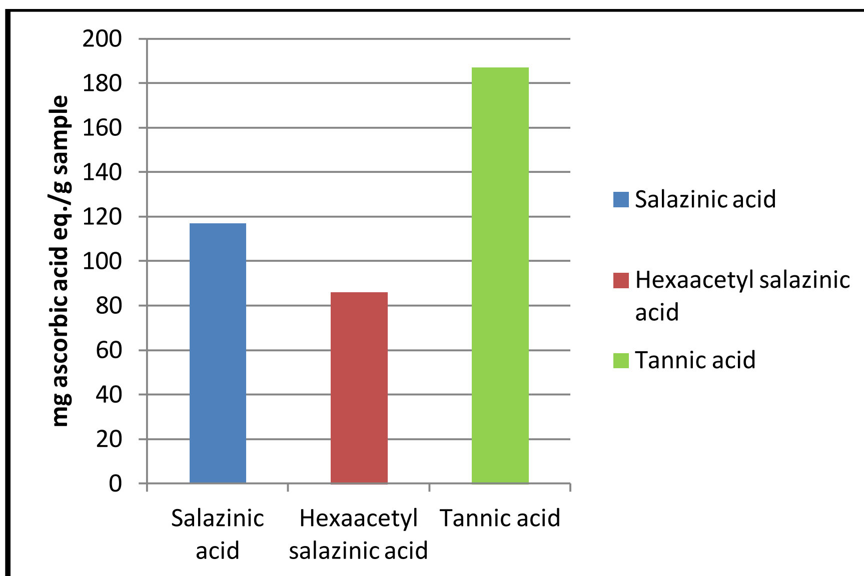

Chart: 9-Phosphomolybdenum activity of salazinic acid, hexaacetylsalazinic acid and Tannic acid

5.8 Ferric Reducing Antioxidant Power

FRAP assay is based on the ability of an antioxidant to reduce Fe^{3+} in the presence of TPTZ, forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593nm. The absorbance decrease is proportional to the antioxidant content. 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: 10. The result shows that among the two test compounds hexaacetyl salazinic acid shows higher activity than salazinic acid, but both the sample less active than the standard tannic acid.

Table: 7-FRAP Assay

Sample	FRAP (mmol(Fe(II)/g) sample
Salazinic acid	37.18±7.00
Hexaacetyl Salazinic acid	166.81±8.44
Tannic acid	1335±160.09

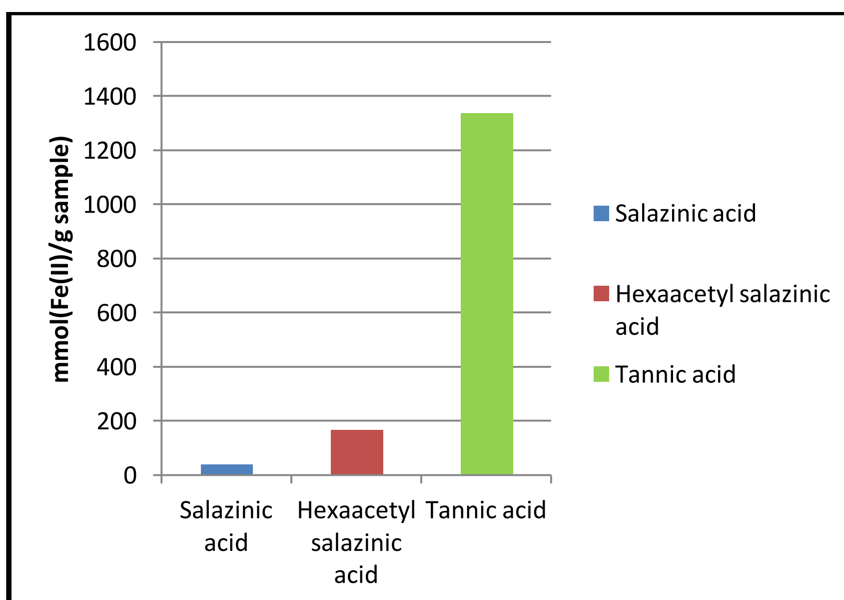

Chart: 10-FRAP activity of salazinic acid, hexaacetyl salazinic acid and Tannic acid

