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Synthesize, characterize and evaluation of anti-bacterial and anti-fungal activity of thiazines

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ABSTRACT

1,3 – Thiazines have been applied as useful starting materials in the stereo selective synthesis of compounds of pharmacological interest and they have served as chiral ligants and auxiliaries in enantioselective transformation. The thiazines are of great importance because they act as precursor for the synthesis of Cephalosporins (3,6-dihydro-2H-,1,3-thiazine) and then converted to the thiazine derivatives (an heterocyclic compounds). Therefore, it was thought to combine chalcones moiety to thiazine derivative together in a molecular frame work to see the biological activities, additive effort of these rings towards biological activities like Anti- bacterial and Anti- fungal activities.

Keywords: Thiazines; 1,3 – Thiazines; Cephalosporins; anti-bacterial; anti-fungal.

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INTRODUCTION

Thiazines a heterocyclic compound having four carbon atoms and one nitrogen atom and sulphur atom at varied positions in the six membered ring exist as 1,2- thiazine,1,3-thiazine and 1,4- thiazine and subsequently their derivatives having N-C-S linkage have been used as anti-tubercular, anti-bacterial, anti-microbial, anti-tumor, insecticidal, fungicidal, herbicidal agents, transquilizers and various dyes etc[5-7], anti-parkinsonian[8], calcium channel modulators, Anti- pyretic, anti-mycobacterial agents, Cannabinoid receptor agonists and Anti- Oxidant agents.

The heterocyclic compounds which contain nitrogen and sulphur posses an enormous significance in the field of medicinal chemistry. Many researchers have synthesized different thiazines derivatives that exhibit various biological activities such as anti-tubercular, anti-fungal, anti-bacterial, analgesic, anti-inflammatory etc. Some thiazine derivatives in the development phase due to their versatility of the thiazine skeleton, it's chemical simplicity and accessibility. Many compounds of thiazines were known as phenothiazines. Phenothiazines are used as vermifuge for live stock and also as an insecticide.[11]

The ability to treat bacterial infections which chemotherapeutic agents represents one of the most important medical achievement of the last millennium. Unfortunately the emergence of micro-organisms resistant to anti- bacterial agents has been continued to the present day. It was well known that bacteria produce beta–lactamases and these enzymes had the natural role of destroying beta–lactam antibiotics. The latest twist to this increasingly alarming situation has been the recent isolation of methicillin resistant strains of Staphylococcus aureus.

The fungal diseases of humans are mainly divided into three groups. The first type is dermatophytoses caused by epidermaphyton species, microsporium species and tycophyton species consist of contagious superficial skin infections that are limited to the epidermal region. The second type is Candidiasis caused by Candida species can affect the skin, nails and mucous membranes and occasionally becomes systemic. The third type is subcutaneous, pulmonary, lymphatic and systemic mycoses caused by

Aspergillus species and Candida species invade the skin, lungs, lymphatic tissue and various organs of susceptible hosts who have accidentally been in contact with the fungal environment. There are increasing detection of systemic mycoses in patients suffering from delilitating diseases such as neoplasias in persons on long team parenteral nutrition.

Additionally, conditions that depress cell mediated immunity, such as the latter half of pregnancy, excessive use of corticosteroids and immunosuppressive and defects in thymus gland tissue, results in marked enhancement of susceptibility to serious fungal diseases. Many remedies have been used against fungal infections but the ideal Anti-fungal agents has not yet been found.

The 1,3-thiazine nucleus is active core of Cephalosporin which are among the widely use beta-lactam anti- biotics. A large group of dyes has phenothiazine structure, including methylene blue thiazine are used for dyes, transquilizers. Thiazine can help to reduce some of that extra water weight you may be holding on to in stomach. Thiazine is a fairly basic diuretic supplement, it reduces water and increase vascularity, so it is use as anabolic agent in medicine.[12]

MATERIALS AND METHODS

Chemicals: Ethanol, Cyclohexane, Benzaldehyde, Potassium hydroxide, Sodium hydroxide, Thiourea, Di methyl formamide, Ethyl acetate, Benzene, Chloroform, Agar- Agar, Sodium chloride, Beef extract, Peptone.

Preparation of 2, 6-di Benzelidene Cyclohexanone

The mechanism involved in the preparation of chalcones is by Claisen Schmidt reaction. The reaction between an aldehyde (R-CHO) or ketone (R=O) and an aromatic carbonyl compound lacking an Alpha-hydrogen is called the Claisen Schmidt reaction. A mixture of 30 ml of 10% sodium hydroxide,50 ml of ethanol and 0.01 mol of cyclohexanone placed in a 250 ml beaker, which was immerse in ice bath as well as equipped with a mechanical stirrer. The stirrer was started, 0.02 mol of benzaldehyde was added to the mixture and stirring continued. After 2 hours, the stirrer was removed and the reaction mixture was kept in an ice cold overnight. The product was filtered, washed with ice cold water, followed by ice cold ethanol, dried and recrystallised from DMF or ethanol.

