Antimicrobial and anti-inflammatory activity of usnic acid and its acetyl derivative usnic acid diacetate

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Abstract-Recent years there is a renewed interest in natural products screening to discover new compounds from living organisms having antimicrobial properties. Among the life forms lichens are unique by their composite nature. The lichen metabolites are well known for their bioactive properties. In this study,usnic acid, a member of dibenzofuran isolated from lichen Usneaimplicata collected from Kodaikanal hills, (South India) which was botanically identified and subjected to chemical investigation. In this study we have taken the usnic acid and usnic acid diacetate for antimicrobial activity and anti-inflammatory activity. The two test compounds were well soluble in acetone was evaluated for antibacterial and anticandidal activity by disc diffusion method. The compound at 150µg/disc and 300µg/disc was tested against Bacillus substilis, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli, Streptococcus faecalis, Salmonella typhi, Salmonella paratyphi, Salmonella typhimurium, Klebsiella pneumonia and Candida albicans. The two test compounds were found to be active against gram positive bacteria than to gram negative bacteria. Candida albicans remained resistant to the compound. The MIC of two test compounds were determined by broth dilution method. Antifungal activity of the usnic acid and usnic acid diacetate were also evaluated against Fusariumoxysporum, Colletotrichumfalcatum, Aspergillusflavus, Aspergillusniger and Curvularialunata. Inhibition of spore germination by usnic acid and usnic acid diacetate were determined using cavity slide technique, Conidal germination was completely arrested by usnicacid andusnic acid diacetate at 300µg/ml concentration. In this study we have also determined the in vitro anti-inflammatory activity of usnic acid and usnic acid diacetate, the results shows that the parent compound usnic acid was more active than usnic acid diacetate.

Index Terms-dibenzofuran, usnic acid, diacetate, antimicrobial, spore germination, anti-inflammatory.

Introduction

A unique association of algae and fungi together comprises the lichen entities. Lichens have been used in traditional medicine in ancient times. The secondary metabolites synthesized by the lichens can act as an antibiotic, pesticide, antiherbivore, insecticide, antioxidant, cytotoxic agent[1,2]. The lichen secondary metabolites called otherwise known as lichen acids, well known for their biological and bio-medicinal values. Lichens are also can be used as bio-indicator, as they are very sensitive to air pollution. They are also used as dyes. The present investigation was undertaken to study the antimicrobial and anti-inflammatory efficacy of usnic acid and its derivative usnic acid diacetate.

Experimental Section

Collection and Identification of the lichen material

The lichen species under investigation was collected from kodaikanal hills, Dindigul district, Tamil Nadu (altitude: 2268m). This lichen species was authenticated as *Usneaimplicata*by Dr. K. P. Singh, Botanical Survey of India, Allahabad.

Extraction of *Usneaimplicata* and isolation of Usnic acid

The lichen thallus was pulverized into a coarse powder. About 27g lichen material was extracted upon reflection with acetone (2l) for about 6hr. The extract was concentrated under vacuum using a rotatory evaporator, it yields (2g) of brown residue.

The concentrated extract (2g) was fractioned on column chromatography with silica gel (60-120 mesh) and benzene. The benzene fraction yielded 150mg of yellowish crystalline solid which was found to be single in TLC (solvent system: Toluene : Acetic acid, 170:30). The compound was recrystallized from benzene, with a

melting point of 202◦C. It produced 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.

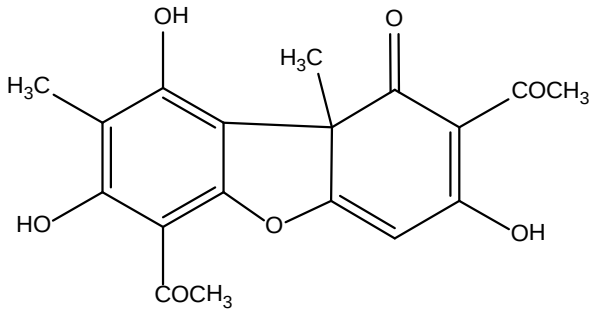

Fig:1. Usnic acid

Preparation of Diacetate derivative for usnic acid

The compound usnic acid (0.2g) upon acetylation with acetic anhydride (2ml) and a drop of perchloric acid (60%), then the mixture was kept at room temperature for 2hr and then poured over ice water yields 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.

