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SYNTHESIS AND SCREENING FOR ANTIMICROBIAL ACTIVITY OF NOVEL BENZIMIDAZOLE CHALCONE

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ABSTRACT
Benzimidazole ring is an important pharmaco-phor in modern drug discovery. The synthesis of novel benzimidazole derivatives remains a main focus of medicinal research as there are wide clinical applications. The benzimidazole derivatives have different pharmacological activities like Antifungal, Neuroleptic, Anti-HIV, Antihistaminic, Antiulcer, Antimicrobial, Anthelmintic, Cardio tonic & Antihypertensives. This article was summarized to know about the chemistry of different derivatives of substituted benzimidazoles along with their pharmacological activities. Novel Benzimidazole Chalcone derivatives were synthesized by using different Aromatic aldehydes like 2-Nitro Benzaldehyde, 4-Nitro Benzaldehyde, and 4-Chloro Benzaldehyde. The reaction between o-phenylenediamine and lactic acid in presence of HCl as catalyst yields first intermediate which upon oxidation with $k_2cr_2o_7$ forms second intermediate and by the addition of different aromatic aldehydes, it yields 2-Nitro Benzimidazole chalcone, 4-Nitro Benzimidazole chalcone, and 4-Chloro Benzimidazole chalcone respectively. All these synthesized compounds were screened for *in vitro* antimicrobial activity and all of them had shown potency against both gram positive and gram negative bacteria.

KEYWORDS: Benzimidazole Chalcone, Benzaldehyde, o-phenylenediamine, lactic acid, Antimicrobial activity.

INTRODUCTION
Benzimidazole is a heterocyclic aromatic organic compound and is bicyclic in nature consisting of a fusion of benzene and imidazole, which has been used as a carbon skeleton for *N*-heterocyclic carbenes, usually used as ligand for transition metal complexes. [1]

Benzimidazole is commercially available. The usual synthesis involves condensation of o-phenylenediamine with formic acid, or the equivalent trimethyl orthoformate: C_6H_4 $(NH_2)_2$ + HC $(OCH_3)_3$ → C_6H_4N (NH) CH + 3 CH_3OH
By altering the carboxylic acid used, this method is generally able to afford 2-substituted Benzimidazoles. [2]

In recent years, considerable attention has been given to the synthesis of Benzimidazole derivatives because of their various pharmacological activities. [3]

Various substitutions on this benzimidazole nucleus were found to have wide applications as Pesticides and herbicides. Compounds bearing Benzimidazole moiety are reported to possess a number of interesting biological activities like Anti-tubercular, Anti-cancer, Anti-helmintic, Anti-allergic, Anti-histaminic, Anti-microbial, Anti-oxidant.

Chalcone is an aromatic ketone and an enone that forms the central core for a variety of important biological compounds, which are known collectively as chalcones or chalconoids. [4]

Despite significant progress in Antimicrobial therapy, infectious diseases caused by bacteria and fungi remain a major worldwide health problem due to the rapid development of resistance to the existing Antimicrobial drugs (Antibacterial and Antifungal). In other words, the increasing use and misuse of existing Antimicrobial drugs has resulted in the development of resistant pathogens.
From this information an innovative thought was created in us to perform work on this Benzimidazole compounds. There by we framed work to synthesize Novel Benzimidazole Chalcone that produces Antimicrobial activity.

MATERIALS

Chemicals

Lactic acid, HCl (4N), Silica gel-G, Peptone, Potassium dichromate, Glacial acetic acid, Ammonium solution, 10% NaOH, 4N NaOH of LR grade from Finar were obtained.

Toluene, 2-Nitro benzaldehyde, 4- Nitro benzaldehyde, 4- Chloro benzaldehyde (Otto chemical, bio chemical Reaction), Nutrient Agar (Qualikem finechem Pvt. ltd.), Beef extract (Molychem), NaCl (NR chem), O-Phenylene diamine, Methanol (Jiangsu huaxi international trade co, ltd), Ethanol (Research lab fine chem. industry).

Equipment: Laminar air flow chamber (Kem), Autoclave (Kadavil electro mechanical industry), Incubator (Bio techniques India), Magnetic stirrer (Remi ZMCH), Hot Air Oven (Kemi), and Antibiotic zone reader.

Microorganism: All these tested strains are reference strains and were collected from NCIM (National Collection of Industrial Microorganisms).

Gram-ve:

Escherichia coli: (NCIM) 2286

Pseudomonas aeruginosa: (NCIM) 2037

Gram +ve:

Bacillus subtilis: (NCIM) 2710

Staphylococcus aureus: (NCIM) 2799

This experiment is performed at Vijaya Institute of Pharmaceutical Sciences for Women in academic year 2013-14.

METHODS: [5, 6, 7, 8]

SYNTHESIS OF BENZIMIDAZOLE CHALCONE[9]

Preparation of 2-hydroxy ethyl benzimidazole

Equi-molar fraction of o-phenylenediamine and lactic acid were refluxed for 7 hr in the presence of 4N HCl and the completion of reaction was determined by performing TLC. Then the product was recrystalized with methanol. Melting point was determined.