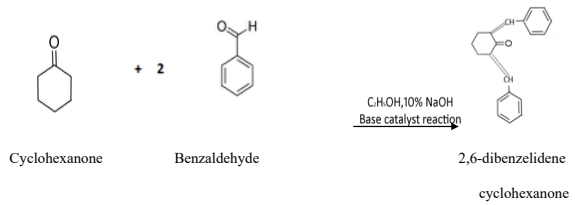


Figure 1: Preparation of 2, 6-di Benzelidene Cyclohexanone

Recrystallization for synthesized chalcone

The product or sample is taken in a conical flask to this add quantity sufficient of ethanol until it gets dissolve on heating, filter the product to remove the impurities in the presence of heating, and cool the solution, yellow colour needle shape crystals are obtained.[38]

Preparation of 2- Imino-8-benzylidene-4-phenyl 5,6-dihydro- 4H, 7H- (3,1) benzothiazine

A mixture of 0.01 mol of 2,6- dibenzylidene-cyclohexanone 0.015 mol of thiourea and 0.01 mol of potassium hydroxide dissolved in 10 ml of water was refluxed in ethanol for 16 hours. Later ethanol was removed under reduced pressure and the residue was treated with ice cold water. The precipitate thus obtained was filtered, washed with water, dried and recrystallized from ethanol.

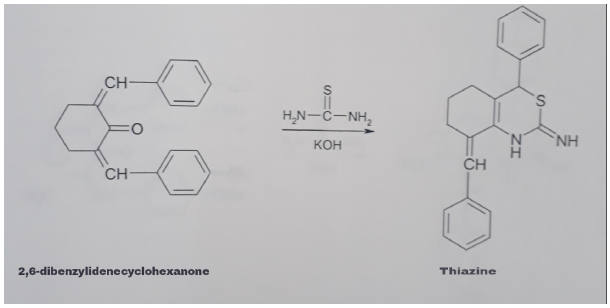


Figure 2: Preparation of 2- Imino-8-benzylidene-4-phenyl 5,6-dihydro- 4H, 7H- (3,1) benzothiazine

Recrystallization for synthesized thiazine

The product or sample is taken in a conical flask to this add quantity sufficient of ethanol until it gets dissolve on heating, filter the product to remove the impurities in the presence of heating, and cool the solution yellow colour needle shape crystals are obtained.[38]

Anti–bacterial Activity

Method followed- Agar diffusion method.

Working Procedure- Stock solutions of the synthesized compounds and standard drugs used were prepared dimethylsulfoxide taken in the concentration of µg/0.1 ml.

Microorganisms used-Standard cultures of gram positive bacteria Staphylococcus aureus and Bacillus subtilus and gram negative bacteria Escherichia coli and Pseudomonas aeruginosa species were taken. The microorganisms were identified by various staining techniques and bio-chemical reactions. The microorganisms were maintained by sub- culturing and used at regular intervals in nutrient agar medium.

Preparation of inoculum- The suspension of all the organisms were prepared as per Mac-Farland Nephlometer standard (Baily and Scott 1990). A 24 hour old culture was used for the preparation of bacterial suspension. Suspensions of organisms were made in sterile isotonic solution of sodium chloride (0.9%w/v) and the turbidity was adjusted.

Preparation of assay medium

Beef extract 4.0, Peptone 5.0, Agar 20.0, Distilled water 1000ml, mentioned quantities of different ingredients were accurately weighed and dissolved in appropriate amount of distilled water. Media so prepared was sterilized by autoclaving at 120°c for 15 minutes. The pH should be maintained at 5.4.

Procedure: The Petri dishes were thoroughly washed and sterilized in hot air oven at 160°c for 1hour. Inoculum was added to 30 ml of sterile nutrient agar medium and was poured into petri dishes for solidifying. Bores were made on the medium using sterile bore. 0.1 ml of test solution was added to the respective bore, 0.1ml of the ampicillin at a concentration of 100 µg/0.1 ml was taken as standard reference. A control having only DMSO in the cup was maintained in each plate. The Petri dishes were kept in the refrigerator at 4°c for 15 minutes for diffusion to take place. After diffusion, the petri dishes were incubated at 37°c for 24 hours and zone of inhibition were observed and measured using a scale. Antibacterial Activity of the compound was carried out against all four microorganisms. The same media was used for sub-culturing and for estimating anti-bacterial activity.

Anti-fungal Activity

Working procedure-Stock solutions of the synthesized thiazines and standard drug were prepared in DMSO in the concentration of 100 µg/0.1ml.

Fungi used-Standard cultures of Candida albicans and Aspergillus niger were taken. The fungi were maintained by subculturing and used at regular intervals.

Sabourd's agar medium: 40mg of Sucrose, 10mg of Peptone, 20 mg of Agar, 1000ml of Distilled water, This medium was found for both sub-culturing and also for estimating the antifungal activity. The pH of the medium plays an important role for the growth of fungi. Acidic medium favours the growth but excess of acid may not come agar to solidify. Hence the pH of a medium was adjusted using 0.1% lactic acid. The pH should be maintained at 5.6.

