SYNTHESIS AND CHARACTERIZATION OF SOME NOVEL DIAZOTIZED COMPOUNDS COMPOSED OF BIS-IMINAMINE MOIETY AND EVALUATION OF THEIR ANTIBACTERIAL PROPERTY

by

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Author
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ABSTRACT

Azo compounds are highly reactive compounds with enormous variety of applications. The azo compounds were marketed mainly in form of azo-disperse, azo-vat, azo-acid, dyes, food, cosmetic medicine, leather, plastics, varnish, automobile etc. These compounds are also more suitable for biocidal treatment of textile fibers due to their greater biological activities, because biocidal template forms a definite type of bonding with fibrous material and forms important structures in the medicinal and pharmaceutical fields. The pharmacological study of these compounds started from the effect of antibacterial action of Prontosil on streptococcal infections by Dog-magk. It is evident from the literature that these kinds of functionalities have great potential to be used as antibacterial as well as antioxidant agents. At the same time these compounds are used as an important synthetic precursor. On this point of view, we have designed and successfully synthesized some novel diazotized compounds composed of bis-iminamine moiety by following diazo coupling reaction between diazonium salts and active methylene compounds. All the compounds are characterized by IR, $^1$H NMR and $^{13}$C NMR spectral evidences. The synthesized compounds are summarized in the following tables.
Table -1: Synthesis of Diazotized compounds composed of bis-imamine moiety.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Amine &amp; Diazinium chloride</th>
<th>Active methylene compounds</th>
<th>Products</th>
<th>mp °C</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="Diagram" /></td>
<td><img src="image2" alt="Diagram" /></td>
<td><img src="image3" alt="Diagram" /></td>
<td>160-164</td>
<td>74</td>
</tr>
<tr>
<td>2</td>
<td><img src="image4" alt="Diagram" /></td>
<td><img src="image5" alt="Diagram" /></td>
<td><img src="image6" alt="Diagram" /></td>
<td>147-151</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td><img src="image7" alt="Diagram" /></td>
<td><img src="image8" alt="Diagram" /></td>
<td><img src="image9" alt="Diagram" /></td>
<td>150-154</td>
<td>68</td>
</tr>
<tr>
<td>4</td>
<td><img src="image10" alt="Diagram" /></td>
<td><img src="image11" alt="Diagram" /></td>
<td><img src="image12" alt="Diagram" /></td>
<td>140-145</td>
<td>76</td>
</tr>
<tr>
<td>5</td>
<td><img src="image13" alt="Diagram" /></td>
<td><img src="image14" alt="Diagram" /></td>
<td><img src="image15" alt="Diagram" /></td>
<td>150-153</td>
<td>74</td>
</tr>
<tr>
<td>6</td>
<td><img src="image16" alt="Diagram" /></td>
<td><img src="image17" alt="Diagram" /></td>
<td><img src="image18" alt="Diagram" /></td>
<td>140-144</td>
<td>60</td>
</tr>
</tbody>
</table>
Antibacterial property

Table: Antibacterial Property.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gram positive</th>
<th>Gram negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td></td>
<td>50 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>Compound 4</td>
<td>0.008 mm</td>
<td>0.02 mm</td>
</tr>
<tr>
<td></td>
<td>0.02 mm</td>
<td>0.013 mm</td>
</tr>
<tr>
<td>Compound 8</td>
<td>0.013 mm</td>
<td>0.16 mm</td>
</tr>
<tr>
<td></td>
<td>0.16 mm</td>
<td>0.003 mm</td>
</tr>
<tr>
<td>Compound 10</td>
<td>0.013 mm</td>
<td>0.020 mm</td>
</tr>
<tr>
<td></td>
<td>0.020 mm</td>
<td>0.020 mm</td>
</tr>
</tbody>
</table>

The synthesized compounds 4, 8, 10 were screened for their antibacterial activity against Gram-positive *Staphylococcus aureus*, *Bacillus cereus*, and Gram negative *Escherichia coli* using different disc diffusion method at 50 µL and 100 µL/disc concentrations. Azithromycin was used as standard but no significant activity was found.
CHAPTER-1

INTRODUCTION
Azo compounds are the compounds which bear the functional group R−N=N−R′, in which R and R′ can be either aryl or alkyl. IUPAC defines azo compounds as: "Derivatives of diazene (diimide), HN=NH, where in both hydrogens are substituted by hydrocarbyl groups, e.g. PhN=NPh azobenzene or diphenyldiazene. The more stable derivatives contain two aryl groups. The N=N group is called an azo group. The name azo comes from the greek word azote.

R
\[ \text{N} \equiv \text{N} \]
R'

General chemical formula of azo compounds

Method of Formation

The aryldiazonium salts are commonly prepared by the diazotization of primary aromatic amines at low temperature in acidic solutions.

\[
\begin{align*}
\text{ArNH}_3\text{Cl} + \text{NaNO}_2 + \text{HCl} & \xrightarrow{0^\circ\text{C}} \text{Ar} - \text{N} \equiv \text{N} + \text{Cl} \equiv \\
\text{ArNH}_2 + \text{NaNO}_2 + 2\text{HX} & \xrightarrow{\text{HONO}/\text{H}_2\text{O}} \text{Ar} - \text{N} \equiv \text{N}:\text{X} + \text{NaX} + 2\text{H}_2\text{O}
\end{align*}
\]

General reaction mechanism of diazonium salt formation

Applications

The aryldiazonium salts involved in a vast number synthetic organic reactions. The reactions may occur either with loss of N=N or without loss of N=N function.
Azo compounds or azo dyes are characterized by the presence of the azo moiety (-N=N-) in their structure conjugated with two distinct or identical mono or polycyclic aromatic or heteroaromatic systems. Because of their specific physico-chemical properties and biological activities, they have a broad spectrum application in the field of pharmaceutical [1], textile industry [2], and in analytical chemistry [3]. However, the most typical and popular field of utility remains as their coloring function. The Azo compounds are applicable for biocidal treatment of textile materials because they exhibit biological activity [4]. Azo compounds are well known for their medicinal importance and are recognized for their applications as anti-diabetics [5], antiseptics [6], antineoplastics [7], antibacterial [8] and antitumor [9]. They are also involved in many biological reactions such as inhibition of DNA, RNA, carcinogenesis, protein synthesis and nitrogen fixation [10-11]. Probably the azo-imine linkage is responsible for the biological activities displayed by some Schiff bases [12-13].

The azo compounds viz. Evans blue, 1 and Congo red, 2 are being studied as HIV inhibitors of viral replications [14]. The existence of azo moiety shows antibacterial and pesticidal activities. Recently, azo group containing compounds as antimicrobial agents has been the subject of study reported by H. N. Chopde [15], A. H. Shridhari [16] and C. J. Patil [17-19]. Synthesis of most azo compounds involves diazotization of a primary aromatic amine, followed by coupling with one or more nucleophiles. Thus, benzoic, phenolic, salicylic and naphtholic compounds undergoes diazotization reactions. Azo compound is often described as a chromogen in the literature [20]. The amino-and hydroxy-groups are commonly used coupling components [21]. The emergence of diverse classes of synthetic dyes including azo dyes occurred due to constant effort to find specific dye for application in diverse materials of industrial importance which include, but not limited to textile fabric [22], ink-jet printer, paper, leather, aluminium sheet [23]. Furthermore, azo compounds also have many applications in photo industry such as photodynamic therapy, photographic or electro-photographic systems and are dominant organic photo conductive [9, 16].
Nowadays, synthetic azo compounds are widely used in different applied fields, such as medicines, cosmetics, foods, paints, plastics, shipbuilding, automobile industry, cable manufacture etc. [24-42]. However, the traditional applied field of the synthetic azo dyes still remains the textile industry, and the finishing of fibrous materials in order to impart simultaneously with coloration, antimicrobial properties is of great interest.

This is due to the fact that textile materials undergo biological degradation, and it seems that about 40 % of the damage is due to the effect of microorganisms. The activity of fungi and bacteria results in the reduced mechanical strength of a material, color change, stains and stale odor. In this regard, the use of materials with antimicrobial properties extends the service life of these materials, and avoids damage caused by biological degradation.
The manufacture of biologically active materials can be performed either by impregnation with antimicrobial compounds, or by chemical reaction (adding antimicrobial compounds by means of chemical bonding to functional groups of the fiber-forming polymers). Dyes are among the compounds which are suitable for biocidal treatment of textile materials due to the fact that some of them exhibit biological activity, resulting from the presence in their molecule of some antiseptic groups that form a definite type of bonding with the molecules of the fibrous material.

Synthesis of two new disazo compounds with structure 3 (derived from 4, 4’-diaminostilbene 2, 2’-disulfonic acid) and structure 4 (derived from 4,4’-diaminobenzanilide) are established as biologically active compounds.

The synthesis of the azo compounds 3 and 4 are performed by a two-step process. The preparation procedure involved the bis-diazotization of the corresponding diamine (4,4’diaminostilbene-2,2’-disulfonic acid and 4,4’-diaminobenzanilide respectively) and there after the coupling reaction (in a 1:2 molar ratio) of the resulting bis-diazonium salt with 4-chlorosalicylanilide.
The bis-diazotization of the two diamines is carried out by the direct method, in a HCl aqueous solution. In the case of 4,4′-diaminostilbene-2,2′-disulfonic acid, the resulting bis-diazone salt is separated by filtration for the complete removal of the salts, unlike the classical method. The coupling reactions are performed in an alkaline aqueous medium (pH around 8), in presence of Na₂CO₃ at a temperature around 10°C, and a 3% excess of coupling component. The direct adding of the alkaline solution of the coupling components to the acid suspension of the bis-diazone salts of both 4,4′-diaminostilbene-2,2′-disulphonic acid and 4,4′diaminobenzanilide, is optimum for the synthesis of the disazo compounds 3 and 4.

The coloristic and the application properties of the disazo compounds 3 and 4 are determined and compared to those exhibited by a classical direct dye, with quite close chemical structure (e.g. C. I. Direct Orange 1).

In the case of the azo compound 3, the untreated dyeing on cotton is reddish-orange, the dye had good migration and good leveling properties. The percentage uptake on cotton is found 68 %. In the case of 4, the untreated dyeing on cotton is orange and the migration is good, Compound 4 exhibited fair leveling properties and the percentage uptake on cotton is 75 %. The obtained results are presented in Table 1. As it can be seen in Table 1, the application properties of the disazo dye 3 are quite similar to the reference dye Direct Orange 1. Moreover, the after-treatment of the dyed samples with CuSO₄ shows improvements in the case of the wet fastness of dye 3. The obtained results indicate that the synthesized dye can be ranged as a direct dye.
Table 1
Application properties on cotton of dye (3) and of C. I. Direct Orange 1 (C. I. 22.250).