Preparation of 2- acetyl benzimidazole

2-hydroxy ethyl benzimidazole was taken and then it was oxidised in presence of potassium dichromate and refluxed for 4 hr with glacial acetic acid. Completion of the reaction was determined by TLC. After refluxing the product it was cooled and neutralized by adding ammonia solution and then it was dried. Recrystalization was performed and the melting point was determined.

Preparation of 3-(1H-benzimidazole-2-yl)-3-hydroxy-1-(1H-indazole-3-yl)-butan-1- one

Acetyl benzimidazole was taken and 10% NaOH was added and then refluxed for 4 hr after refluxing, glacial acetic acid was added, filtered and recrystallization was performed and melting point was also determined.

Preparation of benzimidazole chalcone

This ketone obtained from 3rd step was condensed with aryl aldehydes (2-Nitro benzaldehyde, 4-Nitro benzaldehyde and 4-chloro benzaldehyde). The neutralization of the reaction mixture was carried out by dilute acetic acid followed by recrystallization, gave the final compounds with yields 70%. The chosen aryl aldehydes (10 mmol) was added to 2-acetylbenzimidazole (1.5 g, 10 mmol) in ethanol solution of sodium hydroxide (75 mmol sodium hydroxide in 40 ml of ethanol). The reaction mixture was subsequently stirred at room temperature for 5 h and neutralized with a solution of 30% acetic acid leading to a precipitate. It was filtered, dried and recrystallized in toluene and toluene/EtOH (4:1) to give final compound. Thus, 4-Nitro Benzimidazole chalcone (*Fig.No:1*), 2-Nitro Benzimidazole chalcone (*Fig.No:2*), and 4-Chloro Benzimidazole chalcone (*Fig.No:1*) were synthesized.