Preparation of assay medium: The mentioned quantities of a different ingredients were accurately weighed and dissolved in water. The prepared medium was sterilized in autoclave at 121^0c for 15 minutes.

Working procedure: An inoculum was prepared by suspending a single isolated colony in about 5 ml of normal saline This is mixed slowly to achieve a smooth suspension. Later one drop of tween 20 was added for filamentous fungi and the mould was broken by shaking. A sterile cotton swab was moistened in the inoculums suspension and excess of moisture was removed by rolling the cotton swab on the inside of the tube, above fluid level 30 ml of sterile hot Sabouraud's agar medium was poured in each plate and allow to harden on a level surface. The surface of Sabouraud's agar medium plate was streaked with the help of moistened cotton swab in all the direction ions. The surface of Sabouraud's agar plate was dried out 35^0C. Later 5 bores per plate were made using sterile cork borer. The above operation was carried out in aseptic condition and 0.1 ml test solution was added to the respective bore and 0.1 ml Amphotercin was taken as standard reference. A control having only DMSO was maintained in each plate. The plates are incubated at 35^0 c for 48 hours. Later the values of zones of inhibition were recorded.[41-50][13]

RESULTS AND DISCUSSION

Thin layer chromatography

Solvent front = 4.7 cm

Spot 1: Chalcone = 4.2 cm R_f = 0.93

Spot 2: Thiazine = 4.3 cm, R_f =0.91

Antibacterial activity: structural activity relationship

The antibacterial activity of newly synthesized Chalcone or 2,6-dibenzylidene cyclohexanone and thiazine derivatives were evaluated against gram positive bacteria that is Staphylococcus aureus and Bacillus subtilis and gram-negative bacteria that is Escherchia coli and Pseudomonas aeruginosa. The standard drug used as Ampicillin.

The zone of inhibition shown by the compound 2,6-dibenzylidene cyclohexanone or Chalcone against Bacillius subtilis is 17 mm. Among all the compounds 2-imino-8-benzylidine-4–phenyl-5,6-dihydro 4H,7H(3,1) benzothiazine is 21 mm, had shown highest zone of inhibition indicating that introduction of groups such as N &S enhances the activity against the Bacillus subtilius.

Table 1: Characteristic of Cyclohexanone and 2,6-dibenzylidene cy-clohexanone

S.no	Name of the compounds	Characteristic
UV	Cyclohexanone	287 nm
UV	2,6-dibenzylidene cyclohexanone	328.5 nm
IR (δ_{max})	2,6-dibenzylidene cyclohexanone	1660 cm^{-1} (C=O)
IR (δ_{max})	2,6-dibenzylidene cyclohexanone	1656 cm^{-1} (C=O)
NMR	2,6-dibenzylidene cyclohexanone	δ7.2-7.5(10H, ArH), δ7.6 (1H, proton of benzylidene group), δ7.8(1H, proton of benzylidene group), δ3.5 (1H, proton of C-3), δ2.9-3.1(2H, proton of C-5), δ1.6-1.9(2H, proton of C-4)
UV	2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine	284 nm
IR	2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine	3300-3450 (=NH), 3100-3250 (NH), 1678-1681 (C=N), 1370-1380 (C-N), 1570-1580 (Aromatic)
NMR	2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine	δ7.3-7.5 (10H, ArH), δ7.6 (1H, proton of benzylidene group), δ6.6 (1H, proton of imino group), δ6.8 (1H, proton of =NH group), δ5.0 (1H, proton of S-CH group), δ3.3 (1H, proton of C-7) δ1.8-2.8 (2H, protons of C-5), δ1.3-1.4(3H, protons of C-6)
Mass	2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine	M+331 m/e 303,269,242,91,77

