DOCTORAL DISSERTATION

SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF NOVEL HYDRAZONE COMPOUNDS

ARCHANA BAJRACHARYA

DALIAN MEDICAL UNIVERSITY

SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF NOVEL HYDRAZONE COMPOUNDS

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CERTIFICATE

This is to certify that Miss Archana Bajracharya has done her research study entitled "Synthesis, Characterization and Biological evaluation of Novel Hydrazone Compounds". This is an original work carried out by her under our supervision and guidance. She has completed her dissertation entitled on the same and submitted to College of Pharmacy, Dalian Medical University, in fulfillment of the requirement for the award of Doctorate degree on Biochemistry and Molecular Biology.

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DECLARATION

I humbly declare that this dissertation entitled “Synthesis, Characterization and Biological evaluation of Novel Hydrazones Compounds” is an original research work except as cited in the references, carried out under the supervision and guidance of Prof. Dr. Lin Yuan and Prof. Dr. Diao Yunpeng, College of Pharmacy, Dalian Medical University. This thesis has not been presented previously for any higher degree or examination or in any other publication in home and abroad.

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独创性声明

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SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF NOVEL HYDRAZONE COMPOUNDS

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ABSTRACT

Background and purpose:

Hydrazones are important class of organic compounds with the structure $–C=NNH_2$ and are related to aldehydes and ketones by the replacement of the oxygen with the NNH_2 functional group. Literature studies on these hydrazones compounds have shown that these organic compounds possess various biological activities, such as antibacterial, antifungal, antitumor, anticonvulsant, anti-inflammatory, antiplatelet and antimicrobial. Due to its significant biological activities, these compounds arouse considerable interest. However, the study of hydrazones derivatives of carbonyl compounds on the intestinal smooth muscle contraction was rarely found. Thus, the present study was designed to synthesize the novel hydrazones compounds, characterize its structure and to investigate in-vitro antibacterial activity, ex-vivo intestinal smooth muscle contraction and in-vivo gastrointestinal motility.

Methods:

The novel hydrazones were synthesized by the reaction of 3,5 dihydroxy benzoic acid hydrazide with corresponding aromatic aldehydes in methanolic solution. The structure of the compound was characterized by using IR spectra, thermo gravimetric analysis and single-crystal X-ray diffraction. In-vitro antibacterial activity of the compounds was studied by using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) method. The colorimetric assay was carried out on Caco2 cell culture to evaluate the toxic effects of these new synthesized compounds. The contractility of jejunal smooth muscle (CJSM) of rats was chosen as a major index to investigate the effects of these synthesized

compounds on smooth muscle contraction. The acute oral toxicity of the active compounds was determined by up- and -down experimental design to estimate LD50.The diarrhea predominant rat model was established to explore the role of compound on gastrointestinal motility. Western blot analysis was used to detect the 5HT serotonin transporter (SERT) protein content on intestinal fragments of diarrhea afflicted rats.

Results:

The light yellow crystalline compounds were obtained from the synthesis. The novel synthesized compounds are 3,5 dihydro-N′-(3-Nitrobenzylidene)benzohydrazide(compound 1),3,5dihydro-N′-(4-Nitrobenzylidene)benzohydrazide(compound 2), 3,5dihydro-N′-(2-Nitro benzylidene)benzohydrazide(compound 3), 3,5dihydro -N′- (2-bromo, 4-methoxy, 5-hydroxy benzylidene)benzohydrazide(compound 4),3,5dihydro-N′-(2-hydroxy,3-methoxybenzylidene) benzohydrazide(compound5),3,5dihydro-N′-(2-hydroxy,5-chlorobenzylidene)benzohydrazide (compound6),3,5dihydro-N′-(3,5-ditertiarybutyl,2-hydroxybenzylidene)benzohydrazide (compound 7).

These novel synthesized compounds exhibited mild to moderate antibacterial activities against S. aureus and K. pneumonia. The compounds exhibited the low toxic effects on Caco-2 cell culture. In ex-vivo assay, these new synthesized compounds exhibited the inhibitory effects on CJSM. Among them, 3,5 dihydro-N′-(3-nitrobenzylidene)benzohydrazide(DNBB) showed strong inhibitory effects on contractility of jejunum smooth muscle. They inhibited CJSM in a dose dependent manner, in normal contractile state. They decreased the stimulatory effects on CJSM induced by acetylcholine, high ionic concentration of calcium and potassium respectively. Alpha adrenergic receptor phentolamine and beta adrenergic receptor propanolol partially abolished the inhibitory effects of novel compounds. The oral Lethal Dose 50 (LD50) of the active novel compound DNBB in mice using up-and-down procedure was over 2.0g/kg. In vivo assay indicated that DNBB reduced the feces quantity and its water content of Diarrhea predominant (DP) rat model. SERT protein expression (detected as bands at 70kDa) was faintly detected in jejunum and colon segments of diarrhea afflicted rats.

Discussion and Conclusion:

The structural analysis of these novel hydrazones compounds elucidated that the compounds with nitro, hydroxyl, methoxy, halogen substituent groups on benzene ring were effective on intestinal contractility and in-vitro antibacterial activity. This suggests that the biological activity of the compounds is positively related with different functional groups and its arrangement and position on the benzene ring.

The innovations of the present study are:

1. The novel hydrazones compounds were synthesized by applying simple, reliable and green or sustainable chemical procedure.
2. The compounds (1-7) possessed low toxic effects.
3. Compound (4, 5 and 6) showed mild to moderate antibacterial activities against S. aureus and K. pneumonia.
4. Compound (1-7) exhibited the inhibitory effects on CJSM in a dose dependent manner in normal contractile states. Among them, compound 1(DNBB) containing nitro group at meta position showed the strong inhibitory effects on CJSM.
5. DNBB alleviated the symptoms of diarrhea predominant (DP) rat model.

   Finally, this is the first attempt to study the effects of novel hydrazones compounds on intestinal contractility. The inhibitory effects of novel hydrazones on CJSM and its possible mechanism suggest its clinical implication on the treatment of intestinal hyper contractility.

Keywords: hydrazones, toxicity, intestinal smooth muscle, inhibitory effects, adrenergic α and β receptors, Diarrhea predominant, 5-hydroxytryptamine

新型腙类化合物的合成，表征及生物学评价

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摘要

背景与目的：

腙类试剂是拥有-C= NNH2 结构的一类有机化合物，其结构通常是醛和酮类化合物的氧被 NNH2 官能团替代形成。这些有机化合物具有较为广泛生物活性，如抗菌，抗真菌，抗肿瘤，抗惊厥，抗炎，抗血小板聚集等。基于腙类化合物显著的生物活性，人们对其作用的研究兴趣日益浓厚。然而，羰基化合物的腙衍生物对肠道平滑肌的收缩性作用的研究鲜有报道。本研究拟设计合成新型腙类化合物，鉴定其结构，并探讨其体外抗菌活性，以及对小肠平滑肌收缩的作用，在体对胃肠推动的作用。

方法：

新的腙类化合物由 3,5 二羟基苯甲酰肼在甲醇溶液中与不同的醛类化合物反应合成。合成的化合物经红外光谱(IR),热重分析和单晶 X-射线衍射法鉴定分析其结构特征。MTT 法检测新化合物的体外抗菌活性。比色法测定新合成的化合物对培养的 Caco2 细胞的毒性作用。采用大鼠空肠平滑肌收缩性为主要指标 ,研究合成的化合物对小肠动力的作用。口服毒性经上下法估测合成药物的 LD50。 建立大鼠腹泻模型，探索新合成化合物对胃肠动力的作用。Western blot 法检测新合成化合物对腹泻大鼠特定蛋白质的表达的影响。

结果：

本实验合成得到了淡黄色结晶化合物。新合成的化合物共七种，包括 3-硝基苯甲醛-3,5

二羟基苯甲酰腙（化合物 1），4-硝基苯甲醛--3,5 二羟基苯甲酰腙（化合物 2），2-硝基苯甲醛--3,5 二羟基苯甲酰腙（化合物 3），2-溴-4-甲氧基-5-羟基苯甲醛--3,5 二羟基苯甲酰腙（化合物 4），2-羟基-3-甲氧基苯甲醛-3,5 二羟基苯甲酰腙（化合物 5），3-氯-6-羟基苯甲醛--3,5 二羟基苯甲酰腙（化合物 6），3,5-二叔丁基-1,2-羟基苯甲醛-3,5 二羟基苯甲酰腙（化合物 7）。新合成化合物对金黄色葡萄球菌和肺炎克雷伯菌等两种菌株表现出轻度及中度的抑制活性。该化合物对 Caco-2 细胞表现出低毒性作用。在离体实验中，这些新合成的化合物具有显著的抑制小肠平滑肌收缩的作用。新合成的化合物中，3-硝基苯甲醛-3,5-二羟基苯甲酰腙（DNBB）对小肠平滑肌的抑制作用最为显著。在正常的收缩状态下，新合成的化合物剂量依赖性抑制了平滑肌的收缩，同时显著降低了乙酰胆碱，高钙和高钾克氏液造成的小肠平滑肌高收缩。组胺 H1 和 H2 受体拮抗剂没有影响到新化合物对小肠平滑肌的收缩作用。α 和 β 肾上腺素受体阻断剂一定程度上阻断了新合成化合物的抑制作用。在上下法实验中，新合成化合物对于小鼠的口服致死剂量大于 2g/kg。在体实验中，DNBB 显着减少了腹泻大鼠粪便的数量以及粪便含水量。

讨论及结论：

实验结果提示，新合成的化合物苯环上带硝基，羟基，甲氧基，卤素等官能团在对肠道动力以及抗菌方面具有显著的生物活性。实验结果说明，新合成的化合物的活性依赖于不同的取代基，以及在苯环上的位置。

本实验具有以下创新性：

1、本实验操作简单易行，合成化合物稳定。

2、新合成的七个化合物均拥有毒性较低的特点。

3、新合成的化合物中，化合物四，五以及六对金黄色葡萄球菌和肺炎克雷伯菌等两种菌株表现出轻度及中度的抑制活性。

4、合成的化合物计量依赖性地抑制了小肠平滑肌的收缩。其中含有的硝基在苯环间位时，对小肠平滑肌收缩的抑制作用最为显著。

5、DNBB 较好的缓解了腹泻大鼠的腹泻症状，这种抑制作用和阻断 5 - 羟色胺受体相关。

在实验中，我们首次尝试研究新型对肠道具有活性的腙类化合物。新合成的化合物对小肠的显著的抑制作用以及相关的作用机制，为新合成的化合物应用于治疗肠动力异常增高等疾病提供科学依据。

关键词 腙类化合物，毒性，肠平滑肌，抑制，肾上腺素能 α 和 β 受体，腹泻

INTRODUCTION

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Major: Biochemistry and Molecular Biology

Schiff bases are condensation products of primary amines with carbonyl compounds and were first reported by Hugo Schiff in 1864.The common structural feature of these compounds is the azomethine group with the general formula RCH=NR1,where R and R1 are alkyl,aryl,cyclo alkyl or heterocyclic groups which may be variously substituted. Because of the relative easiness of preparation, synthetic flexibility, and the special property of C=N group, Schiff bases are excellent chelating agents especially when the functional group like –OH and –SH are very close to the azomethine group so as to form a five or six membered ring with metal ions. Versatility of Schiff base ligands and biological, analytical and industrial applications of their complexes make this area highly desirable for further investigation. Nowadays, the research field dealing with Schiff base coordination has expanded enormously. Hydrazones are synonymous of Schiff bases. The azomethine linkage (-NH-N=CH-) of hydrazones is a significant features that makes this ligand the most interesting candidate for the biological activities as well as coordination and chelation with the metal ions. Such ligand represent the major route in designing the new compounds that acts against different types of bacteria, fungi and cancer associated viruses and become resistance to the use of conventional drugs. The C=N bond of hydrazones and terminal nitrogen atom containing a lone pair of electron is responsible for the physical and chemical properties. The C-atom in hydrazones has both electrophilic and nucleophilic character and both the N-atoms are nucleophilic though the amino nitrogen is more reactive. Due to these properties hydrazones are widely used in organic synthesis. The additional C=O group donor site on azomethine linkage provides more flexibility and versatility. These hydrazide derivatives are the rapidly developing field in medicinal chemistry due to its ease of preparation and diverse pharmacological potential. Considerable importance of these hydrazide derivatives is due to its wide variety of biological activities such as antimicrobial, anti-inflammatory, analgesic,

antitumor, antiviral, antitubercular, antiparasitic, antiprotozoal, antiplatelet, anticonvulsant, antidiabetic and antimalarial activities etc.[1-3]

The green or sustainable chemical philosophy, that minimize the use of hazardous substances and maximize the efficiency rate are mostly employed for synthesis of various Schiff base organic ligands.

Sa.Deng et al (2009), Qing Hua jiang et al (2007), Diao et al.(2007),Siti M. S. et al (2010) synthesized and reported the crystal structure of the Schiff base compounds derived from 3,5dihydroxybenzoic acid hydrazide[4-7] and various other author reported synthesis and the crystal structure of similar types of Schiff bases[8-13]. The process applied for synthesis was simple, reliable and sustainable. Sun Zhen Gao et al (2012) have shown the antioxidant activities of Schiff base derived from 3, 5 dihydroxybenzoic acid hydrazide [14]. Ling Z. and BaoHua Z. (2007), Abdul Zameel (2012) reported the antibacterial activities of the Schiff bases ligand and its metal complexes [15, 16]. Sinha et al (2008), Harrop et al (2003), Bhandari et al (2008) describes the various important biological activities of similar types of Schiff base compounds [17-19].

Therefore, it was envisaged that by applying the simple and green chemical procedure the Schiff bases organic ligands from 3,5 dihydroxybenzoic acid hydrazide would be synthesize that have interesting biological activities.

The smooth muscle cell of gastrointestinal sphincter sustained certain tonic contraction. When stimulated by the neurohormonal agents most gastrointestinal smooth muscle generates phasic contraction. The frequency of the phasic contraction produce by the stimulation is a unique property of each gastrointestinal smooth muscle [20].