4-Nitro Benzimidazole Chalcone

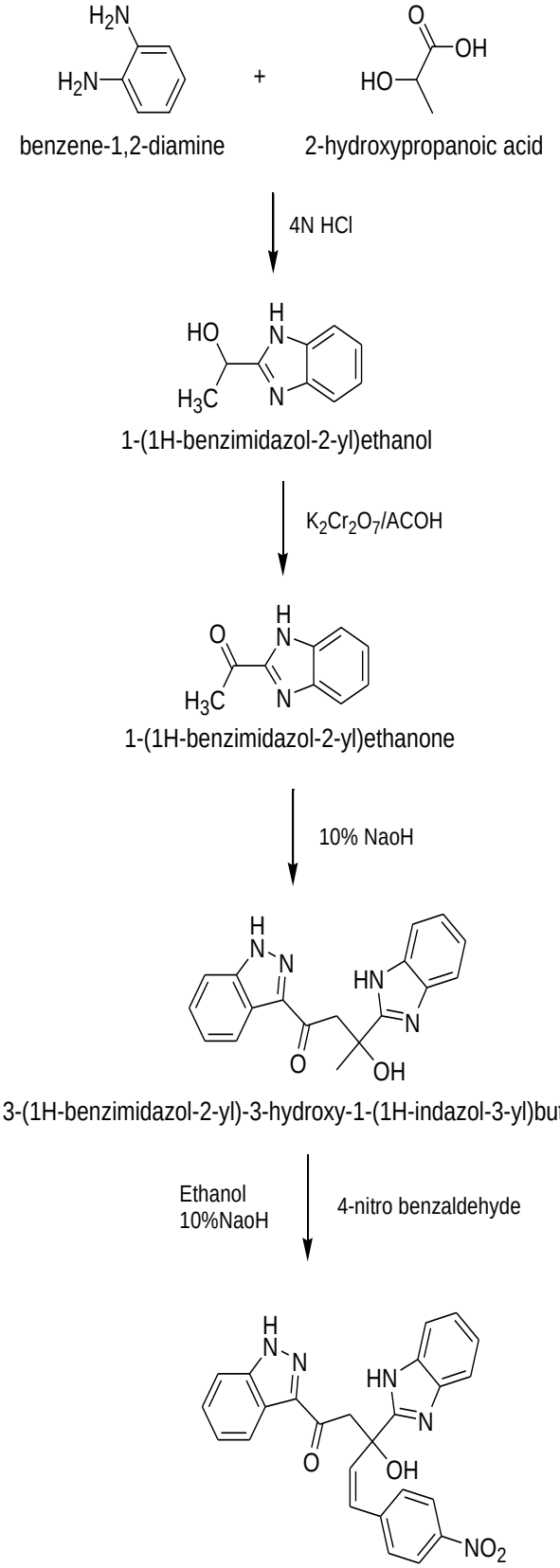


Fig. No: 1- Synthesis of 4-Nitro Benzimidazole Chalcone