<table>
<thead>
<tr>
<th>Fastness:</th>
<th>Disazo dye 3</th>
<th>Disazo dye 4</th>
<th>C. I Direct Orange 1* (C. I. 22.430)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct dyeing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>2-3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Water</td>
<td>3-4</td>
<td>4</td>
<td>3-4</td>
</tr>
<tr>
<td>Wash 40 °C</td>
<td>2-3</td>
<td>3</td>
<td>2-3</td>
</tr>
<tr>
<td>Perspiration (alkaline)</td>
<td>2-3</td>
<td>3</td>
<td>2-3</td>
</tr>
<tr>
<td>Perspiration (acid)</td>
<td>2-3</td>
<td>3</td>
<td>2-3</td>
</tr>
<tr>
<td>Hot pressing</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Further, the azo compounds 3 and 4 are subjected to in vivo imagistic skin evaluation tests. The possibilities of apparition of adverse effect are monitored with an imagistic skin evaluation, in vivo, daily after each application [43-45]. For all the volunteers involved in this study, and for all concentrations applied, any pathological sign is registered. Concerning to skin coloration, no modification was noticed, excepting the transitory color of the studied dyes. As to the skin texture, no peeling effect is registered, and the epidermal desquamative registrations are no more differenced than the blank skin areas. Moreover, during all the period of skin determination, no case of contact dermatitis is registered. The results of screening antimicrobial activity are given in Table 2. The disazo compounds 3 and 4 are subjected to antibacterial activity screening against two gram-positive bacteria.
(Staphylococcus aureus and Streptococcus pyogenes) and three gram-negative bacteria (Pseudomonas aeruginosa, Proteus vulgaris and Escherichia coli) employing the disk diffusion technique. The results show that the studied compounds present antimicrobial activity against most all the tested species, especially against Staphylococcus aureus and Escherichia coli.

Table 2
Antimicrobial activities of compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Diameter of zone of inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Septonex</td>
<td>18</td>
</tr>
</tbody>
</table>

In vivo skin evaluation
The evaluation of the possible skin adverse effects of the disazo compounds 3 and 4 was performed on a study group of 45 healthy volunteers, all women, aged between 25 – 51 years. The disazo compounds 3 and 4 are applied as dilute aqueous solution of 5 %, 2.5 %, 1 % and 0.5 %, on 4 areas of forearms, for 30 days, daily, 1 time a day. In the volunteer selection, the subjects presenting skin pathologic signs (seborrhea dermatitis, psoriasis lesions) are eliminated. However, physiological signs, like photo aging signs, dilatation of sebaceous channel glands, or other physiological signs are admitted. No restrictions regarding to the human skin photo type are considered. For all volunteers, a blank skin area is also chosen. The possibilities of apparition of adverse effect are monetarized with an imagistic skin evaluation, in vivo, daily after the previous application. The tests are performed using a ProDerm II Skin Analyzer. The following parameters are evaluated: the skin coloration (normal or the apparition of a rush); the skin texture (preservation of the normal skin quadrilagde, and eventually irritation signs – a peeling effect), and the possible skin dryness, eventually induced by the disazo compounds (involution of density of sebaceous glands channels).
In vitro antimicrobial investigations

The diazo compounds 3 and 4 are tested for their antimicrobial activity against the following micro-organisms: *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Escherichia coli*, using the cup plate diffusion method [46]. The bacterial organisms are isolated from human beings with characteristic infections and diseases. The disazo compounds 3 and 4 are dissolved in ethanol, yielding solutions of 0.2% concentration. Wells (diameter 6 mm) are loaded with 0, 1 mL solution of the studied disazo compounds. The bacterial suspensions are inoculated on sterile Mueller Hinton agar, in each Petri dish with 9 cm diameter. After 24 hours of incubation, the diameters of the inhibition zones were measured (including the 6 mm diameter of the disc). The results are compared with [1- (ethoxycarbonyl) -pentadecyl] -trimethylammonium bromide (Septonex), a commercial antiseptic agent. Inhibitory effect of the compounds is established at zones greater than 12 mm.

Application as liquid dyes

Azo compounds are well-known important dye compounds [47,48] and have been used not only for dyeing [49-51] but also for high-technology applications [52-54]. As organic dyes have auxochromes such as amino, alkylamino, dialkylamino, hydroxy, alkoxy, and nitro groups in a molecule, they are usually solid. These auxochromes can give strong polarity to the molecule to increase intermolecular interactions. Furthermore, dye molecule is usually large and has electrons to produce strong disperse forces and interactions. In the course of their study on the solid-state fluorescent dyes, they found a few coumarins [55] and (dialkylamino) perfluorophenazines [56]. These results motivated them to find new liquid azo dyes. Some azo compounds without auxochrome such as 2-ethyl- [57], 2,6-dimethyl- 8 [58], and 2,2’-dibutylazobenzenes [59] are liquid. However, no liquid azo dyes which have the auxochrome in a molecule have been reported so far. They reported herein the survey of a series of liquid azo dyes.
Active Methylene Compounds

Compounds possessing a methylene bridge located between two strong electron withdrawing groups (such as nitro, carbonyl or nitrile groups) are sometimes called active methylene compounds. Active methylene compounds are those in which proton can be easily removed from ethylene unit by use of a base. So, basically active methylene compounds are those in which there is a methylene unit having acidic proton, main use is the anion of active methylene compound as a nucleophile, used in different name reactions of organic chemistry, including Knoevenagel Condensation and also used widely in organic synthesis. Active methylene group: as the name suggests it's active means have an acidic hydrogen. "Acidic hydrogen" – reactive. Active methylene, hence, is of great use. Addition of a base it will directly go for that hydrogen. It’s of great use in reactions of unsaturated carbonyl groups. Some are given below

![Dimethyl Malonate](image)
**5**

![Beta-Ketoester](image)
**6**

![Beta-Diketone](image)
**7**

![Malononitrile](image)
**8**

![Malonic Acid](image)
**9**

![Malonic Esters](image)
**10**
Diazotization is a process by which an aromatic primary amine is converted to a diazonium compound. In diazotization, sodium nitrite is added to a solution of the amine in aqueous acid at 0–5°C. Reactions of the amine with nitrous acid give a nitrosamine. Tautomerization and loss of water lead to the diazonium ion, which is stabilized by delocalization of the positive charge at the ortho and para carbon atoms of the ring. Azo dyes form a large structural group of synthetic dyes. An azo dye has the general structure below [60,61].

The azo groups form links or bridges between organic residues of which one is usually an aromatic nucleus and being a chromophore, azo groups impart color to textile fiber. The depth of shade is influenced by the number of azo groups present in the structure of the azo dye. It has been found that the azo dyes are capable of forming hydrogen bonds with the
fibrereby enhancing dye fiber binding forces [62]. Coupling reaction between active methylene and diazonium salts is generally conducted at room temperature and in protic organic solvent in presence of base. Acetyl acetone, benzoyl acetone, diphenylpentanenedione, and other 1,3-diketones including cyclic ketones have been coupled with aromatic diazonium salts in ethanloic sodium acetate to yield corresponding products 17a-c [63,64] and 18a and b [65,66].

![Chemical Structures](image)

Para-amino benzoic acid (PABA) is considered to be in the B-complex vitamin family. PABA has been used as a component of many commercially available sun screens due to its ability to block damaging ultraviolet rays. One very interesting application for this versatile substance is its potential to restore hair to its natural color [67]. From the foregoing literature survey, it has been showed that when the diazonium salt of p-amino benzoic acid allowed to react with different compounds contain methylene group, which act as a nucleophilic center and attack the diazonium salt of p-amino benzoic acid to afford the following : In 2007, Fikret Karel and Aykut Demircall synthesized different derivative through coupling reaction between 4-substituted benzene diazonium salt with malononitrile, ethyl cyano acetate and ethyl acetoacetate in sodium acetate as buffered solution[68].
Compound 19a-c when allowed to react with hydrazine hydrate, cyclization occurs to give compound 22a-c.

R= H, NO₂, OCH₃
Biological evaluation

Antibacterial activities of the synthesized compounds are examined in vitro by the known agar diffusion technique. All compounds are tested for activity against gram-negative bacteria Escherichia coli- Neisseria and Salmonella, compared with antibiotic. All compound under study showed activity against gram-negative bacteria.

The aryldiazonium salt, Ar-N$_2^+$Cl$^-$ are highly reactive compounds. It also plays an important role in synthetic organic chemistry. In chemistry azo dyes of phenolic compounds played a major role in synthesizing many of the commercial dyes and analytical reagent. The dyes are marketed mainly in the form of azo disperse, azo-vat, azo-acid dyes, etc. Due to the simple process of the synthesis, usually an aqueous medium and the almost unlimited choice of starting products, an extremely wide variety of azo dyes skeleton is possible. The number of combination is increased by the fact that a dye molecule can also contain several groups. The practical uses of dyes in various industrial field showed that azo compounds are the largest class of industrial synthesized organic dyes. The azo dyes are a distinct and clearly defined class, characterized by the presence of one or more azo (-N=N-) groups. They are all prepared by a common process involving diazotizing an aromatic primary amine and the formed diazonium salt solution is coupled with a phenol or an aromatic amine. Diazo coupling reaction products are characterized by chromophoric azo group. Azo dyes are used as corrosion inhibitors for the dissolution of carbon steel in HCl acid solution [69]. Azo compounds have received much attention due to their versatile skeleton and uses in many practical applications such as coloring fiber, photo electronic applications, printing systems, optical storage technology and in various analytical techniques [70]. The azo compounds also found their wide applications as a polymer additive [71], also the azo dye-additive was mainly used to color waxes, oils, petrol, solvents and polishes and successful in textile processing, paper, food, cosmetic medicine, leather, plastics, varnish, automobile [72-74]. The aryldiazonium salt are synthesized and reacted upon with AMG containing compound viz. Pentane-2,4- dione or Acetyl acetone(AA). Heterocyclic rings [75-76], which are the reason for the activity of most of the drugs of natural origin lead the discovery of the many synthetic drugs possessing the heterocyclic rings. Heterocyclic nitrogenous [77-78] compounds and their fused analogues represent an important class of heterocyclic compounds exist in numerous natural products displaying a wide range of biological and pharmaceutical activates.
Fig: Diazocoupling reaction with active methylene compounds.
Antibacterial Activity

Antibacterial activity of all the synthesized compounds were screened against gram-negative bacteria, *E. coli* and *B. subtilis* for three different concentrations of 100, 500 and 1000 μg/ml, as per the method described in [79].

There are many classes of organic chemistry like carbonyl compounds [80] viz. aldehyde [81] and ketone [82] and imine [83] etc. likewise aryldiazonium compounds also play an important role in synthetic organic chemistry [84]. There are many chemical reactions like reduction, oxidation, hydrolysis, complexation, and coupling etc. In the present piece of work, they reacted the diazonium salt coupled with a phenolic compound, resorcinol. These synthesized compounds, 30 to 34 are useful for varied applications viz. for synthesis of newer chemicals and pharmaceuticals.
Diazonium Salts
The general formula for Aromatic diazonium salt, is represented as Ar-N₂X⁻ and they are highly reactive and diverse compounds (serves as intermediate in the synthesis of a wide variety of organic transformations). In fact, they are comparable to Grignard reagent in their versatility that is ease of processing.