Intestinal smooth muscle also contracts in absence of nerves; it has certain degree of self regulation so that they contracts independently. Under the ex-vivo state, it can simulate the function of drugs as in whole body condition and reveal the characteristics of drugs in most convenient, spontaneous and quick way. In addition to the extrinsic control exerted by the nerves, cells of intestine secrete the regulating substances including acetylcholine, histamine, serotonin, prostaglandins and several hormones. Ultimately these regulators alters the rate or strength of smooth muscle cell by shifting the ions(Na^+,K^+,Ca^{2+}) across the membrane or/and by changing the intracellular level of second messenger compounds.

The study of various natural, natural derived and non natural products with presence of phenol, quinones, methoxyphenyl moiety etc have shown to possess the stimulatory and/or inhibitory effects on different contractile states of intestinal smooth muscle [21-23]. However, the pharmacological properties of compounds with imine or azomethine groups together with other substituents on intestinal contractility were rarely evaluated.

Therefore, the main objective of this study is to synthesize the variable aroyl hydrazones from 3,5 dihydroxybenzoic acid hydrazide, characterize them by spectral analysis and determine its crystal structure by single crystal X-ray diffraction and to examine the effects of novel hydrazones compounds on intestinal contractility, the isolated jejunal muscle strips of rats used as a research model, the smooth muscle contraction amplitude as indicators. In-vitro antibacterial activity of the compounds against S. aureus and K. pneumonia was also investigated. Cytotoxicity of the synthesized novel compounds were examined against Caco-2 cell culture.

An additional objective is to determine the mechanism by which the novel compounds alters the action of physiological relevant agents such as different neurotransmitter and receptor antagonists [24].In addition oral lethal dose (LD50) of DNBB was also determined. Furthermore, the effect of active novel compound DNBB on the diarrhea predominant (DP) rat model was also illustrated.

Serotonin (5HT) influences motility, visceral perception, and secretion in the gut. The pervasive role of 5-HT in normal and pathological gastrointestinal (GI) conditions is apparent in its pattern of distribution 95% of 5-HT in the body is in the GI tract and approximately 5% is localized in the brain [25-26].Serotonin (5HT) plays an important role in gastrointestinal motility. The various effects of 5HT in the gut and the brain are attributable to the existence of numerous receptor subtypes. By blocking serotonin receptors in the gut; it is possible to treat abnormal bowel action in those afflicted with diarrhea [27-29]. Serotonin transporter or SERT is an extremely important integral membrane transporter protein that is responsible for the reuptake of 5- hydroxytryptamine (5HT) from the synaptic space into presynaptic neurons and terminates the action of 5HT.Serotonin transporter, by recycling serotonin, regulates its concentration in a gap or synapses and thus it effects on a receiving neuron’s receptors.

SERT plays a critical role in modulating serotonin availability and thus has been implicated in

the pathogenesis of various intestinal disorders. Hence, the 5HT serotonin transporter (SERT) protein expression on the DP afflicted rats intestine was determined.

PART ONE

This part describes the synthesis of series of novel hydrazones compounds and structural elucidation of these compounds. The broad ranges of biological activities of hydrazones compounds have shown that their activities were positively related to the functional group present in the compound. This investigation led to the conception, for synthesizing the novel hydrazones using 3,5dihydroxybenzoic acid hydrazide with corresponding aldehydes with similar types of structural elements (Fig 1.1),conceiving they would be biologically active.

1. MATERIAL

1.1 MAIN REAGENTS

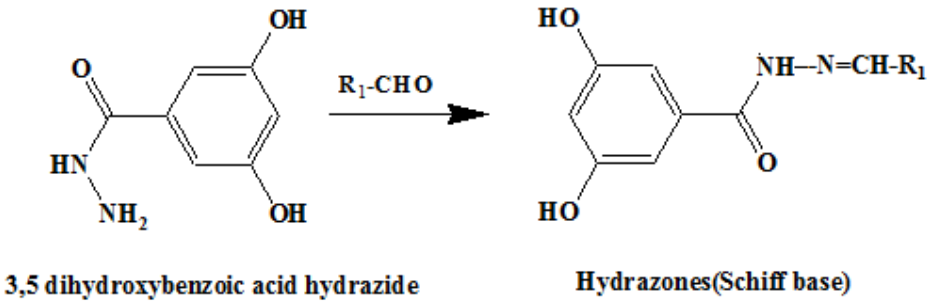
All chemicals and solvents are of reagent grade and commercially available, provided by pharmacology lab of Dalian Medical University. 3,5dihydroxybenzoic acid hydrazide was purchased from SIGMA, USA.

2. METHODS

2.1 SYNTHESIS:

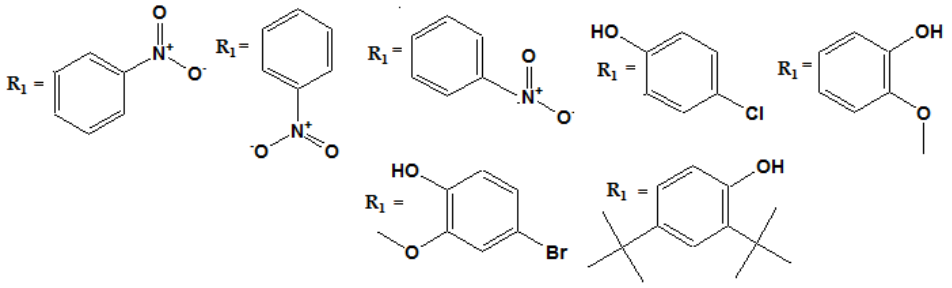
Equimolar quantities of aromatic amine (3, 5 dihydroxybenzoic acid hydrazide) and corresponding aromatic aldehydes were mixed and concentrated in 95% methanolic solution. The mixture was stirred at room temperature until a clear colorless solution was obtained. Light yellow crystals were formed by gradual evaporation of the solvent over a period of five days at room temperature. After washing with methanol, the obtained crystal was dried and the chemical structures of the synthesized compounds were determined.

CHEMICAL REACTION



3,5 dihydroxybenzoic acid hydrazide **Hydrazones(Schiff base)**

GROUP ONE



GROUP TWO

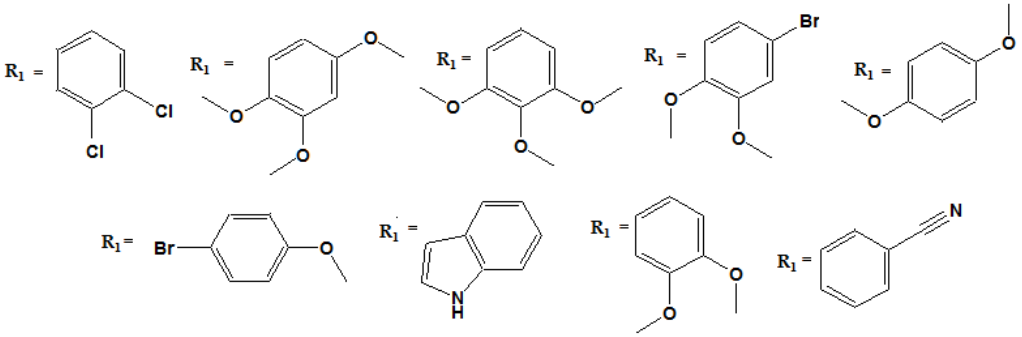
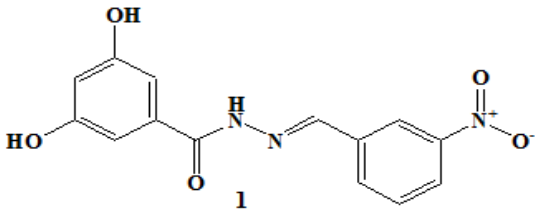


Fig. 1.1: Chemical reaction of novel hydrazones Schiff bases compounds.

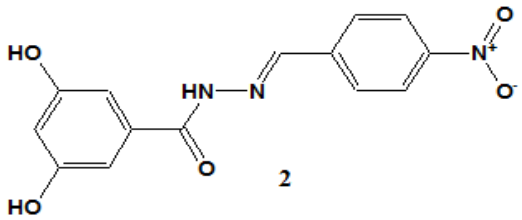
The chemical structure of the hydrazones Schiff bases derived from group one was shown in Fig.1.2.From the biological evaluation, these compounds were found to have prominent bioactivities among all those synthesized compounds. The part two of this study describes the detail activities of these seven novel compounds. The hydrazones derived from group two were found to be less active compare to these seven compounds so they were not studied.

THE NOVEL SYNTHESIZED COMPOUNDS WITH BIOACTIVITY



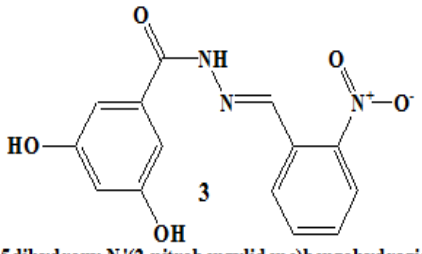
1

3,5dihydroxy-N'(3 nitrobenzylidene)benzohydrazide



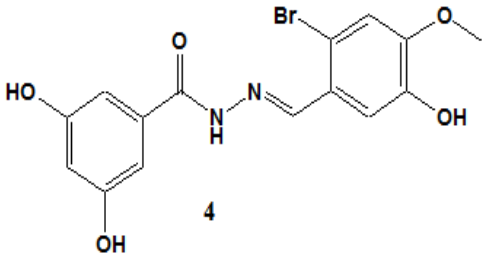
2

3,5dihydroxy-N'(4 nitrobenzylidene)benzohydrazide



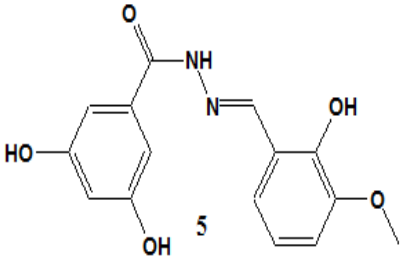
3

3,5dihydroxy-N'(2 nitrobenzylidene)benzohydrazide



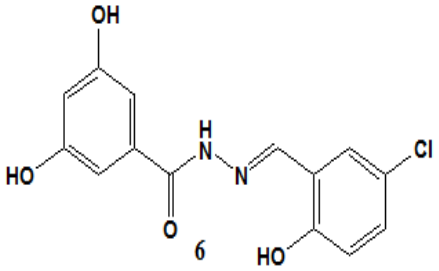
4

3,5dihydroxy-N'(2 bromo,4 methoxy,5 hydroxy benzylidene)benzohydrazide



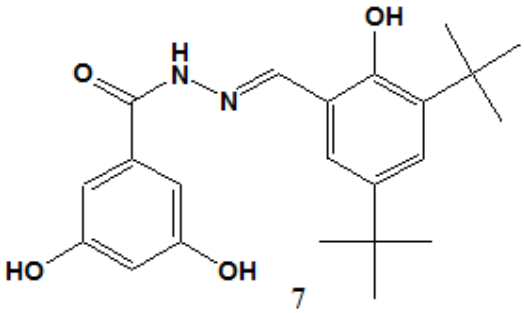
5

3,5dihydroxy-N'(3 methoxy,2 hydroxy benzylidene)benzohydrazide



6

3,5dihydroxy-N'-(2 hydroxy,5 chlorobenzylidene)benzohydrazide



7

3,5dihydroxy-N'(3,5 ditertiary butyl,2 hydroxy benzylidene)benzohydrazide

Fig.1.2: Chemical structure of seven novel synthesized Schiff base hydrazones compounds.

2.2 REPRESENTATIVE STRUCTURE DETERMINATION

The yellow single crystal with dimensions of 0.91 mm×0.90 mm×0.09 mm was put on a Bruker SMART APEX CCD diffractometer equipped with a graphite monochromatic Mo$K\alpha$ radiation (λ=0.7l073Å) by using an ω-φ scan mode at 295(2) K. A total of 4474 reflections were collected in the range of 2.77<θ<26.75º, out of which 3184 were unique with Rint = 0.0133 and 2253 were observed with (I>2σ(I)). All data were corrected by SADABS method. The structure of 3, 5dihydro-N′-(3-nitrobenzylidene) benzohydrazide (DNBB) was established by Patterson methods with SHELX.97 and refined by SHELXL.97 [30]. All non-hydrogen atoms were refined with anisotropic thermal parameters. The H atom bonded to N(3) was located in a different map and refined freely. Other H atoms were placed in the calculated positions. The final full-matrix least-squares refinement gave R = 0.0541, wR=0.1505 w= 1/ [σ2(F02)+(0.0943P)2+0.2252P], where P=(F02 +2Fc2)/3), (Δ/σ)max=0.000, S=1.054, (Δ/ρ)max=0.784, and (Δ/ρ)min=-0.298.

3. RESULTS AND DISCUSSION

3.1PHYSICOCHEMICAL PROPERTIES

The total yields of novel hydrazones compounds were ranges within (51.5-94.2) % vary among each compound. Some compounds were yielded in very minute quantity that they were discarded for the further experiment. All the synthesized compounds were found to be stable in atmospheric condition for extended period of time and were easily soluble in dimethyl sulfoxide (DMSO) and N, N-dimethyl formamide (DMF).They were slightly soluble in ethanol, methanol, chloroform and acetone, however, they were insoluble in water. The melting point of these compounds ranges from 125-268° C.

3.2 REPRESENTATIVE CRYSTAL STRUCTURE DESCRIPTION

The structure of DNBB is shown in Fig. 1.3. According to X-ray data, DNBB formed monoclinic crystals in space group $P2_1/c$. X-ray single-crystal structural analysis revealed that DNBB consists of one Schiff base moiety and one lattice methanol molecule.