2-Nitro Benzimidazole Chalcone

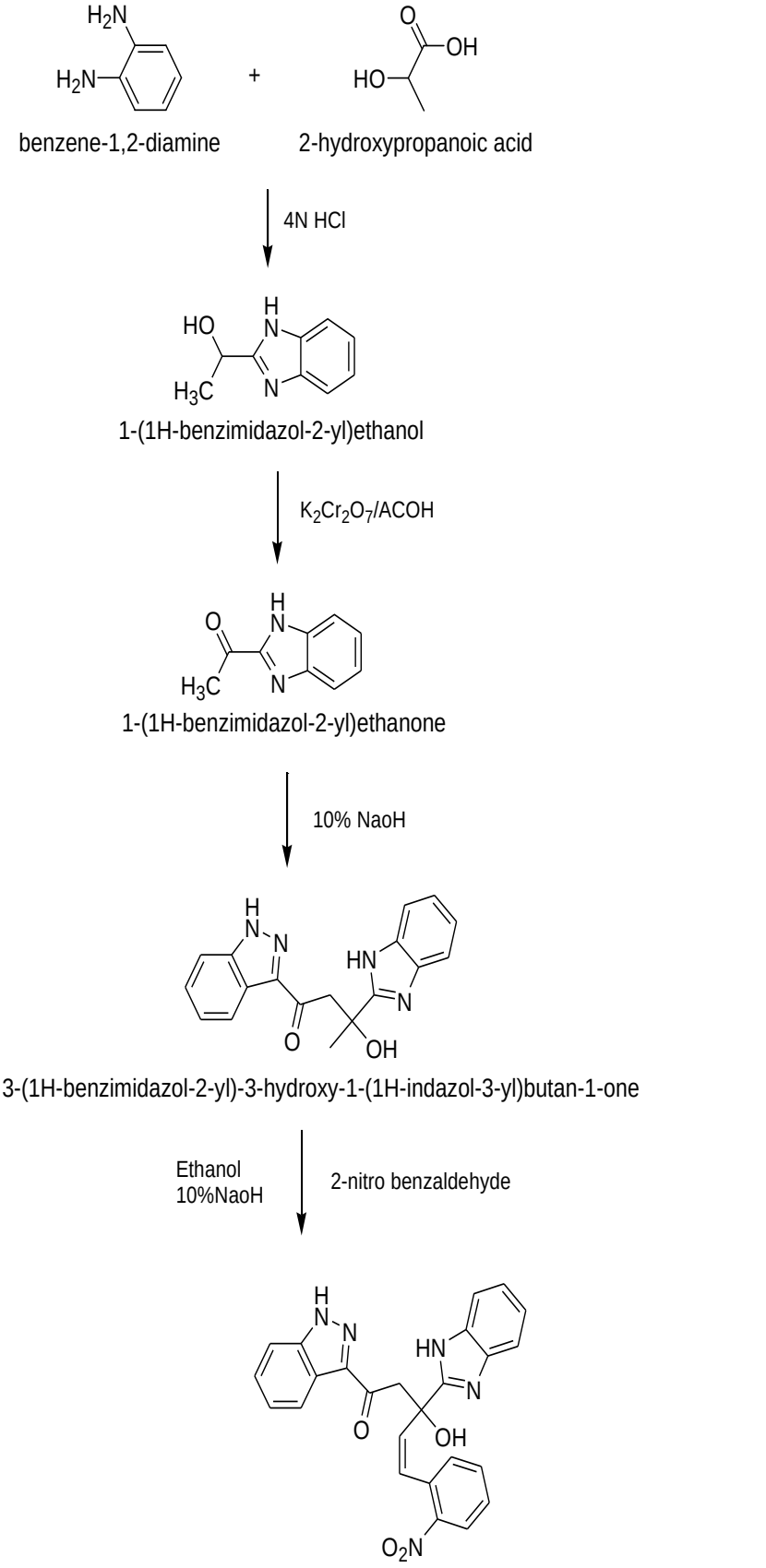
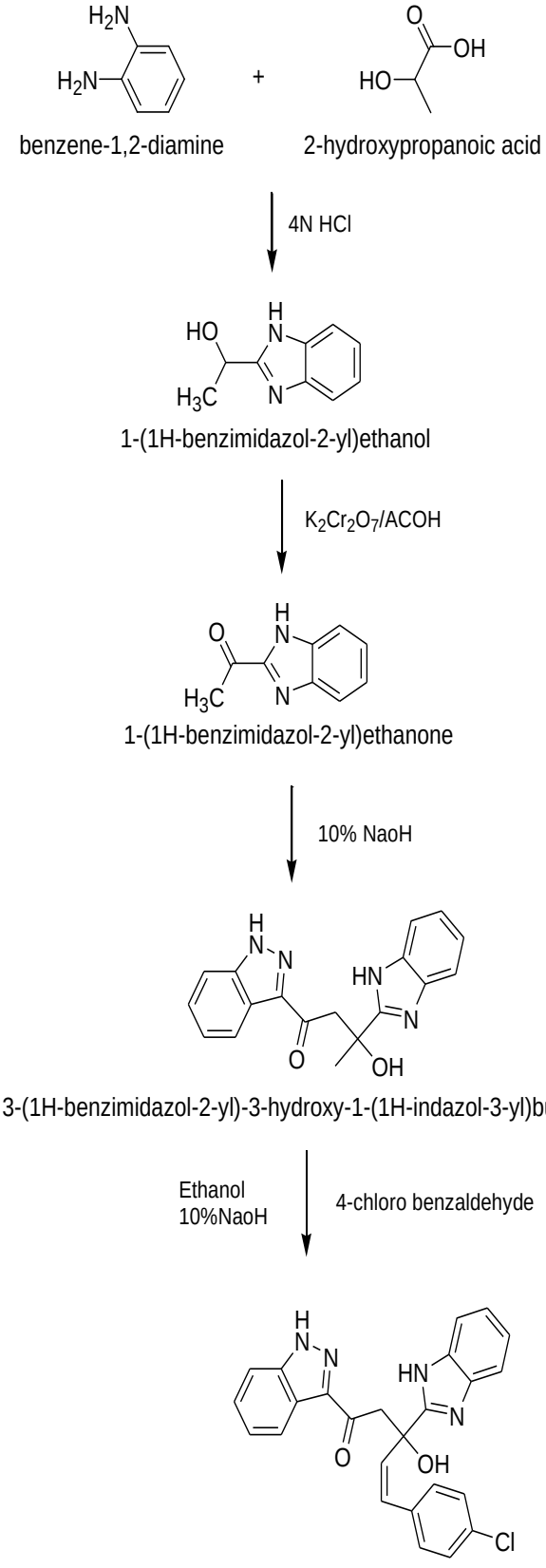