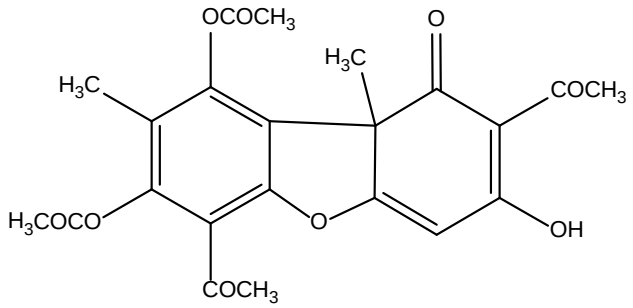

Fig:2. Usnic acid diacetate

Materials and Methods

1. Preparation of stock solution

Six milligram (6mg) of the pure compounds Usnic acid and Usnic acid diacetate were dissolved in 500µl analytical grade (AR) acetone and treated as the stock solution. Required volume of the stock solution was impregnated to sterile 6mm discs. The discs were dried in a laminar airflow chamber to evaporate the solvent. Control discs received equal volume of the solvent. The discs were prepared at two concentrations – 150 and 300µg/disc.

2. Organisms used in this study

A panel of ten bacterial and an unicellular fungus were employed in the antimicrobial activity screening. Additionally five more filamentous fungi were used for spore germination studies. The list of the test organisms taken for our study are*Bacillus subtilis,Staphylococcusaureus, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Streptococcus faecalis, Salmonella typhi, Salmonella paratyphi, Salmonella typhimurium, Klebsiella pneumonia, Candida albicans, Aspergillusflavus, Aspergillusniger, Colletotrichumgloeosporioides, Curvularialunata, Fusariumsolani.*

3. Maintenance of the test organisms

All the bacterial cultures were maintained on Nutrient Agar and the fungi were maintained on Potato Dextrose Agar. All the cultures were sub cultured on fresh slants once every 20days. The media and their compositions were given as **a). Nutrient Agar (g/l):** Peptone- 5.0 g, Beef extract - 3.0 g, Agar- 18.0 g, Distilled water - 1000 ml, pH - 7.0-7.2. **b). Potato Dextrose Agar (g/l):** Potato - 200.0g, Dextrose- 20.0g, Agar - 8.0g, Distilled water - 1000ml, pH - 6.0-6.5.

The media were prepared following standard procedure and the pH was adjusted to the desired level using 1N NaOH/ 1NH_4Cl before sterilization. The media were sterilized in an autoclave at 15psi and 121°C for 20 minutes. Whenever required liquid medium (Broth) was prepared by omitting agar.

4. Determination of antimicrobial activity (Disc diffusion method)

The Kirby and Bauer disc diffusion method (National Committee for clinical Laboratory Standards, 1993) was used to determine the antimicrobial activity.

a. Preparation of inoculum

Five milliliter portions of nutrient broth pH 7.0 and potato dextrose broth, pH 6.5 in separate test tubes were sterilized in an autoclave and inoculated with the test bacteria and Candida. The tubes were incubated over night at 30±2°C. The overnight cultures served as the inoculum for the bioassay.

b. Preparation of the assay plates

The bioassay was performed using PDA. Sterilized and molten (45°C) was dispensed into sterile 10cm dia. petriplates at the rate of 15-20ml/plate and allowed to solidify under aseptic conditions. A 200µl of overnight bacterial Candida cultures containing 10^8 cells/ml were surface inoculated on PDA using sterile cotton swabs. Then the test discs were placed on the surface of the inoculated plates, sealed with parafilm and placed in a refrigerator for 3h to facilitate diffusion of the

compound from the disc into the medium. Control plates received discs impregnated with the solvent. After three hours all the plates were incubated at 35°C for 24-48h. To compare the potency of the test compound a commercial antibiotic gentamycin (10µg/disc, Himedia) was also included in this assay. The antibacterial and anti-candida activity was qualitatively evaluated according to the diameter of clear zone of growth inhibition.

MIC for the two test compounds were also determined by broth dilution method.

b. Antifungal activity (spore germination study)

Antifungal activity [3] of the lichen compound atranorin was evaluated against *Fusariumoxysporum, Colletotrichumfalcatum, Aspergillusflavus, Aspergillusniger*and *Curvularialunata*was determined using cavity slide technique. Conidial germination assay was performed as described by Ganesan and Krishnaraju (1995). Briefly, spores from 5-8 days old PDA plate cultures were harvested in 10ml cold sterile distilled water by gently scraping fungal colony surface using a sterile soft Camlin brush. Spore suspension of each fungus was filtered through two fold cheese cloth to remove mycelial fragments, and washed twice in sterile distilled water by centrifugation. The pellet containing spores was suspended in 5ml cold sterile distilled water. The concentration of spores was adjusted using sterile cold water to give 40-50 spores per microscopic field under low power. The spore suspensions were stored in refrigerator and used within 24h. The test compounds were After 24 hours incubation the slides were examined under microscope and the percent inhibition was calculated.