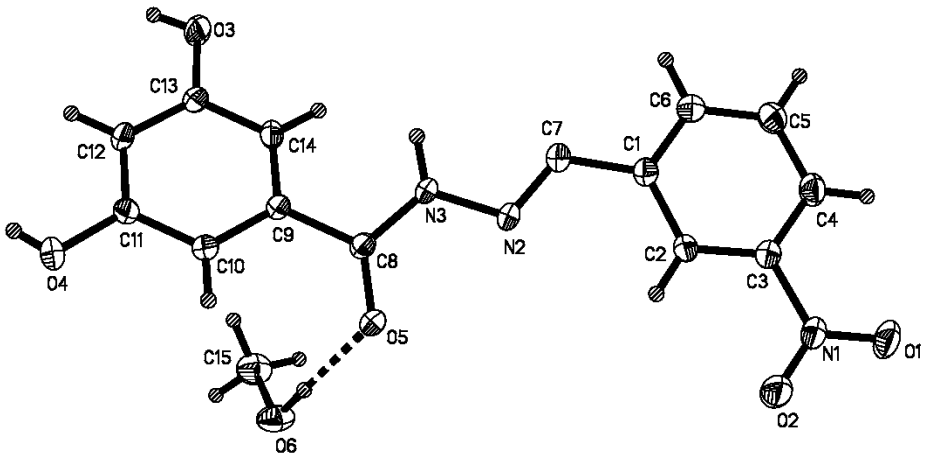


Fig1.3: Structure of DNBB

The bond lengths and bond angles are within normal ranges [31] and comparable to those corresponding in other similar compounds [32-35]. The bond length of 1.276(3) Å between atoms C(7) and N(2) is similar to those observed in other Schiff bases[36-39], indicating it is a double bond. The bond length of C(8)–N(3), 1.344(3) Å, is intermediate between C–N and C=N bonds due to the conjugation effects in the molecule. The mean planes of the two benzene rings make a dihedral angle of 12.89(3)º. As expected, the molecule adopts a *trans* configuration about the C=N double bond. The torsion angles C(9)–C(8)–N(3)–N(2), O(5)–C(8)–N(3)–N(2), C(1)–C(7)–N(2)–N(3)–and C(7)–N(2)–N(3)–C(8) are -175.59(19), 9.4(3), -178.95(15) and 171.7(2)º, respectively. Intramolecular O(6)–H(6a)···O(5) hydrogen bond is observed in the molecular structure. The lattice methanol and nitro group of the Schiff base in the crystal are linked to the Schiff base moieties through intermolecular N–H···O, O–H···O hydrogen bonds (Table 1.1, Figs. 1.3 and 1.4). DNBB extends further to its final three-dimensional network through intermolecular N-H···O, O–H···O hydrogen bonds which interlink molecules that stabilize the structure. (Table 1.1, Fig 1.4).

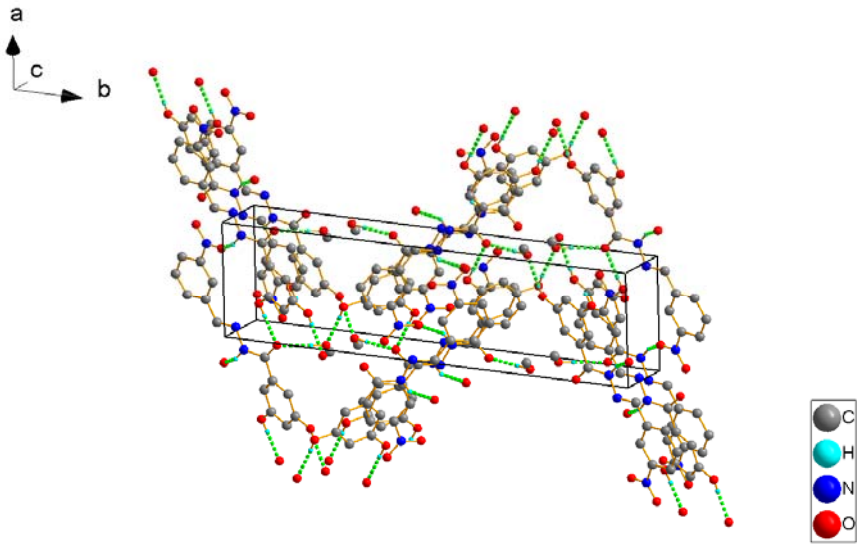


Fig.1.4: Crystal packing of DNBB. Intermolecular N–H···O, N–H···O and O–H···N hydrogen bonds are shown as dashed lines. Hydrogen atoms other than those participating in hydrogen bonding are omitted for clarity

Table 1.1 Hydrogen Bonding Distances (Å) and Angles (°)

D–H···A	d(D–H)	d(H···A)	d(D···A)	<(DHA)
N3—H3A···O2^{i}	0.88 (3)	2.51 (3)	3.258 (3)	143 (2)
O6—H6A···O5	0.85 (3)	1.97 (3)	2.821 (3)	178 (3)
O4—H4A···O6^{ii}	0.75 (4)	1.99 (4)	2.729 (3)	169 (4)
O3—H3···O5^{i}	0.81 (3)	2.00 (3)	2.815 (3)	177 (3)

Symmetry codes: (i) x+1, y, z. ; (ii) x+1, −y+3/2, z+1/2

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PART TWO:

The part two describes the biological evaluation of those seven novel synthesized hydrazones compounds. The in-vitro antibacterial activities of these seven compounds against two species of bacteria were observed. Ex-vivo assay of all those synthesized novel hydrazones compounds derived from group one and two, on the contraction of isolated jejunal smooth muscle of rats were studied. In-vivo assay of compound 1(DNBB) was also carried out to determine the effect of this novel compound on gastrointestinal motility.

1. MATERIALS

1.1 EXPERIMENTAL ANIMALS

Sprague Dawley (SD) Rats of either gender weighing 180-220g were provided by the animal center of Dalian Medical University (Dalian, China). The animals were kept in laboratory under conditions of 22±1°C. All experimental procedures described were carried out in accordance with the Declaration of Helsinki , and approved by Dalian Medical University Animal Care and Ethics Committee.

1.2 THE MAIN APPARATUS, EQUIPMENT

HM-3S-type PH meter: Japan TOA Industries, Ltd. products.

MA240D electronic analytical balance: Shanghai Huxi Analysis Instruments factory

CJ-1 magnetic stirrer: Shanghai Huxi Analysis Instruments factory

CT15RE speed refrigerated centrifuge: Japan Hitachi Ltd.

SCP70H speed refrigerated centrifuge: Japan Hitachi Ltd.

PS-9 type the electrophoresis instrument: Dalian Jie Maike Technology Company.

Vortex mixer: Jiangsu Haimen the unicorn instrument.

The BDY-2 constant voltage current electrophoresis instrument: Broadcom.

Scion-Image scanning software: Scion.

The functional experimental device (including Maxwell bath, McNamara bath tube, thermostatic device), air pump or oxygen cylinders, beakers, thermometers, surgical scissors, biconcave folder, rubber tubing, graduated cylinder

Micro plate reader: Thermo Company, USA

Autoclave instrument: Japan Sanyo MLS-3750.

The integrated automatic water machine: Chengdu Pincheng Technology Co., Ltd.

HW-400S thermostat smooth muscle slot: Chengdu Thai Union Science and Technology Co. Ltd.

BL-420F biological and functional experimental system: Chengdu Thai Union Science and Technology Co. Ltd.

1.3 MAIN REAGENTS AND MEDICINE

Propranolol: Northeast General Pharmaceutical (Shenyang, China),

Cimetidine: Shanghai Biochemical Pharmaceutical (Shanghai, China),

Acetylcholine: Tianjin Jin Yao Amino Acid Co. Ltd. (Tianjin, China)

Diphenhydramine: Tianjin Jin Yao Amino Acid Co. Ltd. (Tianjin, China)

Phentolamine: Tianjin Xinzheng Pharmaceutical Co. Ltd. (Tianjin, China)

Phenylmethylsulfonyl fluoride: SIGMA, USA

Bis-acrylamide: Japan and Wako Pure Chemical Industries.

Tris (hydroxymethyl) amino methane: Japan and Wako Pure Chemical Industries.

Glycine: Shanghai Reagent Factory.

Glycerol: Dalian

Lysate: Western and IP cell lysates, Jiangsu Haimen Biotechnology Institute.

β-actin antibodies: Beijing Biosynthesis Biotechnology Co. Ltd.

5HT SERT [ST (H-115): sc-13997]: Santa Cruz Biotechnology, inc.

Horseradish peroxidase-labeled goat anti-rabbit IgG: Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.

Caco-2 cells: American Type Culture Collection (ATCC)

Cell culture medium: Gibco (Grand Island, NY, US).

Bacteria (S. aureus ATCC25923; K. pneumonia, clinical isolated strains); were provided by pharmacognosy lab of Dalian Medical University.

1.4 CHEMICAL COMPOSITION

Krebs buffer: 118.0mmol/L NaCl, 4.70mmol/L KCl, 2.50mmol/L $CaCl_2$, 42.0mmol/L $NaHCO_3$, 1.20mmol/L KH_2PO_4 , 1.20mmol/L $MgSO_4.7H_2O$ and added distilled water 800ml, adjusted pH 7.4 with concentrated hydrochloric acid then added D-glucose 10mmol/L,

finally adjusted volume to 1000.0ml.

Phosphate buffer solution (PBS): NaCl 8.50g, KCl 0.20g, 2.90g of disodium hydrogen phosphate dodecahydrate, potassium dihydrogen phosphate 0.30g, was dissolved in 1000.0mL distilled water with hydrochloric acid, the pH value was adjusted to 7.2-7.4, after autoclave, stored at 4.0 ° C

Acrylamide stock solution: Weigh acrylamide 29.20g, N, N-methylene-bis-acryl amide 0.80g, dissolved in 100.0mL distilled water, and stored at 4.0° C.

10% Sodium Dodecyl Solution (SDS): Weigh SDS 10.0g, dissolved in 100.0mL of distilled water, and stored at room temperature.

The electrode buffer solution: Weigh 14.40g of glycine, Tris 3.0g, 10.0% SDS 10.0mL, dissolved in 900.0mL distilled water, with concentrated hydrochloric acid adjusted pH value to 8.3, and finally with distilled water adjusted volume to 1000.0mL, stored at room temperature.

Transfer buffer: glycine 14.40g, Tris 3.0g, added methanol 200.0 mL, adjusted volume with distilled water to 1000.0 mL, stored at 4.0 ° C;

Tris HCL Buffer Solution(TBS): Weigh sodium chloride 29.20 g and Tris 2.40g dissolved in 900.0mL distilled water, pH adjusted to 7.5 with concentrated hydrochloric acid, and finally with distilled water adjusted volume to 1000.0mL, and stored at 4.0 ° C;

Tris HCL Buffer and Tween Solution (TBST): TBS solution 500.0mL, add Tween250 μL

Sample buffer solution (2.0 ×): 1.0mol / L Tris-HCl (pH 6.8) 0.80mL ,10% SDS 2.0mL, Bromo Phenol Blue 0.50mg , Glycerol 0.80mL , β-ME solution 0.20mL , 1.20mL distilled water.

Blocking solution (5% nonfat dry milk): skim milk powder, 5.0g, was dissolved in 100.0mL TTBS.

*30.0% Acrylamide-BIS (AABIS) solution:*30.0 g of acrylamide and 0.80 g of N, N'-methylene dioxy-acrylamide were dissolved in a total volume of 60.0 ml water and heated to 37° C till it dissolved then added water to a final volume of 100.0mL. pH of the solution not greater than 7.0, preserved it in brown bottle.

1.5M Tris HCL (pH8.8): Tris 18.20g, dissolved in 80.0mL distilled water, the pH was adjusted to 8.8 with concentrated hydrochloric acid, and finally with distilled water adjusted volume to

100.0mL, and stored at 4.0 ° C.

0.5M Tris HCL (pH6.8): Tris 6.10g, dissolved in 80.0mL distilled water, pH was adjusted to 6.8 with concentrated hydrochloric acid, and finally with distilled water adjusted volume to 100.0mL, stored at 4.0 ° C.

For 2 SDS PAGE:

10% Separating gel buffer: 3.30ml AA Bis, 2.50ml 1.5Tris-HCL (pH8.8),0.10ml 10% SDS, 4.1ml distilled water,0.04ml 10%AP,0.02ml TEMED

10%Stacking gel buffer: 0.83ml AA Bis, 0.63ml 0.5Tris-HCL (pH6.8),0.05ml 10% SDS,3.4ml distilled water, 0.03ml 10%AP,0.015ml TEMED

2. METHODS

2.1 TOXICITY MEASUREMENT:

2.1.1Cytotoxicity

The cytotoxicity assay of above described compound (1-7) was carried out by using standard colorimetric (MTT) analysis [1,2].The cells was diluted to 1 x 10^5/ml with Dulbecco's Modified Eagle Medium (DMEM) plus 10% Fetal Bovine Serum (FBS), 100.0µl of this diluted cells was poured into each wells of the 96-well flat bottomed tissue culture plates and incubated for 24 hour at 37°C and 5% CO_2. Then the culture medium was discarded, 90.0µl new medium without FBS and 10.0µl serial diluted novel compounds (1-7) were added to each well. 90µl medium and 10.0µl menstruum of these compounds were added to the control wells. Each dose of these compounds was repeated five times. After 24 hours of incubation, 10.0µl of MTT reagent was added to each well and further incubated for 4 hours at 37℃. 100.0µl of Detergent Reagent (10%SDS+5%isobutanol+1%HCL) was added to each well and incubated them overnight. Finally, the absorbance in each well was measured at 570 nm in a microtiter plate reader.

2.1.2 Acute toxicity:

Prior to conduct an in vivo assay, the acute oral toxicity of DNBB was tested. The acute toxicity (oral LD50) of the DNBB was determined in mices by using up-and-down procedure [3,4]. Five mice which weighed 18-22g were housed individually in cages. DNBB was diluted

to 0.1g/ml suspension by 0.5% sodium carboxy methyl cellulose (Na-CMC). One mouse was weighed and fasted overnight. DNBB suspension was gastric gavages at a dose of 2.0g/kg. Since no death was observed within 48h, the procedure was repeated to the other four mice. These mice were carefully observed till 14 days.

2.2 DETERMINATION OF ANTIBACTERIAL ACTIVITY

The antibacterial activities of compound (1-7) were assessed by using Thiazolyl Blue Tetrazolium Bromide (MTT) method [5-7]. A stock solution of the synthesized compound (50.0 mg/mL) in DMSO was prepared and graded quantities of the test compounds were incorporated in specific quantity of sterilized liquid (Luria Bertani) LB medium. Bacterial suspensions containing approximately 10^5cfu/ml were prepared. 5.0µl medium containing test compounds were poured into 96-well plates. Compounds in variable concentrations were assayed in 6 wells. The bacteria suspension (95.0µl) was poured into 3 wells and LB medium (95.0µl) was poured into the other 3 wells as control (each compound in each concentration was repeated 3 times). All of them were incubated at 37°C for 18-24h for observing antibacterial activity. After the MICs were visually determined on each of the 96 pore plate, 100.0µl PBS (phosphate buffered saline) with 5.0 mg of MTT/ml was added to each well. Incubation was continued at room temperature for 4hr. 100.0µl DMSO was added into each well and shaken for 5min. The optical density (OD) was measured using a micro plate reader at 570nm.

