"CARDIOPROTECTIVE PROPERTIES OF PYRAZOLONE DERIVATIVES IN MYOCARDIAL ISCHEMIC REPERFUSION INJURY"

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY (PHARMACY)

UNDER

University of North Bengal

By

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UNDER THE SUPERVISION OF

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DEPARTMENT OF PHARMACEUTICALCHEMISTRY

HIMALAYAN PHARMACY INSTITUTE

MAJHITAR, SIKKIM – 737136

2011

DECLARATION

I do here by declare that the thesis entitled **"Cardioprotective Properties Of Pyrazolone Derivatives In Myocardial Ischemic Reperfusion Injury"** submitted by me to **University of North Bengal** for the award of the **Degree of Doctor of Philosophy (Pharmacy)** is a bonafide record of research work carried out by me under the supervision of **Prof. Bishnu Pada Saha**. Neither this thesis nor any part of it has been submitted for any degree/diploma or any academia award anywhere before.

Place: Himalayan Pharmacy Institute

Date: 11-4-11

G. Mariappan.

Mr. G.Mariappan

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Addendum / Corrigendum – 1

Foreword

The thesis has been submitted under the planning and guidance of

Prof.Dr) Bishnu Pada Saha,M.Pharm,PhD,FIC, Ex-Emeritus Fellow, AICTE and Professor and Head, Department of Pharmaceutical Chemistry, Himalayan Pharmacy Institute, Majhitar, East Sikkim, India in consonance with the regulations of North Bengal University for PhD (Pharmacy) Degree.

The co-operation and support received through the Research Promotion Scheme-B of All India Council for Technical Education, Govt. of India, New Delhi, for Mr.G.Mariappan is gratefully acknowledged for completion of this work with the financial support for the procurement of all the instruments, apparatus, glass wares & chemicals etc.

PREFACE

Cardio Vascular Disease (CVD) is the single largest cause for mortality and morbidity in the world as reported in WHO report 2007. The overall burden continues to grow in both developed and developing countries, but there are distinct differences in the pattern of growth between the two, as the expected rate of increase in CVD in developing countries in the next two decades is likely to be almost twice that in the developed countries. Ischemic heart disease is the leading cause of death in developed countries, but third to AIDS and lower respiratory infections in developing countries. The deaths due to CVD in India were 32% in 2007 and are expected to rise from 1.17 million in 1990 and 1.59 million in 2000 to 2.03 million in 2010. In addition, Indians tend to have premature coronary heart disease, at least a decade or so earlier than their counterparts in the developed countries and also have higher case fatality rate. It is most likely that the rapid demographic and health transitions currently occurring in India make a major contribution to gene–environmental interactions and early life influences of fetal malnutrition that may be a cause of increased CVD in India. Although a relatively new epidemic in India, it has quick become a major health issues with deaths due to CVD expected to be double during 1985-2015 (WHO 2004). In India almost 2.6 million individuals are predicted to die due to coronary heart disease (CHD)' constituting nearly 54% of all CVD deaths by 2020.

The work has been divided into the following two parts. Part I: Synthesis of pyrazolone derivatives and Part II: Cardio protective evaluation of those derivatives. Part I of the thesis represent the synthetic part which originates from the observations of cardioprotective activities of pyrazolone derivatives. It covers the synthesis of pyrazolone derivatives from 3-methyl pyrazol-5-one. The structures of the synthesized compounds have been elucidated by UV, IR, ^{1}H NMR, Mass spectral data and elemental analysis. A research paper regarding synthesis, characterization and biological evaluation has been published in **Indian Journal of Chemistry Section B.**

Acute toxicity and gross behavior studies revealed that pyrazolone compounds in present investigation were found to be nontoxic up to 5000 mg/kg body weight in albino mice. All

the animal experiments were performed by the approval of Institutional Animal Ethics Committee, Himalayan Pharmacy Institute, East Sikkim.

The main objective of the investigational work carried out and represented in this thesis, has been to synthesize a number of new organic compounds and to evaluate their cardioprotective effects to furnish a possible cardioprotective agent. The objective has also been to explore and evaluate the activities of those compounds in other areas to provide a suitable lead which may be utilized in the future to pursue a new line of investigations. The work is based upon the combined approaches of both exploitation and exploration, the main stay of investigations in the domain of medicinal chemistry, and more particularly, the synthetic drugs; the former being concerned with the assessment, improvement and extension of a lead and the latter with the search for a new lead.

Part II of the thesis represents the exploration of cardioprotective effect of pyrazolone derivative. This work is based on the reported literature by Yukihito Higash et al 2006 on cardioprotective properties of pyrazolone derivatives. The present study investigates the cardioprotective effect of pyrazolone derivatives (PYZ1-PYZ10) on plasma lipid profile, serum marker enzymes, endogenous enzymatic and non-enzymatic antioxidants in cardiac tissues against isoproterenol (ISO) induced myocardial ischemic injury in rats. Isoproterenol induced myocardial injury in rats is a widely used experimental model for evaluation of cardioprotective effect of various drugs. This is because of pathophysiological changes following ISO administration in rats are similar to those taking place during myocardial injury in humans. Therefore, it is a suitable model to study myocardial ischemic injury.

Lipid metabolism plays an important role in myocardial injury produced by ischemia. Hence the estimation of lipid profile can be directly correlated with the intensity of myocardial injury. In the present study also, isoproterenol administration caused a significant rise in the serum lipids thereby increases lipid biosynthesis and lipid peroxidation. Rats treated with pyrazolone derivatives showed decreased concentration of

total cholesterol, triglycerides, LDL cholesterol in serum indicates the beneficial effects of pyrazolone derivatives in reducing hyperlipidemia caused by isoproterenol.

Pretreatment with the pyrazolone derivatives at 10 mg/kg body weight for 5 days prevented the elevation of serum marker enzymes namely lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) in myocardial injured rats. ISO-induced animals exhibited decreased levels of superoxide dismutase (SOD) and glutathione (GSH) in the heart, which were restored to near normal levels following treatment with pyrazolone derivatives. These derivatives also attenuated lipid peroxidation (LPO) in the heart and improved the imbalance in lipid profile (TG, LDL, VLDL, HDL) caused by ISO. These findings revealed the cardioprotective effect of pyrazolone derivatives against isoproterenol induced myocardial injury. A communication describing the cardio protective properties and anti oxidant activities of pyrazolone derivatives has been published in (i) **Pharmacology Online and (ii) Journal of Advanced Pharmaceutical Technology & Research respectively.**

Since the cardio protective activities of pyrazolone derivatives have been encouraging, the biological evaluation of these derivatives had led to an important conclusion regarding the structure activity relationship and a possible mode of action in this class of compounds. On the other hand, the results obtained in this study have helped to reach a conclusion on structure activity relationship and Yukihito Higash observation on cardio protective properties of pyrazolone derivatives. Moreover the compounds have also been screened for analgesic, anti inflammatory and antipyretic activity since myocardial injury is associated with inflammatory response. Some of the drugs are available in the market as NSAID possess analgesic, anti-inflammatory and antipyretic activity. Hence these pyrazolone compounds also have screened for the same. All these activities have been furnished in concomitant publication in **Saudi Pharmaceutical Journal (Elsevier Publication).** Taking a lead from these activities, the original molecules may be suitably tailored to furnish useful therapeutic agents in future.

List of abbreviations

AA	Arachidonic acid
CAD	Coronary artery disease
CAT	Catalase
CPK	Creatinine phosphokinase
CVD	Cardiovascular disease
e NOS	Endothelial NO Synthase
FFA	Free fatty acid
GPX	Glutathione Peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HOCl	Hypochlorous acid
4-HNE	4-Hydroxyl 2-non-enal
IHD	Ischemic heart disease
IL-1	Interleukin-1
IPC	Ischemic preconditioning
ISO	Isoproterenol
JAK	Janus kinase
LDH	Lactate dehydrogenase

LDL	Low density lipoproteins
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
NADPH	Nicotinamide-adenine dinucleotide phosphate
NF-kB	Nuclear factor-kB
NO	Nitric Oxide
O_2-	Superoxide anions
OA	Osteoarthritis
OFR	Oxygen free radical
OH-	Hydroxyl radicals
PKC	Phospho kinase C
PMN	Poly morphonuclear leukocyte
RA	Rheumatoid arthritis
RISK	Reperfusion injury salvage kinases
ROS	Reactive oxygen species
SOD	Superoxide Dismutase
TGF-β	Transforming Growth Factor
TNF-α	Tumor necrosis factor- α
TBARS	Thiobarbituric acid reactive Substances

Dedicated To My

MENTOR

Professor B.P.Saha

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I feel proud to be under the shade of Prof (Dr). B.P. Saha as my supervisor and framing my project work in the right propulsion without whom I would have been in the mid of an ocean and his role imparts a deep sense in the core of my heart. Without his constant encouragement and insightful guidance, I could not reach this final stage and make this dissertation possible.

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G.MARIAPPAN

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

In connection with the project entitled, it is necessary to describe the normal functioning of the heart and thereby its pathophysiological conditions. The cardiovascular system refers to the heart, blood vessels and the blood. The heart is the muscular organ of the circulatory system that constantly pumps blood with nutrients throughout the body. The heart is composed of cardiac muscle tissue that is very strong and able to contract and relax rhythmically throughout lifetime. The upper chamber on each side of the heart, which is called an atrium, receives and collects the blood coming to the heart. The atrium then delivers blood to the powerful lower chamber, called a ventricle, which pumps blood away from the heart through powerful, rhythmic contractions. Electrical impulse from heart muscle (the myocardium) contracts the heart. This electrical signal begins in the sinoatrial (SA) node, located at the top of the right atrium. The SA node is sometimes called the heart's "natural pacemaker." An electrical impulse from this natural pacemaker travels through the muscle fibers of the atria and ventricles, causing them to contract.

The human heart is actually two pumps in one. The right side receives oxygen-deficient blood from the various regions of the body and delivers it to the lungs. In the lungs, oxygen is absorbed in the blood. The left side of the heart receives the oxygen-rich blood from the lungs and delivers it to the rest of the body. The essential function of the heart is to pump blood to various parts of the body. The mammalian heart has four chambers: right and left atria and right and left ventricles.

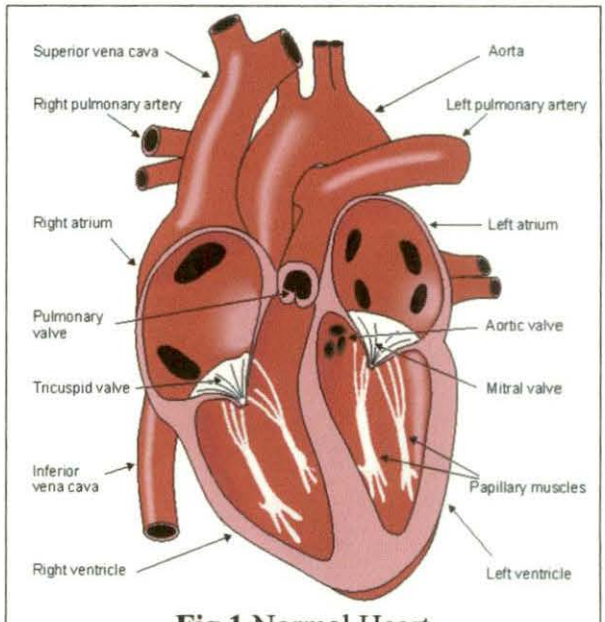


Fig.1. Normal Heart

The two atria act as collecting reservoirs for blood returning to the heart while the two ventricles act as pumps to eject the blood to the body. The heart comes complete with valves to prevent the back flow of blood as in any pumping system. Deoxygenated blood returns to the heart via the major veins (superior and inferior vena cava), enters the right atrium, passes into the right ventricle and from there is ejected to the pulmonary artery on the way to the lungs. Oxygenated blood returning from the lungs enters the left atrium

via the pulmonary veins, passes into the left ventricle, and is then ejected to the aorta which then distributes it to the rest of the body through various arteries and the heart muscle through coronary arteries. Also branching off the aorta as it leaves the heart is a pair of coronary arteries, these arteries supply blood to the heart muscle and are considered as a part of the systemic circulation. After passing through capillaries in the heart, blood in the coronary circuit returns to the right side of the heart through veins that empty directly into the right atrium. Heart attacks are caused by clots in coronary arteries, depriving the heart muscle of oxygen.

1.1. ISCHEMIC HEART DISEASE (MYOCARDIAL ISCHEMIA)

Coronary artery disease is a condition in which fatty deposits (atheroma) accumulate in the cell lining of the coronary arteries. These fatty deposits build up gradually and irregularly in the large branches of the two main coronary arteries which supplies blood to the heart muscle. This process is called atherosclerosis which leads to narrowing or hardening of the coronary arteries and arresting blood supply to the heart muscles. This results in ischemia in heart muscles which can damage them. An obstruction of coronary arteries develops gradually from the accumulation of fatty, fibrous plaques that narrow the lumen of coronary artery, reduce the blood flow and lead to infarction. The complete occlusion of the coronary arteries leads to myocardial infarction and ultimately death (**Fig .2.**)

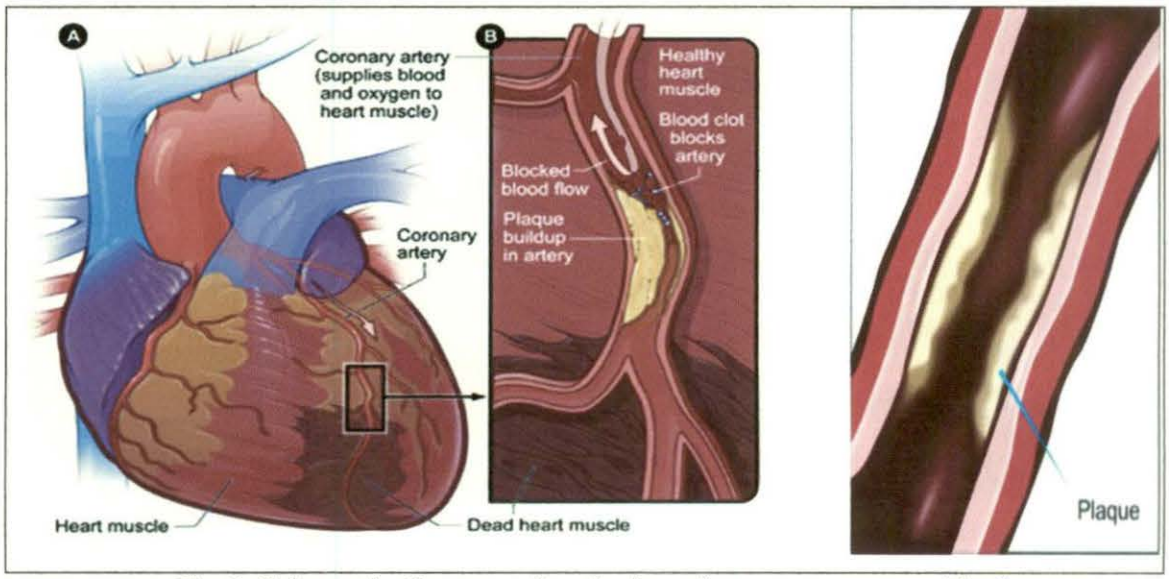


Fig.2. Schematic diagram of occlusion of coronary artery with plaque

An infarct is an area of tissue that has died because of lack of oxygenated blood. Myocardium is affected when a branch of a coronary is occluded. The extent of myocardial damage depends upon the size of the blood vessel and site of the infarct. The damage is permanent because cardiac muscle cannot regenerate and the dead tissue is replaced with non functional fibrous tissue. Speedy restoration of blood flow through the blocked artery using thrombolytic drugs can greatly reduce the extent of permanent damage and improve the prognosis but treatment must be started within a few hours of the infarction occurring. The myocardial infarction is usually accompanied by severe crushing chest pain which radiates to the left arm, jaw and neck and continues even when the individual is at rest.