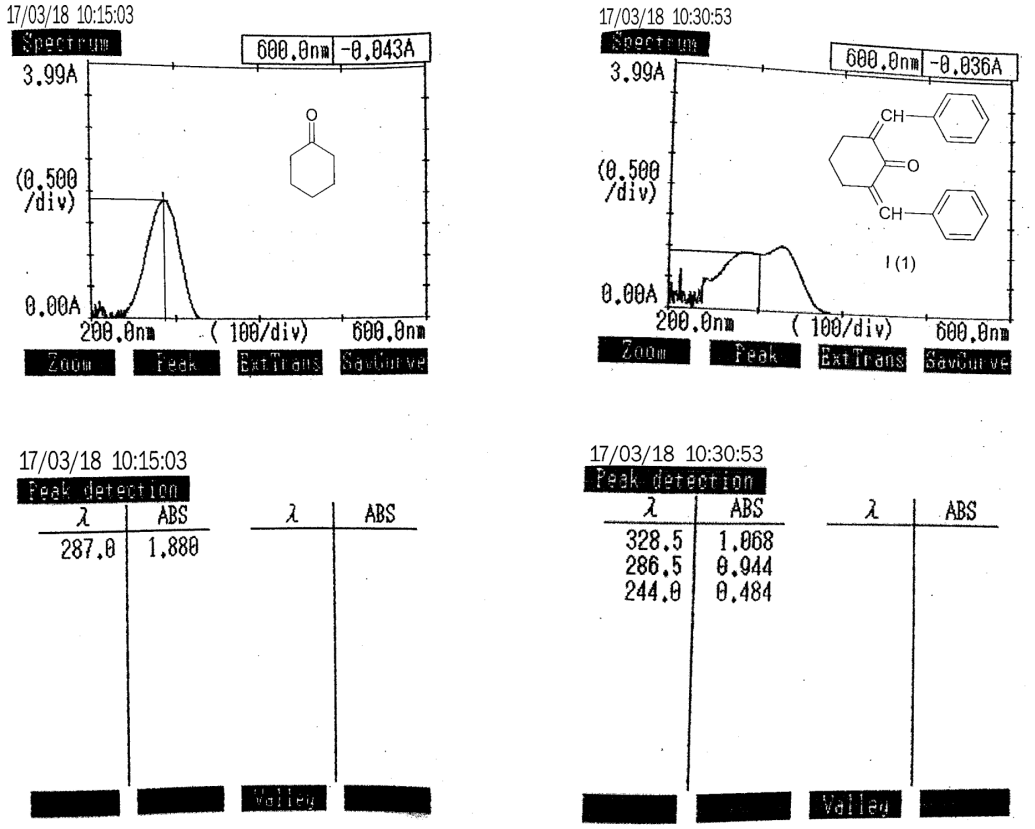


Figure 3: UV spectrum of Cyclohexanone and 2,6-dibenzylidene cyclohexanone

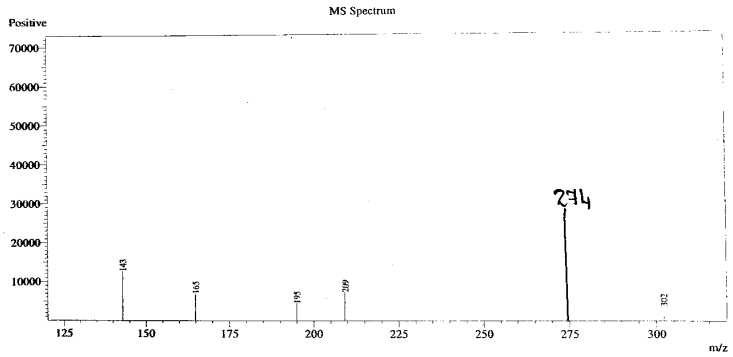


Figure 4: Mass spectrum of Cyclohexanone

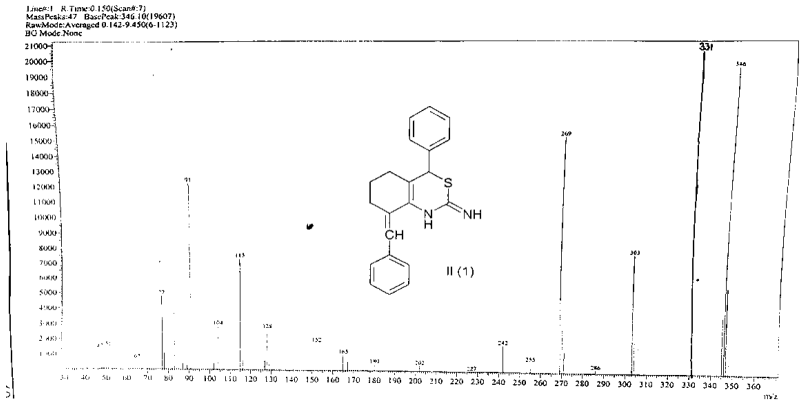
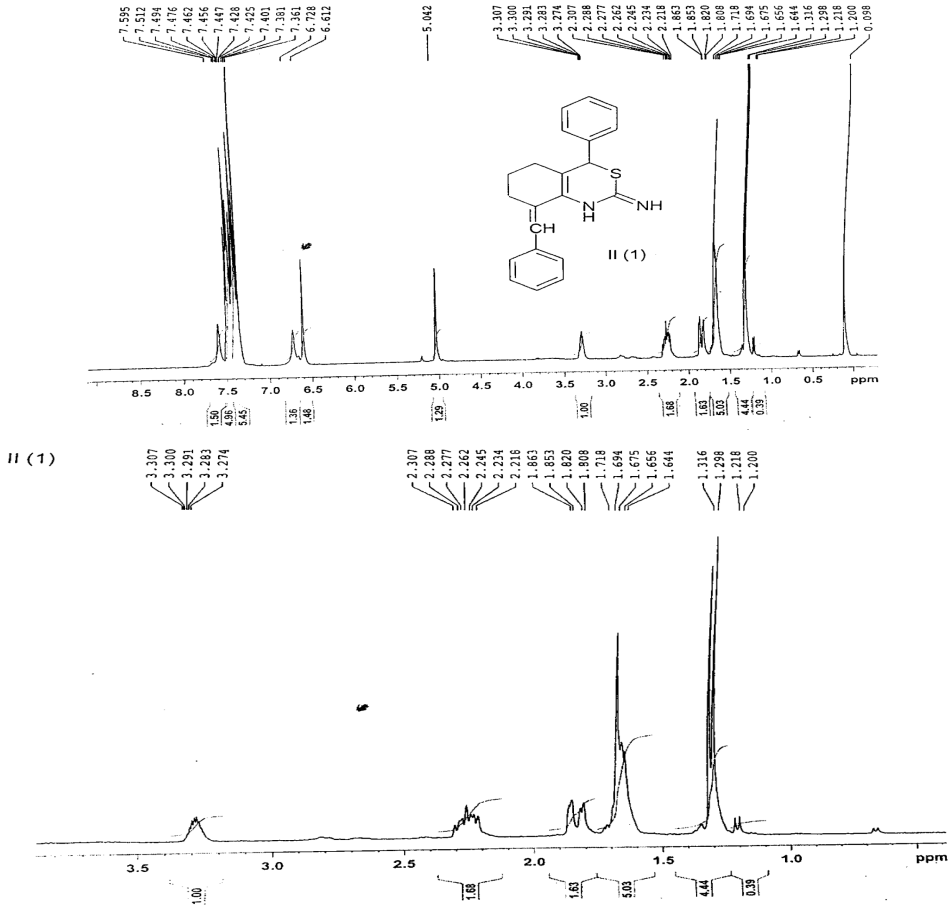