2.3PREPARATION OF INTESTINAL SEGMENTS

Rats of either gender were sacrificed by cervical dislocation; the intact tubular jejunum was removed and placed in ice cold Krebs buffer solution. Subsequently, the jejunal strips were dissected into approximately (1.0-1.2) cm segments in length and luminal content were flushed out using freshly prepared Krebs buffer solution. The isolated strips were mounted in organ bath containing Krebs buffer. One edge of the strips tied with suture was fixed to the bottom of organ bath and the other edge was connected to the isometric force transducer.

2.4 MEASUREMENT OF SMOOTH MUSCLE CONTRACTILITY

Since jejunum is a "typical" region of the small intestine, it was chosen to investigate the effect of novel compounds. Jejunum was prepared with slight modification as described previously [8-10].

The smooth muscle organ bath system was maintained at 37°C and the resting tension was set optimally at 1.0 g per 1cm of tissue. The jejunal strips suspended in longitudinal direction on aerated tube was allowed to equilibrate in Krebs buffer solution for 50 min with washout every 10 min and oxygenated with 95%O_2 and 5%CO_2. The strips were washed at least 3 times (5-10min intervals) with Krebs buffer between each experimental conditions. Smooth muscle amplitude of contraction was measured from the baseline to the peak and was expressed as a percentage of the normal contractile amplitude. Smooth muscle contractile frequency was expressed as a percentage of the normal contractile frequency. The contractile responses were recorded in physiological recording system.

The average peak amplitude and frequency of contractions occurring after administration of each compound was determined and compared to average contraction amplitudes measured prior to administration of novel compounds. The solvent used to dissolve the synthesized compound was dimethyl sulfoxide (DMSO), the effect of it was determined on spontaneous smooth muscle contractions as a drug-free vehicle control. DMSO, in a volume equivalent to that of the synthesized compounds solution, produced not any changes in either the amplitude or frequency of spontaneous muscle contractions. After 50 min equilibration period, the subsequent addition of new synthesized compounds at the concentrations 5.0-160.0μM was made. The contractility outputted from the force transducer was isometrically measured by biological recording system (BL-420F) equipped with amplifier. The process was repeated for all the synthesized compounds to get the concurrent results.

The contraction of jejunal smooth muscle was measured at normal contractile state and high contractile states. The high contractile states were generated by acetylcholine (2.0μM), high ionic concentration of potassium (10.0mM) and calcium (5.0mM) respectively. In experiments, at normal contractile state, the compounds were added starting from 5.0μM up to 160.0μM.The pharmacological blockers, alpha adrenergic receptor antagonist phentolamine (5.0μM) and beta adrenergic receptor antagonist propanolol (5.0μM) as well as histamine H_1

receptor antagonist diphenhydramine (5.0μM) and H_2 receptor antagonist cimetidine (5.0μM) were chosen to investigate the possible mechanism of novel compounds that induced inhibition on the contraction of jejunal smooth muscle. All the neurotransmitters and the receptor antagonists were pretreated for 3min before addition of the novel compounds [11-12].

2.5 PREPARATION OF ANIMAL MODEL FOR IN VIVO ASSAY

Thirty experimental female SD rats weighed (180-200)g were obtained from the animal house of Dalian Medical University. These rats were weighed, well housed and caged randomly into six different groups, five rats in each group and well treated with food and water for one week prior to dosing, for acclimatization to the laboratory condition. After one week, the first control group treated as normal rat without any drug. Except the control group, the other 5 groups were gastrogavage with senna 1.0ml/100g at 25°C and for 3 hour restraint stress on their hind limb for seven successive days. The bottom of each cage of rats were lined with filter paper to examine the feces output. The quality of feces pellets expelled by each animal after 3 hour of restraint stress was examined and counted every day.

The establishment of diarrhea model in rats was identified by examining the feces output, feces water content, the bowel transit time and visceral sensation [13,14].

After the successful establishment of the model by gastrogavage of senna for fourteen days the rats were gastrogavage with DNBB. The high, moderate and low groups were administered DNBB at concentration of 70.0, 40.0,10.0mg/kg respectively and for rest two group one is gastrogavage with positive drug pinaverium bromide [15] (Dicetel) 25.0mg/kg while for the model group only treated with water till fourteen consecutive day. The feces pellets output were observed and counted every day.

After 14 days all six groups rats were sacrificed by cervical dislocation, the gastrointestinal segments jejunum, proximal colon and stomach of each rat were dissected and luminal contents were flushed out using freshly prepared Krebs buffer solution, frozen in liquid nitrogen and preserved in refrigerator at -80 degree centigrade for further experiment.

2.6 WESTERN BLOT ANALYSIS

Solid tissue samples of jejunum and colon were lysed in cell lysis buffer and PMSF. The protein concentration is determined by comparing the target protein with the known standard

BSA (Bovine Serum Albumin) diluted in lysis buffer according to the method of Bradford [16-18]. Proteins were then separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes, which were then blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20. The blots were incubated with the primary antibody ST (H-115) rabbit polyclonal antibody IgG, at a dilution of 1:400, and then further incubated with horseradish peroxidase-conjugated secondary antibody (anti rabbit IgG,1:1000 v/v). The blots were developed by a chemiluminescent substrate. The images of the membranes were obtained using a Scion Image system. All the detected protein bands were normalized to β actin levels.

2.7 STATISTICAL ANALYSIS

The average peak amplitude contractions occurring after administration of compound was determined and compared to average amplitude contraction measured prior to compound administration. The results were expressed as mean ± S.E.M. Statistical difference between control group and group treated with compounds was analyzed by using Student *t* tests. P values less than 0.05 were considered statistically significant.

3. RESULTS:

3.1TOXICITY:

The Caco-2 cell as an in vitro model of the human small intestinal mucosa was chosen to determine the cytotoxicity of novel compound (1-7) [19,20] .All these novel hydrazones did not exhibit lethal effects on Caco-2 cell culture below the concentration of 200.0μM as shown in fig 2.1.

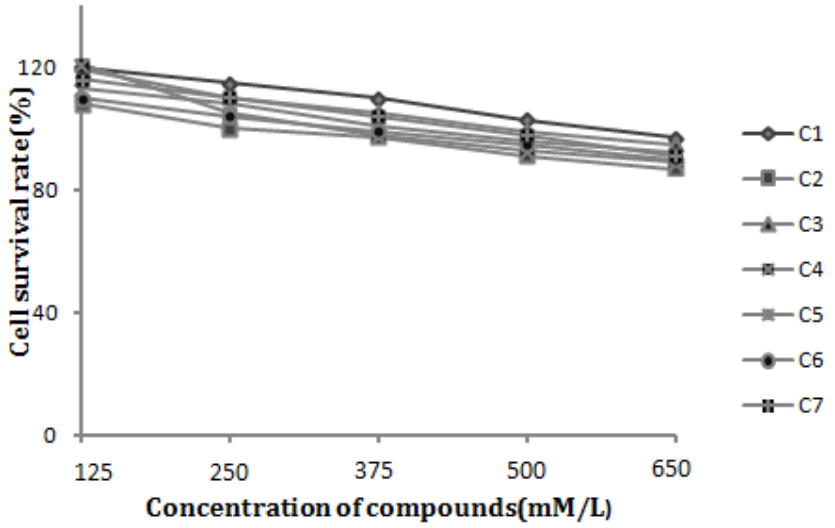


Fig2.1: Represent the cell survival rate (%) at different concentration of compound(1-7).

The acute toxicity (oral LD50) of the compound 1, DNBB was determined in mices by using up-and-down procedure. Five mice were survived within 14 days, indicating that the oral LD50 was over 2.0g/kg.

3.2 ANTIBACTERIAL ACTIVITY

The antibacterial activity of the newly synthesized aroyl hydrazones compound (1-7) was tested against S. aureus and K. pneumonia bacterial strains. The compound (4, 5, 6) exhibited mild to moderate antibacterial activity against Gram-positive (S. aureus) and Gram-negative (K. pneumonia) bacteria. The minimum inhibitory concentration (MIC) of these compounds against bacteria was shown in Table 2.1.Ceftriazone sodium was used as a standard control to compare the antibacterial activity of novel compounds. Compound 5 exhibited moderate activity against both S. aureus (50.0µg/ml) and K. pneumonia (100.0µg/ml).Compound (4, 6) showed mild activities against the growth of S. aureus (200.0µg/ml). Compound (1,2,3,7) showed no antibacterial activity on both bacterial species.

Table 2.1 Minimum Inhibitory Concentration (MIC) of new Schiff bases (µg/ml).

Compounds	S.aureus	K.pneumonia
C1	>250	>250
C2	>250	>250
C3	>250	>250
C4	200	>250
C5	50	100
C6	200	>250
C7	>250	>250
Ceftriazone	0.65	0.30

MIC value>250µg/ml (no antibacterial activity)

3.3 EFFECTS OF NOVEL HYDRAZONES ON JEJUNAL SMOOTH MUSCLE CONTRACTION

3.3.1 Effects of new synthesized Schiff bases on intestinal contractility.

The new synthesized compounds derived from the group two (described above) showed insignificant effects on the contraction of jejunal smooth muscle so they were discarded while those derived from group one compounds (1-7) exhibited the significant inhibitory effects on the contraction of jejunal smooth muscle (CJSM).

These compounds showed an apparent decreased in the force of muscle contractions which remain up to 3min (differs on each compound) and then gradually returned to its pretreatment base line values. These novel compounds decreased the spontaneous contraction of the jejunal strips in a dose dependent manner.

The comparative study of these novel compounds (1-7) on contractility of jejunum, the entire compounds exhibited the inhibitory effects most prominently at the concentration of 40.0µM as shown in Fig 2.2.

The structural analysis revealed that compound with nitro, hydroxyl, methoxy and halogen substituents group on the other benzene ring showed their effects on contraction of jejunal smooth muscle. The compound 1(DNBB) with nitro at meta position, showed strong inhibition at the concentration 40.0µM as shown in Fig.2.2A, table 2.2, while the compound (2,3) having nitro substituent on ortho and para position showed weak inhibition than compound 1 as well as compound(4,5,6).

Compound (6) with chlorine and hydroxyl group showed inhibition less strongly then DNBB but more prominently than other five compounds (fig.2.2F, table 2.2). Similarly, Compound (4,5) with hydroxyl, methoxy and bromine showed their inhibition respectively. Compound (7)

containing hydroxyl group together with two tertiary butyl groups showed the weak inhibition on the contraction of jejunal smooth muscle.

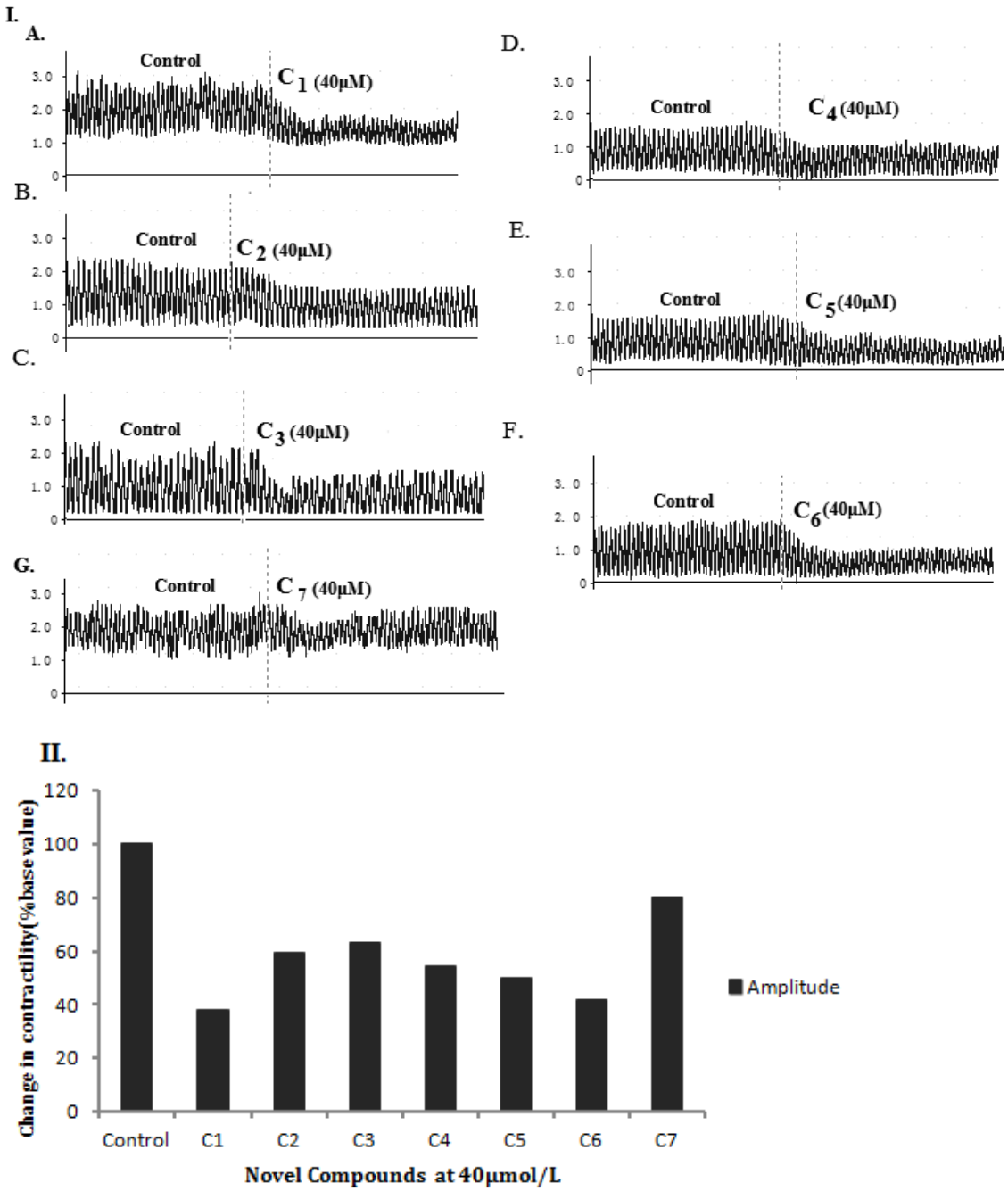


Fig.2.2: Spontaneous smooth muscles contraction of rat jejunum in response to new Schiff bases. Panel I represents the inhibitory effects of compounds (1,2,3,4,5,6,7) fig A,B,C,D,E,F,G respectively. Panel II represents the degree of inhibition of seven novel compounds compare to contractility of control at concentration 40.0µM.