Reaction of Diazonium Salt
The reaction involves loss of nitrogen and reaction involving retention of nitrogen. Aryl diazonium salts are usually synthesized in the presence of a liquid acid dissolve in water at low temperature between 0⁰C and 10⁰C. An initial optimization of the amount of acid is performed by diazotization of substituted aniline as model substrate and diazo coupling with resorcinol. Replacement of diazonium group is the best general way of introducing H, F, Cl, Br and OH into an aromatic ring dizonium salt are valuable in synthesis not only because they react to form so many classes. The diazonium salts are quite different from Grignard reagent. These reactions, either, losses nitrogen containing function or without loss of nitrogen function. They are specially used in nucleophilic substitutions, radical reactions and some cross coupling reactions. Recently the research work on the coupling of aryl diazonium salts with active methylene compounds [85] have been communicated. Recently some published [86, 87] and communicated [88] work on the synthesis of azo compounds, including Sudan and its nitro derivatives [89] have been reported. The coupling of diazonium salt of methyl and methoxy-anilines with resorcinol to yield monoazo compounds including Sudan and its nitro derivatives. These compounds have many applications in the dye as well as other versatile industries. Diazonium intermediates of aniline and substituted anilines are synthesized and reacted with resorcinol.
Disperse dyes are substantially water-insoluble nonionic dyes which are applied to hydrophobic fibres (cellulose acetate, polyester, and nylon) from aqueous dispersions [90-94]. Disperse dyes are characterized by the absence of solubilizing groups and low molecular weight. From a chemical point of view, more than 50% of disperse dyes are simple azo compounds. About 25% are anthraquinones and the rest are methine, nitro, and naphthoquinone dyes [95]. The most dominant group of these dyes are the azo disperse dyes which account for approximately 60–70% of all disperse dyes manufactured [96-105]. The use of malononitrile (propanedinitrile, dicyanomethane; malonic (acid) dinitrile;
propionitrile; methylene cyanide; cyanoacetic acid nitrile; malonitrile; malononitrile) either as a coupling or diazo component or condensing agent with different chromophoric compounds in the synthesis of disperse dyes have been reported [90,94,100-105]. However, there seem to be no literatures on the condensation of disperse dyes derived from 4-amino-3-nitrobenzaldehyde coupled to phloroglucinol, barbituric acid, and α and β-naphthols with malononitrile. The study reports the synthesis of new malononitrile-condensed disperse dyes and their application onto polyester and nylon fabrics. Malononitrile is successfully introduced into the structures of four dyes 35-38 previously prepared by the conventional diazotization and coupling methods to give dyes 39-42. This is achieved by the Knoevenagel condensation in which a molecule of water was eliminated by way of nucleophilic addition of an active hydrogen compound (malononitrile) to a carbonyl group (C=O) contained in 4-amino-3-nitrobenzaldehyde followed by a dehydration reaction. The base in the reaction was piperidine. The products have larger molecular weight with usually higher melting points when compared with their parent compounds.

They used a conventional method for preparing diazotised 4-Amino-3-nitrobenzaldehyde to various Coupling Components. The solution of the various coupling components is cooled to 0–5°C and is treated with a cold (0–5°C) of the diazonium salt as prepared above, to produce dyes 35-38.
Application to Polyester

The dye bath is made up of 2% o.w.f. (dye), 1.5mL chlorobenzene (carrier), and 50:1 (liquor ratio) at 100°C. The fabric (10 cm by 10 cm), after being wetted and thoroughly squeezed to remove excess water, is immersed into the bath at 50°C and allowed to reach the boil within 15 minutes. Dyeing is continued at the boil for 1 hour with constant agitation. At the end of dyeing, the substrate is removed, squeezed, and rinsed thoroughly under a running tap and allowed to dry at room temperature.

Application to Nylon

The dye bath is made up of 2% o.w.f. (dye) and 50:1 (liquor ratio) at 100°C. The fabric (10 cm by 10 cm), after being wetted and thoroughly squeezed to remove excess water, is immersed into the bath at 50°C and allowed to reach the boil within 15 minutes. Dyeing is continued at the boil for 1 hour with constant agitation. At the end of dyeing, the substrate is removed, squeezed, and rinsed thoroughly under a running tap and allowed to dry at room temperature.

Reduction Clearing

The dyed material is treated in a bath containing 1.5 g/L dispersing agent, 2 g/L caustic soda, and 2 g/L sodium dithionite at 60°C for 30 minutes. This is aimed at removing unfixed dye and carrier residues that may be left on the fabric after dyeing.

Aminopyrazoles are very important class of heterocycles due to their biological and pharmacological activities [106,107]. These compounds often exhibit anti-inflammatory, herbicidal, fungicidal, bactericidal, and antipyretic activities, and also can be used as plant growth regulating agents as well as protein kinase inhibitors [108-112]. As derivatives of aminopyrazoles, the condensed heterocyclic compounds especially containing the triazine and tetrazine moiety have received much attention owing to the reported antibacterial, antiviral and antihypertensive activities [113-114]. Moreover, they are used as key starting material for the synthesis of commercial arylazopyrazolone dyes and purine analogues [115-123]. In recent years, with the increase of environmental consciousness in chemical research and industry, efficient, economic and clean procedures have received increased attention. Thus, water has become an intriguing reaction medium, and has particularly captured the interest of organic chemists [124-128]. Reactions previously thought
impossible in water are now a reality. In many cases the catalyst and/or the aqueous medium can be recovered and reused, thereby reducing the environment impact of the reaction process [129-131]. Many Lewis acids work well in aqueous medium [132,133] and even AlCl₃, SnCl₂ and TiCl₄ which are previously used under anhydrous conditions are excellent catalysts in water [125]. Recently, Karci et al. reported that 2-arylhydrazone-3-ketiminobutyronitriles reacted with hydrazine hydrate to afford 5-amino-4-arylazo-3-methyl-1H-pyrazole derivatives [134,135]. The previous work, AlCl₃-catalyzed diazocoupling of 1-phenyl-3-hydroxy-5-pyrazolone in water with different aryldiazonium salts yielded 1-phenyl-3-hydroxy-4-arylazo-5-pyrazolone derivatives. According to the same procedure, they successfully synthesized 5-amino-4-arylazo-3-methyl-1H-pyrazole derivatives 43a–m using AlCl₃-catalyzed diazo coupling of 3-amo-no-5-methyl-1H-pyrazole in water with different aryldiazonium salts with high yields. The compounds 43a–m is diazotized and cyclized into novel 5-aryl-3-methylpyrazolo[3,4-e] [1,2,3,4] tetrazines 44a-m.

There are many classes of compounds in organic chemistry like aldehyde, ketone, nitrile, ester, lactone, anhydride, imine and azo compounds etc. likewise aryldiazonium compounds also plays an important role in synthetic organic chemistry. The aryldiazonium
salt are synthesized and reacted upon with AMG containing compound like Pentane-2,4-dione or acetyl acetone(AA). Heterocyclic rings [136-137], which are the reason for the activity of most of the drugs of natural origin leads to the discovery of the many synthetic drugs possessing the heterocyclic rings. Heterocyclic nitrogenous [138-139] compounds and their fused analogues represent an important class of heterocyclic compounds exist in numerous natural products displaying a wide range of biological and pharmaceutical activities.

Antibacterial activity of all the synthesized compounds are screened against gram-negative bacteria, *E. coli* for two different concentration of 100 μg/ml and 200 μg/ml, as per the method described in [140]. The results of antimicrobial testing are depicted in table.
Table-3. The results of antimicrobial testing against *E. coli*

<table>
<thead>
<tr>
<th>Compound I.D.</th>
<th><em>E. coli</em></th>
<th>100 µg/ml</th>
<th>200 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td></td>
<td>7 mm</td>
<td>9 mm</td>
</tr>
<tr>
<td>46</td>
<td></td>
<td>5 mm</td>
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<td>47</td>
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<td>7 mm</td>
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<tr>
<td>48</td>
<td></td>
<td>6 mm</td>
<td>8 mm</td>
</tr>
<tr>
<td>Positive Control</td>
<td></td>
<td>+Ve</td>
<td>+Ve</td>
</tr>
<tr>
<td>Negative Control</td>
<td></td>
<td>-Ve</td>
<td>-Ve</td>
</tr>
<tr>
<td>Ampicillin</td>
<td></td>
<td>16</td>
<td>24</td>
</tr>
</tbody>
</table>

1,8-napthyridine derivatives are reported to possess a wide spectrum of biological activities such as diuretic [141], antimalarial [142], anti-inflammatory [143], antitumor [144], antihypertensive [145] and antibacterial activities [146-147]. Pyrazolone and Isoxazolone compounds are associated with broad spectrum of biological activities. Antipyrine-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one was the first pyrazolone derivative used in the management of pain and inflammation. In the view of these, they have planned to synthesis of some 1,8-napthyridine containing pyrazolinone, pyrazole, Isoxazolinone, Isoxazole and pyrimidine-2-one derivatives, which have been found to possess an interesting profile of anti-inflammatory, along with analgesic and antimicrobial activities.
Title compounds

<table>
<thead>
<tr>
<th>Title compounds</th>
<th>R-NH-NH₂</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>49a</td>
<td>Hydrazine hydrate</td>
<td>H</td>
</tr>
<tr>
<td>49b</td>
<td>Phenylhydrazine</td>
<td>C₆H₅</td>
</tr>
<tr>
<td>49c</td>
<td>Thiosemicarbazide</td>
<td>CSNH₂</td>
</tr>
<tr>
<td>49d</td>
<td>Chloro phenylhydrazine</td>
<td>Cl-C₆H₄</td>
</tr>
<tr>
<td>49e</td>
<td>Isoniazide</td>
<td>C₅H₄N CO</td>
</tr>
</tbody>
</table>

49: R¹=COCH₃; R²=COOC₂H₅
50: R¹=CN; R²=COOC₂H₅
51: R¹=COCH₃; R²=COCH₃
<table>
<thead>
<tr>
<th>Title compounds</th>
<th>R-NH-NH$_2$ (Chemical name)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>50a</td>
<td>Hydrazine hydrate</td>
<td>H</td>
</tr>
<tr>
<td>50b</td>
<td>Phenylhydrazine</td>
<td>C$_6$H$_5$</td>
</tr>
<tr>
<td>50c</td>
<td>Thiosemicarbazide</td>
<td>CSNH$_2$</td>
</tr>
<tr>
<td>50d</td>
<td>Chloro phenylhydrazine</td>
<td>Cl-C$_6$H$_4$</td>
</tr>
<tr>
<td>50e</td>
<td>Isoniazide</td>
<td>C$_5$H$_4$N CO</td>
</tr>
</tbody>
</table>
### Title compounds

<table>
<thead>
<tr>
<th>Title compounds</th>
<th>R-NH-NH₂ (Chemical name)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>51a</td>
<td>Hydrazine hydrate</td>
<td>H</td>
</tr>
<tr>
<td>51b</td>
<td>Phenylhydrazine</td>
<td>C₆H₅</td>
</tr>
<tr>
<td>51c</td>
<td>Thiosemicarbazide</td>
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</tr>
<tr>
<td>51e</td>
<td>Isoniazide</td>
<td>C₃H₄N CO</td>
</tr>
</tbody>
</table>

![Chemical structure](image1.png)

![Chemical structure](image2.png)

26
Pharmacological screening

Acute toxicity Studies

The acute toxic study is done according to the OECD guidelines on Acute Oral Toxicity under a computer guided statistical programme-AOT423 stat programme, the animals are monitored for the behavioural changes, weight variation, toxicity and death rate.

