In vitro antimicrobial activity of barbatic acid and its acetyl derivative of barbatic acid isolated from usneaflexilis*(south india)*

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Abstract-Exploitation of novel classes of antimicrobial metabolites is increasingly noticeable over recent years. Consequently there is a renewed interest in natural product screening to discover new compounds from living organisms. Among the life forms lichens are unique by their composite nature. They are capable of synthesizing a variety of bioactive secondary metabolites. In this study, antimicrobial activity of barbatic acid, a member of depside isolated from the lichen *Usneaflexilis* collected from Kodaikanal hills, (South India) which was botanically identified by Botanical survey of India, Howrah, India. Barbatic acid, a depsides obtained from the lichen*Usneaflexilis* was spectroscopically identified and subjected to chemical investigation.The barbatic acid was also subjected to acetylation. The two test compounds barbatic acid and its acetyl derivative of barbatic acid are well soluble in acetone were evaluated for antimicrobial activity by disc diffusion method. The test compounds 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, Klebsiellapneumoniae*and *Candida albicans*. The test compounds were found to be active against gram positive bacteria than to gram negative bacteria. The MIC for barbatic acid and its acetyl derivative were evaluated by broth dilution method. Candida albicans remained resistant to both the test compound. Antifungal activity of the barbatic acid was also evaluated against *Fusariumoxysporum,Colletotrichumfalcatum, Aspergillusflavus, Aspergillusnigerand Curvularialunata*. Inhibition of spore germination by barbatic acid and acetyl derivative were determined using cavity slide technique, Conidal germination was completely arrested by barbatic acid and its acetyl derivative of barbatic acid at 300µg/ml concentration.

Index term-*Usneaflexilis*, barbatic acid, acetyl derivativeof barbatic acid, antimicrobial activity, MIC value.

1. INTRODUCTION

Lichens have excited great interest with regard to their biological nature, botanical classification and chemical composition. Lichens are symbiotic association of algae and fungi[1]. Although there are about 20,000knownspeciesof lichens around the worldwide.The lichenare an important food for many animalsincluding human[2].Bioactive secondary metabolites have been isolated from lichens are used in pharmaceutical sciences[3]. Biological activities of some lichens and their components are known, such asantibiotic, antimycobacterial, immunomodulatory, cytotoxic, antioxidant, antiherbivore, anti-inflammatory and antitumour effects (Chand *et al*., 2009)[4]. Lichens are natural products have a long time traditional of being used for decorations, brewing and distilling, perfume, dying industry, food and natural remedies (vide Oksanen, 2006)[5].The aim of our present work is to analyses the antimicrobial activities between the two test compounds ofbarbatic acid and its acetyl derivative ofbarbatic acid.

2. MATERIAL AND METHOD

Collection and identification of lichen material

The lichen specimen *Usenaflexilis* was collected from Kodaidkannal hills, Dindigul district in Tamil Nadu (South India). It was botanically identified by Dr. K.P. Singh, Botanical Survey of India, Allahabad.

Preparation of lichen extracts

Acetone Extraction

The air dried lichen (110g) was crushed and grounded to coarse powder form, it was then placed in a 1000ml flat bottom flask and was extracted with hot acetone. The extract was concentrated to a small volume using a rotary separator. This process was repeated until the last extract was almost colourless (four extraction). On evaporation of

the remaining solvent at room temperature, a powdary mass was obtained (7g). This was found to be heterogeneous by preliminary TLC examination. Hence it was subjected to chromatographic separation as follows.

CHROMATOGRAPHIC SEPERATION:

The crude solid (7g) was adsorbed in silica gel and then transferred to a column (silica gel) of 60-120 mesh, (300g) built in benzene (80^0C).

The column was eluted successively in order with

1) Benzene
2) Benzene with increasing amount of ethyl acetate
3) Ethanol

Fractions of 100ml were collected every time and the monitoring was done with TLC in different solvent systems. Based on the TLC monitoring similar fractions were grouped together. The fraction benzene – ethyl acetate, (9:1-7.5:2.5) were concentrated and evaporated when a colourless solid of barbatic acid (1g) was obtained.

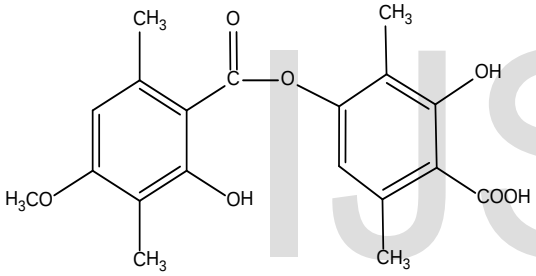

Fig:1-Barbatic acid

Barbatic acid was crystallized from methyl alcohol as colourless prismatic rods melting at 192-193^{0}C. It was sparingly soluble in petroleum ether but easily soluble in ether, acetone and alcohol. It produced violet-red colour with alcoholic ferric chloride and red colour with green fluorescence on heating with aqueous sodium hydroxide solution along with a drop of chloroform. It gave an yellow colour with bleaching powder. Mixed melting point with an authentic sample of barbatic acid from*Usneaalbopunctuata*was undepressed. The co-TLC comparsion with the authentic sample also confirmed its identity. The solvent system used for TLC was Toluene:Acetic acid (170:30). The spectral data H^1 and C^{13} confirmed the structure of barbatic acid (Fig:1).