Table2.2: Effect of new compounds on the amplitude of intestinal contractility,(N=10-15)

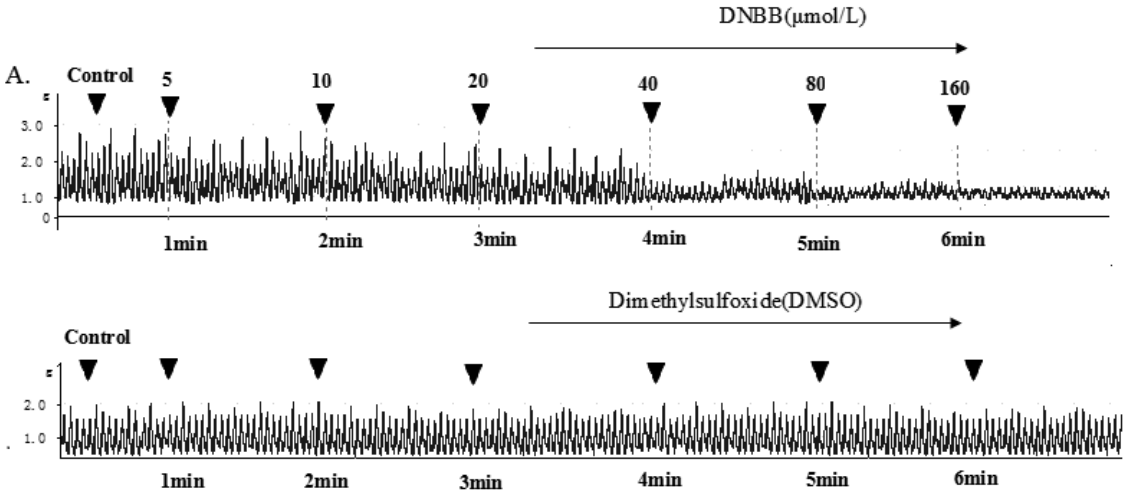
Compounds(40μM)	Before treatment[a]	After treatment[a]
C1	1.36±0.02	0.60±0.03**
C2	2.80±0.09	2.45±0.03*
C3	3.08±0.23	2.69±0.13*
C4	1.84±0.12	1.31±0.00*
C5	1.69±0.09	1.09±0.12*
C6	1.54±0.04	0.90±0.01**
C7	1.78±0.17	1.59±0.06*

[a]Values represent the mean±S.E.M amplitude of spontaneous smooth muscle contractions

**P<0.01, *P<0.05 compared to contractility before treatment (student t- test)

3.3.2 Dose response relationship of compound (1-7) on the contraction of jejunal smooth muscle

The compound (1-7) at dose ranges from (5.0-160.0) μM were treated on the spontaneous contraction of jejunal strips. The dose was increases subsequently in every 2min interval. These compound decreases the force of contraction of smooth muscle in a dose dependent manner. As the dose increases, the inhibition also increases gradually. All these compounds showed apparent inhibition on CJSM at dose of 40.0μM. Among them, DNBB (compound 1) exhibited the strong inhibitory effects on CJSM as shown in Fig. 2.3.



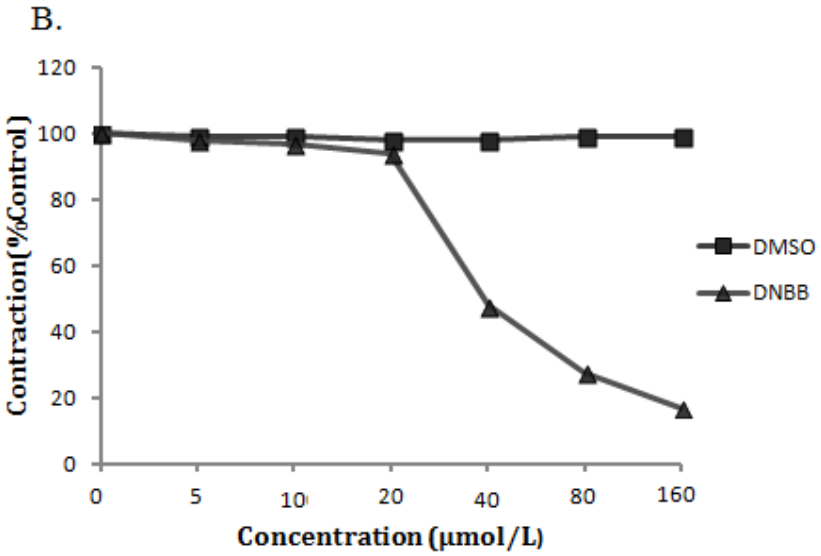


Fig.2.3 Representative traces of DNBB on the contractility of jejunal smooth muscle (CJSM). Panel A represents dose-effects relationship of DNBB and DMSO acting on the CJSM, Panel B graphical representation of inhibitory effects of DNBB and DMSO on the CJSM.

3.3.3 Effects of novel compounds on CJSM in high contractile states.

Based on the evaluation of dose response relationship, compound(1-7) at 40.0µM was chosen to determine its inhibitory effects on contractility of jejunal smooth muscle induced by acetylcholine(ACh),high ionic concentration of calcium(Ca^{+2}) and potassium(K^{+}).

On treatment of ACh (2.0µM), the contraction of jejunal smooth muscle was enhanced significantly, after 3min of its contraction; subsequent treatment of Compound (1-7) did not exhibited significant stimulatory effect of ACh as shown in fig 2.4 and 2.5(B), table 2.3.

Similarly, on bath concentration of 5.0mM and 10.0mM , high Ca^{+2} and K^{+} increased the force of contraction of smooth muscle, the subsequent treatment of novel compounds inhibited the stimulatory effects of high K^{+} and Ca^{+2} as shown in fig 2.4 and 2.5(C, D),table 2.3.

Thus, Compound (1-7) had no significant influence on either the potency or maximal stimulatory action of ACh, high Ca^{+2} and high K^{+} on contraction.

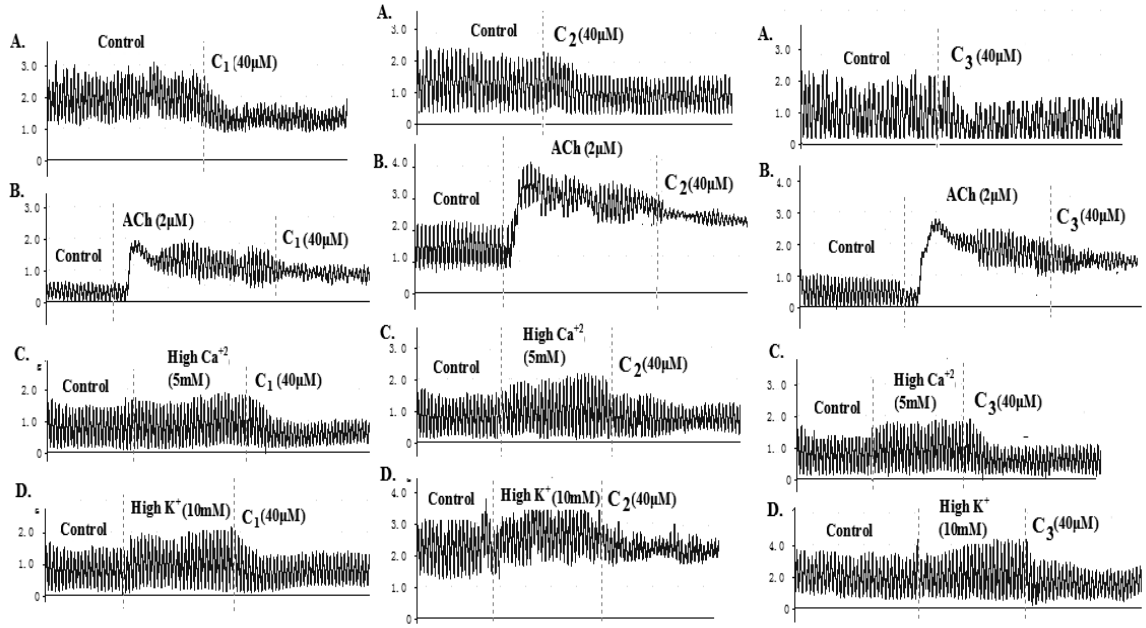


Fig 2.4: Representative traces of compound (1,2,3) on CJSM in different assay conditions. Panel A is the control. Panel B, C, and D represents compound (1,2,3) on the CJSM pretreated with ACh, high Ca2+, and high K+ respectively.

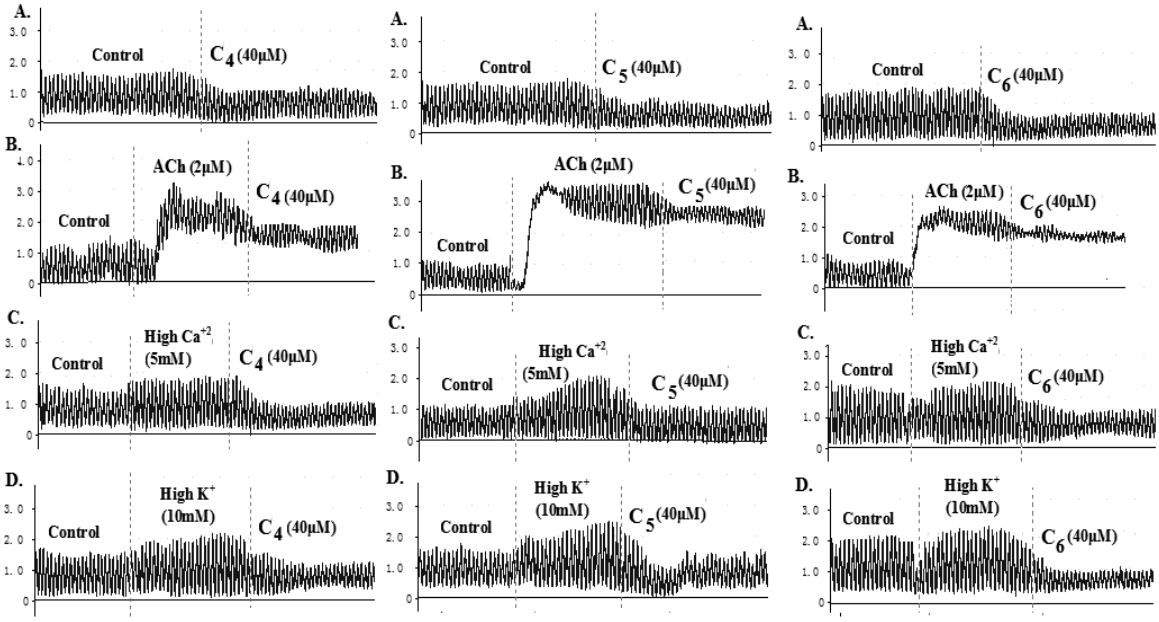


Fig2.5: Representative traces of compound (4,5,6) on CJSM in different assay conditions. Panel A is the control. Panel B, C, and D represents compound (4,5,6) on the CJSM pretreated with ACh, high Ca2+, and high K+ respectively.

Table2.3Analysis of compound(1-7)on CJSM in high contractile state,(N-10-15)

Novel Compounds	NCS	ACh (2μM)	High Ca^{+2}(5mM)	High K^{+}(10mM)
Compound 1(40μM)				
Pretreatment	1.36±0.02	5.84±0.34	3.31±0.07	3.67±0.17
Posttreatment	0.60±0.03**	3.90±0.46	1.78±0.32	2.53±0.27
Compound 2(40μM)				
Pretreatment	2.80±0.09	5.94±0.56	3.45±0.09	3.76±0.53
Posttreatment	2.45±0.03*	3.99±0.05	1.93±0.05	2.69±0.06
Compound 3(40μM)				
Pretreatment	3.08±0.23	5.97±0.54	3.37±0.48	3.59±0.48
Posttreatment	2.69±0.13*	3.97±0.62	1.96±0.49	2.64±0.36
Compound 4(40μM)				
Pretreatment	1.84±0.04	5.92±0.47	3.07±0.63	2.98±0.57
Posttreatment	1.31±0.00*	3.95±0.55	1.61±0.46	2.25±0.43
Compound 5(40μM)				
Pretreatment	1.69±0.09	5.94±0.68	3.08±0.56	2.88±0.59
Posttreatment	1.09±0.12*	3.93±0.42	1.77±0.41	2.19±0.43
Compound 6(40μM)				
Pretreatment	1.54±0.04	5.93±0.52	3.51±0.46	3.12±0.59
Posttreatment	0.90±0.01**	3.88±0.44	2.97±0.39	1.92±0.24
Compound 7(40μM)				
Pretreatment	1.78±0.14	5.03±0.02	3.21±0.26	3.02±0.09
Posttreatment	1.59±0.01*	4.17±0.14	2.77±0.19	2.45±0.24

Values represent the mean±S.E.M, NCS: Normal contractile states

*P<0.05, **P<0.01 compared to contractility before treatment (student t- test)

3.3.4 Mechanism underlying the effects of Compound (1-7) on CJSM

To assess the mechanisms underlying the procontractile action of compound (1-7) on jejunal strips, its action was examined in tissues pretreated with different pharmacological blockers.

At a bath concentration of 5.0μM, α-adrenoceptor blocker phentolamine and the β-adrenoceptor blocker propanolol altered the amplitude of smooth muscle contractions under baseline conditions, and abolished the inhibitory effect of the compound 1(DNBB) after its treatment at 40.0μM [Fig 2.6(B, C), table 2.4].This implicated the involvement of α- and β adrenoceptor in mediating the inhibitory effects of DNBB. Phentolamine and propanolol also partially blocked the inhibitory effects of five other novel compounds respectively [Fig 2.6 and 2.7, table 2.4].The inhibitory effect of compound 7 was not influenced by these α and β adrenergic receptor antagonist, table2.4.