Anti-inflammatory Activity

Oedema is induced by sub planter injection of 0.1 ml of 1% freshly prepared suspension of carrageenan into the right hind paws of the rats of eleven groups of six animals each. The volume of the injected and contra-lateral paws is measured 1, 2, 3 and 4 h after induction of inflammation using a plethysmometer according to the method described by Winter et al. (1962) The test groups received the synthesized compounds (200mg/kg), the standard group received phenylbutazone (100 mg/kg), and the control animals received the vehicle only alone (3% V/V tween-80 10 ml/kg) p.o. All the treatments are given intraperitoneally 30 min prior to the injection of carrageenan an except for the synthesized compounds. Increase of paw oedema thickness was calculated. The results are expressed as mean ± S.E.M. Dennett’s t-test was used to verify the statistical significance at p < 0.05 between the treated and control groups. For comparison purpose, the volume of oedema at various prefixed time intervals was measured. The difference between paw volumes of the treated animals was measured and the mean oedema volume was calculated.
Percentage reduction in oedema volume was calculated by using the formula,

\[
\text{Percentage Reduction} = \frac{V_0 - V_t}{V_0} \times 100
\]

Where, \(V_0\) = Volume of the paw of control at time ‘t’.
\(V_t\) = Volume of the paw of drug treated at time ‘t’.

From the data obtained, the mean oedema volume and percentage reduction in oedema was calculated.

Alfuzosin Hydrochloride (AFZ) 1 is an alpha 1-receptor blocker and is chemically known as N-[3-{(4-amino-6, 7-dimethoxy – quinazolin-2-y1)-methyl-amino}propyl]oxolane-2-carboxamide hydrochloride. Following compound 55 shows the structure of alfuzosin. It is used for the treatment of lower urinary tract symptoms associated with benign prostatic hyperplasia. Literature survey reveals that, few chromatographic 2-6 methods have been reported for the estimation of AFZ. To the best of our knowledge, there is no work in the literature reported about the spectrophotometric method for the analysis of AFZ in either biological fluids or pharmaceutical formulations. Hence the author has made an attempt to develop three simple and rapid spectrophotometric methods for the estimation of AFZ in bulk drugs and in pharmaceutical formulations. The methods are based on the reaction of AFZ with nitrite in acid medium to form diazonium ion, which is coupled with ethoxyethylenemaleic ester or ethylcyanoacetate or acetyl acetone in basic medium to form azo dyes, showing absorption maxima at 440, 465 and 490 nm respectively.

Fig: Structure of Alfuzosin
3H-Quinazolin-4-ones and their derivatives have been reported to possess significant activity as antihypertensive [148], antifibrillatory, choleretic, antiphlogistic [149], antimitotic anticancer [150], antifungal [151,152] and anticonvulsant agents [153]. They have also been successfully tested as CNS depressants [154], muscle relaxants [155] and for their antineoplastic activity [156]. On the other hand, various therapeutic activities have been reported for both pyrazole [157-159] as well as pyrimidine moieties [160,161]. As a part of their continued program on the chemistry of 3H-quinazolin-4-one ring systems, they recently developed a simple and efficient approach to a wide range of such derivatives [162-167]. These results prompted them to synthesize a series of novel 3H-quinazolin-4-one derivatives containing a pyrazolinone, pyrazole or pyrimidinone ring, with the aim of obtaining some novel heterocyclic systems with potentially enhanced biological properties. 3-(4-Aminophenyl)-2-methyl-3H-quinazolin-4-one and its 6-bromo derivative are prepared according to the literature procedures [168,169]. The diazonium chlorides was synthesized by diazotisation of 3-(4-Aminophenyl)-2-methyl-3H-quinazolin-4-one and its 6-bromo derivative using a mixture of sodium nitrite and HCl at 0-5 °C. The diazonium salts thus obtained were treated in ethanol in the presence of sodium acetate with calculated amounts of some active methylene compounds, namely ethyl acetoacetate, ethyl cyanoacetate and acetylacetone to afford the corresponding hydrazono derivatives 56-58.

![Chemical Structure](image)

56a, R¹=COCH₃; R²=COOC₂H₅; X=H
56b, R¹=COCH₃; R²=COOC₂H₅; X=Br
57a, R¹=CN; R²=COOC₂H₅; X=H
57b, R¹=CN; R²=COOC₂H₅; X=Br
58a, R¹=COCH₃; R²=COCH₃; X=H
58b, R¹=COCH₃; R²=COCH₃; X=Br
Hydrazono derivatives 56a, b is cyclized with hydrazine or phenyl hydrazine in boiling ethanol is expected to lead to the formation of the corresponding pyrazolin-5-one derivatives of 3H-quinazolin-4-one 59a-d.

![Chemical Structure](image)

59a $\text{R} = \text{H}$; $\text{X} = \text{H}$
59b $\text{R} = \text{H}$; $\text{X} = \text{Br}$
59c $\text{R} = \text{ph}$; $\text{X} = \text{H}$
59d $\text{R} = \text{ph}$; $\text{X} = \text{Br}$

3-{$[\text{N’-}(3\text{-Amino-5-oxo-1,5\text{-dihydropyrazol-4-ylidene})\text{ hydrazino]}\text{ phenyl}]$-2-methyl-3H quinazolin-4-one 60a and its bromo derivative 60b are synthesized via cyclization of 57a, b with hydrazine.

![Chemical Structure](image)

60a $\text{X} = \text{H}$
60b $\text{X} = \text{Br}$

3-{$[\text{4-(Dimethyl-1-phenyl-1H-pyrazol-4-ylazo)-phenyl}]\text{-2-methyl-3H-quinazolin-4-one}$ 61a and its bromo derivative 61b are obtained by thermal cyclization of 58a, b with phenyl hydrazine in glacial acetic acid.
Reaction of the hydrazono derivatives 58a, b with urea in ethanol under reflux conditions for 5 hours gave solid products of molecular formula \( C_{21}H_{18}N_6O_2 \), which may be formulated as 62a, or \( C_{21}H_{17}BrN_6O_2 \), corresponding to the bromo derivative 62b, respectively.

Azo compounds contain azo groups linked to methine or aromatic sp\(^2\)-hybridized C-atoms. The formation of diazotizing reagent starts with protonation of nitrous acid under strongly acidic conditions, and azo coupling carried out at low temperature in the presence of nucleophilic coupling components, the reactivity of a nucleophilic substrate increases with increasing basicity phenolates and amines [170]. These conventional acidebase catalyzed processes are effective for the near quantitative formation of the desired products. But the main limitation of such synthetic processes is their environmental incompatibility. The acidic and basic effluents from the laboratory and industry produce permanent damage to the environment and disturb the ecological balance [171]. In recent years, clay based catalysts are reported to be effective for performing many of the acid base catalyzed organic reactions in a better, environmentally benign manner [172,173]. Recently, they reported
new azoic dyes containing (1H)-tetrazol and imidoyl azide group [174]. As part of their ongoing research program for exploring the bifunctional catalytic properties, they herein describe a new process for diazotization and diazo coupling reactions using clay based layered silicates as a catalyst toward the synthesis of azo dyes. This paper describes the facile and modified synthesis of azo dyes (this method previously has been reported under acidic and basic conditions) [175] without using conventional acid or base in the presence of clays. In the present synthesis, the sodium sulfanilate or 4-aminobenzene sulfonyl azide is first made into a paste with clay catalyst and its then cooled to 0-5°C. This clay mixture is then diazotized with dilute NaNO₂ solution. The diazonium clay complex formed is subsequently coupled with phenols, naphthols and an aromatic amine. The sodium sulfanilate azo dye formed is separated from the catalyst by extracting it into water or alcohol and sulfonyl azide azo dye is separated from the catalyst by extracting it into acetone and from where it is recovered by removal of the solvent under vacuum. The generality of the process is proved by performing the reaction with all the three catalysts, with sodium sulfanilate or 4-aminobenzene sulfonyl azide and with coupling agents. After the formation of the diazonium clay complex, the edge hydroxyls of the clay platelets are believed to get converted into -ONa.
species by consuming the Na ions from NaNO₂ solution used for diazotization. This -ONa species helps to maintain the pH of the medium neutral or slightly alkaline for a quantitative coupling of the diazonium ion with the coupling agent. In almost all the cases, the isolated yields of the pure products are found to be near quantitative. Control reactions are carried out with the same reagents in the presence of mineral acids like HCl and bases by following the conventional procedure for comparing the yields. All substrate yields for sodium sulfanilate dyes are in the range of 60-85% and for sulfonyl azide dyes in the range of 30-60%. The yields found to be slightly less than the same obtained from the present mineral acids process.

Azobenzene, with two phenyl rings linked by N=N double bond, serves as the parent molecule for a broad class of aromatic azo compounds. The strong absorption of band these compounds can be tailored by ring substitution to fall anywhere from the ultraviolet to red-visible regions, allowing chemical fine-tuning of color. This property, combined with the fact that the azo groups are relatively robust and chemically stable, has prompted extensive study of azo benzene based structures as dyes [176]. Approximately 10,000 different dyes and pigments are used industrially, and over 0.7 million tons of synthetic dyes are produced.
Azoic dyes are the most important group of all synthetic dyes that are used extensively for textile dyeing [178], paper printing and color photography and as additives in petroleum products. In addition, azo benzene moiety is an important structural motif in biological systems [179]. The photo response of azo compounds modifies the activity of enzymes and polypeptides, also is used for more accurate diagnosis of Alzheimer's disease [180]. Moreover, azo benzenes recently have been targeted for potential applications in areas of nonlinear optics [181], optical storage media [182], chemosensory[183,184], liquid crystals [176], photochemical molecular switches [185], molecular shuttles [186], nanotubes [187], in the manufacture of protective eye glasses and filters [188] and as optical switch for in vivo experiments [189]. The synthesis of aromatic azo components has been attracting increasing interest over the past decade because of their utility in various applications. In recent years, several methods have been reported for synthesis of these components [190-192]. Although remarkable developments have been achieved in this field, the search for mild conditions could represent a useful tool in organic chemistry. The most important methods for the preparation of aromatic azo have focused on diazotization-azo coupling reaction. The main limitation of this process is their environmental incompatibility. Since, use of strong liquid acids for diazotization reaction (such as H₂SO₄, HCl) causes permanent damage to the environment and their use is contrary to the principles of green chemistry. In addition, these liquid acids require special processing in the form of neutralization, which involves costly and inefficient catalyst separation from homogeneous reaction mixture, and then results in an unrecyclable waste. The need for a “green” approach to chemical processing has stimulated the use of recyclable strong solid acids as replacements for unrecyclable liquid acid catalysts [193-196]. Magnetic nanoparticles have emerged as a highly valuable substrate for the attachment of homogeneous inorganic and organic containing catalysts. They have advantages such as readily available, high surface area and most importantly magnetically recoverable, which facilitates reaction work-up and rapid sample processing, and reduces solvent consumption [197,198]. They have previously report synthesis of Nano magnetic supported sulfonic acid (nano-g-Fe₂O₃-SO₃H) and its applications as viable alternative to unrecyclable homogenous catalysts in organic synthesis [199-201]. Recently, they have used this catalyst for the synthesis of aryl iodides [202]. As part of their ongoing research program to explore catalytic properties of Nano magnetic supported sulfonic acid, they herein report a new process for diazotization and diazo coupling reaction of aromatic amines using nano-g-Fe₂O₃-SO₃H.
Simple nitrogen-containing heterocycles attached to sulfonamido moieties have received a large amount of attention in the literature, as a consequence of their exciting biological properties and their role as pharmacophores of considerable historical importance. Heterocyclic sulfonamides are used as carbonic anhydrase inhibitors [203-205], antibacterial agents [206], anticancer, anti-inflammatory and analgesic agents [207], β3-adrenergic receptor agonists [208], PC-1 inhibitors [209], antifungal agents [210] and antiviral agents [211]. For these vast biological activities and in continuation of our work [212-220] on the synthesis of novel heterocyclic systems exhibiting biological activity. They undertook the synthesis of a new series of compounds incorporating the abovementioned biologically active moieties in one molecule. They synthesized the precursor of hydrazone 3 by diazotization of sulfamethazine, \([N1-(4,6\text{-dimethyl-2-pyrimidinyl}) sulfanilamide, 1]\) followed by coupling with ethyl cyanoacetate in the presence of sodium acetate at room temperature [221].