Fig. No: 2- Synthesis of 2-Nitro Benzimidazole Chalcone

4-Chloro Benzimidazole Chalcone



3-(1*H*-Benzoimidazol-2-yl)-5-(4-chloro-phenyl)-3-hydroxy-1-(1*H*-indazol-3-yl)-pent-4-en-1-one

Fig. No: 3- Synthesis of 4-chloro Benzimidazole Chalcone

Preparation of TLC Plates: [10, 11]

Principle: Thin layer of chromatography is a method of analysis in which the stationary phase is spread as a thin layer on a rised supporting plate and a mobile phase; a lipid is allowed to migrate across the surface of plate.

TLC is a very commonly used technique in synthetic chemistry for identifying compounds, determining their purity and the progress of a reaction.

Procedure

1. **Coating material:** As stationary phase, a special finely ground matrix (silica gel, alumina or similar materials) was coated on a glass plate, a metal or a plastic film as a thin layer (~0.25mm).
2. **Preparing the plate:** A large number of applicators were commercially available which were used for coating of glass plates with different adsorbent layers of uniform thickness. There were many methods for preparation of TLC plates. The most commonly used method was pouring.
   Pouring: Slurry was poured on the plate and then the plate was tipped back and forth to spread uniformly.
3. **Activation of adsorbent:** The plate were dried in air and then dried in oven at 110-140^{o}c for 30min. On heating, the plates were said to be activated.
4. **Spotting the TLC plate:** Few milligrams of sample material was spotted on the TLC plate with the help of capillary tube. The spotting capillary tube must be extremely small. To spot the plate, simply touch the end of the capillary tube to the coated side of the plate. Do not spot too much sample.
5. **Development:** The prepared TLC plates were placed in development chamber as shown in *Fig.No:4*. The solvent in the chamber must be in contact with the stationary phase. The development chamber was filled with a small amount of the mobile phase and capped with a lid or cork. The origin of spots should not be below the solvent level in the chamber. If the spots were submerged in the solvent, they are washed off and lost.

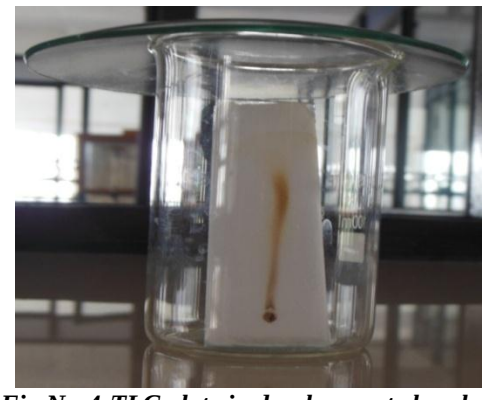


Fig.No:4-TLC plate in development chamber

6. **Visualization:** Some organic compounds are coloured and there is no requirement of using colouring agents, then visualizing of spots is easy.

R_f value

In addition to qualitative results, TLC can also provide a chromatographic measurement known as R_f value. The R_f value is the "retardation factor" or "ratio-to-front" value expressed as a decimal fraction.

The R_f value can be calculated as:

$$R_f = \frac{\textit{Distance travelled by sample}}{\textit{Distance travelled by solvent front}}$$

Solvent system: Mixture of two solvents is used here for determining R_f value. Methanol and water are used in the ratio of 3:1.

Preparation of Nutrient Agar Medium: [12, 13, 14, 15, 16]

Formula for nutrient agar medium

Table. No: 1

NGREDIENTS	QUANTITY TAKEN
Beef extract	0.75gms
Peptone	1.25gms
NaCl	1.25gms
Agar	3.75gms
Distilled water	up to 250ml

Procedure

- Weigh all additives from *Table. No: 1* separately by physical balance.
- Add all the additives in conical flask.
- Heat on water bath with stirring till agar completely dissolved.
- Adjust to pH 8.0-8.4 with 5M NaOH.
- Boil for 10min.
- If necessary, filter it and adjust to pH 7.2-7.4.
- Sterilise by autoclave, using 15*lb* pressure at 115 ^{o}C for 30min.