Cardiovascular disease is a general name for a wide variety of diseases, disorders and conditions that affect the heart and the blood vessels as well. It includes angina, myocardial infarction, atherosclerosis, heart failure, ischemic heart disease and cardiac arrhythmias. The other forms of cardiovascular disease include congenital heart defects, cardiomyopathy, coronary artery disease, heart valve disorders, myocarditis, pericarditis and infections of the heart. Symptoms of cardiovascular disease vary depending on the specific type of cardiovascular disease. A classic symptom of cardiovascular disease is chest pain. It has the characteristic distribution in the chest, left arm, neck, and brought to the shoulder blade.

Risk factors for developing cardiovascular disease are hypertension, diabetes, high cholesterol (hyperlipidemia), obesity, and a sedentary lifestyle. Other risk factors include drinking excessive amounts of alcohol, having a lot of long term stress, smoking and having a family history of a heart attack at an early age.

Ischemic heart disease is the leading cause of death worldwide, and 3.8 million men and 3.4 million women die of the disease each year. After an acute myocardial infarction, early and successful myocardial reperfusion with the use of thrombolytic therapy (eg.streptokinase) or primary percutaneous coronary intervention (PCI) is the most effective strategy for reducing the size of a myocardial infarct and improving the clinical outcome.

1.2. MYOCARDIAL ISCHEMIC REPERFUSION INJURY

Advent of early coronary recanalization for limiting morbidity and mortality due to myocardial infarction has brought forward a menace in the form of reperfusion injury.

Ischemia is the condition in which the organ is derived from blood flow followed by inadequate oxygen and nutrient supply. Although restoration of blood flow to an ischemic organ is essential to prevent irreversible cellular injury, reperfusion itself may augment tissue injury in excess of that produced by ischemia alone. Reperfusion of the previously-ischemic myocardium is often followed by the detrimental changes in coronary arteries and myocardial tissues, which ultimately results in cardiac dysfunction, known as ischemia/reperfusion (I/R) injury.

The process of restoring blood flow to the ischemic myocardium, however, can induce injury. This phenomenon, termed myocardial reperfusion injury (Yello *et al.*, 2007), can paradoxically reduce the beneficial effects of myocardial reperfusion. Healing of myocardial infarcts depends on an inflammatory cascade that ultimately results in clearance of dead cells and matrix debris and formation of a scar. Myocardial reperfusion injury was first postulated in 1960 by Jennings et al. in their description of the histologic features of reperfused ischemic canine myocardium. The observed features are cell swelling, contracture of myofibrils, disruption of the sarcolemma, and the appearance of intramitochondrial calcium phosphate particles. The injury to the heart during myocardial reperfusion causes four types of cardiac dysfunction. The first type is myocardial stunning, a term denoting the "mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or near-normal coronary flow (Barunwald *et al.*, 1982). The myocardium usually recovers from this reversible form of injury after several days or weeks. The second type of cardiac dysfunction, the no-reflow phenomenon, was originally defined as the "inability to reperfuse a previously ischemic region (Krug *et al.*, 1966) it refers to the impedance of micro vascular blood flow encountered during opening of the infarct-related coronary artery. The third type of cardiac dysfunction, reperfusion arrhythmias, is potentially harmful, but effective treatments are available (Mannings *et al.*, 1984). The last type is lethal reperfusion injury (Kloner *et al.*, 1993). The mediators which are responsible for this type are as follows.

1.2.1. Potential mediators of lethal reperfusion injury

1.2.1.1. Oxygen paradox

Experimental studies have established that the reperfusion of ischemic myocardium generates oxidative stress which itself can mediate myocardial injury. Oxidative stress is part of the oxygen paradox in which the reoxygenation of ischemic myocardium generates a degree of myocardial injury that greatly exceeds the injury induced by ischemia alone; the role of oxidative stress in lethal reperfusion injury is clouded by the inconclusive results of animal and clinical studies of cardio protection by antioxidant reperfusion therapy.

Oxidative stress during myocardial reperfusion also reduces the bioavailability of the intracellular signaling molecule, nitric oxide, thereby removing its cardioprotective effects. These effects include the inhibition of neutrophil accumulation, inactivation of superoxide radicals and improvement of coronary blood flow. The Nitric oxide reperfusion therapy to increase nitric oxide levels can reduce the size of a myocardial infarct in animals but clinical studies of the anti anginal nitric oxide donor nicorandil have reported benefit only in terms of improved myocardial reperfusion; results in terms of clinical outcomes after an acute myocardial infarction are mixed (Ono *et al.*, 2004; Ishii *et al.*, 2005).

1.2.1.2. Calcium paradox

At the time of myocardial reperfusion, there is an abrupt increase in intracellular Ca^{2+} which is secondary to sarcolemmal-membrane damage and oxidative stress induced dysfunction of the sarcoplasmic reticulum. These two forms of injury overwhelm the normal mechanisms that regulate Ca^{2+} in the cardiomyocyte, this phenomenon is termed the calcium paradox. The result is intracellular and mitochondrial Ca^{2+} overload and this excess of Ca^{2+} induces cardiomyocyte death by causing hypercontracture of the heart cells and mitochondrial PTP opening. Attenuating intracellular Ca^{2+} overload with pharmacologic antagonists of the sarcolemmal Ca^{2+} ion channel, the mitochondrial Ca^{2+} uniporter or the sodium hydrogen ion exchanger decreases myocardial infarct size by up to 50% in experimental studies. However, the results of the corresponding clinical studies have been negative that inhibition of sodium-hydrogen ion exchange at the time

of PCI does not protect the myocardium. During an acute myocardial infarction is consistent with the results of experimental studies in which the beneficial effects of inhibiting sodium-hydrogen ion exchange were shown to occur during myocardial ischemia and not reperfusion, the new class of agents that reduce intra cellular Ca^2+ loading by inhibiting the sodium hydrogen exchanger and promoting Ca^2+ uptake by the sarcoplasmic reticulum has also not influenced infarct size when given during reperfusion.

1.2.1.3. Inflammation

After an acute myocardial infarction, the release of chemo attractants draws neutrophils into the infarct zone during the first 6 hours of myocardial reperfusion and during the next 24 hours they migrate into the myocardial tissue. This process is facilitated by cell-adhesion molecules. These neutrophils cause vascular plugging and release degradative enzymes and reactive oxygen species. Experimental studies have shown reductions in infarct size of up to 50% with several interventions aimed at neutrophil during myocardial reperfusion. These interventions include leukocyte depleted blood; antibodies against the cell-adhesion molecules P-selectin 64, CD11 and CD18, 65 and the intercellular adhesion molecule 166; and pharmacologic inhibitors of complement activation. However, the corresponding clinical studies have not shown any meaningful cardio protective effect of such interventions.

After inconclusive experimental studies, clinical studies of the anti-inflammatory agent adenosine as an adjunct to PCI have shown an 11% reduction in the size of myocardial infarcts, but benefits in terms of clinical outcomes were limited to patients presenting within 3 hours after the onset of symptoms (**Fig.3**)

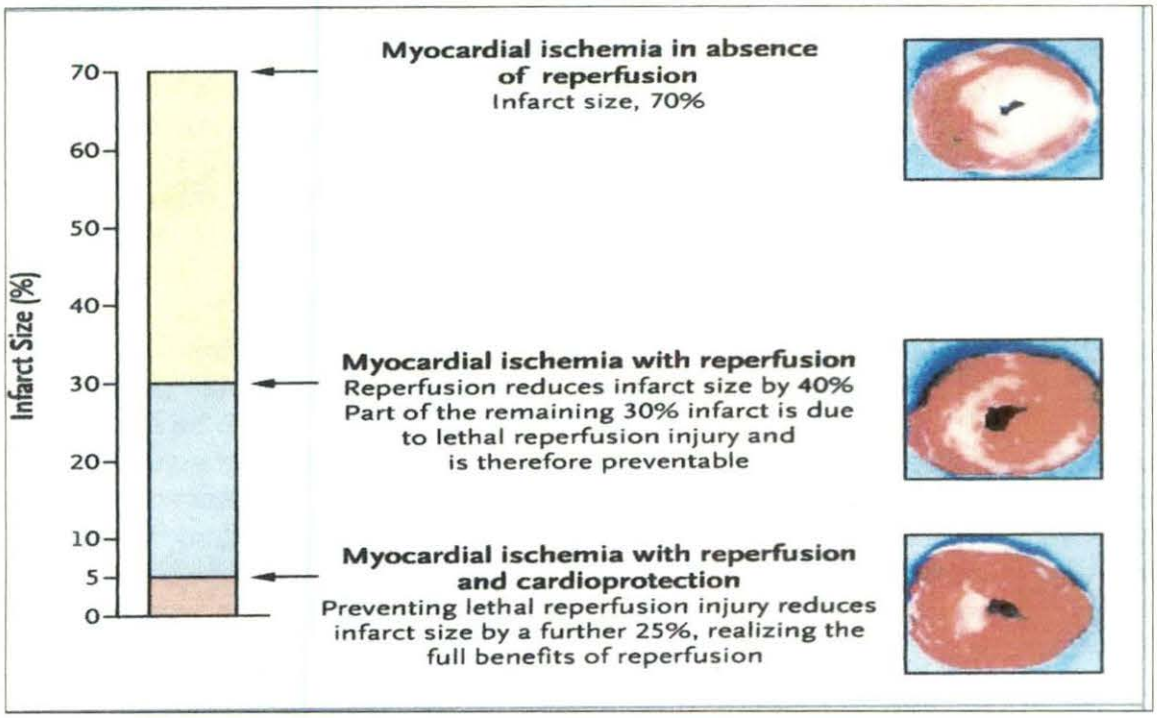


Fig.3. Contribution of lethal reperfusion injury to final myocardial infarct size.

1.2.2. Cardioprotective strategies for preventing lethal myocardial reperfusion injury

Targeting individual mediators of lethal reperfusion injury has produced discrepant findings in studies in animals and clinical studies that use this strategy have not been successful. A more effective approach may be to target more than one mediator at a time. The recently described interventional strategy of ischemic post conditioning which by its nature targets several mediators of lethal reperfusion injury and it has been shown to reduce myocardial injury in patients with acute myocardial infarction who are undergoing PCI (Staat *et al.*, 2005). These findings along with a number of preclinical studies have not only re-ignited interest in the myocardial reperfusion phase as a target for cardio protection, but they also have provided confirmatory evidence of the existence of lethal re perfusion injury in humans Further more, the RISK pathway (Hausenloy *et al.*, 2006), and the mitochondrial PTP are emerging as new targets for preventing lethal reperfusion injury.

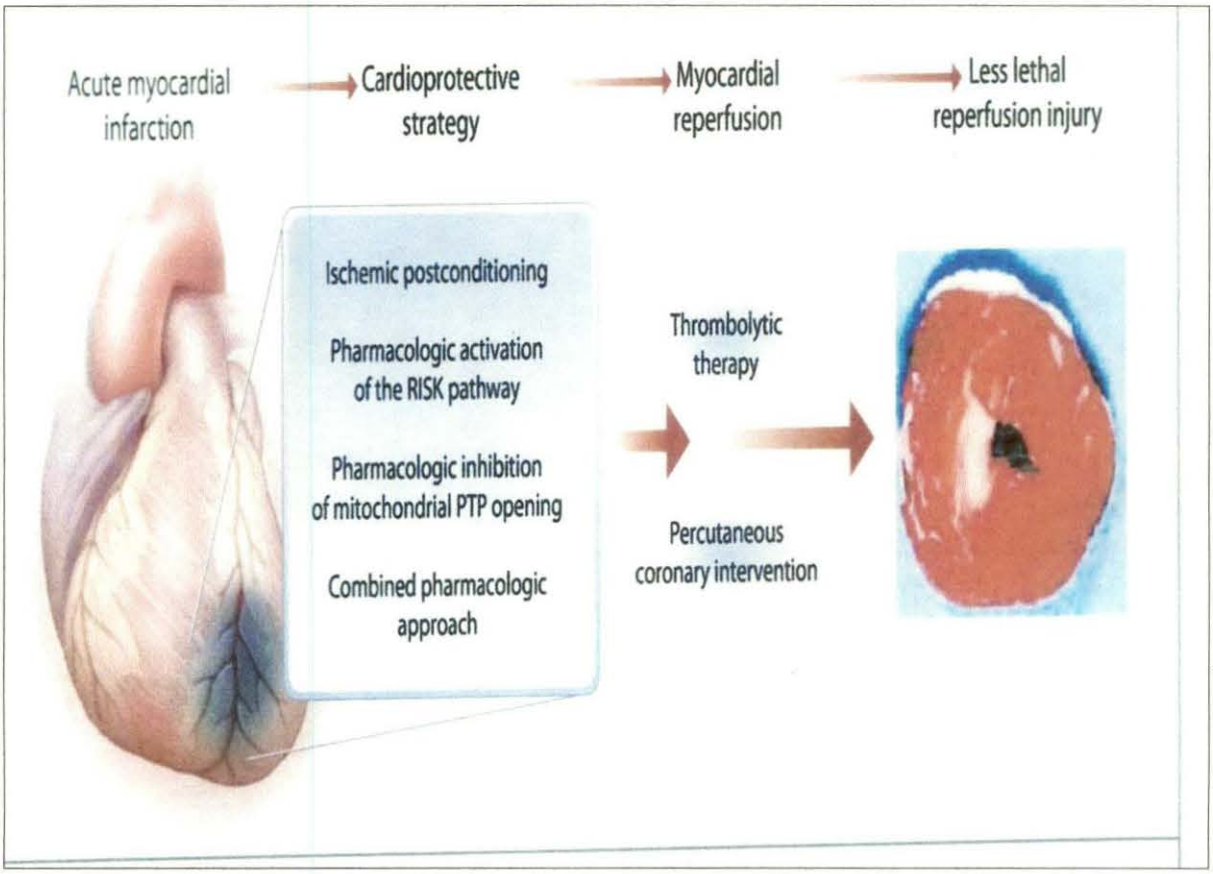


Fig.4. New cardioprotective strategies for reducing lethal reperfusion injury

including thrombolysis and primary coronary angioplasty improve survival after myocardial infarction (MI) (Ryan *et al.*, 1996). Unfortunately, these reperfusion therapies are rarely carried out before considerable myocardial injury has occurred. Moreover, reperfusion after prolonged ischemia also produces a paradoxical myocardial injury (ischemia-reperfusion injury) (Ambrosio *et al.*, 1991; Forman *et al.*, 1990) potentially limiting the efficacy of reperfusion therapies. This has provided impetus for identifying therapies that protect against ischemia-reperfusion injury. Acute interventions including ischemic preconditioning (Jenkins *et al.*, 1995; Parratt, 1995) and infusion of adenosinergic agents (Downey *et al.*, 1993; Forman *et al.*, 1993) reduce ischemia-reperfusion injury in animal models and human myocardium. Unfortunately, the timing of an MI cannot be predicted clinically thus we need to develop therapies that produce sustained protection against ischemia-reperfusion injury in vulnerable patients.

Several lines of evidence suggest that nucleoside transport inhibitors, which increase extracellular adenosine levels by inhibiting uptake into myocytes and endothelial cells might offer sustained protection against ischemia-reperfusion injury (**Fig.4**). For example, we recently found that chronic exposure to ethanol, an adenosine uptake inhibitor, reduces ischemia-reperfusion injury in guinea pig hearts, requiring adenosine A_1 receptor signaling at the time of ischemia to effect cardioprotection (Miyamae *et al.*, 1997). Other nucleoside transport inhibitors including dipyridamole (Amrani et al., 1992; Auchampach and Gross, 1993; Gokgoz *et al.*, 1992) also reduce ischemia-reperfusion injury when infused immediately before experimental MI. However, still it is unknown whether these agents offer sustained protection against ischemia-reperfusion injury when given chronically.

1.3. TREATMENT FOR ISCHEMIC HEART DISEASE

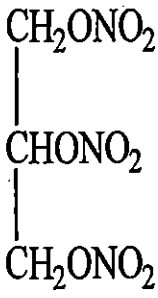
There are currently three types of medicines used to treat stable angina are as follows.