Figure 5: Mass of 2,6-dibenzylidene cyclohexanone



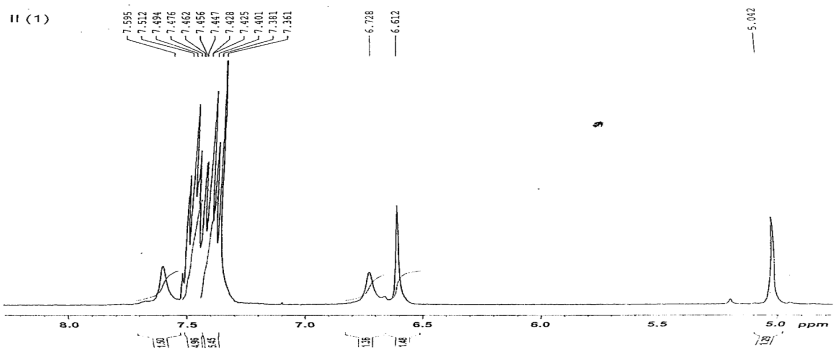


Figure 6: NMR spectrum of 2,6-dibenzylidene cyclohexanone

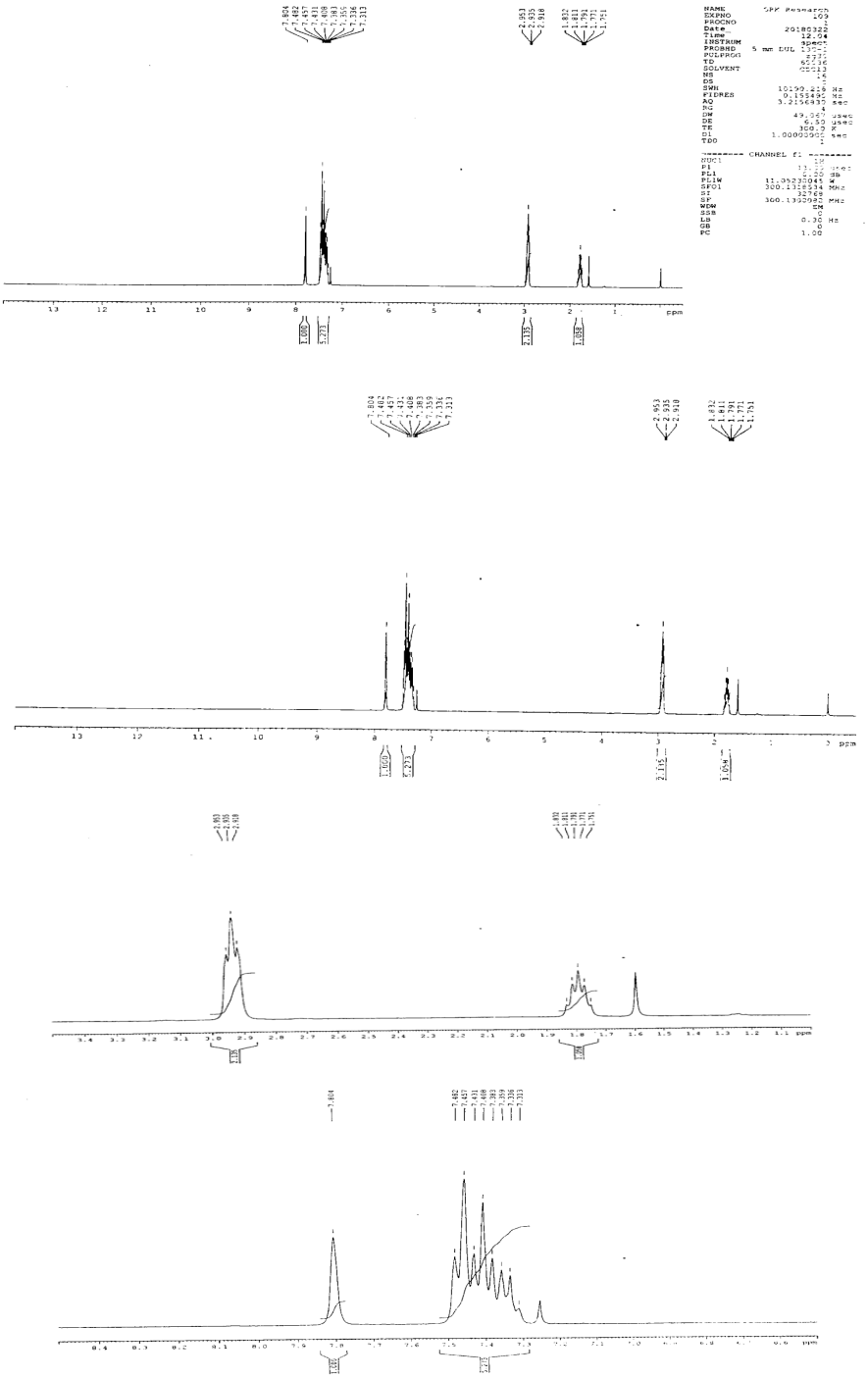


Figure 7: NMR spectrum of Cyclohexanone

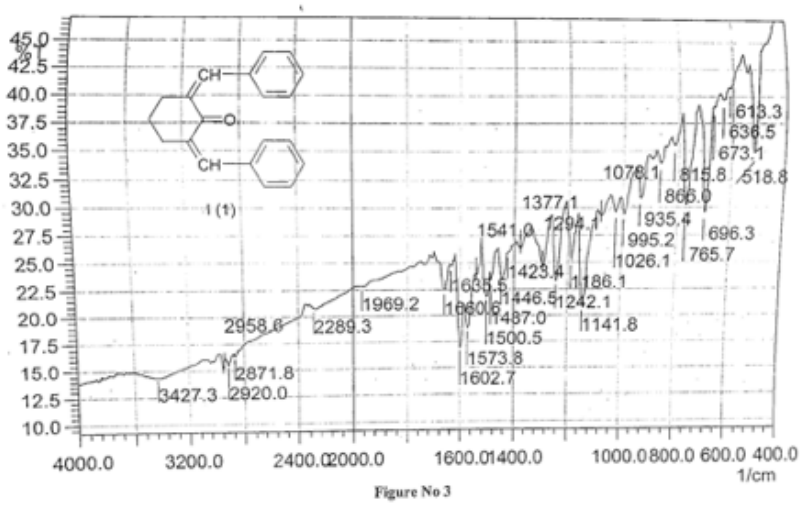


Figure 8: IR spectrum of Cyclohexanone

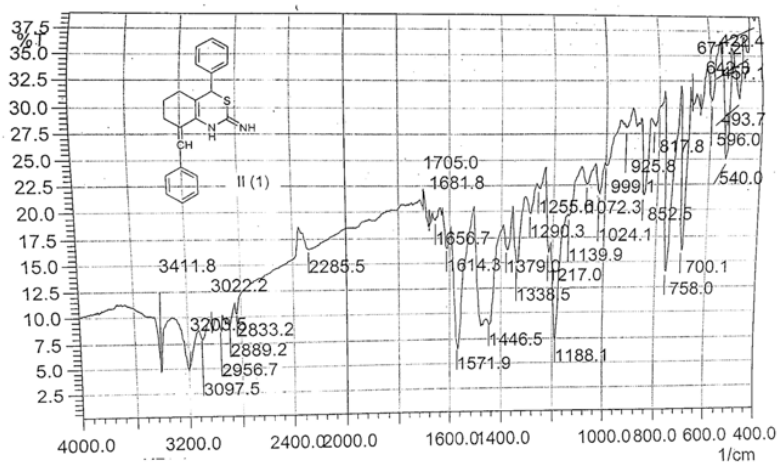


Figure 9: IR spectrum of 2,6-dibenzylidene cyclohexanone

Table 2: Antibacterial activity of Compounds

Sl.no	Name of the Compound	Zone of inhibition of bacillus subtilis	Zone of inhibition of staphylococcus aureus	Zone of inhibition of escherichia coli	Zone of inhibition of pseudomonas aeruginosa
1	2,6-dibenzylidene cyclohexanone	17	15	12	13
2	2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4h,7h-(3,1) benzothiazine	21	18	15	17
Control	Dmso	-	-	-	-
Std	Ampicillin	37	30	32	31