Preparation of Agar Plate

- Aseptically transfer the sterile nutrient agar in to the Petri plates.
- This process is performed on the table top of laminar air flow bench.
- Flame the neck of culture flask.
- Lift half of the lid of sterile Petri-dish and add nutrient agar into it, so that it is equally distributed throughout the plate.
- Replace the closure and allow the plate to solidify.

Screening For Antimicrobial Activity: [17]

Theory:

This method is based on the diffusion of an antibiotic from a vertical cylinder or filter disc through the solidified agar layer of a Petri-dish. Growth of inoculated microorganism is inhibited in a circular area "zone", around the filter disc containing a solution of antibiotics.

Procedure:

- Microbial inoculum was prepared with the required quantity of test organism.

- Add prepared microbial suspension in the media and mix it and transfer into Petri-dish.
- Prepare the solutions of known concentration of the standard preparation with respect to the concentrations of the antibiotics to be examined.
- Apply the solutions to the filter discs and place them on the prepared agar plate.
- Leave the plates for 1-4hrs at room temperature.
- Incubate the plates in inverted position at 20-30^{o}c for 18hrs.

OBSERVATION

Observe the zone around the filter disc by antibiotic zone reader or visually.

Preparation of Sample Solutions:

Prepare three dilutions of each synthesized compounds (2-nitro benzimidazole chalcone, 4-nitro benzimidazole chalcone, 4-chloro benzimidazole Chalcone) that differ in their concentrations. Also prepare another dilution of known concentration of Tetracycline (standard drug). The procedure for these dilutions is as follows:

- Dissolve 1mg of the synthesized compounds in 10ml of DMF (Dimethylformamide), those concentrations will be 1000µg/ml.
- Take 0.5ml of the above dilution and make up to the volume of 10ml with DMF as solvent, those concentration is 500µg/ml.
- Take 0.25ml of the above dilution and make up to the volume of 10ml using DMF as solvent, those concentration is 250 µg/ml.

RESULTS

During the synthesis of Benzimidazole Chalcone, the end point for each step was identified by performing TLC (*Fig. No: 5)*. The R_f value was measured for every 20min till two consecutive R_f values are obtained as shown in following

Table No: 2.

S.NO	STEPS INVOLVED	R_f (cm)	R_f (cm)	R_f (cm)
1	Preparation of 2-hydroxy ethyl benzimidazole	0.87	0.80	0.80
2	Preparation of 2-acetyl benzimidazole	0.92	0.76	0.76
3	3-(1H-benzimidazole-2-yl)-3-hydroxy-1-(1H-indazole-3-yl)-butan-1- one	0.90	0.86	0.86

Fig.No:5-R_f Values during synthesis

Antimicrobial activity

Fig.No:6, 7&8 represents antimicrobial activity of synthesized Benzimidazole chalcones.

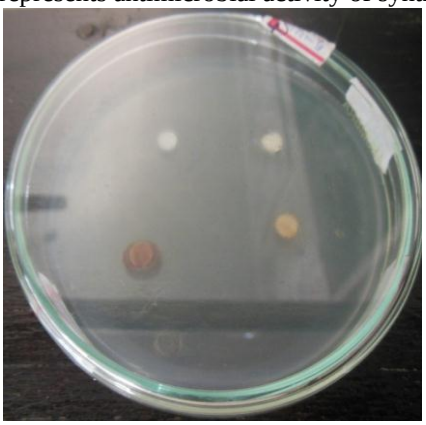


***Fig. No.6:* 2-Nitro Benzimidazole Chalcone**

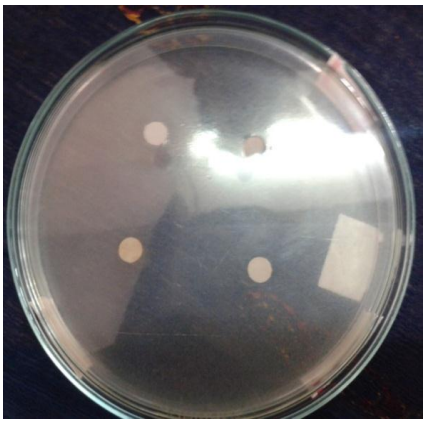


***Fig. No: 7*4-Nitro Benzimidazole Chalcone**

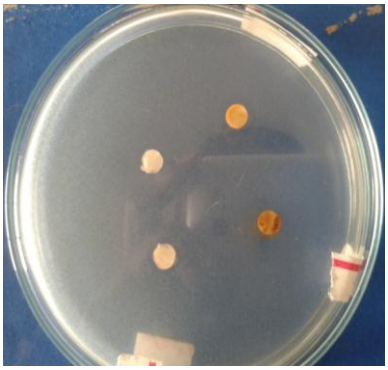


Fig .No.8: **4-Chloro Benzimidazole Chalcone**

The antimicrobial activity for three synthesized compounds was evaluated and the results are tabulated as follows (*Table. No 3, 4&5*):