- Nitrates
- Beta blockers
- Calcium channel blockers
- Drugs from natural origin sources/ Herbal remedies

1.3.1. Nitrates

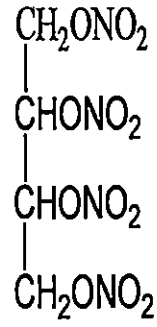
Nitrates improve the blood flow by relaxing and dilating veins and arteries including the coronary arteries. Some of the nitrate preparations available in the market are as follows.

- Short acting: Glyceryl trinitrate (GTN, Nitroglycerine)
- Long acting: Isosorbide dinitrate, Isosorbide mononitrate and Erythirtyl Tetranitrate



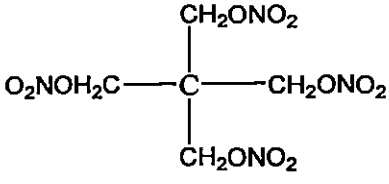
Nitroglycerin

Drugs of choice for Unstable Angina



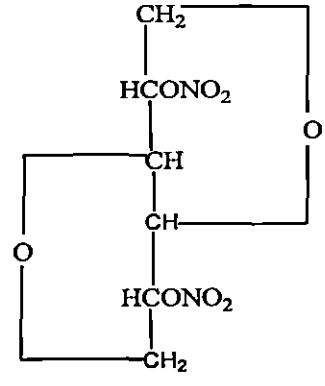
Erythrityl Tetranitrate

Drug used in the prophylaxis of Angina pectoris,cyanide poisoning &CHF



Pentaerythritol Tetranitrate

Drug used in the prophylaxis of Angina pectoris



Isosorbide Dinitrate

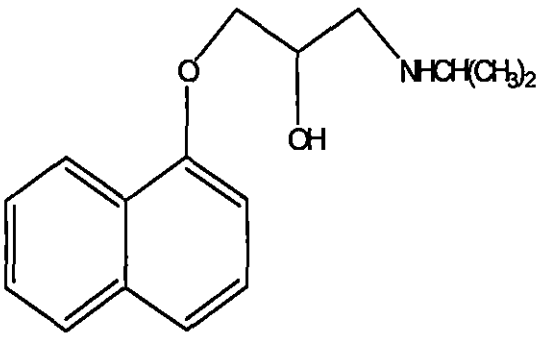
Drug of choice for Angina pectoris only for prophylactic use

1.3.2. Beta blockers

Beta blockers reduce the heart rate, blood pressure and the force of contractions, thereby decreasing the amount of oxygen which the heart requires to pump blood. Along with nitrates, beta blockers are usually the first choice for the treatment of stable angina. There are different types of beta blockers and, although all are equally effective in the

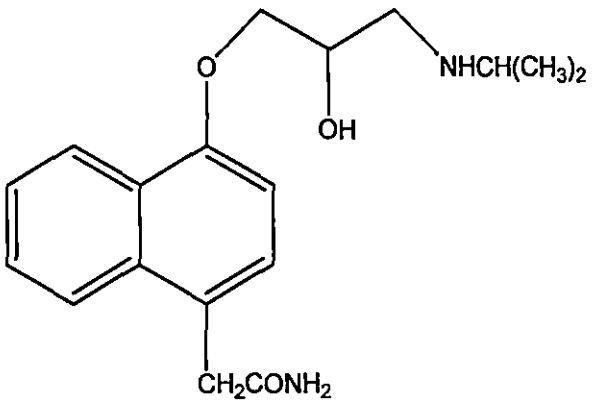
treatment of angina.Nonselective beta blockers (propranolol) block all types of beta receptors throughout the body and are therefore more likely to cause side effects.

Cardio selective beta blockers (atenolol and metoprolol) selectively block the beta receptors found in the heart. Some beta blockers (acebutolol and pindolol) are less likely to depress cardiac function or cause a slow resting heart rate and may be a better choice for people who have specific cardiac conditions or more sensitive to the effects of beta blockers. Some beta blockers (labetalol or carvedilol) also block alpha receptors, which are another type of receptor found in the blood vessels. These medications have the added benefit of dilating blood vessels.



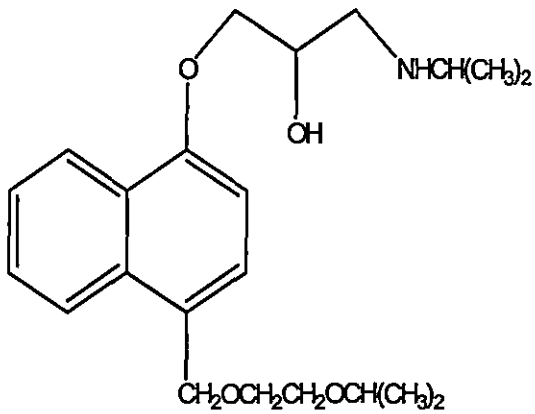
Propranolol

Drug used for the treatment of Hypertension, Angina, Myocardial Infarction and Arrhythmia

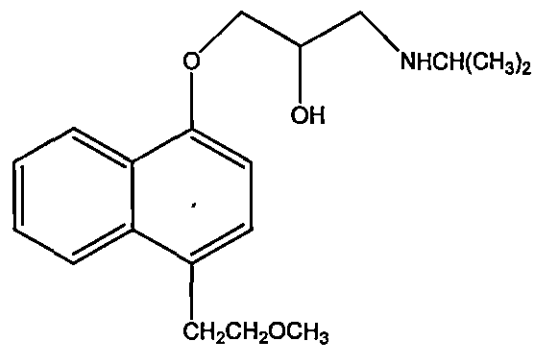


Atenolol

Drug used in the treatment of Angina and Hypertension



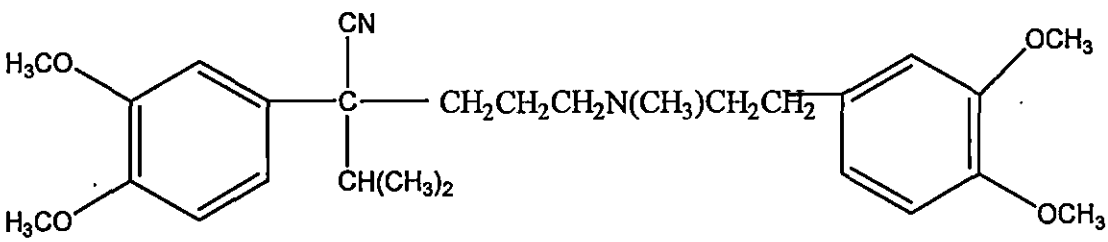
Bisoprolol



Metoprolol

It is used in Congestive Heart Failure & Hypertension

Drug of choice for Angina, Cardiac Arrhythmia & CHF



Verapamil

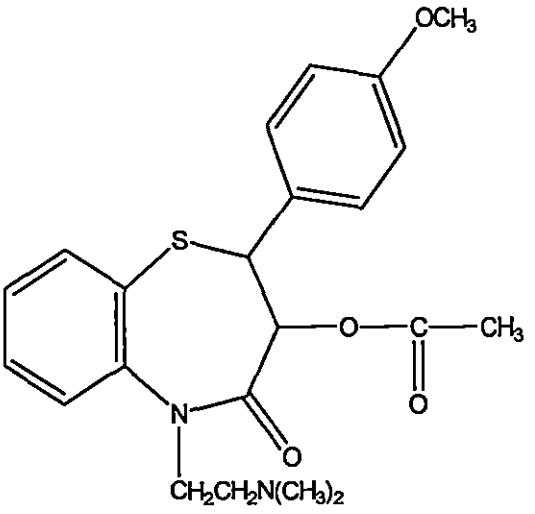
Drugs used for the treatment of Hypertension, Angina & Arrhythmia

1.3.3. Calcium channel blockers

Calcium channel blockers dilate arteries and lower blood pressure which decreases the force of the heart's contractions. They also dilate veins, reducing the amount of blood returning to the heart, which reduces the workload of the heart. It includes dihydopyrimidine derivatives, diltiazem and verapamil. The dihydropyridine includes nifedipine, nicardipine, Felodipine, Amlodipine, Nitrendipine, Nimodipine, Lacidipine, Lercanidipine and Benidipine etc.

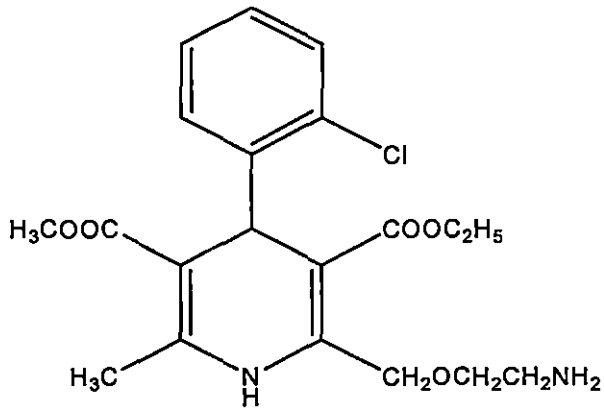
Verapamil slows the heart's conduction of electrical impulses, decreases the force of the heart's contractions, and dilates blood vessels. Although it is less effective than beta blockers for slowing the heart rate, it is a safe and effective alternative.

Diltiazem dilates blood vessels (especially coronary arteries), decreases the heart's force of contraction, and slows the heart's conduction of electrical impulses. It is available in sustained release form that is taken once per day. Anti-cholesterol drugs called statins, which help to lower blood cholesterol may also be used to reduce greater plaque buildup in the coronary arteries.



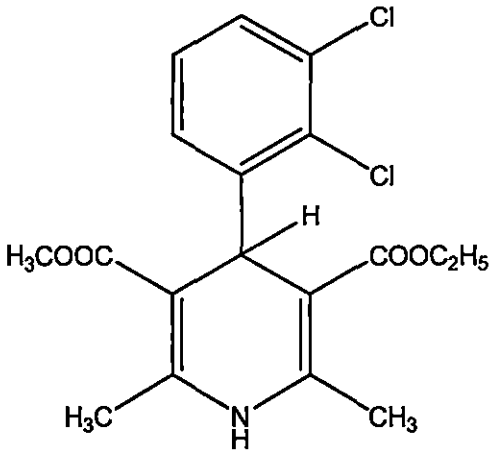
Diltiazem

It is a potent coronary vasodilator

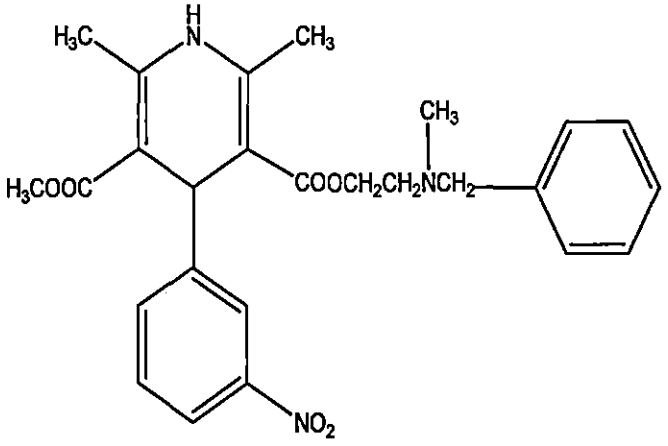


Amlodipine

Drug of choice for Angina pectoris



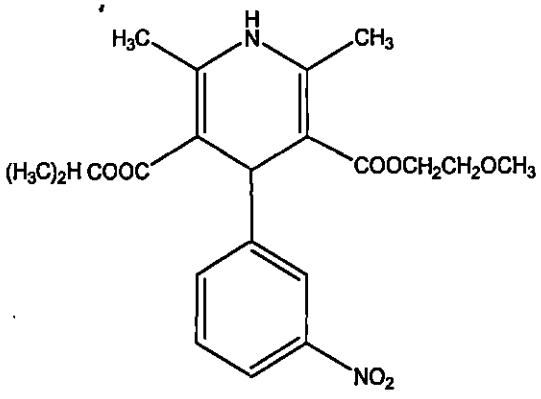
Felodopine



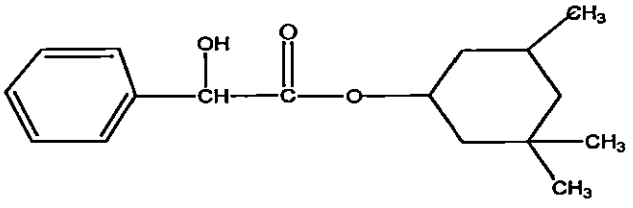
Nicardipine

Used in the treatment of Hypertension

Drug of choice for Myocardial infarction



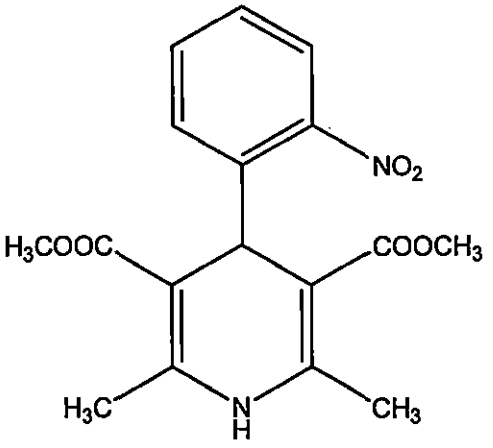
Nimodipine



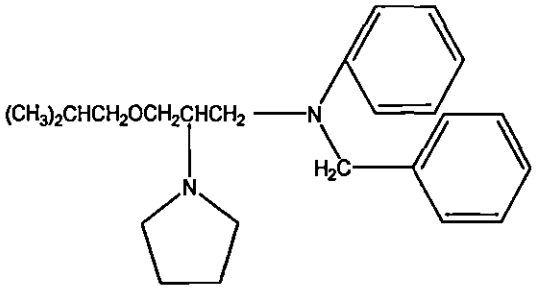
Cyclandelate

Drug of choice for myocardial infarction

Drug used for the treatment of Thrombophlebitis & Raynaud's Disease



Nifedipine

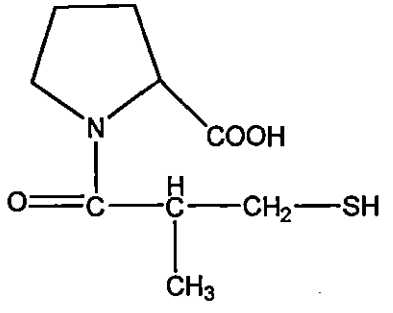


Bepridil

Used in the treatment of Angina, Hypertension & Arrhythmia

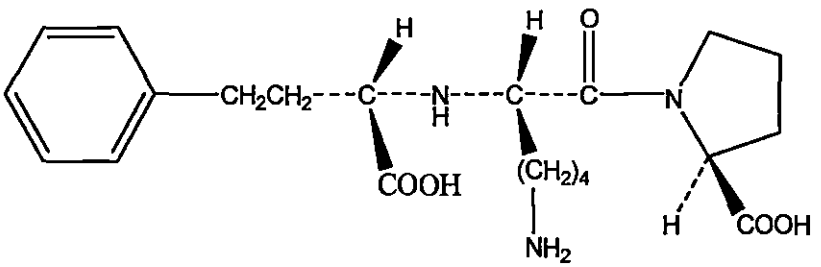
Drug of choice for Angina & Arrhythmia

Angiotensin converting enzyme inhibitors includes the following drugs captopril, enalpril, lisinopril, benazepril, ramipril, fosinopril, trandolapril, imidapril and perindopril. ACE inhibitors prevent the conversion of angiotensin-I to angiotensin- II (active octapeptide).