![Chemical structure of compound 95](image)

They investigated the reactivity of compound 95 towards active methylene reagents. Firstly, reaction of hydrazone 95 with dicarbonyl compounds is studied. Thus, when compound 95 reacted with acetylacetone in refluxing dioxane in the presence of catalytic amounts of triethylamine, the pyranone derivative 96 is obtained. Similarly, reaction of 95 with acetoacetanilide under the same reaction conditions afforded the pyridinone derivative 97 which is formed through the intermediate followed by loss of an ethanol molecule.
Secondly, they investigated the behavior of 95 with other active methylene compounds, such as ethyl acetoacetate, ethyl benzoylacetate, ethyl cyanoacetate and malononitrile. This investigation resulted in the synthesis of polyfunctional substituted pyridazine derivatives 98a–c.

The behaviour of 95 towards hydrazine derivatives are examined in order to prepare pyrazoles. Thus, treatment of 95 with hydrazine hydrate [222] (98%), phenylhydrazine, benzoylhydrazine or thiosemicarbazide [223] furnished the aminopyrazole derivatives 99a–d, respectively. On the other hand, reaction of 3 with hydroxylamine hydrochloride in the presence of sodium acetate produced the isoxazole derivative 100. The structure of the prepared compounds is in accordance with their spectral data. Pyrazolopyrimidine [224] derivative 103 is obtained when the hydrazone 95 was treated with cyanoacetohydrazide in refluxing dioxane in the presence of triethylamine. As a speculative mechanism for the formation of compound 103, the intermediate 101 is firstly formed followed by an internal nucleophilic attack by the NH group on the cyano group. Then a migration of the two NH protons to the negatively charged nitrogen atom takes place to form the second intermediate 102. Finally, 102 cyclized via nucleophilic attack by the NH₂ group on the cyano group to
produce the pyrazolopyrimidine derivative 103. Treatment of compound 95 with urea, thiourea and guanidine in the presence of ethanolic sodium ethoxide [225] produces the pyrimidine and thiazine derivatives 104, 105a-b. The formation of these compounds is assumed to be occur via the addition of the NH₂ or SH groups to the cyano group followed by cyclization with elimination of an ethanol molecule.
Diazotized 1 H-aminoazoles are versatile reagents and their synthetic potentialities has received considerable recent attention [226-230]. As a part M. H. Elnagdi et al. programs directed for development of new procedures for the synthesis of bridge-head azoles [231-237] they have previously [231,236] reported the synthesis of a variety of pyrazolo[1,5-c]-as-triazines and pyrazolo-[1,5-c]-1,2,4-triazoles based on the coupling reaction of 3-phenylpyrazole-5-diazonium chloride with active methylene compounds. In continuation of this work they report here the results of their further investigation on the reaction of diazotized 1H aminoazoles. The work has resulted, in addition to synthesis of several new pyrazolo[1,5-c]-as-triazines in clarification of the mechanistic path ways for reaction of diazotized 5-aminopyrazoles with active methylene compounds. Thus, diazotization of 5-amino-3-phenylpyrazole has afforded 3-phenyl -pyrazole-5-diazonium chloride which coupled with benzoylacetetonitrile and with phenacylthiocyanate to yield the corresponding hydrazon derivatives 106a, b, respectively. Compound 3 a readily cyclised into the pyrazolo-[1,5-a]-as-triazine derivative 108a upon treatment with concentrated sulphuric acid. On the other hand, when 106b was similarly treated, compound 108b, the structure of which was inferred from analytical and spectral data, was formed. The formation of 108b from 106b and concentrated sulphuric acid might be assumed to proceed via initial elimination of thiocyanic acid leading to the formation of a resonance stablised nitrile imine intermediate which cyclises then to afford the final reaction products. Previously they have reported [236] that 3-Phenylp vzazole-5-diazonium chloride reacts with acrylonitrile, ethyl acrylate and with dimethyl acetylene-dicarboxylate to yield the pyrazolo[1,5-c]- as-triazine derivatives 107a, b respectively. Intermediacy of 3-phenyl-5-diazopyrazole has been suggested to account for the formation of these products. Now, they would like to report the isolation of 3-phenyl-5-diazopyrazole as well as its reactions with dipolarophiles [238]. Thus, when 3-Phenylp vzazole-5-diazonium chloride was treated with aqueous sodium acetate solution it was converted almost quantitatively into the diazobetaien 3-phenyl-5-diazoazylpyrazole. When 3-phenyl-5-diazopyrazole was treated with acrylonitrile ethyl acrylate or with dimethyl acetylenedicarboxylate, products identical in all aspects with those previously formed on treatment of 3-Phenylp vzazole-5-diazonium chloride with the same reagents are formed in excellent yields. 3-phenyl-5-diazopyrazole readily added less reactive dipolarophiles namely chalcone and methyl cinnamate to yield the pyrazolo[1,5-c]-as-triazine derivatives 108c, d, respectively. Compound 3-phenyl-5-diazopyrazole reacted with benzoylacetetonitrile, ethyl acetoacetate, 3-iminobutyrtonitrile, ethyl cyanoacetate and with malononitrile to yield the pyrazolo[1,5-c]-as-triazine derivatives
108a, d-g. Under similar conditions compound 3-phenyl-5-diazopyrazole failed to react with phenacylthiocyanate.

Arylhydrazones of methylene active compounds (AHMACs) are very interesting molecules and versatile starting materials for a number of organic syntheses, leading to compounds which are biologically active [239], possess liquid crystal properties [240], can be applied as analytical reagents [241], indicators [242], ionophores [243], or hydrazone dyes [244].
The first methylene-active arylhydrazones were reported as early as 1883, by Richter and Müntzer, then designated as "Benzolazoacetone" [245] which is five years later shown by Japp and Klingemann to be a hydrazine [246]. The synthesis of AHMACs consist in a coupling of a MAC (methylene active compound) with an aromatic diazonium salt, mostly performed in methanolic or ethanolic solution containing acetate [247]. The AHMAC products can be used as intermediates in further syntheses of organic molecules or as a promising ligands in coordination chemistry.

Despite their ready accessibility by diazotisation of the corresponding amino-heterocycles, the chemical reactivity of five-membered heterocyclic diazonium salts has, in comparison with their benzenoid counterparts, been little investigated [248]. Diazonium salts (2) derived from 1H-aminoazoles are of particular interest as sources of 1,4-dipoles of the type. Thus, diazonium salts derived from 3-amino-2H-pyrazoles and 5-axnino-IH-1,2,3-triazoles are converted in neutral or weakly basic solution into the relatively stable diazonium betaines [249,250]. 1,4-Dipoles may also be invoked as reactive intermediates in the coupling reactions of 2H-indazole-3-diazonium salts and pyrazole-5-diazonium salts with active methylene compounds [251,252] and phenols [249,253] to afford hydrazones convertible by cyclisation into indazolo[3,2-c] [1,2,4] triazines and pyrazolo[5,1-c] [1,2,4] triazines, respectively. As part of a general investigation of the chemistry of diazonium salts and the related 1,4-dipoles they described [254] a general synthesis of the chemistry of the previously unknown 1,2,3-triazolo[5,1-c] [1,2,4] triazine ring system based on the coupling reactions of 4-phenyl-1,2,3-triazole-5-diazonium chloride with active methylene compounds. Analogous coupling reactions of 1,2,4-triazole-5-diazonium nitrate are now shown to provide a new, general synthesis of otherwise not readily accessible [1,2,4] triazolo- [5,1-c] [1,2,4] triazine derivatives. The ready replacement of the diazonium group in 1,2,4-triazole-5-diazonium salts by chloride ion [255,256] precluded the study of the
reactions of 1,2,4-triazole-5-diazonium chloride with active methylene compounds. Coupling reactions were therefore carried out in situ by using solutions in dilute nitric acid of the relatively stable [256] 1,2,4-triazole-5-diazonium nitrate. Previous work they had shown [257] that 3-alkyl-1,2,4-triazole-5-diazonium nitrates coupled with acetylacetone and ethyl acetoacetate to give products which were not characterized but gave correct analytical data for the corresponding 1,2,4-triazol-5-ylhydrazones 110. In their present studies, which are smoothly converted in warm aqueous ethanolic sodium acetate or glacial acetic acid, in high yield, into the corresponding [1, 2,4] triazolo [5.1-c]- [1,2,4] triazine derivatives 111-112.

[Chemical structures are shown here, but not transcribed.]

Pyrazolo[3,4-b] pyridinediazonium chlorides react with a variety of active methylene-containing reagents (e.g., cyanoacetic acid arylidenehydrazide derivatives) to afford the corresponding 3-hydrazonopyrazolo[3,4-b] pyridine derivatives. The diazonium chlorides react with N'-acyl-2-cyanoaceto-hydrazide derivatives to give the corresponding 3-hydrazonopyrazolopyridine derivatives. The latter affords the corresponding 3-hydrazonopyrazolo[3,4-b] pyridine derivatives on reflux in acetic acid. Diazo coupling of 2,4-dimethylpyrazolo[3,4-b] pyridinediazonium chloride with ketoester, e.g., ethyl benzoyleacetate, is followed by cyclization to afford pyrido [2',3':3,4] pyrazolo[5,1-c]triazine derivative. Diazo coupling of the same dimethylpyrazolo[3,4-b] pyridinediazonium chloride with unsymmetrical β-diketone, e.g., benzoylacetonate, was also
studied to afford the corresponding hydrazono derivative, which undergoes in situ cyclization to furnish the pyrido [2',3':3,4] pyrazolo[5,1-c] triazine derivative.