Acetylation of Barbatic acid

The barbatic acid (0.1g) was acetylated with acetic anhydride (2mL) and 3 drops of perchloric acid, kept aside for 2hrs at room temperature. To that mixture, ice water was poured. A white mass thus obtained was kept overnight and filtered. The solid obtained was recrystallized with methanol. The structure of acetyl derivative of Barbatic acid (Fig:2) was confirmed with the spectral data H^1 and C^{13}.

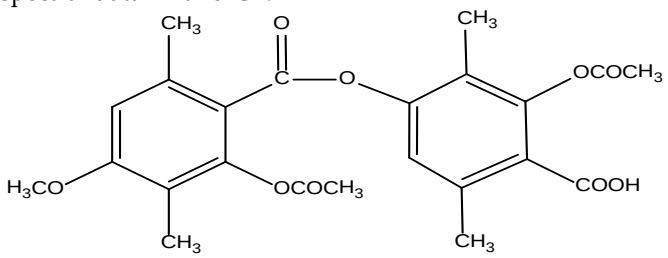

Fig: 2-Acetyl derivative of barbatic acid

Test organisms

Organism used in this study are- two Gram positive bacteria (*Staphylococcus aureus*MTCC 96), *Bacillus subtilis* MTCC 121) and eight Gram- negative bacteria (*Proteus vulgaris* MTCC 424), *Pseudomonas aeruginosa* MTCC 7299) *Escherichia coli*, *Streptococcus faecalis*, *Salmonella typhi,Salmonella paratyphi, Salmonella typhimurium,Klebsiella pneumonia* and unicellular fungus (*Candida albicans* MTCC 183). Bacteria were maintained on nutrient agar. The composition of the media used in the study is nutrient agar (g/l) Peptone-5.0g, Beef extract-3.0g, Agar-18.0g, Distilled water-1000ml, pH-7.0-7.2. Potato dextrose agar (g/l) Potato-200.0g, Dextrose-20.0g, Agar-18.0g, Distilled water-1000ml, pH-6.0-6.5.

Preparation of stock solution:

Six milligram (6mg) of the pure compound barbatic acid and its acetyl derivative of barbatic acid were dissolved in 500µl analytical grade (AR) acetone and treated as the stock solution. Required volume of the stock solution was impregnated to sterile 6 mm 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 different concentrations 150 and 300 µl/discs. The media were prepared following standard procedure and the pH was adjusted to the desired level using 1N NaOH/ 1 N HCl before sterilization. The media were sterilized in an autoclave at 15psi and 121^{0}C for 20 minutes. Whenever required liquid medium (broth) was prepared by omitting agar.

Determination of antimicrobial activity (Disc diffusion method)

The Kirby and Bauer disc diffusion method (Bauer *et al*.,1966)[6].(National committee for Clinical Laboratory

Standards, 1993)[7] was used to determine the antimicrobial activity.

1. 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^{0}C. The overnight cultures served as the inoculum for the bioassay.

2. Preparation of the assay plates

The bioassay was performed using PDA. Sterilized and molten (45^{0}C) PDA was dispensed into sterile 10cm dia. Petri plates 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 3hrs to facilitate diffusion of thecompound from the disc into the medium. Control plates received discs impregnated with the solvent. After three hours all the plates were incubated at 35^{0}C for 24-48 hours. To compare the potency of the test compound a commercial antibiotic gentamycin (10µg) disc, (Himedia) was also included in the assay. The antibacterial and anti-candida activitywas qualitatively evaluated according to the diameter of clear zone of growth inhibition.

3. Antifungal activity (spore germination study)

Antifungal activity of the compound barbatic acidand its acetyl derivatives of barbatic acid was evaluated against *Fusariumoxysporum, Colletotrichumfalcatum, Aspergillusflavus, Aspergillusnigar* and *Curvularialunata* was determined using cavity slide technique. Conidial germination assay was performed as described by Ganesan and Krishnaraju (1995)[8]. Briefly, spores from 5-8 days old PDA plate culture 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 distillled water. The concentration of spores was adjusted using sterile cold water to give 40-50 spores permicroscopic filed under low power. The spore suspensions were stored in refrigerator and used within 24hours. After 24 hours incubation the slides were examined under microscope and the percent inhibition was calculated.