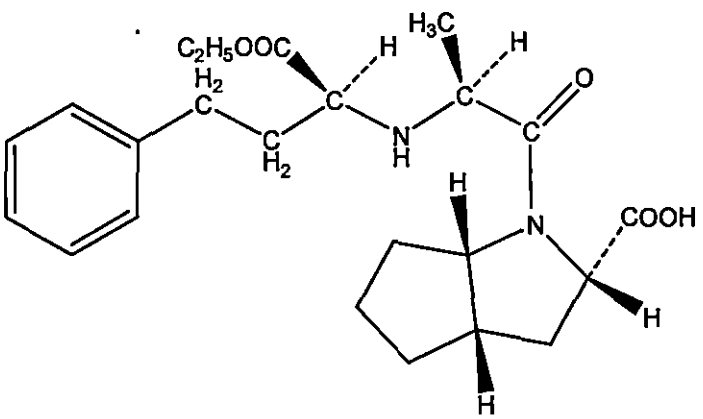
Captopril

It is effective in renal & malignant hypertension



Lisinopril

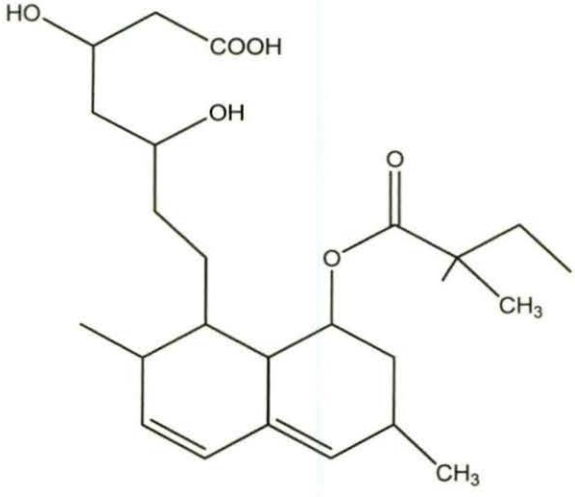
It is used in CHF & Heart Failure



Ramipril

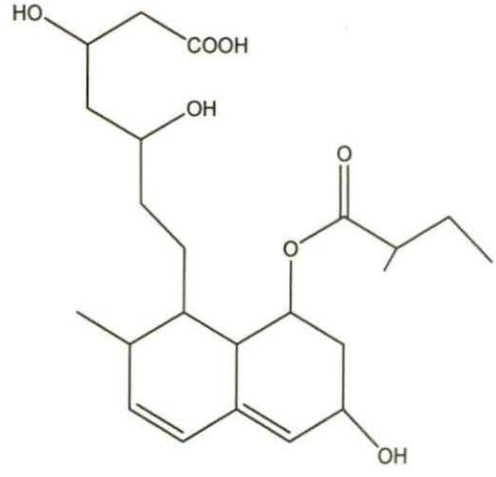
It is used in CHF & Heart failure

Lovastatin are the drugs called HMG CoA reductase inhibitors. They inhibit the synthesis of cholesterol which is responsible for the formation of plaque in coronary arteries.



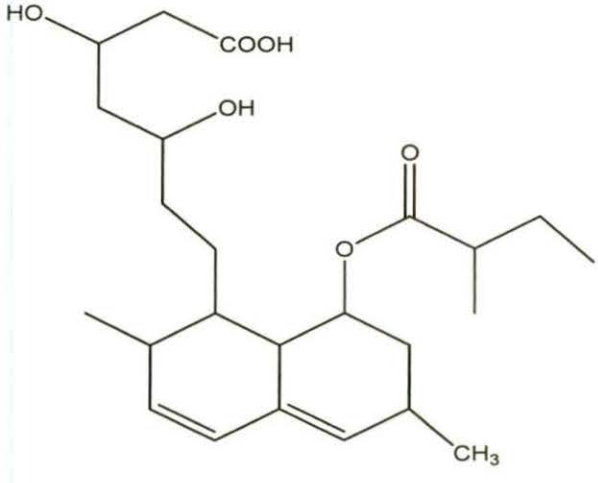
Simvastatin

It is used in diabetes mellitus for its LDL Lowering effects



Pravastatin

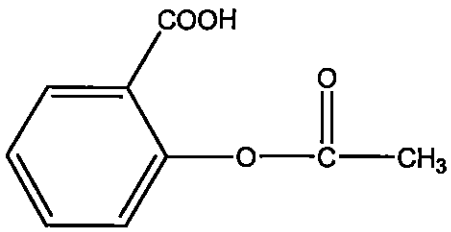
It is used in treatment of diabetes mellitus for its LDL Lowering effects



Lovastatin

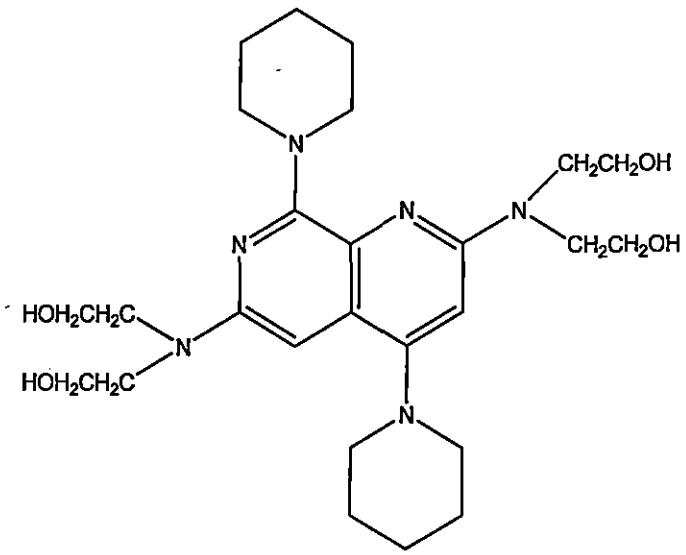
It is used in treatment of diabetes mellitus for its LDL Lowering effects

Anti-platelet drugs- such as aspirin are recommended for patients with coronary artery disease. Aspirin binds irreversibly to platelets and prevents them from clumping on blood vessel walls- thus preventing platelets from forming a clot on the fatty plaques which could block an artery and result in heart attack.



Aspirin

It is used as fibrinolytic agents



Dipyridamole

It is used as antiplatelet agent

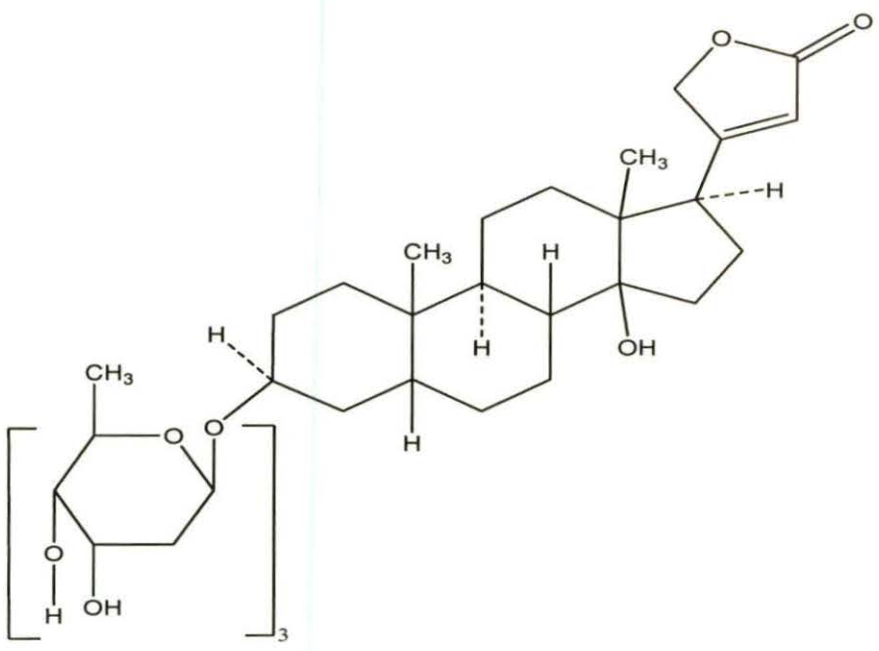
1.3.4. Herbal remedies

Numerous literatures related to cardioprotective activity of phyto molecules from herbal sources have shown that there is a vast array of phytoconstituents having cardio protective efficacy. Resveratol is one of the potent molecules reported for their cardioprotective activity. The cardioprotective activity of resveratol is associated with the inhibition of platelet aggregation and LDL oxidation and the promotion of artery vasorelaxation. The natural sources such as sesame oil, abana, terminalia arjuna and allium sativum etc are being used to treat cardiovascular disease ((Szmitko *et al.*, 2005; Mukherjee *et al.*, 2003; Kaneez *et al.*, 2007).

The history of herbal medicines is as old as human civilization. The documents many of which are of great antiquity revealed that plants were used medicinally in China, India, Egypt and Greece long before the beginning of the Christian era. A large portion of the Indian population even today depends on the Indian system of medicine -Ayurveda "An ancient science of life" a discipline under Atharvaveda since the Vedic age dating nearly 2000 B.C.The Hindu materia medica like "Charak Samhita" and Sushrata Samhita and the later the Egyptian document "Ebers Papyrus" are the earliest written treaties to record the disease along with its symptoms and a number of possible remedies (Holcomb, 1963)

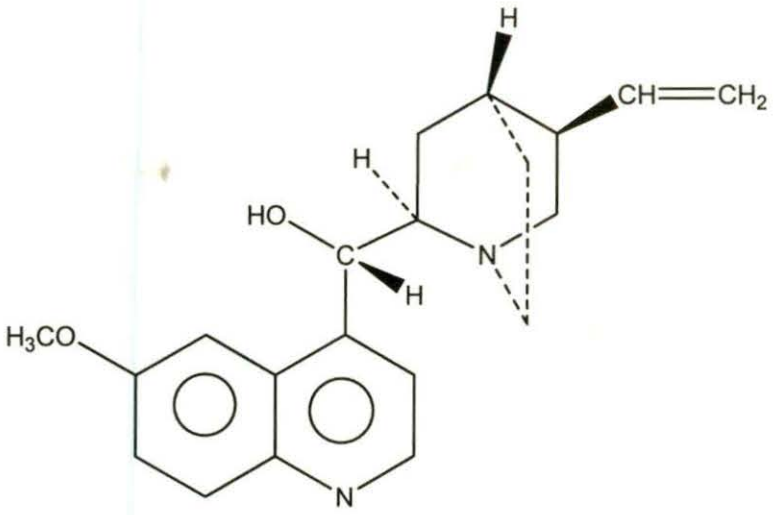
Some examples of common herbal /ayurvedic cardiovascular drugs are mentioned below.

Digoxin,a steroid glycoside from Digitalis lanata used in the treatment of congestive heart failure, it also finds use in the treatment of arrthymia.



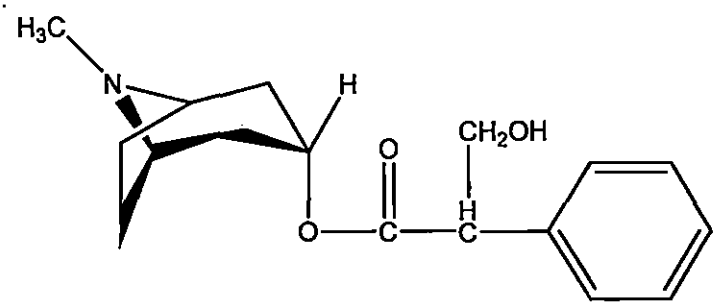
Digoxin

Quinidine, a stereo isomer of the antimalarial quinine, found in cinchona bark, is acting as an adequate antiarrythimic agent but has recently been replaced by pace maker and newer drugs.



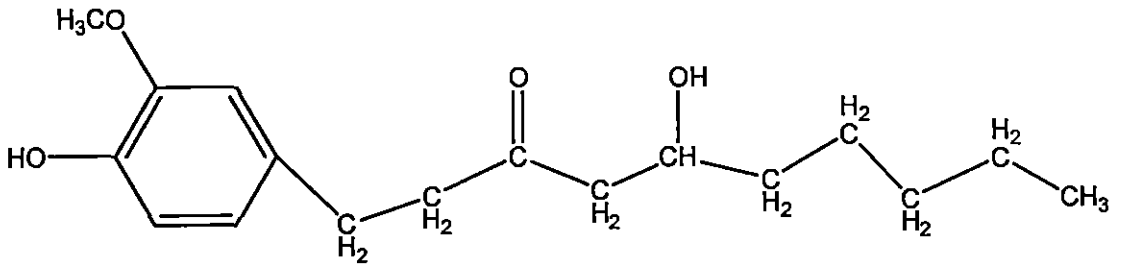
Quinidine

Slow heart beat (bradycardia) is treated with Atropine, a metabolite of Solanaceus plant Atropa belladonna.



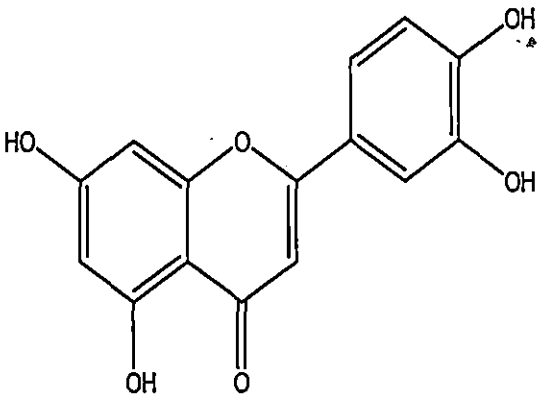
Atropine

The relatively simple phenol-gingerol from the rhizomes of Zingeber officinale (Zingiberaceae) appears to have new cardiotonic action.

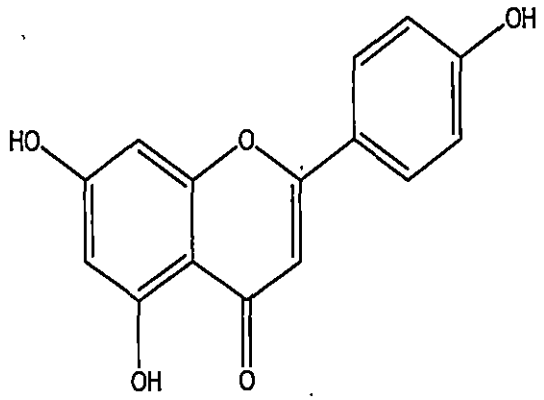


Gingerol

There are many Ayurvedic drugs such as as Arjuna (*Terminalia arjuna*) used as cardio tonic. Many drugs containing flavones are also showing cardio tonic activity.

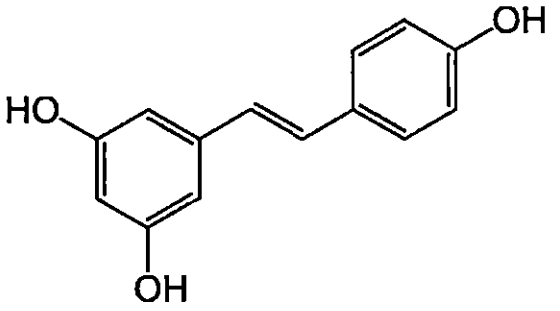


Flavone



Flavonol

This 'french paradox' has been assigned in part to consumption of red wine which contains resveratol, thought to be responsible for the cardiovascular benefits(Hung *et al.*, 2000). A vast number of pharmacological investigations have substained the therapeutic potential of Resveratol, a phyllotoxin group of polyphenol found naturally in grapes (King *et al.*, 2006).Resveratrol has been shown to dose-dependently inhibit induced production of PGE2 in human peripheral blood leukocytes (Richard *et al.*, 2005, while significantly decreased levels of rat PGD2 in vivo models (Martin *et al.*, 2004). Further, it has antioxidant activity associated with chemo-preventive and cardioprotective activity (Lin and Sai, 1999).