Azo compounds are the oldest and the largest class of industrial synthetic organic dyes due to their versatile applications. Azo compounds are characterized by the presence of the azo moiety (-N=N-) in their structure conjugated with two, distinct or identical, mono or polycyclic aromatic systems. They have been most widely used in dyeing textile fibers, papers and coloring agents for foods and cosmetics [258,259]. The pharmacological use of azo compounds originates from the discovering of the antibacterial action of prontosil on streptococcal infections by Domagk [260]. Furthermore, azo compounds such as salicylazosulfapyridine, known drug with name azulfidine (sulfasalazine), used to treat rheumatoid arthritis [261]. These azo compounds display multiple biological and pharmaceutical applications and are known for their antimicrobial [261-265], anti-inflammatory [267,268], antioxidant [269] and enzyme inhibitors [270]. There has been considerable attention in the development of nitrogen containing heterocyclic compounds in both medicinal and industrial chemistry. 1,2,3-triazole derivatives, which are remarkable class of heterocycles on account of their wide range of applications as pharmaceuticals due to their wide spectra of biological activities such as antimicrobial [271-273], anticancer [274-276], anticonvulsant and analgesic activity [277]. In view of these findings, they aimed to synthesize simpler substances that can have antibacterial activity. For this aim they synthesized some new novel diazotized compounds [258-261] as well as 1,2,3-triazole derivatives [263-264] with help of 4-aminoacetophenone and active methylene compounds.
1-(4-(4-acetyl-5-methyl-1H-1,2,3-triazol-1-yl) phenyl) ethanone

1-(4-acetylphenyl)-5-methyl-1H-1,2,3-triazole-4-carboxilic acid

Methyl-1-(4-acetylphenyl)-5-amino-1H-1,2,3-triazole-4-carboxylate
CHAPTER-2

EXPERIMENTAL
2.1 Synthesis of 3,3'-(1,4-phenylenebis(hydrazin-2-yl-1-ylidene)) bis(pentane-2,4-dione)

*p*-Phenylendiamine (0.01 mol, 1.0814 gm) was dissolved into a mixture of concentrated hydrochloric acid (8 ml) and water (6 ml). The mixture was cooled to 0-5°C on ice bath. A cold aqueous solution of sodium nitrite (0.04 mol, 2.76 gm) was added to that mixture. Then the cooled diazonium salt solution was added dropwise into a cooled solution of acetyl acetone (0.02 mol, 2.0 gm) and sodium acetate (0.1 mol, 8.0 gm) in ethanol (25 ml) and stirred for 2 hours. The progress of the reaction was monitored by TLC. Then the resulting solid mass was filtered, dried and purified by recrystallization from ethanol. After drying an orange solid compound with 74% yield was obtained and the melting point was recorded as 160-164°C.
IR (KBr, cm\(^{-1}\), Fig. 1): 3400-3500 (w, N-H), 3010 (C-H, aromatic), 2928 (C-H, aliphatic), 1674 (C=O, conjugated), 1621 (C=N), 1519,1439 (C=C, aromatic).

\(^1\)H NMR (400 MHz, CDCl\(_3\), ppm, Fig. 2-3): \(\delta\) 2.50 (s, 6H, -CH\(_3\)), 2.62 (s, 6H, -CH\(_3\)), 7.47 (s, 4H, aromatic), 14.84 (s,2H, N-H).

\(^{13}\)C NMR (100 MHz, CDCl\(_3\), ppm, Fig.4): \(\delta\) 198 (2C, C=O), 196 (2C, C=O), 139 (2C, C=N), 133 (2C, N-C-), 117 (4C, aromatic), 31 (2C, COCH\(_3\)), 26 (2C, COCH\(_3\)).
2.2 Synthesis of diethyl 2,2’-(1,4-phenylenebis(hydrazin-2-yl-1-ylidene)) (2,2’)-bis(3-oxobutanoate)

*p*-Phenylendiamine (0.01 mol, 1.0814 gm) was dissolved into a mixture of concentrated hydrochloric acid (8 ml) and water (6 ml). The mixture was cooled to 0-5°C on ice bath. A cold aqueous solution of sodium nitrite (0.04 mol, 2.76 gm) was added dropwise to that mixture. Then cooled diazonium salt solution was added into a cooled solution of ethylacetoacetate (0.02 mol, 2.26 gm) and sodium acetate (0.1 mol, 8.0 gm) in ethanol (25 ml) and stirred for 2 hours. The progress of the reaction was monitored by TLC. Then the resulting solid mass was filtered, dried and purified by recrystallization from ethanol. After drying an orange solid compound with 65% yield was obtained and the melting point was recorded as 147-151°C.

![Chemical Structure](image-url)
IR (KBr, cm\(^{-1}\), Fig. 5): 3200-3300 (w, N-H), 3064 (C-H, aromatic), 2983 (C-H, aliphatic), 2935 (C-H, aliphatic), 1715 (C=O, COCH\(_3\)), 1684 (C=O, COC\(_2\)H\(_5\)), 1607 (C=N), 1555, 1525, 1490 (C=C, aromatic).

\(^1\)H NMR (400 MHz, CDCl\(_3\), ppm, Fig. 6): \(\delta\) 1.40 (m, 6H, 2COCH\(_2\)-CH\(_3\)), 2.5 (m, 6H, COCH\(_3\)), 4.4 (m, 4H, 2CH\(_2\)CH\(_3\)), 7.3 (s, 4H, aromatic), 12.76 (s, 1H, N-H), 14.76 (s, 1H, N-H).
2.3 Synthesis of \(2,2'-(1,4\text{-phenylenebis(hydrazin-2-yl-1-ylidene)bis(5,5-imethylcyclohexane-1,3-dione)}}\)

\(p\)-Phenylene diamine (0.01 mol, 1.0814 gm) was dissolved into a mixture of concentrated hydrochloric acid (8 ml) and water (6 ml). The mixture was cooled to 0-5\(^{\circ}\)C on ice bath. A cold aqueous solution of sodium nitrite (0.04 mol, 2.76 gm) was added dropwise to that mixture. Then the cooled diazonium salt solution was added into a cooled solution of dimerdone (0.02 mol, 2.8 gm) and sodium acetate (0.1 mol, 8.0 gm) in ethanol (25 ml) and stirred for 2 hours. The progress of the reaction was monitored by TLC. Then the resulting solid mass was filtered, dried and purified by recrystallization from ethanol. After drying a deep orange solid compound with 68% yield was obtained and the melting point was recorded as 150-154\(^{\circ}\)C.
**IR (KBr, cm$^{-1}$, Fig. 7):** 3300-3500 (w, N-H), 3050 (C-H, aromatic), 2961 (C-H, aliphatic), 2869 (C-H, aliphatic), 1682 (C=O), 1622 (C=N), 1570,1500,1435 (C=C, aromatic).

**$^{1}$H NMR (400 MHz, CDCl$_3$, ppm, Fig. 8-9):** δ 7.62 (bd, s, 4H, Aromatic), 2.63 (s, 8H, CO-CH$_2$-CO), 1.25 (bd, s, 12H, 2 –C(CH$_3$)$_2$).

**$^{13}$C NMR (100 MHz, CDCl$_3$, ppm, Fig.10-12):** δ 197 (2C, C=O), 193 (2C, C=O), 139 (C=N), 130 (C=N), 128.95 (1C, Ar), 128.56 (1C, Ar), 118 (4C, Ar), 52.58 (2C, aliphatic), 52.55 (2C, aliphatic), 30 (2C, aliphatic), 28 (4C, aliphatic).
2.4 Synthesis of 3,3′-(1,3-phenylenebis(hydrazin-2-yl-1-ylidene)) bis(pentane-2,4-dione)

*m*-Phenylenediamine (0.01 mol, 1.0814 gm) was dissolved into a mixture of concentrated hydrochloric acid (8 ml) and water (6 ml). The mixture was cooled to 0-5°C on ice bath. A cold aqueous solution of sodium nitrite (0.04 mol, 2.76 gm) was added dropwise to that mixture. Then cooled diazonium salt solution was added into a cooled solution of acetylaceetone (0.02 mol, 2.0 gm) and sodium acetate (0.1 mol, 8.0 gm) in ethanol (25 ml) and stirred for 2 hours. The progress of the reaction was monitored by TLC. Then the resulting solid mass was filtered, dried and purified by recrystallization from ethanol. After drying a deep orange solid compound with 76% yield was obtained and the melting point was recorded as 140-145°C.
IR (KBr, cm⁻¹, Fig. 13): 3370-3460 (N-H), 3062 (C-H, aromatic), 2927 (C-H, aliphatic), 2869 (C-H, aliphatic), 1682 (C=O), 1615 (C=N), 1600,1530,1510 (C=C, aromatic).

¹H NMR (400 MHz, CDCl₃, ppm, Fig. 14-15): δ 2.53 (s, 6H, COCH₃), 2.64 (s, 6H, COCH₃), 7.15 (d, 1H, Ar-H, J=2.0), 7.17 (d, 1H, Ar-H, J=2.0), 7.44 (t, 1H, Ar-H, J=8.0), 7.64 (bd, s, 1H, Ar-H), 14.67 (s, 2H, N-H).
2.5 Synthesis of diethyl 2,2'-(1,3-phenylenebis(hydrazin-2-yl-1-ylidene))(2,2')-bis(3-oxobutanoate)

*m*-Phenylendiamine (0.01 mol, 1.0814 gm) was dissolved into a mixture of concentrated hydrochloric acid (8 ml) and water (6 ml). The mixture was cooled to 0-5°C on ice bath. A cold aqueous solution of sodium nitrite (0.04 mol, 2.76 gm) was added dropwise to that mixture. Then the cooled diazoniim salt solution was added into a cooled solution of ethylacetoacetate (0.02 mol, 2.26 gm) and sodium acetate (0.1 mol, 8.0 gm) in ethanol (25 ml) and stirred for 2 hours. The progress of the reaction was monitored by TLC. Then the resulting solid mass was filtered, dried and purified by recrystallization from ethanol. After drying a deep red solid compound with 74% yield was obtained and the melting point was recorded as 150-153°C.
IR (KBr, cm$^{-1}$, Fig. 16): 3200-3300 (N-H), 3064 (C-H, aromatic), 2983 (C-H, aliphatic), 2937 (C-H, aliphatic), 1715 (C=O), 1608 (C=N), 1570, 1500, 1484 (C=C, aromatic).