At a bath concentration of 5.0μM, neither the Histamine H_1 receptor antagonist (diphenhydramine) nor the Histamine H_2 receptor antagonist (Cimetidine) influenced the

amplitude of smooth muscle contractions under baseline conditions or after treatment with compound(1-7) at 40.0µM , table 2.4, (Fig. not shown).

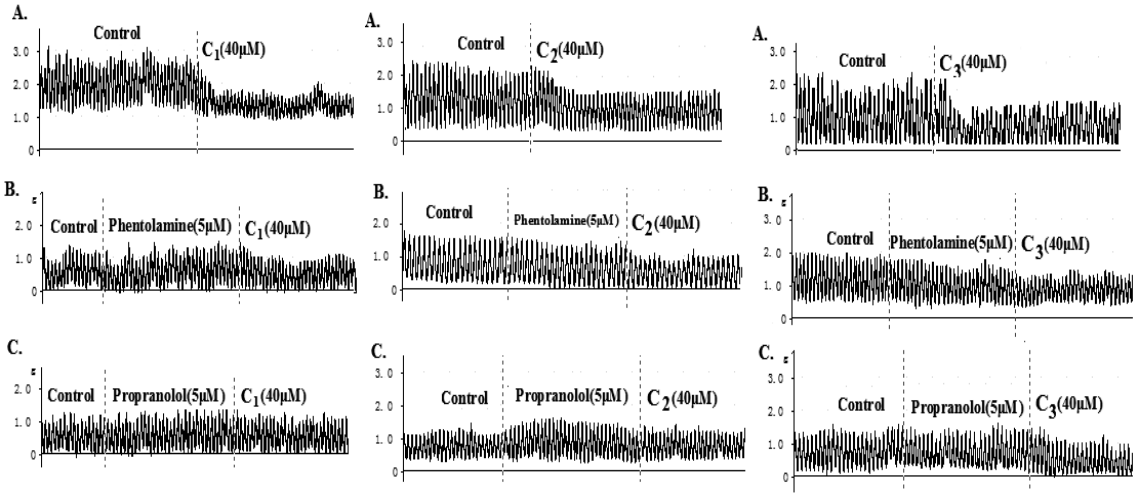


Fig 2.6: Representative the traces of compound (1, 2, 3) on the CJSM in the presence of different antagonists respectively. Panel A is the control. Panel B and C represents compound (1, 2, 3) on the CJSM pretreated with phentolamine, propranolol respectively.

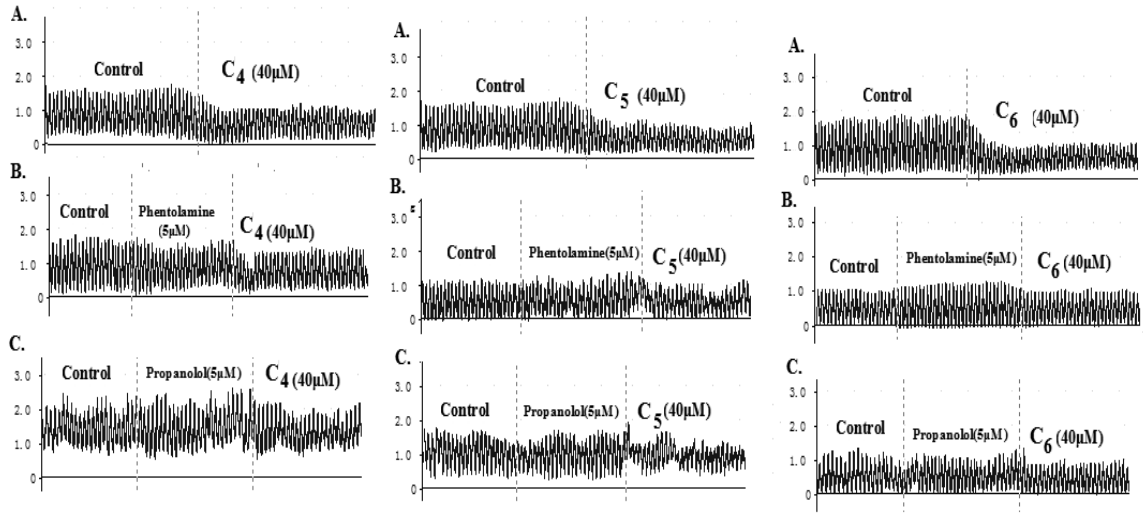


Fig 2.7: Representative the traces of compound (4, 5, 6) on the CJSM in the presence of different antagonists respectively. Panel A is the control. Panel B and C represent compound (4, 5, 6) on the CJSM pretreated with phentolamine, propranolol respectively.

Table 2.4: Analysis of Compound(1-7) on CJSM with or without α and β antagonist(n=10-15)

Novel Compounds	NCS	Propanolol (5μM)	Phentolamine (5μM)	Cimetidine (5μM)	Diphenhydramine (5μM)
Compound 1(40μM)					
Pretreatment	1.36±0.02	2.87±0.07	2.96±0.04	3.07±0.05	2.55±0.07
Posttreatment	0.60±0.03**	2.86±0.02**	2.91±0.08**	2.53±0.17	1.86±0.12
Compound 2(40μM)					
Pretreatment	2.80±0.09	2.99±0.42	2.98±0.48	3.66±0.22	2.86±0.12
Posttreatment	2.45±0.03*	2.65±0.39*	2.71±0.45*	2.99±0.06	2.25±0.09
Compound 3(40μM)					
Pretreatment	3.08±0.23	2.98±0.47	2.61±0.53	3.09±0.08	2.48±0.07
Posttreatment	2.69±0.13*	2.61±0.53*	2.49±0.54*	2.64±0.16	2.01±0.03
Compound 4(40μM)					
Pretreatment	1.84±0.04	2.77±0.41	2.59±0.58	2.58±0.05	2.78±0.41
Posttreatment	1.31±0.00*	2.58±0.48*	2.32±0.57*	2.05±0.03	2.18±0.28
Compound 5(40μM)					
Pretreatment	1.69±0.09	2.72±0.58	2.88±0.49	2.76±0.09	2.57±0.18
Posttreatment	1.09±0.12*	2.59±0.28*	2.53±0.31*	2.19±0.04	2.09±0.08
Compound 6(40μM)					
Pretreatment	1.54±0.04	2.76±0.28	2.63±0.39	3.02±0.59	2.26±0.38
Posttreatment	0.90±0.01**	2.59±0.38*	2.42±0.24*	2.52±0.14	1.79±0.18
Compound 7(40μM)					
Pretreatment	1.78±0.14	2.46±0.06	2.44±0.13	3.12±0.09	2.44±0.16
Posttreatment	1.59±0.01*	1.40±0.08	1.72±0.03	2.55±0.14	1.80±0.08

Values represent the mean±S.E.M, NCS: Normal contractile states

*P<0.05, **P<0.01 compared to contractility before treatment (student t- test)

3.4 EFFECTS OF DNBB ON GASTROINTESTINAL MOTILITY

3.4.1 Defecation during the stress

The restraint stress significantly increased feces pellet output on diarrhea predominant rats. During 3hr of stress, there were sufficient increased in fecal pellets output i.e. 84.14±5.86 in the stressed rats *vs.* 66.57±3.57 in the control (P<0.05).In addition, stressed rats showed a higher incidence of poorly formed fecal pellets, which appeared to contain more fluid.

3.4.2 Effects of DNBB on gut motility

DNBB exhibited its effects on model rats in a dose dependent manner. The feces pellets output decreased greatly in high dose group than moderate and low group. The quality of feces pellets also appeared to change after administration of DNBB on 7 successive days.

3.4.3 Defecation after treatment of DNBB

There was reduction of fecal pellets on model rats after treatment with DNBB. After 7 days of DNBB treatment, treated groups 110.0±18.7 feces pellets output vs. 159.4±21.98 model and 130.0±12.1 dicetel. Similarly after 14 days, treated groups 71.6±13.35 vs. 204.0±48.21model.After 14 days, Dicetel also exhibited sharp reduction in fecal pellets output i.e.77.4±5.68.There was statistically significant differences in feces pellets output among these groups($p<0.05$).This indicated that DNBB plays some role to alleviate the symptoms of Diarrhea predominant(DP) rat model.

3.4.4 SERT protein

The protein of interest 5HT serotonin transporter (SERT) protein at 70kDa and β-actin at 40kDa was detected by western blot analysis, which was shown in Figure 2.8.

From the figure 2.8, the density and size of protein bands in each treated groups can be visualized. The intensity of SERT at 70kDa in entire model groups including control group was relatively very low; almost absent in some groups, in both jejunum and colon segments. However, the β-actin 40kDa protein bands can be distinctly observed in both jejunum and colon segments. This result indicated that there were no significant differences on the 5HT serotonin transporter (SERT) level in jejunum and colon of diarrhea afflicted rats before and after treatment with DNBB and positive drug pinaverium bromide.

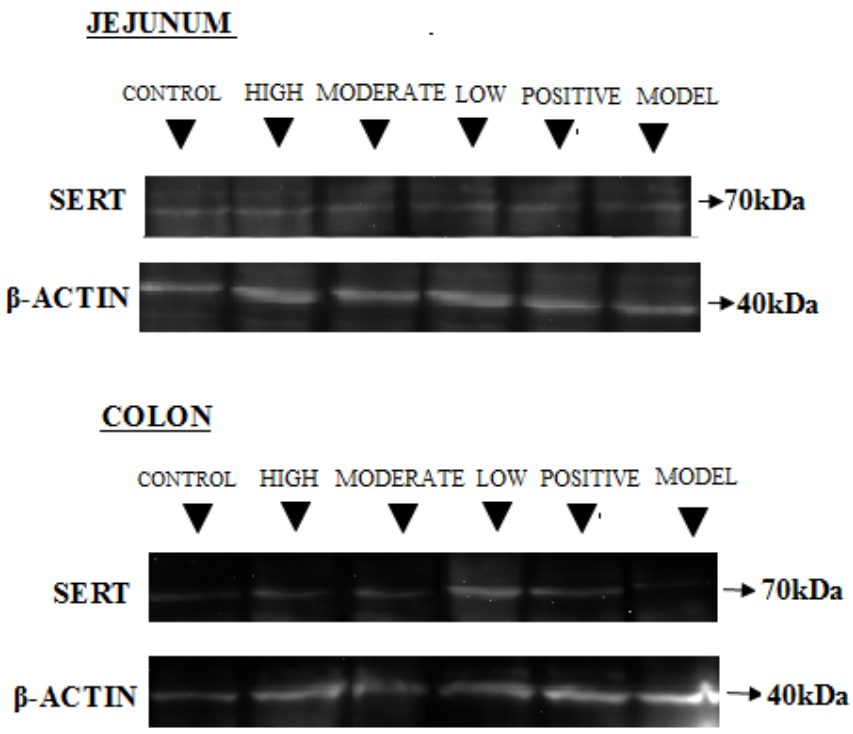


Fig 2.8: Shown as proteins bands onto a nitrocellulose membrane, at MW 70kDa and 40kDa, digested 20µl each groups of proteins of tissue sample jejunum and colon.

4. DISCUSSION

Schiff bases are most widely developing organic compounds that have biological, analytical and industrial applications. Because of the relative easiness of preparation, synthetic flexibility, and the special property of C=N group, Schiff bases are generally excellent chelating agents. The most importance of the Schiff base complexes for bioinorganic chemistry, biomedical application, supramolecular chemistry, catalysis and material science, separation and encapsulation processes has been well recognized and reviewed.[21]

The present study synthesized the novel Schiff bases compounds by applying simple, reliable and green or sustainable chemical procedure. These synthesized compounds are organic ligands that have capability to coordinate or chelate with transition metal ions. The present study observed the biological activity of this synthesized novel Schiff base organic ligands. These Schiff base organic ligands were biologically evaluated on two different perspectives,

in-vitro antibacterial activity and ex-vivo intestinal contractility.

This study observed that the biological activity and mechanism of action of chemical compounds mostly depend on their molecular structure. The structure of a molecule, its composition and arrangements of chemical functional groups, determines the compounds overall pharmacological effects. However, not all the compounds with similar chemical structure have the same biological action.

The antibacterial activities of novel synthesized hydrazones compounds showed that, the inhibition on bacterial growth is highly influenced by the different functional groups present on each compound. The compound (5) 3,5-dihydro-N′-(2-hydroxy,3-methoxybenzylidene) benzohydrazide showed moderate activities against S. aureus and K. pneumonia, while the compound (4) 3,5-dihydro-N′-(2-bromo,4-methoxy,5-hydroxybenylidene)benzohydrazide and compound (6) 3,5-dihydro-N′-(2-hydroxy,5-chlorobenzylidene)benzohydrazide exhibited mild activities against S. aureus. The study on the structure-activity relationship on similar Schiff base compounds reported that hydrophilicity is most important for antibacterial activity. All three compounds (4, 5 and 6) are hydrophilic in nature as they possesses additional hydroxyl group on aromatic ring. Karthikeyan MS. et al. (2006) described that the halogen substituent phenyl moiety are effective in inhibition of bacterial growth [22].Compound 4 and 6 contain bromine and chlorine substituent phenyl moiety indicated that this halogen substituent phenyl moiety influence on inhibition of bacterial growth. Diao et al. 2010, (ref. 5) reported the significant antibacterial activity of the compound which was similar in structure with compound 5 vary only in the position of one hydroxyl group in hydrazide benzene ring. The study on the Schiff bases derived from the 5-chlorosalicyldehyde and 3-methoxysalicyldehyde reported to possess significant antimicrobial activities [23,24].In this study, the compound 5 and 6 are Schiff bases derived from respective salicyldehydes. Zhu X.Y. et al (2009) reported the potent antibacterial activities of Schiff bases that have halogen and hydroxyl group benzohydrazide [25]. The results elicited from this study on antibacterial activities are related to those of previous reports on similar types of Schiff bases. The comparative studies on the structure of the compounds show that the antibacterial activity increases with increase in hydrophilicity, halogen substituent phenyl moiety, and presence of methoxy group.

The activity of these novel compounds coordinated with transition metal remains to be

determined but it is conceivable that, the compound activities on microorganisms could increases when it complexes with metal ions. There are sufficient evidences on similar types of Schiff bases and its transition metal complexes that possess better activity compared to that of ligand [26-29].