Resveratrol

It is effective against atherosclerosis & CHF

1.3.5. YOGA AND CARDIOVASCULAR DISEASE

The ancient marvel of yoga is the priceless gift of India to the world. Yoga is beneficial in health as well as disease as it is holistic and has promotive, preventive as well as curative potential. Our body, mind and spirit are intricately interrelated and constantly influence one another. The holistic science of yoga has been designed to have subtle effect on our whole being, body, mind as well as spirit. The all pervasive stress and stress-induced disorders like hypertension and angina are fast growing epidemics and bane of "modern" society. The holistic science of yoga is the best method for prevention as well as management of stress and stress-induced disorders. The psycho physiological responses to yoga are opposite to the stress response. Shavasan, yoganidra, meditation and slow, rhythmic pranayam breathing are very effective in calming the mind and

promoting psychosomatic health. Cardiac patients are sensitive and reactive. Yoga relaxation techniques calm the mind and make one emotionally balanced. Consequently, minor disturbances do not cause emotional upsets and cardiovascular problems. Throughout the world, hypertension is a common condition and many patients are on life-long medication as a way of life. Drugs are expensive and have many adverse side effects. Hence, nondrug management like yoga should be the first choice. If diagnosed early, majority of the cases of essential hypertension can be managed effectively by yoga alone. In more advanced cases, yoga can decrease drug dosage and improve the overall quality of life. Besides being inexpensive, safe and effective, yoga improves overall health and can be combined with allopathic or ayurvedic medication. For best results, yogic lifestyle should be adopted early in life as it has been demonstrated that atherosclerotic plaques in coronary arteries form early in life.

Moreover, in a recent study, it has been demonstrated that the levels of total and LDL cholesterol are higher in pre hypertensive as compared to normotensive subjects (Pavithran *et al.*, 2007). Hence, yogic relaxation and yogic diet should be adopted early in life to prevent progression of the condition and development of hypertension. The effectiveness of yoga in the management of hypertension has been demonstrated from our laboratories (Vijayalakshmi *et al.*, 2004) and also by earlier workers (Datey *et al.*, 1969. Hence, it is recommended that yogic relaxation techniques should be adopted as the first line of treatment for pre hypertension, borderline hypertension and mild hypertension. Yoga has therapeutic potential in other conditions also.

In an interesting study, it was recently demonstrated that yoga relaxation training is beneficial in patients with benign ventricular ectopies (Ravindra *et al.*, 2005). Therapeutic effect of yoga may be due to i) management of stress ii) improvement of cardio respiratory function and overall fitness and iii) modulation of autonomic function. Stress is an important causative factor in cardiovascular diseases like hypertension and angina. In an interesting work from research laboratories, it was demonstrated that subjects trained in yoga can achieve a state of deep psychosomatic relaxation associated with highly significant decrease in oxygen consumption within five minutes of practicing savitri pranayam (a slow, rhythmic and deep breathing) and shavasan. These findings are consistent with the report that yoga training not only produces a significant decrease in

basal anxiety level, but also attenuates the change in anxiety score in stressful situations such as examination (Malathi and Damodaran, 1999). It has also been reported that yoga training helps in development of resistance against stress (Udupa and Singh, 1972). Practice of asans and pranayams results in overall improvement in physical fitness and cardio-respiratory functions. In a study conducted on medical students, it was found that yoga training of 12 weeks duration produces a significant increase in respiratory pressures, breath holding time and handgrip strength. This indicates an improved physical strength and cardio-respiratory function. It was reported that after yoga training, exercise-induced stress to cardiovascular system is less severe (Madanmohan *et al.*, 2004). Yoga training promotes emotional and physiological balance. In an interesting study, it was found that a brief (15 min) yoga based relaxation training normalizes the function of autonomic nervous system by deviating both sympathetic and parasympathetic indices towards more "normal" middle region of the reference values. These studies show that yoga has a great potential to improve our physiological functions, psychosomatic health and overall performance.

1.4. EPIDEMIOLOGY OF CARDIO VASCULAR DISEASE

Coronary artery disease (CAD) is a leading cause of death in the western world. It is responsible for one-third of all global deaths. Nearly 85% of the global mortality and disease burden from CAD is borne by low and middle income countries. In India, approximately 53% of CAD deaths are in people younger than 70 years of age; in China, the corresponding figure is 35%. The majority of the estimated 32 million heart attacks and strokes that occur every year are caused by one or more cardiovascular risk factors like hypertension, diabetes, smoking, high levels of blood lipids, physical inactivity and most of these CAD events are preventable if meaningful action is taken against these risk factors(WHO Report 2002).

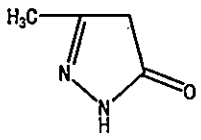
Ischemic heart disease (IHD) is a major cause of death in industrialized countries and is rising at an alarming rate in many developing countries. Coronary artery disease (CAD) is a leading cause of death in the western world. It is a leading cause of mortality and is responsible for one third of all global deaths. Nearly 85% of the global mortality and disease burden from CVD is borne by low and middle income countries. According to the World Health Organization projections, it is predicted that morbidity and mortality

due to cardiovascular diseases in India will reach an epidemic proportions by the middle of this century due to rapid changes in life style and a significant segment of which will be due to ischemic heart disease In India approximately 53% of CVD deaths are in people younger than 70years of age. By 2020 CVD will be the leading cause of death in developing countries like India (WHO, 2007).

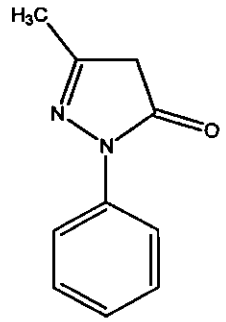
Awareness of the rising incidence of IHD in India coupled with prohibitive cost of treatment particularly for developing country generated urgency for the rapid development of a novel drug molecule with fewer side effects in the amelioration of IHD. Thus the present study is emphasized to synthesize some novel molecule by using pyrazolone as a heterocyclic scaffold and to evaluate the cardioprotective effect of pyrazolone derivatives since it has been proved that these molecules have preventive effects on myocardial injury following ischemia and reperfusion in the rat heart (Yangisawa *et al.*, 1994) and in patients with acute myocardial infarction (Tsujita *et al.*, 2004).

1.5. PYRAZOLONE DERIVATIVES IN ISCHEMIC INJURY

Pyrazolone was first synthesized in 1887 as antipyrine to reduce fever. It is a key structure in numerous compounds of therapeutic importance. The drugs containing pyrazolone nucleus are known to display diverse pharmacological activities such as antibacterial, antifungal, anti-inflammatory, analgesic and antipyretic activities. The compounds like 3-Alkyl-4-arylmethylpyrazol -5-ones are reported to exhibit potent antihyperglycemic activity while 1-phenyl-3-tetrafluoroethylpyrazol- 5-one is an anxiolytic. Thus, the biological activities of pyrazol-5-ones depend on the nature of the substituent. For instance Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a strong novel free radical scavenger is used for treatment of patients with acute brain infarction. It has preventive effects on myocardial injury



3-methyl pyrazol-5-one



3-methyl-1-phenyl pyrazol-5-one(Edaravone)

following ischemia and reperfusion in patients with acute myocardial infarction. The antioxidant actions of edaravone include enhancement of prostacyclin production, inhibition of lipoxygenase metabolism of arachidonic acid by trapping hydroxyl radicals, inhibition of alloxan-induced lipid peroxidation, and quenching of active oxygen, leading to protection of various cells, such as endothelial cells, against damage by reactive oxygen species (ROS). Recently, it has been proved that edaravone improves endothelial function through a decrease in ROS in smokers. From a clinical perspective, it is important to select an appropriate drug that is effective in improving endothelial function in patients with cardiovascular diseases. The novel free radical scavenger edaravone may represent a new therapeutic intervention for endothelial dysfunction in the setting of atherosclerosis, chronic heart failure, diabetes mellitus and hypertension. This study focused on clinical findings and on putative mechanisms underlying the beneficial effects of the anti oxidative agent i.e edaravone on the atherosclerotic process in patients with cardiovascular diseases.

Pyrazolone derivatives have been used in patients with acute brain infarction since April 2001 in Japan (Otomo, 2003). These derivatives have been shown to be effective against brain edema after ischemia and reperfusion injury in animal models (Nishi et al., 1989) and in stroke patients (Toyoda *et al.*, 2004). Several lines of evidence have demonstrated that oxidative stress plays an important role in the pathogenesis and development of cardiovascular diseases, including hypertension, hypercholesterolemia, diabetes mellitus, atherosclerosis, myocardial infarction, angina pectoris and heart failure (Chien, 2009).

The lipid peroxidation is found to cause formation of atherosclerotic plaques, neurological disorders, cancer, diabetes mellitus, myocardial infarction and ageing. It is associated with ischemia-reperfusion injury and hyperoxic lung injury. The peroxides derived from lipid peroxidation such as MDA (TBARS) and 4-HNE have been strongly associated with myocardial ischemic reperfusion injury (Blasig *et al.*, 1995; Ski et al., 2008). It is expected that pyrazolone derivatives have beneficial effects on coronary artery and myocardial cells after ischemic and post ischemic myocardial injury in patients with ischemic heart diseases, including acute myocardial infarction and angina pectoris. Some animal studies using acute myocardial ischemia-reperfusion models have suggested the protective effects of pyrazolone derivatives on myocardial damage.

From the above mentioned facts and figures, it can be ascertained that the oxidative stress plays an important role in the etiopathogenesis of atherosclerosis and ischemic heart disease. Previous studies have shown that compounds like pyrazolone derivatives played a vital role in the above mentioned diseases. It may be hypothesized that the overall beneficial effects of pyrazolone derivatives in ischemic heart disease may be due to their protective properties by either acting as an anti oxidant itself or by increasing the endogenous anti oxidant levels. There have been a few studies, which have indicated the cardioprotective potential of the same. Since limited studies have been conducted in this context, the present study is in sighted to elucidate the cardio protective property of pyrazolone derivatives in terms of plasma lipid profile, myocardial injury markers and induction of endogenous anti oxidant enzymes in myocardial ischemic reperfusion injury. Hence forth the present study intends to concentrate on the mechanism of cardioprotective action of pyrazolone derivatives in myocardial IR injury.

1.6. EVALUATION OF CARDIO PROTECTIVE PROPERTIES

Cardio protective action can be evaluated by

i) Chemical methods

(a) Isoproterenol induced myocardial ischemic injury model.

(b) Doxorubicin induced myocardial ischemic injury model.

ii) Instrumental method using Langendorff isolated-perfused heart model.

Isoproterenol (ISO) is a ß-adrenergic agonist that causes severe stress in myocardium resulting in the infarct like necrosis of heart muscle (Welexer, 1978). Some of the mechanisms proposed to explain ISO induced damage in cardiac myocytes include hypoxia due to myocardial hyperactivity, coronary hypotension, calcium overload, depletion of energy reserves and excessive production of free radicals due to oxidative metabolism of catecholamine's (Mohanty *et al.*, 2004). ISO induced myocardial necrosis in rats is a widely used experimental model for evaluation of cardioprotective effect of various herbal drugs (Naik and Panda, 2008; Nandave *et al.*, 2009), because pathophysiological changes following ISO administration in rats are comparable to those taking place during myocardial injury in

humans (Nirmala and Puvanakrishnan, 1996). Therefore, it is a suitable model to study myocardial ischemic injury.

Doxorubicin an anthracycline is well established and highly efficacious drug in the fight against many kinds of cancers like solid tumors, leukemia, soft tissue sarcoma, breast cancer, small cell carcinoma of the lung and esophageal carcinomas (Blum and Carter, 1974; Chabner *et al.*, 2001).But, its clinical usefulness is still restricted due to its specific toxicities to cardiac tissue. Congestive heart failure, cardiomyopathy and electrocardiographic changes were demonstrated after cumulative doxorubicin administration (Lenaz and Page, 1976).The mechanisms proposed for cardio toxic effects of doxorubicin includes free radical induced myocardial injury, lipid peroxidation (Myers *et al.*, 1977),mitochondria damage (Bier and Jaenke, 1976),decreased gravity of Na^+K^+ATPase, vasoactive amine release(Bristow *et al.*, 1980), impairment in myocardial adrenergic signaling/regulation, increase in serum total cholesterol, triglyceride and low density lipoproteins (Ilskowic and Singal, 1997).Generation of reactive oxygen species like superoxide anion and hydrogen peroxide by doxorubicin leads to causing impairment of cell functioning and cytolysis (Daoud, 1992).Due to the presence of less developed antioxidant defense mechanisms, heart is particularly vulnerable to injury by anthracycline induced reactive oxygen species.

Liberation of free radicals is central to the mechanism of doxorubicin induced damage to the myocardium (Potemski *et al.*, 2006).It also causes the elevation of serum enzymes like lactate dehydrogenase (LDH) and creatinine phosphokinase (CPK) (Abd-Alah *et al.*, 2002).Endogenous antioxidant deficits have been suggested to play a major role in doxorubicin induced cardiomyopathy and heart failure (Hanaa *et al.*, 2005). Hence it is also suitable model to evaluate myocardial injury

The concept of the isolated perfused heart system was introduced and established by Oskar Langendorff in 1895. It is now a predominant technique in pharmacological and physiological research. The technique allows the examination of cardiac contractile strength (inotropic effects), heart rate (chronotropic effects) and vascular effects without the neuronal and hormonal complications of an intact animal model. (Frederich *et al.*, 2005). The technique originally required elevated reservoirs to provide a constant, gravity supplied pressure but the technique and equipment have evolved to include both

constant pressure and constant flow models in both recirculating and non-recirculating modes.

After a midline sternotomy, the hearts were rapidly excised and perfused retrogradely at a constant perfusion pressure of 80 mmHg with a modified Krebs' solution containing NaCl (120 mM), $NaHCO_3$(25mM), $MgSO_4$(1.2mM), KH_2PO_4(1.2 mM), $CaCl_2$ (1.2 mM), and glucose (11 mM). The perfusate buffer was saturated with a 95% O_2 and 5% CO_2 gas mixture at 37^0C. In the Langendorff preparation, the aorta is cannulated and the heart is perfused in a retrograde (reverse) fashion, usually with a nutrient rich, oxygenated solution. The pressure of the solution causes the aortic valve to shut and the perfusate is then forced into the ostium and into the coronary vessels. This allows the heart to beat for several hours. Then the heart is subjected to evaluate the cardio protective effect as per the experimental protocol.

Cardio vascular diseases have been linked to oxidative stress which is initiated by the reaction of free radicals with biological macromolecules such as proteins, lipids and DNA (Martinez-Cayuela, 1995). The serum enzymes namely LDH, AST, ALT and ALP serve as sensitive indices to assess the severity of myocardial infarction (Sheela-Sasikumar *et al.*, 2000). The increased activities of these enzymes following injection of ISO as observed in this study confirmed the onset of myocardial necrosis (Parithaithayarasi *et al.*, 1997).

Most tissue damages are mediated by free radicals which attack membranes through peroxidation of unsaturated fatty acids (Stringer *et al.*, 1989). Myocardial injury induced by ISO in rats results in increased lipid peroxidation, which is an evidence of intensified free radical production (Lefer and Granger, 2000). These free radicals initiate lipid peroxidation of the membrane bound polyunsaturated fatty acids, leading to impairment of membrane structural and functional integrity. In this study, malondialdehyde (MDA) level was elevated in the heart of myocardial injured rats which is an indication of oxidative stress in the tissue. Recently another lipid peroxidation marker 4- HNE has been proposed as an important marker of radical induced myocardial injury (Blasig *et al.*, 1995).

Endogenous antioxidant enzymes such as SOD, CAT, GS peroxidase (GPX) and GST are the first-line cellular defense against oxidative stress, decomposing oxygen and H_2O_2

before they interact to form the more reactive hydroxyl radical (OH). The equilibrium between these enzymes is an important process for the effective removal of oxygen stress in intracellular organelles. SOD and CAT are important antioxidant enzymes in mitigating free radical-induced cell injury. A decrease in the activity of SOD and CAT can result in the decreased removal of superoxide ion and H2O2 radicals that brings about a number of reactions, which are harmful to myocardium. Superoxide is inactivated by SOD, the only enzyme known to use a free radical as a substrate. An increase in SOD activity is beneficial in the event of increased free radical generation. However, it has been reported that a rise in SOD activity, without a concomitant rise in the activity of CAT and/or GPX, may be detrimental because SOD generates H_2O_2 as a metabolite, which is more cytotoxic than oxygen radicals, and must be scavenged by CAT or GPX (Yim *et al.*, 1990). Thus, a simultaneous increase in CAT and/or GPX activity is essential for an overall beneficial effect of an increase in SOD activity (Harman, 1991). The endogeneous anti oxidative enzyme Glutathione (GSH) is an important antioxidant which plays the role of an intracellular radical scavenger and is a substrate for many xenobiotic elimination reactions. Decreased level of GSH observed in the heart tissue may be a result of increased oxidative stress. Glutathione has the ability to manage oxidative stress with adaptional changes in enzymes regulating its metabolism (Arulselvan and Subramanian, 2007).