Table 3: Antifungal activity of Compounds

S.no	Name of the compound	Zone of inhibition of candida albicans	Zone of inhibition of aspergillus niger
1	2,6-dubenzylidene cyclohexanone	13	14
2	2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4h,7h-(3,1) benzothiazine	15	18
Control	Dmso	-	-
Standard	Amphotericin b	19	17

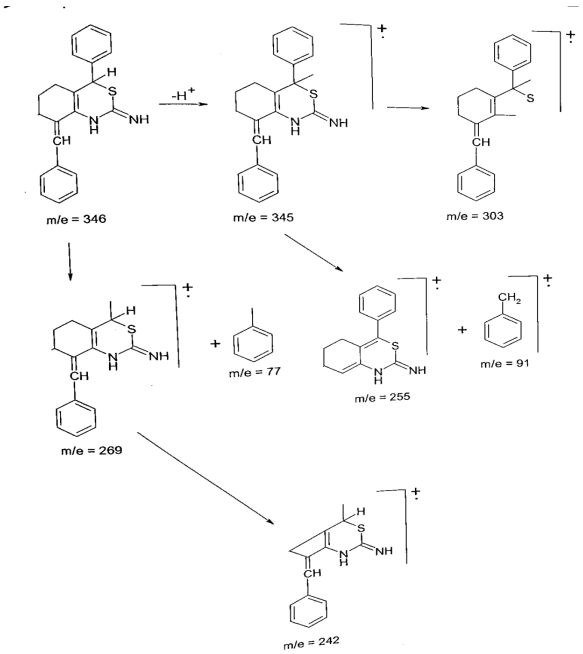


Figure 10: fragmentation pattern of 2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine

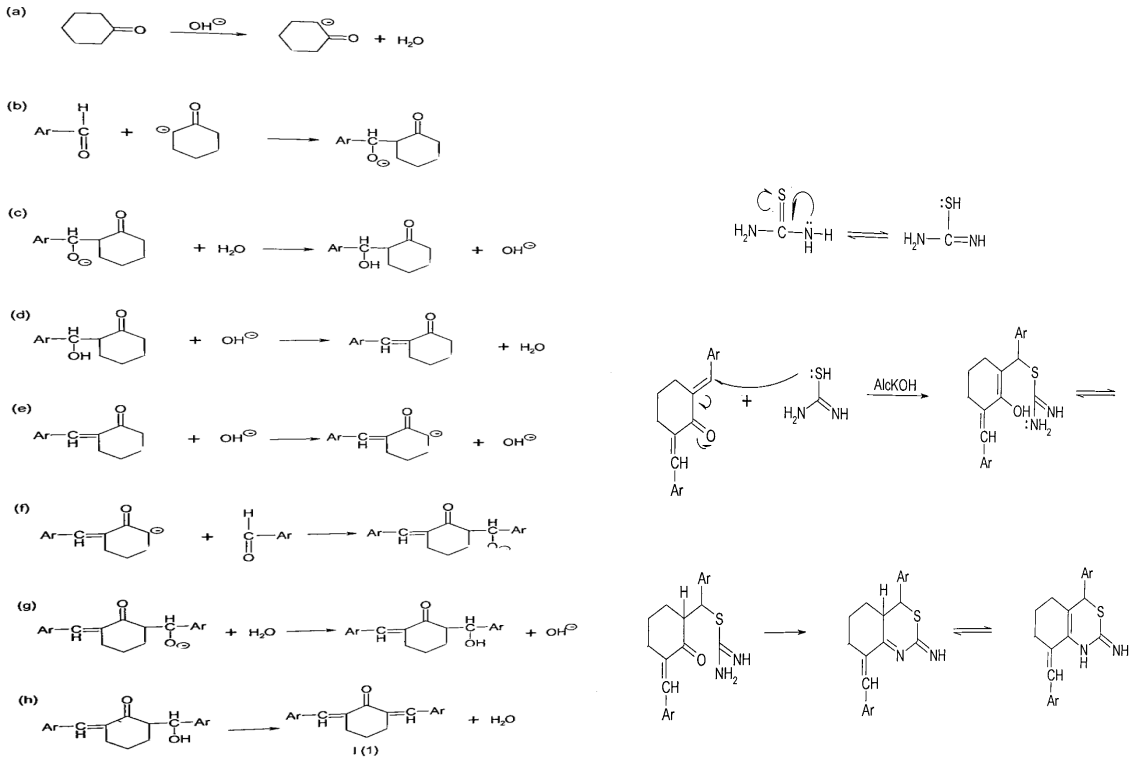


Figure 11: Preparation of 2-imino-4-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazinefrom cyclo-hexanone

The zone of inhibition exhibited by the compound against staphylococcus aureus is 15 mm. Among all the compounds one derivative that is had shown the activity against Staphylococcus aureus indicating the introduction of nitrogen and sulphur in enhances the activity .

The zones of inhibition shown by the compound against Escherchia coli and Pseudomonas aeruginosa are 12 and 13 mm respectively. Among all the compounds exhibited maximum zones of inhibition against Escherchia coli and Pseudomonas aeruginosa by 15 and 17 mm respectively, indicating the introduction of nitrogen and sulphur in enhances the activity against gram negative bacteria.