The result from ex-vivo study indicated that the compound (1-7) decreased the amplitude of spontaneous smooth muscle contraction in the isolated rat jejunum in a dose dependent manner. It was also observed that the position of each substituent group on benzene ring of each compound variably influence or interfere on CJSM. The activities of these compounds were solely influenced by a substituent's electronic effect, as the position of same functional group (nitro) on the benzene ring of compound (1, 2 and 3) exhibited inhibitory effects on CJSM inconsistently. Result also implicated that the inhibitory effects on the CJSM were positively related to the presence of imine or azomethine group as well as other substituent hydroxyl, nitro, and methoxy group. The weak inhibition showed by compound 7 may be due to the presence of two bulky tertiary butyl groups at meta position, though it also contain hydroxyl group. This bulky substituent may act like a shield and hinder the ideal influence on the CJSM. The imine or azomethine group present in the compound has shown to be critical for the antibacterial activities [30-32], this study found that the compound with such group also plays vital role to produce the inhibitory effects on CJSM. The inhibitory effects on CJSM of the compound 1(DNBB) was correlated with the α- and β-adrenoceptor antagonist phentolamine and propanolol since they abolished the inhibitory effects of DNBB. Similarly, the results implicated the involvement of α- and β-adrenoceptor in mediating the inhibitory effects of other five compounds as the inhibitory effects of these compounds were also partially blocked by phentolamine and propanolol. The contractile responses elicited by the entire seven novel compounds in the high contractile states generated by the ACh, high Ca^{+2} and high K^{+} directed their potential exploitation in the intestinal hyper contractility.

The improvement in defecation, association with change in frequency and change in feces form by the oral administration of DNBB on DP rat model suggested some role of this novel compound DNBB on gastrointestinal motility. The result obtained from the western blot analysis indicated that there was not any effects of 5HT serotonin transporter (SERT) on the gastrointestinal movement of diarrhea afflicted rats.

In physiological studies of the gastrointestinal smooth muscles, 5-HT can produce different effects such as muscle contractions or relaxations depending on the experimental conditions [33], however the results obtained in this study suggested that DNBB induced intestinal muscle relaxants was not mediated by 5HT receptor pathways.

In summary, though the precise mechanism underlying the motility action of these novel compound require further characterization, the results of this study clearly demonstrated that the novel synthesized compound (1-7) are bioactive substances having some theoretical and practical values.

Although the antibacterial activities of these compounds on various other Gram positive and Gram negative bacterial species remains to be investigated, this study put-forth considerable antibacterial activities of compound 4, 5 and 6 against S. aureus and K. pneumonia.

The exact mechanism involved in effects of DNBB on reducing the symptoms of diarrhea afflicted rat model needs further study, but it may be concluded from this study that DNBB may have clinical importance, particularly in dysmotility states, involving chronic idiopathic diarrhea.

To our knowledge, this study reports for the first time relaxant effects of novel aroyl hydrazones compounds on contractility of intestinal smooth muscle. This might provide some important information for the future design of novel aroyl hydrazones compounds in relieving the intestinal hyper contractility. The results presented here, possibly explain the therapeutic effects of hydrazones compounds on functional disorders of GI tracts.

Limitation of this study:

1. The spectral and elemental analysis of the entire novel hydrazones compounds need to be determined.
2. The antibacterial activities of these novel compounds on the various other Gram positive and Gram negative bacterial species need to be determined.
3. The immunohistochemical analysis of the intestinal segments of diarrhea afflicted rats need to be determined.

5. CONCLUSION:

In conclusion, novel findings of this study are:

The novel Schiff base compounds were synthesized and characterized. Representative Compound 1(DNBB) crystal structure was determined.

The antibacterial studies showed mild to moderate activities of novel compound (4, 5 and 6) against S. aureus and K. pneumonia. No lethal effects of compound (1-7) on Caco2 cell culture were observed when their concentration were below 200.0μM.Intestinal contractility analysis confirmed that the compound (1-7) produced inhibitory effects on the contraction of jejunal smooth muscle significantly at concentration of 40.0μM.The concentration of the compounds plays vital role in the degree of inhibition. Compound1 (DNBB) showed the strongest inhibition and compound 7 showed the weak inhibition among them. On analyzing the structure-activity relationship, in-vitro antibacterial activities and ex-vivo contractility of jejunal smooth muscle of the novel compounds were mainly related to azomethine and other substituent groups.

These entire compounds (1-7) decreased the stimulatory action on the contractility of jejunal smooth muscle induced by acetylcholine, high ionic concentration of calcium and potassium implicated their potential values on relieving the intestinal hypercontractility. The inhibitory effects of compound 1(DNBB) were correlated to stimulation of α and β adrenergic receptor since α receptor antagonist phentolamine and β receptor antagonist propanolol partially abolished the inhibitory effects of DNBB on CJSM. Acute oral toxicity (LD50) of DNBB in mice was over 2.0g/kg indicating its low toxic effects. DNBB also alleviated the symptoms of diarrhea predominant (DP) rat model indicating its role on gastrointestinal motility.

Finally, the present study provides useful information of novel hydrazones, its relaxant activity on CJSM could be an important finding for the application in treating gastrointestinal diseases. However, further study is needed for its detail mechanism and possible practical uses.

The part of results from this study was published in the SCI cited journal.

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LITERATURE REVIEW

Hydrazones are organic compounds characterized by the presence of –NH-N=CH- group, formed by condensation of substituted hydrazines with aldehyde and ketones.

These are widely studied compounds due to its diverse pharmacological potential as well as ease of preparation. Researchers throughout the world have done immense work on these hydrazones derivatives of carbonyl compounds. Literature studies on these organic compounds portrayed that these derivatives possess a wide variety of biological activities such as antitumor, anti-bacterial, antiviral, antihypertensive, anticonvulsant, anti-Inflammatory, analgesic, vasorelaxant, anticoagulant, anti protozoal activities etc. [1-3]

The common structural feature of these hydrazones is the azomethine –NH-N=CH- group, constitutes the important class of compounds for the new drug development. Hydrazones are synonymous of Schiff bases with -HC=N- double bond. They are more stable and possess considerable chemical and biological importance than original Schiff base. The C=N bond of hydrazones and terminal nitrogen atom containing a lone pair of electron is responsible for the physical and chemical properties. Acylhydrazones have additional C=O donor site, which provides more flexibility and versatility of these compounds. Hydrazones are important component in drug design as they act as ligands for metal complexes, organocatalysis and synthesis of organic compound. Due to these properties, hydrazones derivatives represent rapidly developing field in modern medicinal chemistry. Aroyl hydrazones derived from the condensation of benzaldehyde and its derivatives with alkyl and aroyl hydrazones play very important role in coordination chemistry. Hydrazones compounds display a versatile behavior in metal coordination and their biological activity is often increased by bonding to transition complexes[4-9].The importance of hydrazones complexes for bioinorganic chemistry, biomedical applications, supramolecular chemistry, catalysis and material science, separation and encapsulation processes, and formation of compounds with unusual properties and structures has been well recognized and reviewed.

The introduction of functional groups in the hydrazone molecules expands the scope of its use in organic synthesis. Moreover, the combination of the hydrazono group with other functional groups leads to compounds with unique physical and chemical properties. Hydrazones containing halo and hydroxy atom in α- or β-positions have been explored for many years,

which are active intermediates in cycloaddition chemistry. The amidrazones and thiosemicarbazones are well documented because of their biological activity and use in the synthesis of heterocyclic compounds. Synthetic approaches and chemical reactivity of the hydrazones substituted with ester, cyano, methoxy groups were also reported [10].

SYNTHETIC METHOD

The Green or sustainable chemistry, is the chemical procedure that minimize the use and generation of hazardous substances and maximize the efficiency of any chemical products. In recent times, researchers focus on synthesis of the chemical compounds by applying this green chemistry. Recently, there are sufficient articles on Schiff bases compounds, where the synthetic method applied was simple, reliable and sustainable. Xiao Yang qui et al (2006), Diao Yun-peng et al (2007),Hong Bo Ma et al(2008), Sa.Deng et al(2009),Wei-Guang Zhang et al (2012) and various other authors synthesize the Schiff base compounds by applying green or sustainable chemical philosophy[11-15].

Schiff base reactions are widely used in the field of chemistry. With the advantages such as mild reaction conditions and high reaction rates, they were employed for protecting various functional groups and synthesizing a series of organic ligands.

The most general and oldest method for the synthesis of hydrazones Schiff base is the reaction of hydrazines with carbonyl compounds [16-22]. Because of the high nucleophilicity of both hydrazine nitrogen atoms the use of this method is difficult for the preparation of hydrazones bearing carbonyl and other electrophilic substituents.

The amide group is less reactive than the ester and ketone functions. Therefore, amidohydrazones may be selectively prepared by reaction of alkyl- or arylhydrazines with 2-oxo- 2-phenylacetamides [23].

The use of the benzyl protecting group allowed to obtain the *bis*-hydrazones, the reaction of N-methyl-α-chloroacetoacetamide with ethyl carbazate in ethanol affords N-acylhydrazones as the only product [24].

The indole derivatives exhibit interesting biological properties, a series of hydrazones bearing an amide group incorporated into an indole moiety were prepared by reactions of isatines with hydrazines [25].

The coupling of diazonium compounds with active methylene compounds is one of the oldest method for the synthesis of aryl hydrazones.[26,27] The reaction is usually carried out in a cold aqueous solution buffered with sodium acetate, but the pH of the medium can be lowered for strongly activated methylene compounds.

A series of arylhydrazonothioacetanilides were prepared by the Japp-Klingemann reaction, which is an azo coupling accompanied by the elimination of one of the two electron withdrawing groups such as COOH, COMe, CONH2 and COOEt19.The use of Japp-Klingemann reaction is also a convenient method for the synthesis of hydrazonoacetamides bearing halogen atom[28].

Isatine hydrazones were prepared either by condensation of isatine with hydrazines or by the alternative process of azo coupling of aryldiazonium salts with indole-2-one. The azo coupling reaction is also used to prepare 4-arylhydrazonopyrazol-5-ones and - imines [29].

The synthetic method for hydrazones by the azo coupling reaction is more suitable to prepare compounds bearing carbamoyl, thiocarbamoyl and amidine groups than the alternative method based on the reaction of carbonyl compounds with hydrazines [30].

In recent years, stannous chloride is frequently used in organic synthesis as a catalyst due to its properties such as nontoxic nature, easy availability, inexpensiveness and easiness for work up procedures. It played a great role for the synthesis of biologically active heterocycles such as benzimidazoles, Quinoxalines and functionalization of 4, 5-diaminopyrazoles.

Classical method for the synthesis of hydrazones is the reaction of hydrazine with slight excess of carbonyl compounds in refluxing conditions using ethanol or toluene as a solvent. Recently RS Varma utilized the polystyrene sulphonic acid as a catalyst for the synthesis of hydrazones under microwave conditions. DJ Brondani reported the synthesis of some aryl hydrazones in aqueous media under ultrasound irradiation. And the very recent development in the synthetic methods is the use of Ball-Mill for the solvent free synthesis of hydrazones by F Lamaty [31].

Synthesis of hydrazones by modification of the substituents

Hydrazono-amides can be prepared by reaction of hydrazono-esters with amines or by hydrolysis of a cyano group of hydrazonomalononitriles. The reaction is carried out in sulfuric

acid accompanied by sulfonation to give final products. These reactions are not widely used in organic synthesis because of the availability of hydrazono-amides by other methods.

Reaction of hydrazono-amides with Lawesson's reagent represents an alternative method to prepare thioamides. Tetradentate *bis*-hydrazones were prepared successfully in a similar way. 2-Chloro-2-arylhydrazonoacetamides can be prepared by the reaction of acid with thionyl chloride followed by substitution with amines. In a second stage, the chlorine of chlorohydrazone can be substituted by the amino moiety as a result of its reaction with various amines to form 2-amino-2-phenylhydrazonoacetamides exhibiting interesting biological properties.

4-Arylhydrazono-1*H*-pyrazoles can be easily prepared by the reaction of arylhydrazono malononitrile, arylhydrazonocyanoacetate, and arylhydrazonoacetoacetate and arylhydrazono -3- ketimino butyronitriles with hydrazine [32].

The reactions of carbonyl compounds with hydrazines, coupling of diazonium salts with malonamides, malonthioamides and malonamidines and elaboration of preformed hydrazones constitute an arsenal of synthetic methods to hydrazones containing amide, thioamide and amidine groups [33].

The 4-hydroxy benzohydrazide and all the other benzaldehyde hydrazones are so far unknown synthones which could be used for preparing various heterocyclic systems. The Vilsmeier-Haack reaction is widely used for formylation. It can be applied to introduce an aldehyde group on activated aromatic compounds and olefinic compounds. Formylation of benzaldehyde hydrazones using DMF/POCl3 formed C-terminal or N-terminal formylated products without cyclisation with comparatively low yields [34].

Hydrazones from hydrazines bearing electron withdrawing groups, and aromatic or aliphatic aldehydes form rapidly hydrolyze in water at neutral pH. Hydrazones, acyl hydrazones, semicarbazones and oximes formed by hydrazines, hydrazides, semicarbazides or hydroxylamines are stable even at low pH. The delicate combination of hydrazine nitrogens and electron withdrawing substituents allows the rapid formation and hydrolysis of some hydrazone derivatives under neutral conditions. The equilibrium constants vary significantly with the nature of the substituents may strongly bias dynamic combinatorial mixtures in favor of the most stable products [35].

Hydrazones are reactants in hydrazone iodination, the Shapiro reaction and the Bamford -Stevens's reaction to vinyl compounds. A hydrazone is an intermediate in the Wolff–Kishner reduction. Another method to synthesis a hydrazone is the Japp–Klingemann reaction (from β-keto-acids or β-keto-esters and aryl diazonium salts).Aryl hydrazones were easily obtained by nucleophilic addition of lithium reagents or Grignard reagent and were converted into indoles in good yields [36].

Hydrazones exhibit a varied reactivity, taking part in reactions with nucleophiles, electrophiles and other chemical reagents. Being ambient nucleophiles, hydrazones react with electrophilic reagents with participation of either the nitrogen atom or the carbon atom of the azomethine group. Strong bases deprotonate hydrazones to form anions, whereas nucleophiles attack the azomethine carbon atom to form products of both addition and substitution. Furthermore, hydrazones can be reduced to amines or oxidized to diazo compounds.