The decreased activities of SOD and GR in the heart as observed in this study may be due to increased production of reactive oxygen radicals which are capable of reducing the activities of these enzymes (Basheeruddin Asdaq and Prasannakumar, 2009). SOD is an important defense enzyme which catalyses the dismutation of superoxide radicals (Lin et al., 2005) while GR is required for the conversion of oxidized (GSSG) to reduced (GSH) glutathione (Zhang and Tan, 2000).

Lipid peroxidation is associated with ischemia-reperfusion injury and hyperoxic lung injury. The peroxides derived from lipid peroxidation such as thiobarbituric acid reactive substances (TBARS) and hydroxyl 2-non-enal (4-HNE) has been strongly associated with myocardial ischemic reperfusion injury (Blasig et al., 1995: Ski Kami *et al.*, 2008). Based on the above reports, the cardio protective activity will be evaluated by estimating the serum lipid profile, serum marker enzymes, and myocardial tissue endogenous markers and anti oxidant parameters.

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CHAPTER 2

LITERATURE REVIEW

2. REVIEW OF LITERATURE

2.1. ETIOLOGY OF CORONARY HEART DISEASE (CHD)

The risk factors for the development of CHD and angina pectoris are genetic predisposition, age, sex and a series of reversible risk factors: The most important factors include high fat and cholesterol rich diets, lack of exercise, inability to retain normal cardiac function under increased exercise tolerance due to tobacco, smoking, excessive alcohol drinking, carbohydrate, fat metabolic disorders, diabetes, hypertension obesity and the use of drugs that produce vasoconstriction or enhanced oxygen demand. The increased cholesterol levels caused by the consumption of a diet rich in saturated fat stimulates the liver to produce cholesterol; a lipid needed by all cells for the synthesis of cell membranes and in some cells for the synthesis of other steroids. This is the principal reversible determinant of risk of heart disease. Low density lipoproteins (LDLs, also referred to as "bad" cholesterol) transport cholesterol from liver to other tissues whereas high density lipoproteins (HDLs, also referred as "good" cholesterol) transport cholesterol from tissues back to the liver to be metabolized. Triglycerides are transported from the liver to the tissues mainly as very low density lipoproteins (VLDLs). VLDLs are the precursors of the LDLs. The LDLs are characterized by high levels of cholesterol; mainly in the form of highly insoluble cholesteryl esters.
However, there is a strong relationship between high LDL levels and coronary heart disease and a negative correlation between HDL and heart disease. In general, for people who have total cholesterol levels lower than 200mg/dL, heart attack risk is relatively low. If the total cholesterol level is 240 mg/dL, the person has twice the risk of heart attack as someone who has a cholesterol level of 200 mg/dL. The cholesterol levels of 240 mg/dL are considered high and the risk of coronary heart disease.

The reduction of LDL cholesterol levels lower the cardiovascular risk. During the last few years, there has been reliable evidence that coronary artery disease is a complex genetic disease. In fact, a number of genes associated with lipoprotein abnormalities and genes influencing hypertension, diabetes, obesity, immune and clotting systems play important roles in atherosclerotic cardiac disorders. Researchers have identified genes regulating LDL cholesterol, HDL cholesterol and triglyceride levels based on common genetic variation. Many genes linked to CAD are involved in determining how the body removes

low density lipoprotein (LDL) cholesterol from the bloodstream. If LDL is not properly removed, it initiates ischemia-reperfusion syndrome which is defined as myocardial injury caused by the restoration of coronary flow. This phenomenon has a complex pathophysiology and results in a paradoxical reduction of the beneficial effect of myocardial reperfusion. Studies suggest that ischemia-reperfusion injury may account for up to 50% of the final size of a myocardial infarct.

The technique of reperfusion during acute myocardial infarction has led to a dramatic decrease in the morbidity and mortality associated with coronary artery disease in recent decades. The restoration of blood flow within the "golden hours" has resulted in a reduction in myocardial infarct size. Although greatly beneficial overall, the abrupt restoration of blood flow in the coronary arteries after occlusion was also, surprisingly, found to be associated with an additional and accelerated myocardial injury beyond that generated by ischemia alone, an observation first reported by Jennings *et.al.* This phenomenon has been called "ischemic-reperfusion injury".

The process has a complex pathophysiology leading to cardiomyocyte death that is distinct from that associated with ischemic injury. Because of the deleterious effects of ischemia-reperfusion injury, several treatments aiming to prevent or limit this process have been proposed. Trimetazidine (Vastarel MR) is an antianginal drug that acts by switching the energy substrate from fatty acid metabolism to glucose metabolism, thus making possible the increased formation of ATP with a decreased need for oxygen .These properties are of great potential interest to the reduction of ischemic-reperfusion injury.

2.1.1. Role of oxidative stress in cardiovascular diseases

Several lines of evidence have demonstrated that oxidative stress plays an important role in the pathogenesis and development of cardiovascular diseases including hypertension, hypercholesterolemia, diabetes mellitus, atherosclerosis, myocardial infarction, angina pectoris and heart failure (Chien, 1999; Griendling and Fitzgerald, 2003; Stocker and Keaney, 2004; Benjamin and Schneider, 2005). The susceptibility of vascular cells to oxidative stress is a function of the overall balance between the degree of oxidative stress and the antioxidant defense capability **(Fig.1).**

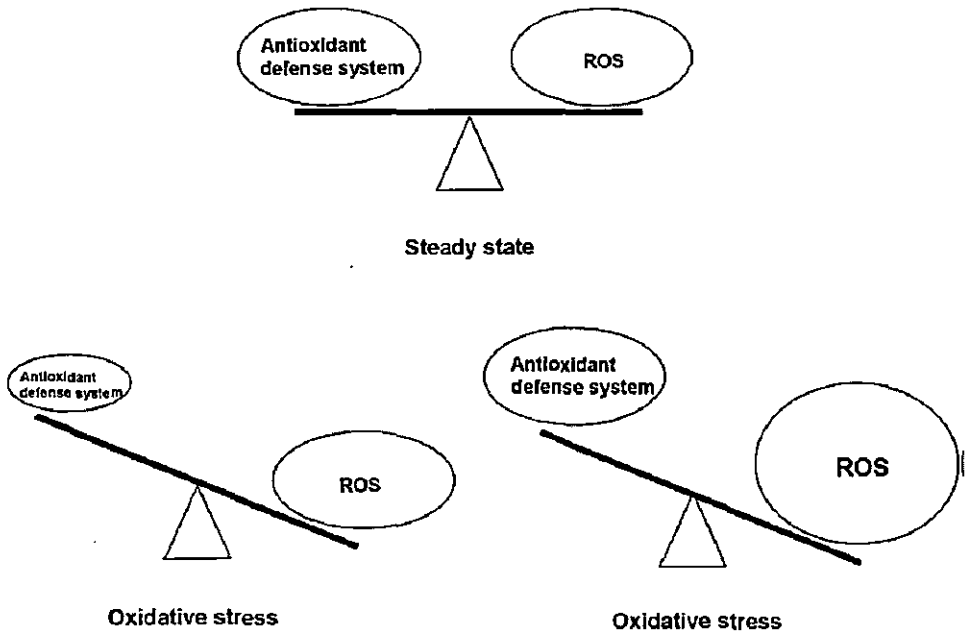

Fig.1. Steady state and oxidative stress: a balance between the degree of oxidative stress and the antioxidant defense capability. ROS indicates reactive oxygen species.

Protective antioxidant mechanisms are complex and multifactorial. The antioxidant defense systems such as Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX) and catalase(CAT) scavenge ROS in the vasculature resulting in inhibition of Nitric Oxide (NO) degradation. Although SOD rapidly converts superoxide to hydrogen peroxide, hydrogen peroxide itself is involved in vascular remodeling, inflammation, apoptosis and growth of vascular smooth muscle cells as an intracellular second messenger (Fukai *et al.,* 2000). The production of lipid peroxidation and protein oxidation induce over expression of redox genes, intracellular calcium overload and DNA fragmentation, resulting in damage of vascular smooth muscle cells, endothelial cells or myocardial cells.

2.2. PATHOPHYSIOLOGY OF ISCHEMIA-REPERFUSION INJURY

The pathophysiology of ischemia-reperfusion injury is complex. In this process, the re-oxygenation of ischemic myocardium generates a high degree of myocardial injury as a result of the generation of potent oxygen-derived free radicals. This phenomenon is known as the "oxygen paradox". The oxidative stress also reduces the bioavailability of nitric oxide which is critical to the improvement of coronary blood flow and inactivation of

superoxide radicals. Reperfusion is also associated with an abrupt increase and overload of intracellular calcium which cause hyper contracture of cardiomyocytes, leading to cell death. The activation and accumulation of polymorphonuclear neutrophils occur in the damaged myocardium and contribute to the ischemia-reperfusion injury. The neutrophils are important for the development of reperfusion injury, releasing oxygen free radicals, proteases and pro inflammatory mediators that further amplify the infiltration of neutrophils into the jeopardized myocardium. The other processes involving platelets and complement also participate in ischemia-reperfusion injury. Acting together, these effects may last hours or months after reperfusion and participate in sustained cardiomyocyte death.

The exact contribution of ischemia-reperfusion injury to infarct size is difficult to determine. However, on the basis of the observed reduction in infarct size associated with treatments preventing ischemia reperfusion injury, it is postulated that up to 50% of the final size of the myocardial infarct is linked to the ischemia reperfusion injury. The reduction of this phenomenon should provide great clinical benefit, and is therefore currently the subject of extensive experimentation.

Myocardial injury has been implicated in the pathology of peripheral vascular insufficiency (Muller *et al.,* 2002), angina (Verma *et al.,* 2002), myocardial infarction (Mc Donough *et al.,* 1999) and stroke (Oliver *et al.,* 1990). Brief intermittent periods of ischemia followed by reperfusion at a time prior to prolonged ischemia known as ischemic preconditioning or immediately after a period of ischemia before the onset of reperfusion known as ischemic post conditioning, have been shown to reduce I/R-induced myocardial injury (Murry *et al.,* 1986; Zhao *et al.,* 2003).

The basic mechanisms involved in the pathophysiology of I/R injury and the pharmacology of pre-conditioning and post conditioning are discussed hereunder.

The important consequences of ischemic reperfusion are reversible contractile dysfunction known as myocardial stunning and impairment of blood flow at micro vascular level known as no reflow with neutrophil plugging and vasoconstriction. Myocardial stunning is the contractile dysfunction of heart that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or nearly normal coronary flow (Bolli, 1990). The impairment in resynthesis of high energy phosphates, alteration in sympathetic

responsiveness, damage to collagen matrix, leukocyte activation, transient calcium overload, decreased sensitivity of myofilaments to calcium and generation of oxygen free radicals have been implicated in the pathogenesis of prolonged contractile dysfunction in myocardial stunning (Ferrari *et al.,* 1995). The ischemic myocardium reduces its metabolic needs and tends to adopt itself to survive with minimal requirements by reducing its own contractility. Such state is referred to as hibernating myocardium in which unlike myocardial stunning, the contractility is restored immediately once the blood flow is restored. The mechanisms responsible for the development of myocardial hibernation in which the heart reduces the contractile function in proportion to reduced blood flow are yet to be identified. The calcium responsiveness in experimental myocardial hibernation has been noted to be reduced and this reduction has not been related to decreased calcium sensitivity. Another important event of prolonged post ischemic reperfusion is no-reflow phenomenon in which no blood flow occurs through coronary blood vessels due to increased leukocyte-endothelial cell adhesion, platelet leukocyte aggregation, interstitial fluid accumulation and loss of endothelium dependent vasorelaxation, which all together result in mechanical blood flow obstruction (Maxwell and Lipp, 1997). The cellular and vascular effects due to prolonged ischemia are shown in **Fig. 2.**

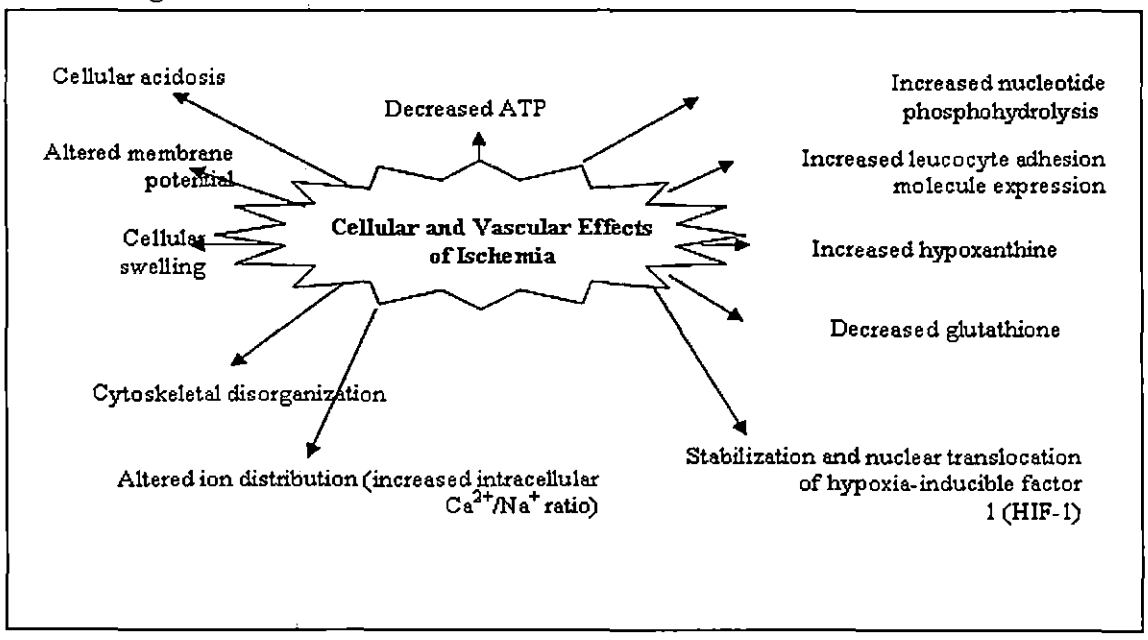

Fig.2.The cellular and vascular effects due to prolonged ischemia

Ischemia reduces cellular oxidative phosphorylation and thus the synthesis of energy rich phosphates is decreased, which alter the membrane ATP-dependent ionic pump function. This alteration favors the entry of calcium, sodium and water into cell which ultimately leads to cellular swelling. The reduced mitochondrial oxidative phosphorylation results in loss of major source of ATP production for energy metabolism. A compensatory increase in anaerobic glycolysis for ATP production leads to accumulation of hydrogen ions and lactate, resulting in intracellular acidosis (Buja, 2005). Moreover, ischemia promotes the expression of pro inflammatory genes, leukocyte adhesion molecules, endothelins and thromboxane A2 (Carden and Granger, 2000), which all together may affect the integrity of coronary vascular endothelium.