The synthesized compound 2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1)benzothiazine

shown anti-bacterial activity but none of them had greater activity than standard reference, Ampicillin.

Anti-fungal activity: structure activity relationship

The antifungal activity of newly synthesized chalcone and thiazine derivatives were evaluated against two fungi that is Candida albicans and Aspergillus niger. The standard reference drug was Amphotericin B.

The zone of inhibition shown by the compound 2,6-dibenzylidene cyclohexanone against Candida albicans is 13mm. The compound 2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine is 15 mm and had shown the highest zone of inhibition indicating that the introduction of nitrogen and sulphur enhances the activity.

The zone of inhibition shown by the compound Aspergillus niger is 15mm. The compound 2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H(3,1) benzothiazine is 18 mm and shown the maximum activity against Aspergillus niger indicating the introduction of nitrogen and sulphur indicating that introduction of nitrogen and sulphur enhances the activity.

The thiazine had shown the antifungal activity against Candida albicans and Aspergillus niger but none of the compounds had greater potency than the standard reference, Amphotericin B

DISCUSSION

The compound 2,6-dibenzylidene cyclohexanone has been prepared by condensing one mole of cyclohexanone with two moles of benzaldehyde. The formation of 2,6-dibenzylidene cyclohexanone has been indicated by its UV spectrum. The starting material cyclohexanone exhibited λ_{max} at 287 nm. The compound 2,6-dibenzylidene cyclohexanone exhibited λ_{max} at 328.5 nm. This clearly indicate that the bathochromic shift may be attributed because of =CH-AR chromophore.

The compound 2,6-dibenzylidene cyclohexanone has been indicated by its infrared spectra spectrum. The starting material exhibit γ max at 1680 cm^{-1} due to C=O group. The appearance of 2,6-dibenzylidene cyclohexanone exhibited γ max at 1660 cm^{-1} due to C=O group. The appearance of a characteristic bond at C=O is mainly due to α,β, and α΄,β' unsaturation. This clearly indicate the formation of 2,6-dibenzylidene cyclohexanone.

The formation of 2,6-dibenzylidene cyclohexanone has also been indicated by its nuclear magnetic resonance spectrum. The presence of signals at δ7.2-7.5 (10 H, aromatic protons, δ7.6 (1H, proton of benzylidene group), δ7.8 (1H, proton of benzylidene group), δ3.5 (1H, proton of C-3), δ2.9-3.1 (2H, protons of C-5) and δ1.6-1.9 (2H, protons of C-4) clearly indicate the formation of 2,6-dibenzylidene cyclohexanone.

The compound 2- imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine has been prepared by cyclocondensation of 2,6- dibenzylidene cyclohexanone with thiourea in the presence of potassium hydroxide. The formation of 2- imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine has been indicated by its UV spectrum. The compound 2,6-dibenzylidene cyclohexanone exhibited λ_{max} at 328.5 nm. The compound 2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine exhibited λ_{max} 284 nm. This clearly indicate that the hypsochromic shift may be attributed because of cyclocondensation.

The formation of 2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine has been indicated by its infrared spectrum. The compound 2,6-dibenzylidene cyclohexanone exhibited a characteristic bond at 1660 cm^{-1} (C=O). The absence of 1660 cm^{-1} and presence of 3411 (=NH) and 3203 (=NH) clearly indicate the formation of 2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine. The formation of 2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1)benzothiazine has also been indicated by its nuclear magnetic resonance spectrum. The presence of signals at δ7.3-7.5 (10H, aromatic proton of benzylidene group), δ6.6 (1H, proton of imino group), δ6.8 (1H, proton of =NH group), δ5.0 (1H, proton of S-CH group), δ3.3 (1H, proton of C-7), δ1.8-2.8 (2H,protons of C-5), δ1.4-1.5 (2H, protons of C-6) clerly show the formation of 2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine.

The formation of 2-imino-8-benzylidene-4phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine has also been confirmed by its mass spectrum. The molecular ion peak of 2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine has been at 331, which is in good agreement with the calculated molecular weight of the compound. The probable fragmentation pattern of 2-imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine has been depicted as shown below.

CONCLUSION

The method followed for the synthesis of Chalcone / 2,6-dibezylidene cyclohexanone is by Claisen Schmidt reaction and the yield was found to be 2.67 grams. The method followed for the synthesis of Thiazine/ 2-Imino8-benzylidene-4-phenyl -5,6-dihydro-4H,7H-(3,1) benzothiazine is by Michael addition mechanism and the yield was found to be 3.43 grams. The Anti-microbial activity such as Anti- bacterial activity and Anti- fungal activity of Chalcone/ 2,6-dibenzylidene cyclohexanone was evaluated. The Anti-microbial activity such as Anti- bacterial activity and Anti- fungal activity of Thiazine or 2- Imino-8-benzylidene-4-phenyl-5,6-dihydro-4H,7H-(3,1) benzothiazine was evaluated.

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