Anti-inflammatory activity

Inhibition of protein denaturation

The reaction mixture (0.5mL) consisted of 0.45mL bovine serum albumin (5% aqueous solution) and 0.05ml of the sample (100-500µg/mL). pH was adjusted to 6.3 using a small amount of 1N HCL. The samples were incubated at 37°C for 20 min and then heated at 57°C for 3min. After cooling the samples,2.5ml phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660nm. For control tests 0.05ml of distilled water was used instead of extracts while product control tests lacked bovine serum albumin[4]. The percentage inhibition of protein denaturation was calculated as follows.

Percentage inhibition= 100-((O.D of test-O.D of product control)/O.D of Control)x100

The IC_{50} value was defined as the concentration of the sample extract to inhibit 50% of protein denaturation under assay condition.

Proteinase inhibitory activity

The reaction mixtures (2.0ml) contained 0.06mg trypsin, 1.0ml of 25mM tris-HCL buffer (pH-7.4) and 1.0mL aqueous solution of the sample (100-500µg/ml). The mixtures were incubated at 37°C for 5min then 1.0mL of 0.8% (w/v) casein was added. The mixtures were incubated for an additional 20 min. 2.0mL of 70% (v/v) perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged. Absorbance of the supernatant was read at 280nm against buffer as blank[4]. The percentage of inhibition was calculated as follows.

Percentage inhibition= 100-((O.D of test-O.D of product Control)/O.D of Control)x100

The IC_{50} value was defined as the concentration of the sample extract to inhibit 50% of proteinase inhibition activity under assay condition.

Results and Discussion

In our present investigation, we have analysed the in vitro antibacterial efficacy of Usnic acid and Usnic acid diacetate on some human pathogens such as *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Streptococcus faecalis, Salmonella typhi, Salmonella paratyphi, Salmonella typhimurium, Klebsiella pneumonia, Candida albicans, Aspergillusflavus, Aspergillusniger, Colletotrichumgloeosporioides, Curvularialunata, Fusariumsolani.* Antimicrobial activity of usnic acid and usnic acid diacetate were given in the table:1 and table-2, out of all test organisms gram-positive bacteria *Bacillus subtilis, Staphylococcus aureus* were found to be more sensitive than gram-negative bacteria *Pseudomonas aeruginosa, Proteus vulgaris*. The yeast *Candida albicans* was completely resistant to the Usnic acid and Usnic acid diacetate. Maximum inhibition zone of 25mm and 20mm was recorded for *Bacillus subtilis*and *Staphylococcus aureus*by Usnic acid while Usnic acid diacetate shows the maximum inhibition zone of 24mm and 20mm at 150µg/disc. At higher dose, 300µg/disc only a slight increase in zone of inhibition was recorded. Increasing the dose did not show any enhancement in activity. The two gram-negative bacteria *Pseudomonasaeruginosa and Proteus vulgaris* were sensitive with 12mm inhibition zone and 10mm for Usnic acid and Usnic acid diacetate. But other gram-negative bacteria such as *Escherichia coli, Streptococcus faecalis, Salmonella typhi, Salmonella paratyphi, Salmonella typhimurium, Klebsiella pneumonia* were completely resistant to the two test compounds. The antimicrobial potency of usnic acid and Usnic acid diacetate towards the gram-positive bacteria were compared with antibiotic gentamycin. Though sensitivity is comparatively less than the standard gentamycin, usnic acid and Usnic acid diacetate were less toxic than the commercial antibiotic gentamycin. Spore germination for Filamentous fungi *Aspergillusflavus, Aspergillusniger,*

*Colletotrichumgloeosporioides, Curvularialunata and Fusariumsolani*were completely inhibited at 300µg/ml concentration. The Minimum Inhibitory Concentration of Usnic acid and Usnic acid diacetate were also determined to the test organism which shows sensitivity. The MIC for Usnic acid and Usnic acid diacetate were shown in the table:3.