Polymorphonuclear leucocytes (PMNs) are mobilized from intravascular space to the interstitium during hypoxia and such responses may contribute significantly to tissue damage during subsequent reperfusion (Collard et al., 2002; Eltzschig *et al.,* 2003). The migration of PMNs through the endothelial barrier may disrupt such tissue barriers and create the potential for extra vascular fluid leakage and edema formation (Luscinskas *et al.,* 2002). The adenine nucleotide catabolism during ischemia leads to intracellular accumulation of hypoxanthine, which subsequently generates ROS upon reperfusion **Fig.3.**

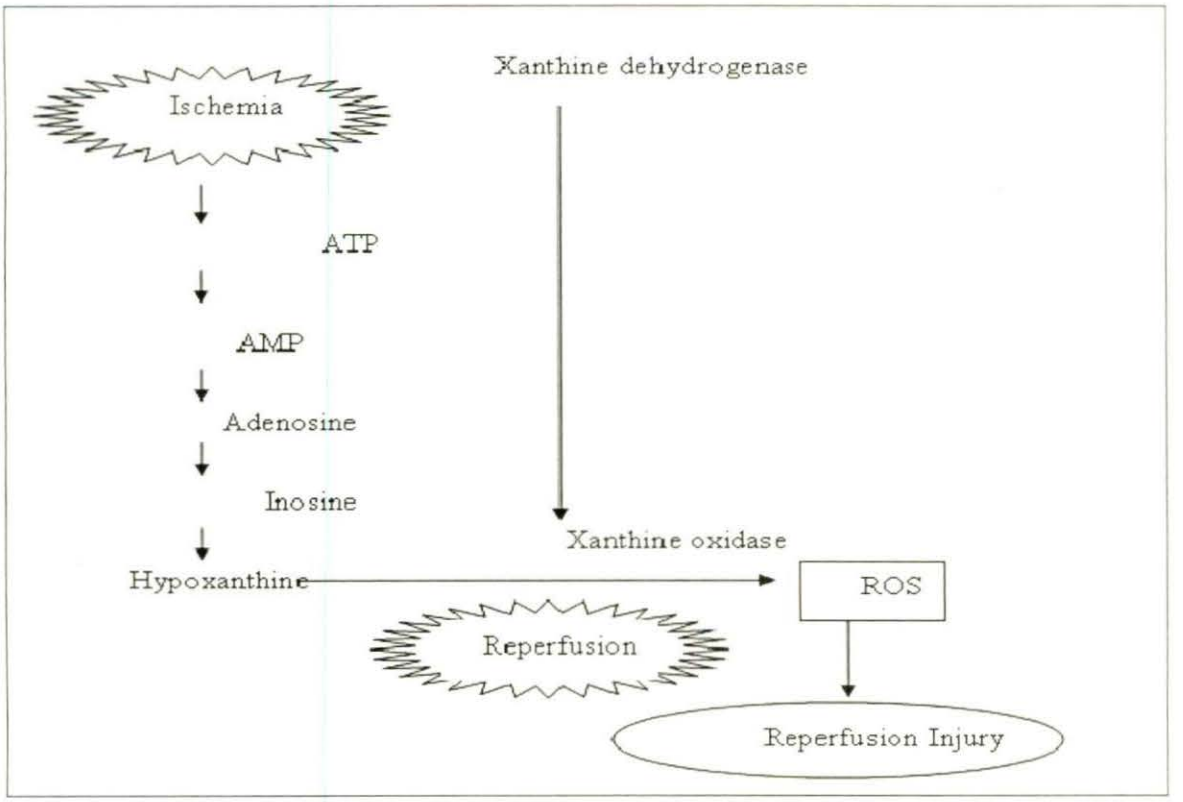

Fig.3. Formation of ROS in ischemia–reperfusion injury

During ischemia, cellular ATP is degraded to form hypoxanthine. Under normal condition, hypoxanthine is oxidized by xanthine dehydrogenase to xanthine, but during ischemia, xanthine dehydrogenase is converted to xanthine oxidase. Unlike xanthine dehydrogenase, which uses nicotinamide adenine dinucleotide as its substrate, xanthine oxidase uses oxygen and therefore, during ischemia, is unable to catalyze the conversion of hypoxanthine to xanthine, resulting in a buildup of excess tissue levels of hypoxanthine. When oxygen is reintroduced during reperfusion, conversion of the excess hypoxanthine by xanthine oxidase results in the formation of ROS (Charles et al., 2001) including superoxide anions (O_2-), hydroxyl radicals (OH-), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2) and peroxynitrite. ROS directly damage cellular membranes through lipid peroxidation. Further, ROS stimulate leukocyte activation and chemotaxis by activating plasma membrane phospholipase A2 to form arachidonic acid, an important precursor for synthesis of eicosanoids such as thromboxane A2 and leukotriene B4. Moreover, ROS stimulate leukocyte adhesion molecule and cytokine gene expression *via* activation of transcription

factors such as nuclear factor-kB (NF-kB). The multiple mechanisms have been postulated for the leukocyte-mediated tissue injury that occurs after ischemia/reperfusion. Micro vascular occlusion, increased vascular permeability (Bjork *et al.,* 1982) and release of oxygen free radicals (Fujita *et al.,* 1996), cytotoxic enzyme (Weiss, 1989) and inflammatory cytokines (Chaumoun *et al.,* 2000) have been demonstrated to contribute to leukocyte-induced tissue injury. I/R-induced leukocyte activation has been noted to release ROS, proteases and elastases, which result in increased micro vascular permeability, edema, and thrombosis and cell death.

Various signaling systems such as tumor necrosis factor- α (TNF-α, Rho-kinase, NF-κB, Janus Kinase (JAK), poly (ADP-ribose) polymerase (PARP), p38 mitogen activated protein kinase (MAPK), Caspases, interleukin-1 (IL-1) and IL-6 have been implicated in the pathophysiology of I/R injury. Further, polymorphonuclear leukocyte (PMN) and factor associated with neutral sphingomyelinase activation (FAN) have been noted to play a pivotal role in affected myocardium. Moreover, resident cardiac mast cells play a key role in I/R injury. The mast cells originate from pluripotent progenitor cells in bone marrow and are major players in the inflammation process. The degranulation of mast cells releases various cytotoxic mediators, which have been noted to be involved in the pathophysiology of ischemia/reperfusion injury.

2.3. PRECONDITIONING

In 1986, Murry and colleagues described an endogenous protective strategy in which multiple brief ischemic episodes in canine hearts limited infarct size from a subsequent sustained ischemic insult termed as ischemic preconditioning (IPC). The IPC has two phases of protection in which an early phase is lasting from few minutes to hours known as early preconditioning, and a late phase starts after 12 hours and lasts up to 3 days is referred to as delayed preconditioning (Yellon and Baxter, 1995; Bolli, 2000).

Following the finding of IPC by Murry and colleagues, several studies have investigated the mechanisms involved in its organ protective effects. By determining the mechanisms by which IPC confers myocardial preservation may eventually lead to the development of therapies to reduce cardiomyocyte injury following cardiopulmonary bypass. These studies led to the discovery that preconditioning could be induced by pharmacological means (Teoh

et al., 2002; Kevelaitis *et al.,* 2001) in which drugs are administered before the ischemic event with an intervening washout period before ischemia known as true pharmacological preconditioning or without a washout period known as pharmacological pretreatment.

The preconditioning may be triggered by substances like adenosine, bradykinin, NO, diazoxide, a mitochondrial ATP sensitive K^+ channel (KATP) opener, phospho kinase C (PKC) activators, opioids and prostaglandins (Post and Heusch, 2002). In addition, anesthetics were investigated for their potential to precondition the heart before ischemia. All halogenated, volatile substances were found to be protective and their actions were comparable to that of ischemic preconditioning (Chiari *et al.,* 2005; Przyklenk *et al.,* 2003)

Short periods of ischemia in remote vessels or even distant organs protected the myocardium from injury induced by coronary artery reperfusion. Thus, the substances must have been released from the remote ischemic reperfused tissue that protected the jeopardized myocardium. The occlusion of circumflex artery has produced the protection of myocardium supplied by left anterior descending coronary artery and this phenomenon is termed as intra cardiac preconditioning (Przyklenk *et al.,* 1993). The short occlusions of renal artery (Mclahan *et al.,* 1993; Pell *et al.,* 1998) abdominal aorta (Weinbrenner *et al.,* 2002; Singh *et al.,* 2004) and mesenteric artery have been documented to protect myocardium against I/R-induced injury. This phenomenon is termed as remote preconditioning (Heusch and Schulz, 2002; Wang *et al.,* 2001) or intra organ preconditioning or preconditioning at a distant site (Schoemaker, 2000).

2.4. POSTCONDITIONING

The brief intermittent episodes of ischemia and reperfusion, at the onset of reperfusion after a prolonged period of ischemia confer cardio protection; a phenomenon is termed as ischemic post conditioning. This concept was first introduced by Zhao *et al.,* 2006. In a canine left anterior descending coronary artery ligation (LAD) model, they compared the protective effects of IPC to that of post conditioning. The brief ischemia and reperfusion of 30 seconds each after prolonged ischemia significantly reduced infarct size and endothelial dysfunction (Zhao *et al.,* 2006). The word post conditioning was given since the stimulus (10-30 seconds for 3-6 times) is applied after a period of ischemia. It has been proposed that passive and active phases are involved in cardio protective mechanisms of post-

conditioning. The passive phase is initiated via stepwise reperfusion that reduces the delivery of oxygen radicals and mitochondrial Ca^{2+} overload.

In active phase, the reperfusion injury salvage kinases (RISK) pathways which include phosphatidylinositol-3-OH kinase (PI3K), akt(protein-Ser/Thr kinase) and extracellular signal-regulated kinase (ERK-1/2) are activated by endogenous stimulators such as adenosine, opioids and some unidentified endogenous substances (Tsang *et al.,* 2004; Yang *et al.,* 2004; Morrison *et al.,* 2007). Post-conditioning mediated activation of PI3 kinase, Akt and subsequently endothelial NO synthase (eNOS) inhibit the opening of mitochondrial permeability transition pore (mPTP) to afford cardio protection (**Fig. 4.**) (Gross, 2006).

Further, post conditioning activates p70s6K through mitogen-activated protein kinase (MEK ½) and ERK ½ signaling systems that initiates protein translation to mediate cardio protection. It has been suggested that post conditioning mediated cardio protection is likely produced via the ERK1/2 pathway rather than PI3 kinase/Akt pathways. On the other hand, it has been noted that Akt and ERK activated during post conditioning do not protect myocardium from reperfusion injury (Schwartz and Lagranha, 2006).

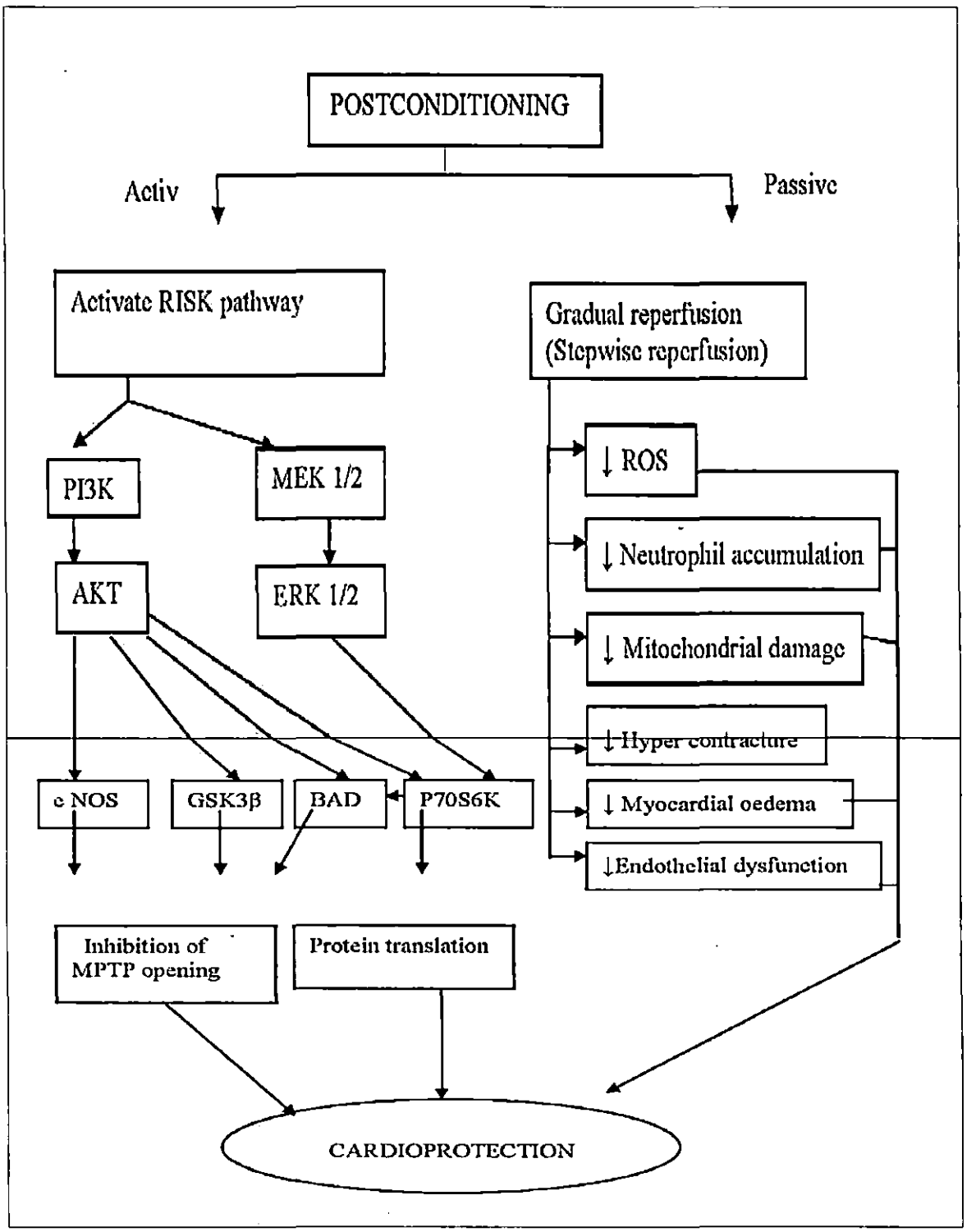

Fig.4. Post conditioning mediated cardio protection

2.5. CLINICAL RELEVANCE OF PRECONDITIONING AND POST CONDITIONING

Numerous *in vitro* findings suggest that the human myocardium can be protected by ischemic preconditioning. Ischemia required for the insertion of coronary artery bypass grafts during cardiac surgery has been shown to provide cardio protection (Jenkins *et al.*, 1995). The findings from many preclinical studies in which cardio protection has been seen in healthy animal hearts might not be reproducible in the human myocardium since human ischemic heart disease is frequently associated with various disorders such as diabetes mellitus and its complications and left ventricular hypertrophy etc or with other contributing factors like older age. The presence of these conditions might interrupt with the protection induced by ischemic preconditioning (Schulman *et al.*, 2001; Tsang *et al.*, 2005). The use of pharmacologic agents to target different components of the signaling pathway that mimic the protection induced by ischemic preconditioning known as pharmacologic preconditioning, might enable this approach to be recognized as a clinical therapy (Ramzy *et al.*,2007; Vinten *et al.*, 2007). Nicorandil has cardio protective effects when given as an adjunctive therapy at the time of reperfusion in cardiac patients during surgery (Ono *et al.*, 2004; Kloner *et al.*, 2007).

Preclinical studies demonstrated that pharmacologic inhibition of Na^+/H^+ exchanger before myocardial ischemia could reduce infarct size through a reduction in myocardial calcium accumulation, to a level comparable to ischemic preconditioning (Avkiran and Marber, 2002; Yellon and Hausenloy 2005). Adenosine has been shown to be a great promising cardio protective agent in different clinical settings of myocardial I/R (Quintana *et al.*, 2004). The post conditioning has been shown to be effective in patients with coronary artery disease. Marked improvement in coronary blood flow has been noted in post conditioned patients (Statt *et al.*, 2005). Taken together, post conditioning would be a safe cardio protective intervention to reduce reperfusion injury in patients with ischemic heart diseases.