Bacterial species	Barbatic acid (150µg/disc)	Barbatic acid (300µg/disc)	Acetyl derivative of barbatic acid (150µg/disc)	Acetyl derivative of barbatic acid (300µg/disc)	Gentamycin (10µg/disc)
I. Bacteria					
a.**Gram Positive**					
Bacillus subtilis	22mm	27mm	21mm	26mm	30mm
Staphylococcus aureus	16mm	18mm	15mm	17mm	18mm
b.Gram Negative					
Escherichia coli	-	-	-	-	-
Pseudomonas aeruginosa	14mm	15mm	12mm	12mm	21mm
Proteus vulgaris	11mm	16mm	12mm	12mm	13mm
Streptococcus faecalis	-	-	-	-	-
Salmonella typhi	-	-	-	-	-
Salmonella paratyphi	-	-	-	-	-
Salmonella typhimuriumKlebsiella pneumonia	-	-	-	-	-
	-	-	-	-	-
II.**Unicellularfungus**					
Candida albicans	-	-	-	-	-

Table:1-Antibacterial activity of barbatic acid and its acetyl derivative of barbatic acid

Results and Discussion

In the present work antibacterial and antifungal activity of barbatic acid and its acetyl derivative was investigated against human pathogens like *Staphylococcus aureus*, *Bacillus subtilis* and eight Gram- negative bacteria *Proteus vulgaris* , *Pseudomonas aeruginosa* , *Escherichia coli*, *Streptococcus faecalis*, *Salmonella typhi,Salmonella paratyphi, Salmonella typhimurium,Klebsiellapneumoniae* and an unicellular fungus (*Candida albicans*). Also studied the MIC value for barbatic acid and its acetyl derivative barbatic acid for the test organisms which showed sensitivity. The results are shown in the Table: 1&2. The experimental results of MIC value of barbatic acid were given in the Table: 3. Amongthe test organisms gram positive bacteria *Bacillus subtilis* and *Staphylococusaureus,*were found to be more sensitive than gram negative bacteria *Proteus vulgaris*, *Pseudomonas aeruginosa*. The yeast, *Candida albicans* was

completely resistant to barbatic acid as well as acetyl derivative of barbatic acid. Maximum inhibition zone of 22mm and 16mm was recorded for *Bacillus subtilis* and *Staphylococcus aureus* respectively at 150µg/disc while the acetyl derivative of Barbatic acid showed maximum inhibition zone of 21mm and 15mm were recorded. At the 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 gram negative bacteria *Pseudomonas aeruginosa, Proteus vulgaris,* were sensitive with 14mm and 11mm inhibition zones for barbatic acid while its acetyl derivative of barbatic acid shows 12mm and 12mm. Growth of the other gram- negative bacteria such as *Escherichia coli, Streptococcus faecalis, Salmonella typhi, Salmonella paratyphi, Salmonella typhimurium, Klebsiella pneumonia* was not affected by both test compounds.The potency barbatic acid and its acetyl derivative of barbatic acidtowards the gram positive bacteria was compared with a standard antibiotic gentamycin (10µg/disc). Barbatic acid and acetyl derivative of barbatic acid were less toxic than the commercial antibiotic gentamycin. The MIC of barbatic acid and acetyl derivative of barbatic acid were determined for the sensitive organisms. The spore germination of all the five filamentous fungi *Aspergillusflavus*, *Aspergillusniger*, *Colletotrichumgloeosporiodes*, *Curvularialunata*and *Fusariumsolani*were arrested completely at 300µg/mL concentration.

Table: 2-Spore germination study

Test Organism	% Germination		
	Control	Treatment	
		Barbatic acid	Acetyl derivative of Barbaticacid
Filamentous fungi			
Aspergillusflavus	96	0	0
Aspergillusniger	95	0	0
Colletotrichumgloeosporioides	98	0	0
Curvularialunata	97	0	0
Fusariumsolani	100	0	0

Table: 3-MIC for barbatic acid and its acetyl derivative of barbatic acid

Test organism	MIC for barbatic acid	MIC for acetyl derivative ofbarbatic acid
Bacillus subtilis	1000µg/mL	>1000µg/mL
Staphylococcus aureus	750µg/mL	>800µg/mL
Pseudomonas aeruginosa	20µg/mL	>40µg/mL
Proteus vulgaris	700µg/mL	>900µg/mL

Conclusion

The present study revealed the selective activity of barbatic acid and its acetyl derivative of barbatic acid towards certain bacterial species and inactive on other bacteria. From the result it was found that barbatic acid and acetyl derivative of barbatic acid were active against gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, and gram negative bacteria *Pseudomonas aeruginosa, Proteus vulgaris.* The MIC value for barbatic acid and its acetyl derivative ofbarbatic acid were also reported. Also the barbatic acid andits acetyl derivatives ofbarbaticacid arrested the spore germination of filamentous fungi *(Usneaflexilis)*.From this we suggest that barbatic acid and its acetyl derivative of barbatic acid shows more or less similar activity, they can be used as antibiotic.

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