Table:1-Antibacterial activity of Usnic acid and Usnic acid diacetate

Bacterial species	Usnicscid (150µg/disc)	Usnic acid (300µg/disc)	Usnic acid diacetate (150µg/disc)	Usnic acid diacetate (300µg/disc)	Gentamycin (10µg/disc)
I. Bacteria					
a. Gram Positive					
Bacillus subtilis	25mm	26mm	24mm	25mm	30mm
Staphylococcus aureus	20mm	22mm	20mm	22mm	18mm
b.**Gram Negative**					
Escherichia coli	-	-	-	-	-
Pseudomonas aeruginosa	12mm	12mm	10mm	12mm	21mm
Proteus vulgaris	12mm	12mm	10mm	12mm	13mm
Streptococcus faecalis	-	-	-	-	-
Salmonella typhi	-	-	-	-	-
Salmonella paratyphi	-	-	-	-	-
Salmonella typhimuriumKlebsiella pneumonia	-	-	-	-	-
	-	-	-	-	-
II.**Unicellularfungus**					
Candida albicans	-	-	-	-	-

Table:2-Spore germination study on Usnic acid and Usnic acid diacetate

Test Organism	% Germination		
	Control	Treatment	
		Usnic acid	Usnic acid diacetate
III. Filamentous fungi			
Aspergillusflavus	96	0	0
Aspergillusniger	97	0	0
Colletotrichumgloeosporioides	95	0	0
Curvularialunata	94	0	0
Fusariumsolani	100	0	0

Table:3-MIC for Usnic acid and Usnic acid diacetate

Test organism	MIC for Usnic acid	MIC for Usnic acid diacetate
Bacillus subtilis	>1000µg/mL	>1000µg/mL
Staphylococcus aureus	>800µg/mL	60µg/mL
Pseudomonas aeruginosa	30µg/mL	>800µg/mL
Proteus vulgaris	>800µg/mL	>800µg/mL

The in vitro anti-inflammatory activity of usnic acid and usnic acid diacetate were studied under Protein denaturation inhibiting activity and proteinase inhibiting activity. For this study the two test compounds are taken in different concentrations 100µL, 200µL,300µL,400µL,500µL. The results were given in the table:4 and table:5. In both the studies, the parent compound usnic acid shows higher activity than usnic acid diacetate. Eventhough the two test compounds are being active, they are less active than the commercial anti-inflammatory drug asprin.

Table:1-Protein denaturation inhibiting activity of Usnic acid and Usnic acid diacetate

Sample	Concentration	Percentage activity %	IC_{50} (µg/mL)
Usnic acid	100	6.37±1.72	189.32±0.12
	200	16.48±0.65	
	300	25.84±2.25	
	400	34.83±1.12	
	500	46.44±2.34	
Usnic acid diacetate	100	7.12±0.65	210.46±10.81
	200	12.73±0.65	
	300	22.10±1.72	
	400	31.46±2.25	
	500	42.70±2.25	
Aspirin	25	15.53±1.74	43.37±0.58
	50	31.06±1.75	
	100	43.56±2.37	
	150	60.61±1.31	
	200	68.56±1.74	

Table:2-Proteinase inhibiting activity of Usnic acid and Usnic acid diacetate

Sample	Concentration	Percentage activity %	IC_{50} (µg/mL)
Usnic acid	100	18.46±0.93	107.15±1.34
	200	24.43±0.93	
	300	29.41±0.25	
	400	35.21±0.62	
	500	43.71±0.93	

Usnic acid diacetate	100 200 300 400 500	5.56±0.62 11.36±0.37 18.22±0.37 24.92±0.37 30.47±0.99	164.85±1.58
Aspirin	25 50 100 150 200	12.35±0.46 14.99±0.15 28.19±0.15 42.81±0.21 54.06±0.21	35.18±0.15

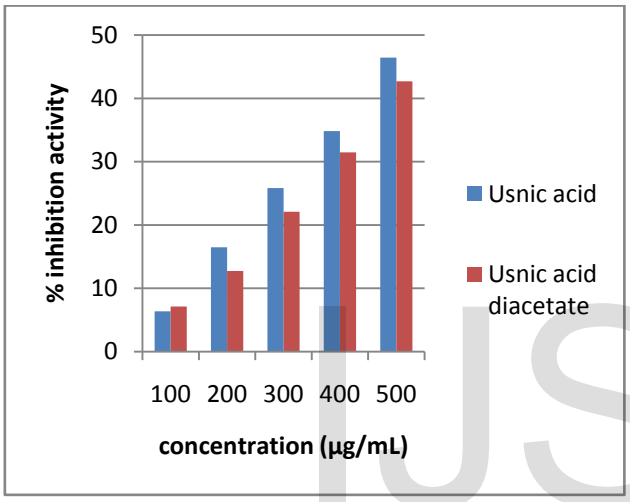

Chart:1-Protein denaturation inhibiting activity of Usnic acid and Usnicdiacetate