Table: 8-IC_{50}value of the test compounds and the standard

Method of antioxidant assay	Salazinic acid IC_{50} Value (μg/mL)	Hexaacetyl salazinic acid IC_{50} Value (μg/mL)	Standard Tannic acid IC_{50} Value (μg/mL)
DPPH Radical Scavenging	974.66±30.69	127.78±2.76	4.31±0.02
Superoxide Dismutase activity	294.07±6.57	133.40±0.67	6.40±0.05
Hydroxyl radical Scavenging	193.36±0.12	161.87±1.67	21.51±0.19
Lipid Peroxidation	20.25±0.04	24.98±0.50	13.37±0.21

6. CONCLUSION

In our present work, we have studied the in-vitro antioxidant activity of salazinic acid and hexaacetyl salazinic acid. But among the two test compounds hexaacetyl salazinic acid gives lower IC_{50} value and shows higher inhibition activity than salazinic acid for DPPH, Superoxide Distmutase activity, Hydroxyl radical scavenging activity. It was already known that lower the IC_{50} value higher is the inhibition activity. In lipid peroxidation both the test compounds shows nearly equivalent activity.In Phosphomolybdenum assay salazinic acid shows better activity than hexaacetyl salazinic acid. In FRAP assay hexaacetyl salazinic acid shows better activity than salazinic acid.The metal chelating capacity of both the test compounds were found to be less. From the results, it has been found that the both the test compounds shows lesser activity than the standard Tannic acid. Our test compounds are obtained naturally and are free from side effects.

REFERENCES

[1] Rosana S.S Barreto, Richardo L.C, Albuquerque-Junior, Rose NelyPereia-Filho, Jullyana S.S, Quintans, Andre S. Barreto, Josimari M. Desantana, Valter J. Santana-Filho, Marcio R.V. Santos, Leonardo R. Bonjardim, Adriano A.S. Araujo, Lucindo J. Quintans-Junior. "Evaluation of wound healing activity of atranorin, alichen secondary metabolite, on rodents". 2013; *Brazilian Journal of Pharmacognosy*; 23(2); pp.310-319.

[2] Mohammad Ali Abrahimzadeh, Seyed Mohammad Nabavi, SeyedFazelNabavi, FathemehBahramian and Ahmad Reza Bekhradnia. "Antioxidant and free radical scavenging activity of H. Officinalis L. Var. Angustifolius, V. Odorata, B. Hyrcana and C.Speciosum". 2010; Pak. J. Pharm. Sci; 23(1); pp.29-34.

[3] IlhamiGulcin, ZubeyrHuyut, MahfuzElmastas, Hassan Y. Aboul-Enein. Radical Scavenging and antioxidant activity of tannic acid. Arabian Journal of Chemistry 2010; 3, pp.43-53.

[4] Blois MS. Antioxidant determinations by the use of a stable free radical. *Nature*. 1958; 26: pp.1199-1200.

[5] Klein SM, Cohen G and Cederbaum AI. Production of formaldehyde during metabolism of dimethyl sulphoxide by hydroxyl radical generating system. *Biochem.* 1991; 20: pp.6006-6012.

[6] Beauchamp C and Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 1971; 44: pp.276-277.

[7] Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant power". The FRAP assay. *Anal Biochem*. 1996; 239: pp.70-76.

[8] Dinis TCP, Maderia VMC and Almeidia MCM. Action of phenolic derivatives (acetoaminophen, Salycilate and 5-amino salycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavenger. *Arch. Biochem. Biophys*. 1994; 315: pp.161-169.

[9] Prieto P, Pineda M and Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal Biochem*. 1999; 269: pp.337-341.

[10] Ohkawa, H., Ohishi, N., Yagi, K. Assay forlipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, 1979; 95: pp.351-358.