2-Nitro Benzimidazole Chalcone

Table. No: 3

Concentration (µg/ml)	Bacillus subtilis (mm)	Pseudomonas aeruginosa(mm)	Staphylococcus aureus(mm)	E. coli (mm)
1000	10	10	9	11
500	9	8	9	10
250	8	8	8	10
Tetracycline	9	17	16	9

4-Nitro Benzimidazole Chalcone

Table No: 4

Concentration (µg/ml)	Bacillus subtilis (mm)	Pseudomonas aeruginosa (mm)	Staphylococcus aureus (mm)	E. coli (mm)
1000	11	11	10	11
500	10	10	10	10
250	9	9	7	9
Tetracycline	19	19	17	9

4-Chloro Benzimidazole Chalcone

Table No: 5

Concentration (µg/ml)	Bacillus subtilis (mm)	Pseudomonas aeruginosa (mm)	Staphylococcus aureus (mm)	E. coli (mm)
1000	10	10	9	9
500	10	9	11	8
250	7	7	9	7
Tetracycline	11	17	18	9

The histograms were plotted representing zone of inhibition along Y-axis and concentration on X-axis, for four different microorganisms.

Gram- ve: *Escherichia coli:*

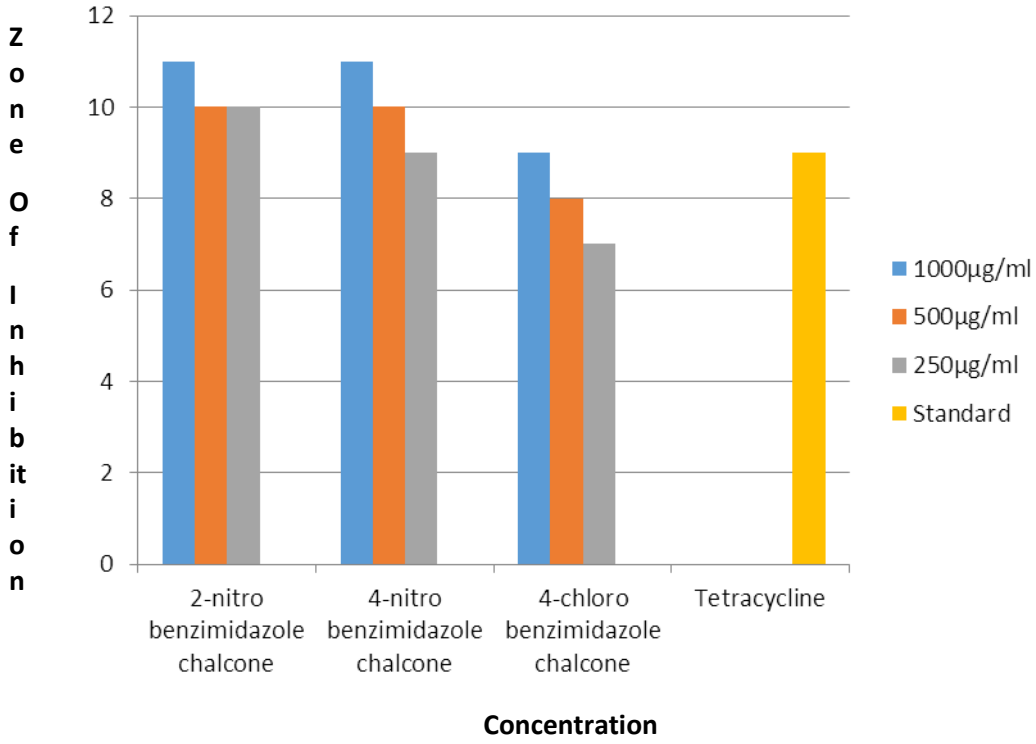


Fig .No:9

Pseudomonas aeruginosa

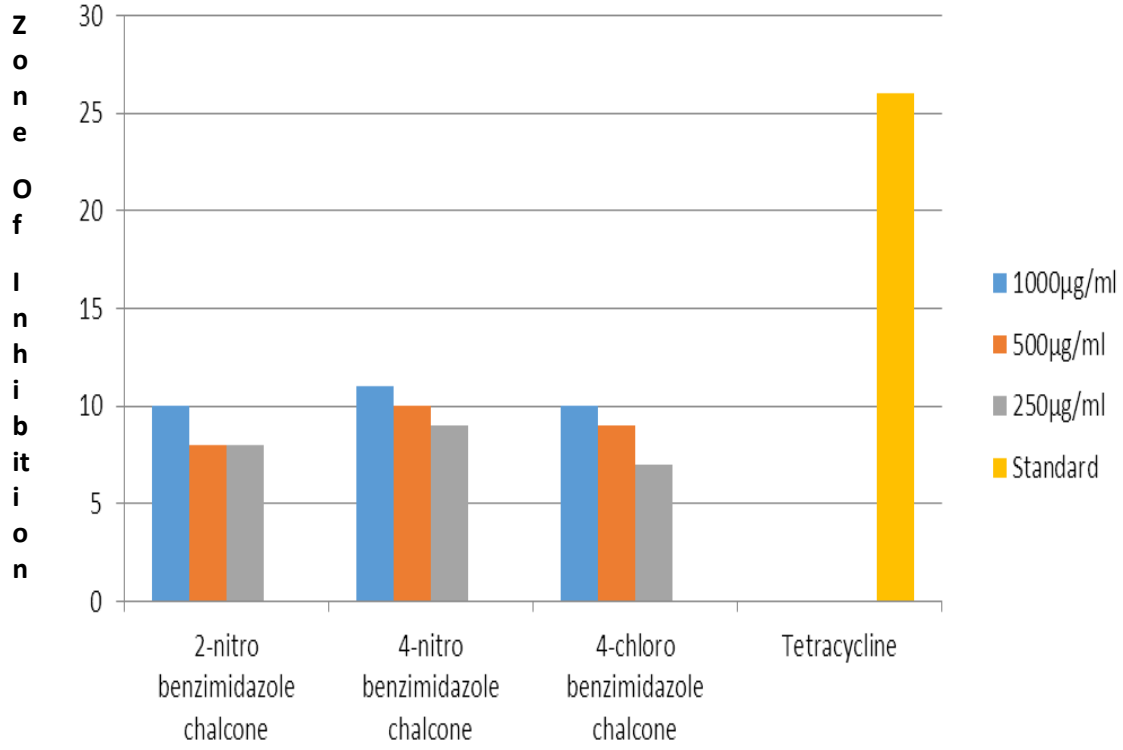


Fig. No: 10

Gram +ve:
Bacillus subtilis:

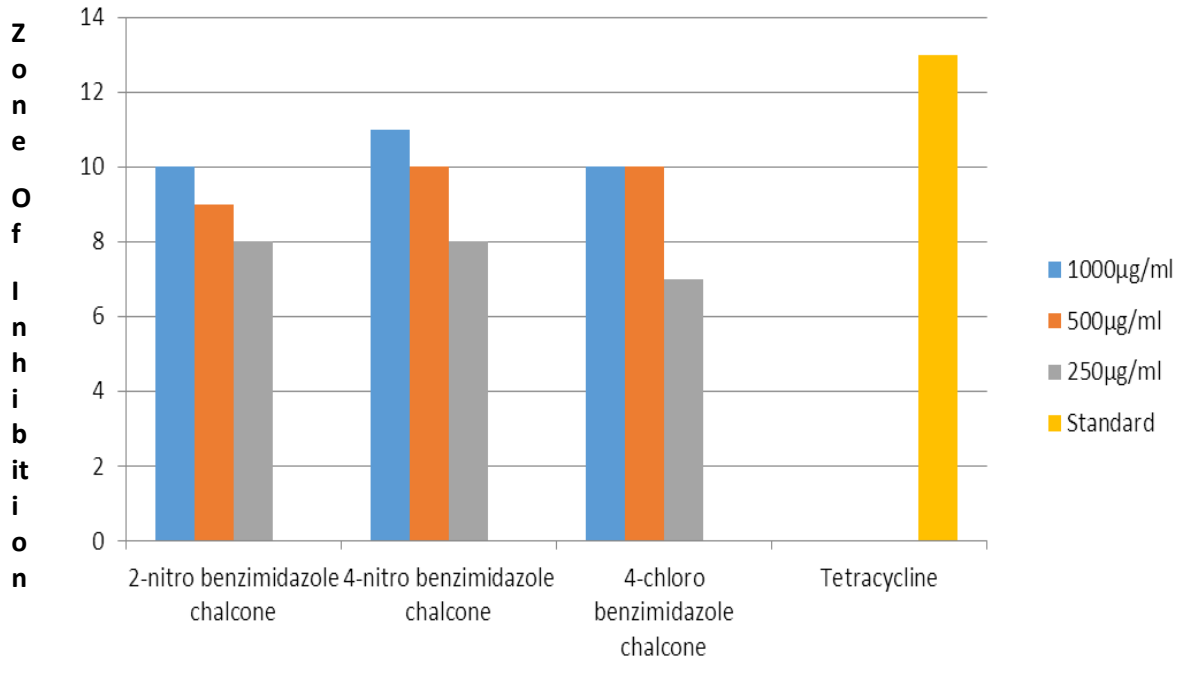


Fig. No: 11

Staphylococcus aureus

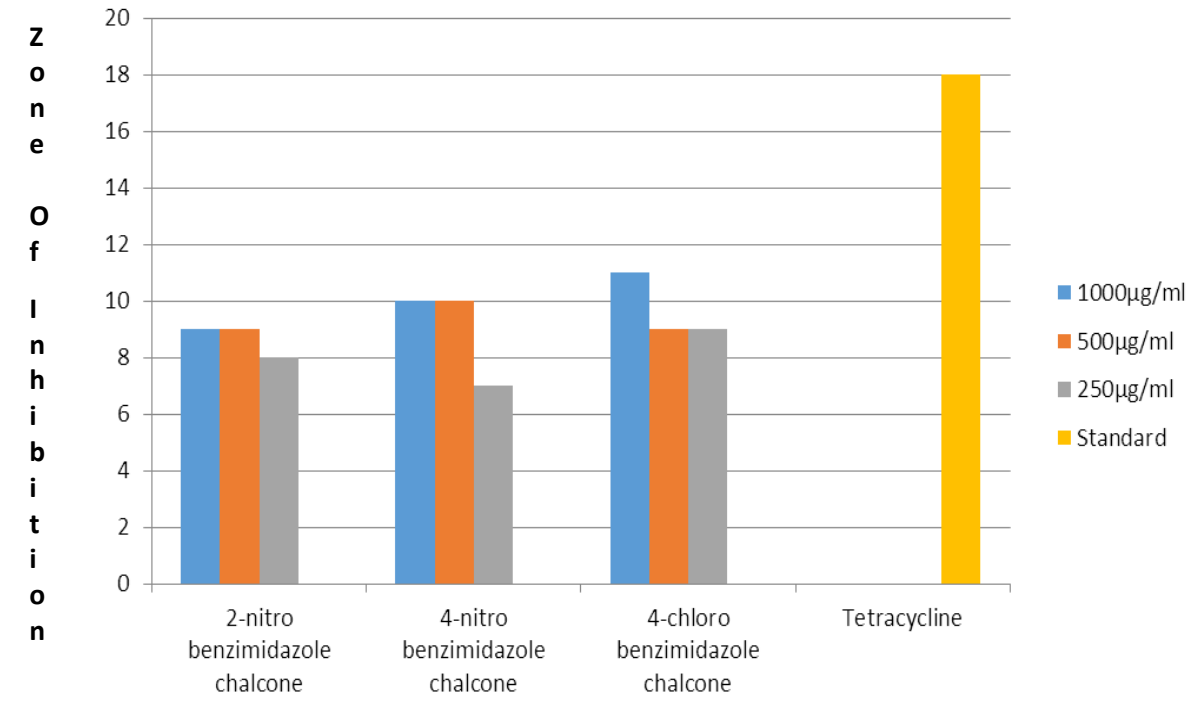


Fig. No: 12

DISCUSSION

The R_f values were used to determine the end point of each step involved in the synthesis of Benzimidazole Chalcone. Two consecutive constant R_f values represent that the reaction is complete.

When the Antimicrobial activity was performed by using different dilutions of synthesized compounds (2-Nitro Benzimidazole Chalcone, 4-Nitro Benzimidazole Chalcone, 4-Chloro Benzimidazole Chalcone) and Tetracycline (as standard) on both gram-ve and gram +ve zone of inhibition was observed around the paper discs placed on the nutrient agar in petri dish. This zone was measured by Antibiotic zone reader.

These can be easily interpreted using histograms. Four histograms were plotted that represent, the effect of different concentrations of synthesized compounds on different microorganisms.

Effect on *Escherichia coli*

- As the concentration of the drug increased, the zone of inhibition also increased.(from *Fig.No:9*)
- From *Fig.No:9,* it is observed that 2-Nitro Benzimidazole Chalcone and 4-Nitro Benzimidazole Chalcone produces more Antimicrobial activity when compared to 4-Chloro Benzimidazole Chalcone and Tetracycline.

Effect on *Pseudomonas aeruginosa:*

- As the concentration of the drug increased, the zone of inhibition also increased.
- From *Fig.No:10,* it is observed that 4-Nitro Benzimidazole Chalcone produce more Antimicrobial activity when compared to 2-Nitro Benzimidazole Chalcone and 4-Chloro Benzimidazole Chalcone.

Effect on *Bacillus subtilis:*

- As the concentration of the drug increased, the zone of inhibition also increased.
- From *Fig.No:11,* it is observed that 4-Nitro Benzimidazole Chalcone produce more Antimicrobial activity when compared to 2-Nitro Benzimidazole Chalcone and 4-Chloro Benzimidazole Chalcone.

Effect on *Staphylococcus aureus:*

- As the concentration of the drug increased, the zone of inhibition also increased.
- From *Fig.No:12,* it is observed that 4-Chloro Benzimidazole Chalcone produce more Antimicrobial activity when compared to 2-Nitro Benzimidazole Chalcone and 4-Nitro Benzimidazole Chalcone.

CONCLUSION

The screening of literature of Benzimidazole Chalcone synthesis by using 2-Nitro Benzaldehyde, 4-Nitro Benzaldehyde, 4-Chloro Benzaldehyde, revealed that no Antimicrobial work has been done so far.

The Antimicrobial activity test answered positively for gram-ve (Escherichia coli and Pseudomonas aeruginosa) and gram +ve (Bacillus subtilis and Staphylococcus aureus) Microorganism.

The Novel Benzimidazole Chalcone showed significant difference from standard drug in zone of inhibition especially against gram-ve microorganism of Pseudomonas aeruginosa was found to be satisfactory.

It can be observed that the Antimicrobial activities are compared with the standard drug of Tetracycline.

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