$^1$H NMR (400 MHz, CDCl$_3$, ppm, Fig. 17): $\delta$ 1.27 (s, 6H, 2CH$_2$CH$_3$), 1.40 (s, 6H, 2COCH$_3$), 4.41 (s, 4H, 2-OCH$_2$-CH$_3$), 7.94-7.16 (m, 4H, Ar-H), 13.15 (s, 1H, N-H), 14.69 (s, 1H, N-H).
2.6 Synthesis of 2,2′-(1,3-phenylenebis(hydrazin-2-yl-1-ylidene))bis(5,5-dimethylcyclohexane-1,3-dione)

*m*-Phenylendiamine (0.01 mol, 1.0814 gm) was dissolved into a mixture of concentrated hydrochloric acid (8 ml) and water (6 ml). The mixture was cooled to 0-5°C on ice bath. A cold aqueous solution of sodium nitrite (0.04 mol, 2.76 gm) was added dropwise to that mixture. The cooled diazonium salt solution was added into a cooled solution of dimedone (0.02 mol, 2.8 gm) and sodium acetate (0.1 mol, 8.0 gm) in ethanol (25 ml) and stirred for 2 hours. The progress of the reaction was monitored by TLC. Then the resulting solid mass was filtered, dried and purified by recrystallization from ethanol. After drying a deep orange solid compound with 60% yield was obtained and the melting point was recorded as 140-144°C.
**IR (KBr, cm\(^{-1}\), Fig. 18):** 3300-3490 (N-H), 3054 (C-H, aromatic), 2956 (C-H, aliphatic), 2925 (C-H, aliphatic), 1681 (C=O), 1623 (C=N), 1514,1500,1450 (C=C, aromatic).

**\(^1\)H NMR (400 MHz, CDCl\(_3\), ppm, Fig. 19):** \( \delta \) 1.10 (s, 3H, CH\(_3\)), 1.14 (s, 6H, 2CH\(_3\)), 1.40 (s,3H, CH\(_3\)), 2.60 (s, 8H, CH\(_2\)), 7.21-7.62 (m, 4H, Ar-H), 15.25 (s,1H, N-H), 15.43 (s,1H, N-H).
CHAPTER-3

RESULTS AND DISCUSSION
3.1 Characterization of (3,3'-(1,4-phenylenebis(hydrazin-2-yl-1-ylidene))
bis(pentane-2,4-dione))

A mixture of ethanolic solution of acetyl acetone and aqueous solution of sodium nitrite
was stirred for 30 minutes at 0-5°C. To this mixture, a diazonium salt solution of p-
phenylenediamine was added dropwise and the stirring was continued for two hours at the
same temperature. The progress of the reaction was monitored by TLC. After completion
the reaction an orange solid compound was isolated and found 74% yield. The melting
point of the product was recorded as 160-164°C.

The IR spectra (Fig.1) of the compound 2 showed a wide absorption band at 3400-3000
\text{cm}^{-1} for the N-H groups. The aromatic C-H stretching absorption was assigned at 3010 \text{cm}^{-1}. The aliphatic C-H band was ascribed at 2928 \text{cm}^{-1}. The conjugated carbonyl absorption peaks were distinctive at 1674 \text{cm}^{-1}. The sharp peak at 1621 \text{cm}^{-1} was designated for C=N moiety. The characteristic peaks at 1519,1439 \text{cm}^{-1} was distinguishable for aromatic C=C
double bonds.
The $^1$H NMR spectra (Fig.2-3) of compound 2 showed a sharp singlet for six protons of 2CH$_3$ at 2.50 ppm. The similar signal at 2.62 ppm was designated for six protons of another 2CH$_3$. The sharp singlet at 7.47 ppm was attributed for four aromatic protons. The downfielded singlet of two N-H protons was detected at 14.84 ppm.

The $^{13}$C NMR spectra (Fig.4) of the compound 2 showed signal at 198 ppm for 2C of C=O group. Another 2C of C=O was distinctive at 196 ppm. The signal at 139 ppm was designated for 2C of C=N, the signal at 133 ppm was assignable for 2C of nitrogen attached aromatic carbons. The four equivalent aromatic carbon were attributed at 117 ppm. The signal at 31 ppm was designated for 2C of COCH$_3$ and another 2C of COCH$_3$ was distinctive at 26 ppm.

All the spectral evidences expressed harmony with the structure of the compound 2 as

![Compound Structure](image)

The compound 2 was the isomerized product of 1 which was justified by the presence of N-H proton in the $^1$H NMR spectra.
Figure 1: IR spectrum of compound 2
Figure 2: 1H NMR spectrum of compound 2
Figure 3: $^1$H NMR (extended) of compound 2
Figure 4: $^{13}$C NMR spectrum of compound 2
3.2 Characterization of diethyl 2,2’-(1,4-phenylenebis(hydrazin-2-yl-1-ylidene)) (2,2’)-bis(3-oxobutanoate)

A mixture of ethanolic solution of ethyl acetoacetate and aqueous solution of sodium nitrite was stirred for 30 minutes at 0-5°C. To this mixture, a diazonium salt solution of p-phenylenediamine was added dropwise and the stirring was continued for two hours at the same temperature. The progress of the reaction was monitored by TLC. After completion the reaction, an orange solid compound was isolated and found 65% solid. The melting point of the product was recorded as 147-151°C.

The IR spectra (Fig.5) of the compound 4 showed a wide absorption band at 3200-3300 cm\(^{-1}\) for the N-H bond. The aromatic C-H stretching was assignable at 3064 cm\(^{-1}\). The aliphatic C-H stretching bands were designated at 2983 and 2935 cm\(^{-1}\) for -COCH\(_3\) and -CH\(_2\)CH\(_3\) respectively. The saturated C=O peak was distinctive at 1715 cm\(^{-1}\). The esteric C=O peak was identified at 1684 cm\(^{-1}\). The peak at 1607 cm\(^{-1}\) was assigned for C=N moiety. The characteristic aromatic C=C vibrations were distinguishable at 1555, 1525 and 1490 cm\(^{-1}\).
The $^1$H NMR spectra (Fig.6) of compound 4 showed a multiplet at 1.40 ppm for six protons of 2COCH$_3$. The multiplet signal at 2.50 ppm was designated for six protons of 2 –COCH$_3$. Another multiplet signal at 4.4 ppm was indicated for four protons of 2 –OCH$_2$CH$_3$. The singlet at 7.30 ppm was ascribed for four equivalent aromatic protons. The singlet at 12.76 ppm for one proton was assignable for N-H group. The other singlet for one proton was distinguished for N-H moiety.

All the spectral evidences supported the structure of the compound 4 as

The compound 4 was the isomerized product of compound 3 which was proved by the presence of N-H proton in the $^1$H NMR spectra.
Figure 5: IR spectrum of compound 4
Figure 6: $^1$H NMR of compound 4
3.3 Characterization of diethyl 2,2’-(1,4-phenylenebis(hydrazin-2-yl-1-ylidene))bis(5,5-dimethylcyclohexane-1,3-dione)

A mixture of ethanolic solution of dimedone and aqueous solution of sodium nitrite was stirred for 30 minutes at 0-5°C. To this mixture a diazonium salt solution of p-phenylenediamine was added dropwise and the stirring was continued for two hours at the same temperature. The progress of the reaction was monitored by TLC. After completion the reaction an orange solid compound was isolated and found 68% solid. The melting point of the product was recorded as 150-154°C.

The IR spectra (Fig.7) of the compound 6 showed a wide absorption band at 3300-3500 cm\(^{-1}\) for the N-H groups. The weak aromatic C-H stretching frequency was detected at 3050 cm\(^{-1}\). The aliphatic C-H stretching vibrations were indicative at 2961 and 2869 cm\(^{-1}\) for -CH\(_3\) and -CH\(_2\)- groups respectively. The peak at 1682 cm\(^{-1}\) was assigned for C=O group. The C=N moiety was designated at 1622 cm\(^{-1}\). The characteristic bands at 1570, 1500 and 1435 cm\(^{-1}\) were distinguishable for aromatic C=C bonds.
The $^1$H NMR spectra (Fig.8-9) of the compound 6 showed a broad singlet of aromatic for four protons at 7.62 ppm. A sharp singlet at 2.63 ppm was ascribed for eight protons of –COCH$_2$–CO–. A broad singlet signal at 1.25 ppm was distinctive for twelve protons of 2–C(CH$_3$)$_2$.

The $^{13}$C NMR spectra (Fig.10-12) of the compound 6 showed a signal at 197 ppm for 2C of 2C=O groups. Another two carbons of 2C=O groups were designated at 193 ppm. The signal at 139, and 130 ppm were distinctive for 2 C=N carbons. The signal for aromatic six carbons were ascribable at 128.95 ppm for 1C, 128.56 ppm for 1C, 118 ppm for 4C. The aliphatic carbons were attributed at 52.58 ppm for 2C, 152.55 ppm for 2C. The other aliphatic carbons were identified at 30 ppm for 2C and 28 ppm for 4C.

All the spectral evidences expressed harmony with the structure of the compound 6 as
Figure 7: IR spectrum of compound 6
Figure 8: $^1$H NMR of compound 6
Figure 9: $^1$H NMR (Extended) of compound 6
Figure 10: $^{13}$C NMR spectrum of compound 6
Figure-11: $^{13}$C NMR spectrum (Extended) of compound 6
Figure-12: $^{13}$C NMR spectrum (Extended) of compound 6
3.4 Characterization of 3,3'-(1,3-phenylenebis(hydrazin-2-yl-1-ylidene)) bis(pentane-2,4-dione)

A mixture of ethanolic solution of acetyl acetone and aqueous solution of sodium nitrite was stirred for 30 minutes at 0-5°C. To this mixture, a diazonium salt solution of m-phenylenediamine was added dropwise and the stirring was continued for two hours at the same temperature. The progress of the reaction was monitored by TLC. After completion the reaction, a yellow solid compound was isolated and found 70% solid. The melting point of the product was recorded as 140-145°C.

The IR spectra (Fig.13) of the compound 8 showed a wide absorption band at 3370-3460 cm\(^{-1}\) for the N-H groups. The aromatic C-H stretching frequency was absorbed at 3062 cm\(^{-1}\). The peak at 2927 cm\(^{-1}\) was indicative for aliphatic C-H vibrations. The conjugated C=O band was distinctive at 1682 cm\(^{-1}\). The C=N moiety was assignable at 1625 cm\(^{-1}\).
characteristic bands at 1600, 1530 and 1510 cm$^{-1}$ were distinguished for aromatic C=C bonds.

The $^1$H NMR spectra (Fig.14-15) of compound 8 showed a sharp singlet for six protons of 2COCH$_3$ at 2.53 ppm. Another singlet signal at 2.64 ppm was attributable for six protons of 2COCH$_3$. The doublet at 7.15 ppm was designated for one aromatic proton with the gem coupling constant J=2.0. The another doublet at 7.17 ppm was assigned for one aromatic proton with the gem coupling constant J=2.0, The triplet signal for one aromatic proton was identifiable at 7.44 ppm with the coupling constant J=8.0. The broad singlet for one proton was detected at 7.64 ppm. The down fielded singlet for two N-H protons were designated at 14.67 ppm.

All the spectral evidences supported the structure of the compound 8 as

![Chemical Structure of Compound 8]

The compound 8 was the isomerized product of compound 7 which was justified by the presence of N-H proton in the $^1$H NMR spectra.
Figure 13: IR spectrum of compound 8
Figure 14: $^1$H NMR of compound 8
Figure-15: $^1$H NMR (Extended) of compound 8
3.5 Characterization of diethyl 2,2'-(1,3-phenylenebis(hydrazin-2-yl-1-ylidene))(2,2')-bis(3-oxobutanoate)

A mixture of ethanolic solution of ethyl acetoacetate and aqueous solution of sodium nitrite was stirred for 30 minutes at 0-5°C. To this mixture, a diazonium salt solution of \textit{m}-phenylenediamine was added dropwise and the stirring was continued for two hours at the same temperature. The progress of the reaction was monitored by TLC. After completion the reaction, a red solid compound was isolated and found 74% solid. The melting point of the product was recorded as 150-153°C.