BIOLOGICAL ACTIVITY

Antimicrobial activity

There are immense researches reported on aryl hydrazones as antimicrobial agents. Yung D.K. et al (2010) reported in vitro antimicrobial evaluation of novel hydrazones aminopiperazines that showed broad spectrum of activity [37]. Y Harinath et al (2011) studied the heteroaromatic hydrazones derivatives against various bacterial strains [38]. Pramilla Sah and Chandra Prakash Gharu (2012) synthesized some novel hydrazones bearing thiadiazole moiety and evaluated antimicrobial activities against different bacterial strains [39]. Angelusiu MV et al (2010), synthesized the metal complexes with aroyl hydrazones based ligand and evaluated its antibacterial activities [40]. Ozkay et al. (2010) synthesized novel benzimidazole derivatives bearing hydrazones moiety and evaluated their antibacterial activity against different bacterial strains [41].

Novel chloropyrrole derivatives of aroylhydrazone developed by Rane et al. (2010) have been evaluated for antibacterial activity against different bacterial strains [42].Lee et al.(2012) synthesized various hydrazones as selective inhibitors of Staphylococcus aureus β-ketoacyl carrier proteinsynthase III[43].

Analgesic and Anti-inflammatory Activity

Several researchers have reported hydrazones derivatives as analgesic and anti-inflammatory agents. Moldovan et al. (2011) synthesized various hydrazones derivatives and reported them to have promising in-vivo anti-inflammatory activity [44]. El-Sayed et al. (2011) synthesized hydrazones derivatives with selective COX-2 inhibition [45]. The compound is reported to have an ED50 value of 0.2 mmol/Kg.

Kavita C.S.et al (2010) synthesized the novel benzimadazole derivatives and reported them as potent analgesic and anti-inflammatory activities [46].

Anticancer Activity

Cancer, having high level of penetrating potential affecting every organ of the body, is life threatening disease. There are immense investigations on novel hydrazones that are potent as anticancer agent.

Wei Yong Liu et al (2009) synthesized the novel ribavirin hydrazones derivatives and showed its antiproliferative activity against A549 lung cancer cells [47].Tarek Aboul Fadl et al (2012) studied an antiproliferative activity on Schiff bases of isatin derivatives [48].

Basavaraj R. Patil et al (2011) synthesized cyclotriphophazene hydrazones derivatives and evaluated anti proliferative activity in vitro against the human liver carcinoma cell line (HepG2) and Human cervix carcinoma cell line (HeLa)[49]. Vogel S. et al (2008) investigated the aroyl hydrazones of 2phenyl indole 3 carbaldehydes as antimitotic agents [50]. Kumar et al. (2012) synthesized various bis (indolyl) based hydrazones active against multiple cancer cell lines [51].

Antihypertensive Activity

Gil Longo J. et al studied the antihypertensive activity of hydrazones derivatives which reported that the compounds owe the antihypertensive activity to direct the relaxation of vascular smooth muscle [52].

M. Minami et.al elucidated the effects of a new vasodilating antihypertensive drug, budralazine 4, mesityl oxide -1- phthalazinyl hydrazones and suggested budralazine is active on renin angiotensin [53].

Anticonvulsant and Antidepressant Activity

Manav Malhotra et al (2011) synthesized the isonicotinohydrazide derivatives as potent anticonvulsant agents [54]. Jain J. (2011) reported the methane aryl acyl hydrazones as better and safer anticonvulsant agents [55]. Gokce M. et al (2008) synthesized and evaluated some hydrazone derivatives of both 2-oxobenzoxazoline and 2-oxobenzothiazoline that exhibited potent anticonvulsant activity [56]. Reema Sinha et al (2011) evaluated the aryl acid hydrazones of nicotinic acid hydrazide possesses anticonvulsant activity, the compounds displayed excellent protection in maximal electroshock screen [57]. Compound N [1]-(4-chlorobenzylidene) nicotinohydrazide as most potent analog with ED_{50} value of 16.1 mg/kg and protective index ($PI = TD_{50}/ED_{50}$) value of >20, which was much greater than that of the prototype drug phenytoin (PI = 6.9).

De Oliveira KN et al (2011) synthesized a series of sulphonyl hydrazones derivatives and evaluated its antidepressant effects by forced swimming test in mice. The sulphonyl hydrazones cyclic imides derivatives are potential for the treatment of depression [58]. Özgür Devrim Can et al (2011) reported the novel thiodiazole derivatives bearing hydrazones moieties possess the antidepressant like effects [59].

Antitubercular Activity

There are several novel hydrazones derivatives identified that played an effective role as antitubercular agents.

Basak Oral and Sevim Rollas (2012) synthesized substituted hydrazones, 2-pyrazoline-5-one and 2-isoxazoline-5-one derivatives possessing 1,3,4 thiadiazole moiety which showed the highest inhibition that acts as good antitubercular properties[60].Mahajan A et al.(2011)synthesized and tested in vitro antitubercular activity of ferrocene based hydrazones that exhibited the significant antitubercular activity against M. tuberculosis (MIC 2.5-5μg/ml)[61].

Antimalarial Activity

Some of the novel aryl hydrazones were identified that showed their antimalarial activities.Walcourt A. et al synthesized and evaluated the antimalarial activities of novel aryl

hydrazones, which revealed that the compounds antimalarial activity correlates with the antiproliferative activity against neoplastic cells [62]. Fattorusso C. et al (2008) reported the structure activity relationships study of new series of hydrazones derivatives as potent antimalarial agents [63]. Sahu NK et al (2012) performed the QSAR analysis of some substituted hydrazones derivatives that exhibited antimalarial activities [64]. Khankischpur M et al (2011) synthesized and reported that α hydroxyl hydrazonates possesses strong antimalarial properties [65].

AntiHIV Activity

Hydrazones are also reported as antiHIV agents. Vicini P et al (2009) evaluated an isothiazole hydrazones derivatives possesses antiHIV activity [66]. Xiao dong Ma et al (2011) synthesized the new series of hydrazones and evaluated their antiviral activity against human immunodeficiency virus in MT-4 cells [67].

Leishmanicidal Activity

Hydrazones are also characterized as leishmanicidal drugs. Savornin B. synthesized and tested the new hydrazones of thiophene carbaldehydes against three leishmania strains that exhibited the significant lieshmanicidal activity. The minimum inhibitory concentrations were evaluated against pentamidine, as a reference drug. Several compounds exhibited significant leishmanicidal activity; only one compound was ten times more active than pentamidine [68].

Antiprotozoal activity

Protozoal diseases are highly prevalent in tropical countries affecting large population of human, causing suffering and death. Caputto *et al.* (2011) [69] reported the inhibitory activity of hydrazones against cruzipena major cysteine protease of *T.cruzi*. Hayat *et al.*(2010) reported the *in-vitro* antiamoebic activity of hydrazones against the HM1:IMSS strain of *Entamoeba histolytica*. The compounds are reported to have IC50 value of 0.03 and 0.04μM respectively [70]. Vaio *et al.* (2009) [71] synthesized hydrazone derivatives and described them to be of high utility in Chagas disease. Siddiqui *et al.* (2012) described the antiamoebic activity of hydrazone derivatives [72]. Romeiro *et al.* (2009) developed hydrazone derivatives

as cruzin inhibitors [73]. Aponte *et al.* (2010) evaluated the antitrypanosomal activity of hydrazone derivatives [74].

Antioxidant activity

Oxidation reactions are crucial for sustenance of life but they can also be damaging. Oxidative stress is the cause of different pathological states. Hydrazone derivatives synthesized by Musad *et al.* (2011) are reported to have radical scavenging activity at the concentration of 10μg/mL [75]. Abdel-Wahab *et al.* (2011) evaluated imidazoline based hydrazones by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS)assay method and reported them to have promising antioxidant activity [76].

Jagadeesh *et. al*.(2010) synthesized a new series of ketone 1-(5- (pyridin-3-yl) -1,3,4-thiadiazol-2-yl)-hydrazone derivatives by the condensation of 1-(5- (pyridin-3-yl)- 1,3,4 -thiadiazol-2-yl) hydrazine with substituted and unsubstituted ketones. They evaluated their antioxidant property by using 1, 1-diphenyl-2-picrylhydrazil (DPPH) method. All the compounds demonstrated good antioxidant activity [77].

Antiplatelet activity

Antiplatelets decrease platelet aggregation, hold back the formation of thrombus. Jordao *et al.* (2009) synthesized hydrazone derivatives and evaluated them for *in-vitro* antiplatelet activity. The antiplatelet activity of novel tricyclic acylhydrazone derivatives was evaluated by their ability to inhibit platelet aggregation of rabbit platelet-rich plasma induced by platelet activating factor (PAF) at 50nM. Benzylidene- / 4′-bromobenzylidene 3-hydroxy -8-methyl -6-phenyl pyrazolol [3,4-b]thieno-[2,3-d]pyridine-2-carbohydrazide were evaluated at 10μM, presenting, respectively, 10.4 and 13.6% of inhibition of the PAF-induced platelet aggregation [78].

Silva et al(2004) reported the evaluation of platelet anti aggregating profile for identification of a new potent prototype of antiplatelet derivative, that is benzylidene 10H- phenothiazine -1-carbohydrazide (IC_{50}=2.3μM),which acts in the arachidonic acid pathway probably by inhibition of platelet COX-1 enzyme. Additionally, the change in *para*-substituent group of acylhydrazone framework permitted to identify a hydrophilic carboxylate derivative and a

hydrophobic bromo derivative as two new analgesics that are more potent than dipyrone, which is the standard, possessing selective peripheral or central mechanism of action [79].

Antiparasitic activity

Ali *et al.* (2010) evaluated the *in-vitro* anti parasitic activity of hydrazone derivatives against *Ctenocephalides felis* and *Rhipicephalus sanguineus*as LD50 of 0.39 and 0.28µg/tick has been reported [80]. Aslam *et al.* (2011) synthesized hydrazone derivatives as urease inhibitors. Urease catalyzes the hydrolysis of urea to ammonia and carbamate. This is beneficial for the pathogenesis of urolithiasis, pyelonephrities, ammonia and hepatic encephalopathy, hepatic coma and urinary catheter encrustation [81].

Antidiabetic activity

Girges MM. et al. (1993) synthesized and pharmacological evaluated the new series of substituted alpha-picolinium p-dimethyl amino benzalhydrazine derivatives and their o-hydroxy analogues and reported their efficacy as potential hypoglycemic agents [82]. Oellerich M and Haekel R.(1980) reported phenylethylhydrazono-propionic acid and cyclo hexyl-ethyl hydrazono-propionic acid that can lower the blood glucose level[83].

Cardioprotective activity

Despite the intensive drug research cardiovascular diseases still remain the leading cause of mortalities worldwide. El-Sabbagh *et al.* (2010) synthesized octahydroquinazoline hydrazones and reported them to be potential hypotensive agents. The activity was attributed to α-blockage [84].

Vasodilator Activity

Conventional therapy to treat hypertension often involves arterial vasodilation. It is important to find new vasodilators with a potential for clinical use. Silva et al(2005)[85] reported a new bioactive compound of the *N*-acylhydrazone class, 3,4-methylene dioxybenzoyl -2-thienyl hydrazone named LASSBio-294, was shown to have inotropic and vasodilatory effects.

New derivatives of LASSBio-294 were designed and tested on the contractile responses of rat vascular smooth muscle *in vitro*. Phenylephrine-induced contractions of aorta was inhibited by the derivatives *N*-methyl-2-thienylidene-3,4-methylenedioxy-benzoyl hydrazine, named LASSBio-785 and *N*-allyl-2-thienylidene-3,4-methylenedioxy-benzoyl hydrazine, named LASSBio-788.Vasodilation induced by both derivatives is likely to be mediated by a direct effect on smooth muscle because it was not dependent on the integrity of vascular endothelium. LASSBio-785 was seven times more potent than the reference compound LASSBio-294 (IC50 = 74μM) in producing an endothelium- independent vasodilator effect.

Schistosomiasis activity

Schistosomiasis is a parasitic disease caused by several species of flatform. Schistosomiasis causes debilitating nutritional, hematologic and cognitive deficits, with substantial morbidity and mortality in populations.

9-Acridanone hydrazones have been developed by Hoffmann-La Roche. One of these compounds (RO 15-5458/000) was administered at two dose levels 25 mg and 15mg/kg body-weight to *S. mansoni* infected vervet-monkeys [86].In addition, same compounds were found to be effective against *S. mansoni* in mice, killing almost all the skin chistosomules, when administered at the dose of 100mg/kg. In experiments carried out with Cebus monkeys, the compound RO 15-5458 /000 was shown to be fully effective at 25 mg/kg [87].

Besides above mentioned activities, hydrazones reported to have various other biological activities such as enzyme inhibitors, herbicides, insecticides, nematocides, rodenticides, and plant growth regulators as well as plasticizers and stabilizers for polymers etc [88-93].

During this literature survey, the study on activities of hydrazones compounds on the intestinal contractility was hardly found. The present study is the first attempt to study the effects of the novel hydrazones compounds on contraction of intestinal smooth muscle.

In summary, hydrazones derivatives possess the array of biological activities. With the proper synthesis and the precise analysis on structure-activity relationship there is high potential to develop the novel hydrazones as drug that provides the great value in medicinal world.

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ABBREVIATIONS:

DNBB	3, 5 dihydro -N′ - (3-nitrobenzylidene) benzohydrazide
DP	Diarrhea predominant
CJSM	Contractility of jejunal smooth muscle
LD50	Lethal Dose 50
DMSO	Dimethyl sulfoxide
DMF	N,N-dimethylformamide
SERT	Serotonin transporter
5HT	5- hydroxytryptamine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PMSF	Phenyl Methyl Sulfonyl Fluoride
BSA	Bovine Serum Albumin
BCA	Bicinchonninic Acid
SDSPAGE	Sodium Dodecyl (lauryl) Sulphate-PolyacrylAmide Gel Electrophoresis
MIC	Minimum Inhibitory Concentration
Na-CMC	Sodium Carboxy Methyl Cellulose
FBS	Fetal Bovine Serum
LB	Luria-Bertani
DMEM	Dulbecco's Modified Eagle Medium

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