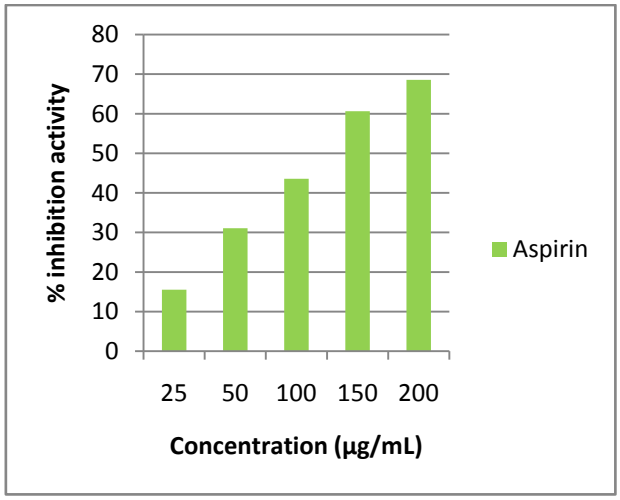

Chart:2-Protein denaturation inhibiting activity of Asprin

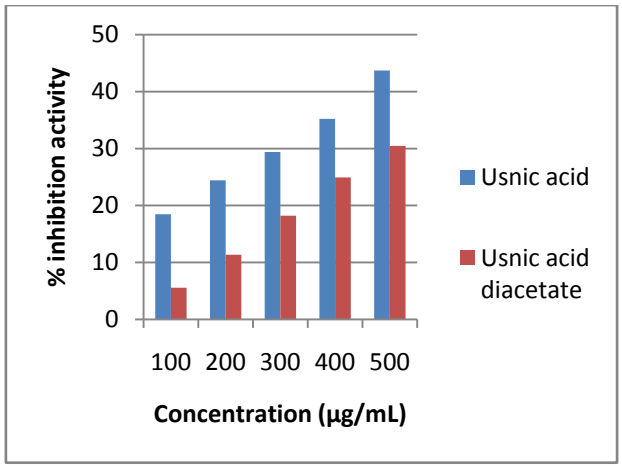

Chart:3-Proteinase inhibiting activity of Usnic acid and Usnic acid diacetate

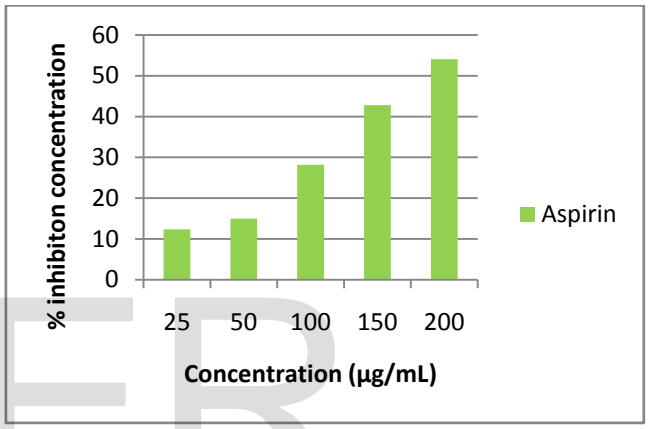

Chart:4-Proteinase inhibiting activity of Aspirin

Conclusion

The present investigation proved that both the test compounds possess activity against the growth of *Bacillus subtilis, Staphylococcus aureus, Proteus vulgaris, Streptococcus faecalis.* The MIC value of Usnic acid and Usnic acid diacetate were also determined. It has been noted that Usnic acid and Usnic acid diacetate both shows similar activity, therefore we have suggested that both the test compounds can be used as antibiotic agents in oinments. And it was found that the spore germination of certain filamentous fungi such as *Fusariumoxysporum, Colletotrichumfalcatum, Aspergillusflavus, Aspergillusniger* and *Curvularialunata*was completely arrested by the effect of Usnic acid and Usnic acid diacetate. Also the results regarding the in vitro anti-inflammatory activity of usnic acid and usnic acid diacetate under protein denaturation inhibiting activity and proteinase denaturation activity were shown significant efficacy, the substitution of acetyl group in the parent compound usnic acid lowers the efficacy of usnicdiacetate, therefore it may concluded that the anti-inflammatory activity mechanism depends on the presence of more available –OH group.

Reference

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