![Reaction Scheme]

The IR spectra (Fig.16) of the compound 10 showed a wide absorption band at 3200-3300 cm\(^{-1}\) for the N-H groups. The aromatic C-H stretching frequency was observed at 3064 cm\(^{-1}\). The peaks of 2983 and 2937 cm\(^{-1}\) were assigned for aliphatic C-H stretching vibrations.
The sharp peak at 1715 cm\(^{-1}\) was assigned for C=O. The C=N peak was distinctive at 1608 cm\(^{-1}\). The characteristic bands at 1570, 1500, 1489 cm\(^{-1}\) were distinguishable for aromatic C=C bonds.

The \(^1\)H NMR spectra (Fig.17) of the compound 10 showed a singlet at 1.27 ppm for six aliphatic protons of 2 CH\(_2\)CH\(_3\). Another singlet at 1.40 ppm was ascribed for six protons of 2 COCH\(_3\). The four proton of 2 –OCH\(_2\)CH\(_3\) was assigned as singlet at 4.41 ppm. The multiplet signal at 7.94-7.16 ppm was ascribed for four aromatic protons. The singlet at 13.15 ppm for one proton was distinctive for N-H. The another N-H proton was designated in the downfield at 14.69 ppm.

All the spectral evidences expressed harmony with the structure of the compound 10 as

![Chemical Structure](image)

The compound 10 was the isomerized product of compound 9 which was justified by the presence of N-H proton in the \(^1\)H NMR spectra.
Figure 16: IR spectrum of compound 10.
Figure-17: $^1$H NMR of compound 10
3.6 Characterization of 2,2′-(1,3-phenylenebis(hydrazin-2-yl-1-ylidene))bis(5,5-dimethylcyclohexane-1,3-dione)

A mixture of ethanolic solution of dimedone and aqueous solution of sodium nitrite was stirred for 30 minutes at 0-5°C. To this mixture, a diazonium salt solution of \( m \)-phenylenediamine was added dropwise and the stirring was continued for two hours at the same temperature. The progress of the reaction was monitored by TLC. After completion the reaction a yellow solid compound was isolated and found 60% solid. The melting point of the product was recorded as 140-144°C.

\[ \text{2,2'}-(1,3\text{-phenylenebis(diazene-2,1-diyl)})\text{bis}(5,5\text{-dimethylcyclohexane-1,3-dione}) \]

\[ \text{2,2'}-(1,3\text{-phenylenebis(hydrazin-2-yl-1-ylidene)})\text{bis}(5,5\text{-dimethylcyclohexane-1,3-dione}) \]
The IR spectra (Fig.18) of the compound 12 showed a wide absorption band at 3300-3490 cm\(^{-1}\) for the N-H moiety. The aromatic C-H stretching was assigned at 3054 cm\(^{-1}\). The peaks at 2956 cm\(^{-1}\) and 2925 cm\(^{-1}\) were distinctive for aliphatic C-H stretching vibration. The conjugated C=O frequency was detectable at 1681 cm\(^{-1}\). The C=N moiety was identified at 1623 cm\(^{-1}\). The characteristic bands at 1514, 1500,1450 cm\(^{-1}\) were distinguished for aromatic C=C bonds.

The \(^1\)H NMR spectra (Fig.19) of compound 12 showed a sharp singlet at 1.10 ppm for the three protons of CH\(_3\). The singlet signal at 1.14 ppm was attributable for six protons of 2CH\(_3\). The another singlet signal at 1.40 ppm was designated for three protons of CH\(_3\). The eight CH\(_2\) protons were detected by the sharp singlet signal at 2.60 ppm. The multiplet signal at 7.21-7.62 ppm was identified for four aromatic protons. The down fielded singlet signals at 15.25 ppm and 15.43 ppm were vividly indicative for two N-H protons.

All the spectral evidences expressed the same tone with the structure of the compound 12 as

![Diagram of Compound 12]

The compound 12 was the isomerized product of compound 11 which was justified by the presence of N-H proton in the \(^1\)H NMR spectra.
Figure 18: IR spectrum of compound 12
Figure 19: $^1$H NMR of compound 12
CHAPTER-4

BIOLOGICAL ACTIVITY TEST
Biological activity test of the synthesized compound by agar diffusion Technique.

Principle
An antibiotic refers to a substance produced by a microorganism or to a similar substance produced wholly or partly by chemical synthesis, which in low concentration inhibit the growth of other microorganisms. Many antibiotics are used as chemotherapeutic agent but other is too highly toxic for use. In case of chemotherapeutic use, an antibiotic should, ideally be toxic only for pathogenic microorganisms and should be harmless to the host. Assay of antibiotics include certain methods such as microbiological assays, radio enzymatic assays, high performance of high-pressure liquid chromatography (HPLC), immunoassay etc. There are three main methods of microbiological assay by which the potency of an antibiotic can be compared with that of a standard antibiotic.

A. Dilution method
B. Diffusion method
C. Turbidimetric, titrimetric and gravimetric methods.

Among these methods the agar diffusion method is most commonly used to assay the potency of an antibiotic. In agar diffusion assays, the response of a growing population of microorganism to the antimicrobial agent is measured. Potency is a term used to express the strength of chemical. Antibiotics, originated from microorganism, also undergo chemical formulation and thereby need to be evaluated in terms of potency for clinical use as well as for market value. While assaying the potency of an antibiotic, proper calibration is essential to express the result in terms of absolute units. Thus a pure sample of the drug or a sample of known potency is required for the preparation of calibrator solutions.

The agar diffusion assay may be one dimensional, two-dimensional or three-dimensional. Two or three-dimensional assay is the commonest form of microbiological assay. This experiment employs the three-dimensional agar diffusion assay. In this system samples are applied in reservoir (well) to a thin layer of agar seeded with indicator organism. The drug diffuses into the medium. After incubation a zone of growth inhibition forms (a circle
around the reservoir). The diameter of the zone is related to the concentration of antibiotic in the reservoir.

The edge of a zone is formed when the minimum concentration of antibiotic which will inhibit the growth of the organism on plate (Critical Concentration) reaches for the first time (when the population density is too great for the antibiotic to inhibit). The position of the zone edge is thus determined by:

1. The initial population density.
2. Growth rate of the organism.
3. The rate of diffusion of antibiotic.
4. Agar thickness.

A balance design is one in which all controllable variables have been accommodated and such design is called “Latin square design”. A Latin square arrangement is acceptable for pharmacokinetic or clinical assays. In situations where the concentration range of tests will lie in a narrow range and where high precision is sought, a Latin square design with tests and calibrators at two or three levels of concentration may be used. As a result, this assay ensures maximum precision. Different types of Latin square designs are known. In this experiment, the potency of the supplied antibiotic was determined by three-dimensional agar diffusion assay using 4*4 (2+2) Latin square design.

The 4*4 (2+2) Latin square design is used for the assay of one sample and one standard at two concentrations each and allows four zones for each concentration in a true Latin square design where each treatment appears once in every row and in every column. The plate size was (20*20) inches.
Material and Method

Sample
Supplied three synthesized sample

Culture
*S.aureus, B.cereus, E.coli*

Medium
Nutrient agar

Reagents
Phosphate buffer and 0.1 N HCl

Equipment
Large Petri-dish, micropipette, electric balance, pipette, puller, conical, flask, borer, incubator, inoculating loop, burner and test tubes.

Procedure
1. **Preparation of test sample**: 0.01mg of sample dissolves in 0.9 ml of solvent; prepare total 1ml stock sample. This experiment employs the three-dimensional agar diffusion assay. In this system samples are applied in reservoir (well) to thin layer of agar seeded with indicator organism. The drug diffuses in to the medium. After incubation a zone of growth inhibition forms (a circle around the reservoir). The diameter of the zone is related to the concentration of antibiotic in the reservoir.
2. After finishing all these preparations, 0.75 ml bacterial suspension was added to 150 ml nutrient agar media (in liquid state) in conical flask and by swivel to avoid bubble.
3. The petri-plates were placed on a level bench and precaution was taken to keep the plate sterile. Then media seeded with bacterial suspension was poured in the petri-plate using a circular motion to ensure the even distribution of medium over the plate and allowed to stand for a few minutes for solidification.
4. Then using a sterile borer, well were designed in such a way that every well is apart from another by the same distance.
5. Two drops of each of the standard and the test solutions were dropped in wells.
6. After dropping the plate was kept undisturbed for about half an hour and then incubated at 37\(^{0}\)C for 24 h.
7. After overnight incubation, formation of any zone of growth inhibition was monitored.
8. The diameter of zone of inhibition was then measured and the potency of drug was calculated as below.
Effect of Bacterial growth:

**Compound 4**

![Chemical structure of Compound 4](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gram positive</th>
<th>Gram negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S.aureus</em></td>
<td><em>B.cereus</em></td>
</tr>
<tr>
<td></td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td></td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>0.8 mm</td>
<td>0.55 mm</td>
</tr>
<tr>
<td></td>
<td>1.2 mm</td>
<td>0.9 mm</td>
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</tbody>
</table>

**Compound 8**

![Chemical structure of Compound 8](image)

<table>
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<th>Gram negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S.aureus</em></td>
<td><em>B.cereus</em></td>
</tr>
<tr>
<td></td>
<td>50µl</td>
<td>50µl</td>
</tr>
<tr>
<td></td>
<td>100µl</td>
<td>100µl</td>
</tr>
<tr>
<td></td>
<td>1 mm</td>
<td>0.6 mm</td>
</tr>
<tr>
<td></td>
<td>1.6 mm</td>
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### Compound 10

![Chemical Structure of Compound 10](image)

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</thead>
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<tr>
<td></td>
<td><em>S. aureus</em></td>
<td><em>B. cereus</em></td>
</tr>
<tr>
<td>Compound 10</td>
<td>50 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>0.9 mm</td>
<td>1.4 mm</td>
</tr>
<tr>
<td></td>
<td>1 mm</td>
<td>1 mm</td>
</tr>
<tr>
<td>Standard Azithromycin</td>
<td>50 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>19 mm</td>
<td>23 mm</td>
</tr>
<tr>
<td></td>
<td>25 mm</td>
<td>12 mm</td>
</tr>
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</table>

### Control (DMSO)

<table>
<thead>
<tr>
<th>E. Coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>0 mm</td>
<td>0 mm</td>
</tr>
</tbody>
</table>
The synthesized compounds 4,8,10 were screened for their antibacterial activity against gram-positive *S.aureus*, *B.cereus*, and gram negative *E.coli* using different disc diffusion method at 50 µL and 100µL/disc concentration. Azithromycin was used as standard but no significant result was found.
References:


170. 


173. 


175.


116


255. Thiele J. and Manchot W., Annalen, 33, P.808, (1898).


triazolo[4,3-a] pyrimidin-5(1H)-ones Incorporating Triazole Moiety”, *Molecules*, 20, PP.1357-1376, *(2015).*