2.6. MOLECULAR AND CELLULAR MECHANISMS OF MYOCARDIAL ISCHEMIA REPERFUSION INJURY

Reperfusion injury manifestations include reperfusion arrhythmias, endothelial cell damage leading to micro vascular dysfunction, myocardial stunning, myocyte death and infarction. Normal cardiac function is predicated on a continuous supply of oxygen and nutritive substances. When coronary perfusion is interrupted, profound myocardial damage can occur at both microscopic and macroscopic levels. Clinically, this scenario gives rise to acute coronary syndromes manifesting as angina, or in the most severe form, acute myocardial infarction. The onset of ischemia triggers homeostatic processes geared at limiting damage, but which may act in concert with processes associated with reperfusion, to actually exacerbate injury.

As a result of intensive investigation over decades, a detailed understanding is now available of the complexity of the response of the myocardium to an ischemic insult. Myocardial ischemia results in a characteristic pattern of metabolic and ultra structural changes that lead to irreversible injury. Recent studies have explored the relationship of myocardial ischemic injury to the major modes of cell death, namely, oncosis and apoptosis. The evidence indicates that apoptotic and oncotic mechanisms can proceed together in ischemic myocytes with oncotic mechanisms and morphology dominating the end stage of irreversible injury. Reperfusion of ischemic myocardium leads to severe damage, which is indicated by free radicals, intracellular calcium overloading and loss of membrane phospholipids (Maxwell and Lip, 1997; Dobsak et al., 2003). Malondialdehyde (MDA), a stable metabolite of the free radical-mediated lipid peroxidation cascade, is widely used as marker of oxidative stress. Glutathione (GSH) is an important endogenous antioxidant the levels of which are influenced by oxidative stress.

Myocardial infarcts evolve as a wave front of necrosis, extending from sub endocardium to sub epicardium over a 3 to 4 hour period. A number of processes can profoundly influence the evolution of myocardial ischemic injury. Timely reperfusion produces major effects on ischemic myocardium, including a component of reperfusion injury and a greater amount of salvage of myocardium.. Preconditioning by several short bouts of coronary occlusion and reperfusion can temporarily salvage significant amounts of myocardium and extend the

window of myocardial viability. Ongoing research into the mechanisms involved in reperfusion and preconditioning is yielding new insights into basic myocardial pathobiology.

2.7. ENDOTHELIAL FUNCTION IN CARDIOVASCULAR DISEASES

The vascular endothelium is involved in the release of various vasodilators, including NO, prostaglandins and endothelium-derived hyperpolarizing factor as well as vasoconstrictors. The NO plays an important role in the regulation of vascular tone, inhibition of platelet aggregation and suppression of smooth muscle cell proliferation. Impaired endothelium-dependent vasodilation has been found in the forearm, coronary, and renal vasculature in patients with cardiovascular diseases. Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis. In patients with coronary artery diseases, (Suwaidi *et al.,* 2000), found that severe coronary endothelial dysfunction is associated with increased cardiovascular events. Schachinger *et al.,* 2000 demonstrated a link between coronary endothelial dysfunction and subsequent cardiovascular events in patients with coronary artery disease. These clinical studies have shown that endothelial function can serve as an independent predictor of cardiovascular events. From a clinical perspective, it is important to select an appropriate intervention that is effective in improving endothelial function in patients with cardiovascular diseases. Several investigators (Ress DD *et. al.*, 1989, Cai H *et. al.*, 2000 Delles C *et. al.*, 2002) have reported possible mechanisms of impairment of endothelial function in cardiovascular diseases; abnormalities of shear stress, increase in the amount of endogenous endothelial NO synthase (eNOS) inhibitor, asymmetrical dimethylarginine, increases in the amount of vasoconstrictors, such as angiotensin II (Ang II), endothelin-1, and nor-epinephrine, and inactivation of NO by ROS. The growing evidence has shown an interaction between oxidative stress and endothelial function. Enhanced production of ROS and an attenuated antioxidant system may contribute to endothelial dysfunction in cardiovascular diseases. In other words, enhanced NO inactivation caused by excess ROS production, rather than decreased NO production, may play an important role in the impaired endothelium-dependent vasodilation in cardiovascular diseases. These findings suggest that a decrease in NO inactivation contributes to the improvement in endothelial function in patients with cardiovascular diseases.

2.8. MECHANISMS OF ISCHEMIC INJURY

Early observations on the mechanisms of ischemic injury focused on relatively simple biochemical and physiological changes which were known to result from interruption of circulation. Examples of these changes are loss of high-energy compounds, acidosis due to anaerobic generation of lactate. Subsequent research has shown the problem to be far more complex than was previously thought, involving the action and interaction of many factors which are discussed as follows.

2.8.1. Free Radicals

The radicals which have unpaired electrons and potent ability of oxidation are called as free radicals. The sources and metabolism of reactive oxygen species (ROS) are as follows.

ROS includes superoxide anion (O^{2-}.), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), hypochlorous acid (HOCl), nitric oxide (NO), and peroxynitrite (ONOO-). Amongst these O^{2-}, OH and NO are classified as free radicals whereas H_2O_2, HOCl, and ONOO- are classified as non-free radicals that also have the ability to oxidize. The sources of ROS are a variety of cell types such as vascular smooth muscle cells, endothelial cells, and mononuclear cells. Potential sources of ROS production include nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, NO synthase, mitochondrial electron transport, cyclooxygenase, glucose oxidase, and lipooxygenase (**Fig. 5**). These various oxidase enzymes produce superoxide. The antioxidant enzyme superoxide dismutase (SOD) rapidly dismutates superoxide to H_2O_2. Then H_2O_2 is eliminated by glutathione peroxidase (GPX) and catalase to water.

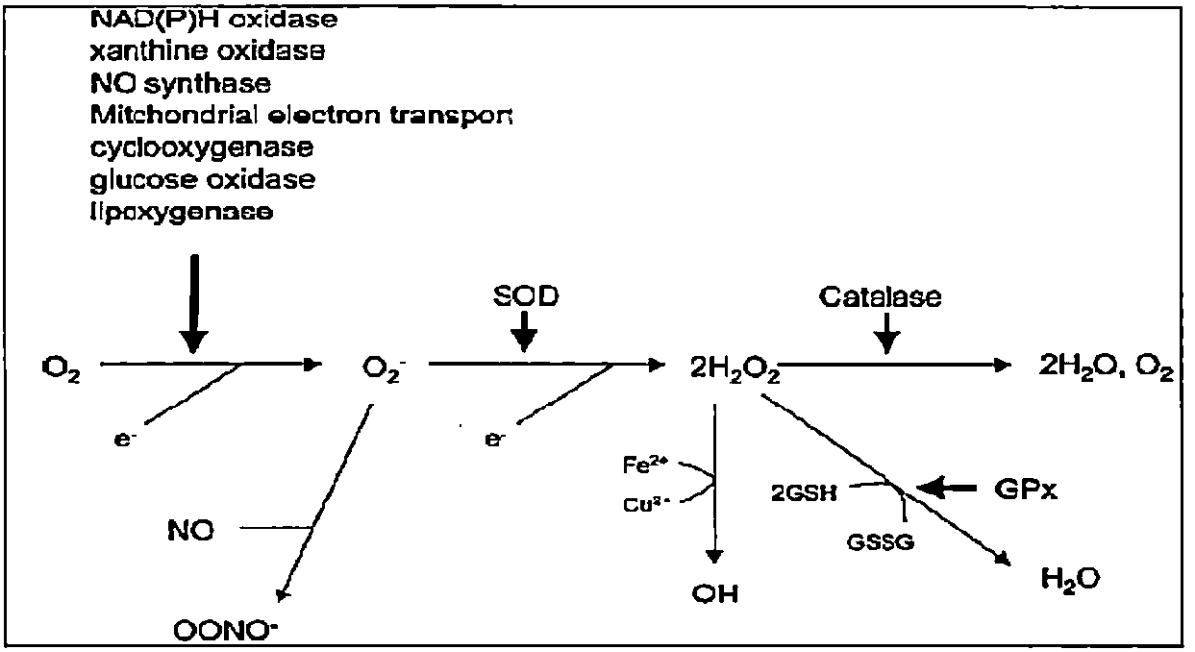

Fig.5. Metabolism of reactive oxygen species.

NADPH indicates nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; O_2, oxygen; O^{2-}, superoxide anion; e, electron; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; H_2O, water; OONO-, peroxynitrite; OH, hydroxyl radical; GSH,glutathione; GSSG, disulfide of glutathione ,GPX, glutathione peroxidase.

During ischemia, the hydrolysis of ATP via AMP leads to an accumulation of hypoxanthine. The increased intracellular calcium enhances the conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO). Upon reperfusion and reintroduction of oxygen, XO may produce superoxide and xanthine from hypoxanthine and oxygen. Even more damaging free radicals could conceivably be produced by the metal catalyzed Haber-Weiss reaction as follows:

O_2- + H_2O ----Fe^{3+} ------> O_2 + OH-+ OH-

Iron, the transition metal needed to drive this reaction, is present in abundant quantities in bound form in living systems in the form of cytochromes, transferrin and hemoglobin. Anaerobic conditions have long been known to release such normally bound iron. Indirect experimental confirmation of the role of free iron in generating free-radical injury has come from a number of studies which have confirmed the presence of free-radical breakdown products such as conjugated dienes and low molecular weight species of iron.

During reperfusion and re-oxygenation, significantly increased levels of several free-radical species that degrade cell and capillary membranes have been postulated: O2-, OH-, and free lipid radicals (FLRs). The super oxide ion may be formed by the previously described actions of XO and/or by release from neutrophils which have been activated by leukotrienes. The re-oxygenation also restores ATP levels and this may in turn allow active uptake of calcium by the mitochondria, resulting in massive calcium overload and destruction of the mitochondria.

2.8.2. Calcium

Normally, calcium is present in the extracellular milieu at a concentration of 10,000 times greater than the intracellular concentration. This 10,000:1 differential is maintained by at least the following four mechanisms:

i. Active extrusion of calcium from the cell by an ATP-driven membrane pump.

ii. Exchange of calcium for sodium at the cell membrane driven by the intracellular to extracellular differential in the concentration of Na+ as a result of the cell membrane's Na^+ - K^+ pump.

iii. Sequestration of intracellular calcium in the endoplasmic reticulum by an ATP-driven process.

iv. Accumulation of intracellular calcium by oxidation-dependent calcium sequestration inside the mitochondria.

The loss of cellular high-energy compounds during ischemia causing the loss of the Na^+ -K^+ gradient, virtually eliminates three of the four mechanisms of cellular calcium homeostasis. This, in turn, causes a massive and rapid influx of calcium into the cell. Mitochondrial sequestration, the remaining mechanism, causes overloading of the mitochondria with calcium and diminished capacity for oxidative phosphorylation. Elevated intracellular Ca^{++} activates membrane phospholipases and protein kinases. A consequence of phospholipase activation is the production of free fatty acids (FFA's) including the potent prostaglandin inducer, arachidonic acid (AA). The degradation of the membrane by phospholipases almost certainly damages membrane integrity, further reducing the efficiency of calcium pumping and leading to further calcium overload and a failure to regulate intracellular calcium levels

following the ischemic episode. Additionally, FFAs almost certainly have other degradative effects on cell membranes. The production of AA as a result of FFA release causes a biochemical cascade ending with the production of thromboxane and leukotrienes. Both these compounds are profound tissue irritants which can cause platelet aggregation, clotting, vasospasm, and edema, with resultant further compromise to restoration of adequate cerebral perfusion upon restoration of blood flow.

2.8.3. Mitochondrial Dysfunction

Calcium loading and free-radical generation are no doubt major contributors to the mitochondrial ultra structural changes which are known to occur following cerebral ischemia. In addition to the structural alterations observed, there are biochemical derangements such as a marked decrease in adenine nucleotide translocase and oxidative phosphorylation. There is also an accumulation of FFAs, longchain acylCoA, and long-chain carnitines. Of these alterations, the accumulation of long-chain acylCoA is perhaps most significant, since intramitochondrial accumulation of long chain acylCoA is known to be deleterious to many different mitochondrial enzyme systems.

2.8.4. Lactic Acidosis

Lactic acidosis does apparently contribute to the pathophysiology of ischemia while it is clearly not the sole or even the major source of injury in ischemia. It has been shown, for instance, that lactate levels above a threshold of 18 - 25 micromol/g result in currently irreversible injury. Decrease in pH as a consequence of lactic acidosis has been shown to injure and inactivate mitochondria. Lactic acid degradation of NADH (which is needed for ATP synthesis) may also interfere with adequate recovery of ATP levels post ischemically. Lactic acid can also increase iron de compartmentalization, thus increasing the amount of free-radical mediated injury.

2.8.5. Neutrophil Activation

Since the late 1960s, polymorphonuclear leukocytes (PMNLs) and monocytes/ macrophages have been implicated as significant causes of pathology in ischemia. During the last decade there has been a veritable explosion of research documenting the role of PMNLs in reperfusion injury. Most of the initial work done in this area focused on PMNL-mediated

reperfusion injury to the myocardium, establishing that PMNL activation and subsequent plugging and degranulation (resulting in release of oxidizing compounds) is responsible for the no-reflow phenomenon following myocardial ischemia. In particular, the work of Engler has demonstrated that PMNL activation is responsible for plugging at least 27% of myocardial capillaries and is further responsible for the development of edema and arrhythmias upon reperfusion

To what extent leukocyte plugging occurs in the brain following global ischemia remains controversial. Recent study answered the question of how rapidly leukocyte plugging occurs following ischemia. It was noted that no leukocyte plugging after 3 hours of reperfusion following a 40-minute ischemic episode. However, it is clear from a growing body of work that neutrophils are a major mediator of ischemic injury in a variety of organ systems and that their acute activation is responsible for many of the effects of ischemia observed in the brain and other body tissues, including the loss of capillary integrity and the degradation of ultra structure upon reperfusion.

2.8.6. TNF-α

Recent studies have focused their attention on the role of the proinflammatory cytokine tumor necrosis factor (TNF) in the development of heart failure. In addition, both in vivo and in vitro studies demonstrate that TNF effects cellular and biochemical changes that match those seen in patients with congestive heart failure. Furthermore, in animal models, the development of the heart failure phenotype can be abrogated at least in part by anti cytokine therapy. Based on information from experimental studies, investigators are now evaluating the clinical efficacy of novel anti cytokine and anti-TNF strategies in patients with heart failure.

Healing of myocardial infarcts depends on an inflammatory cascade that ultimately results in clearance of dead cells and matrix debris and formation of a scar. Myocardial necrosis activates complement, Nuclear Factor (NF)-κB and Toll-like Receptor (TLR)-dependent pathways, and generates free radicals, triggering an inflammatory response. Chemokines and cytokines are markedly induced in the infarct and mediate recruitment and activation of neutrophils and mononuclear cells. Extravasation of platelets embedded in a mesh of cross linked fibrin. This provisional matrix provides a scaffold for migration of cells into the

infarct. Monocytes differentiate into macrophages and secrete fibrogenic and angiogenic growth factors inducing formation of granulation tissue, containing myofibroblasts and neovessels. Repression of proinflammatory cytokine and chemokine synthesis, mediated in part through Transforming Growth Factor (TGF)-β and Interleukin (IL)-10, is critical for resolution of the inflammatory infiltrate and transition to fibrous tissue deposition. Infarct myofibroblasts deposit extracellular matrix proteins and a collagen-based scar is formed. As the wound matures, fibroblasts undergo apoptosis and neovessels regress, resulting in formation of a scar with a low cellular content containing dense, cross-linked collagen. The pathologic and structural changes associated with infarct healing directly influence ventricular remodeling and affect prognosis in patients with myocardial infarction. Understanding the mechanism involved in the regulation of the post-infarction inflammatory response, and the spatial and temporal parameters of wound healing is necessary in order to identify specific molecular targets for therapeutic intervention.

2.8.7. Opportunities for Intervention

i. Numerous studies have suggested a cardio protective effect for a variety of calcium channel blockers administered post-insult.

ii. Free radical damage: Free radicals have long been understood to be a major source of ischemic pathology. Similarly, there have been a number of studies which suggest that free radical associated ischemic injury can be reduced greatly or eliminated by pre or post insult treatment with nutritional antioxidants such as vitamin E, selenium, vitamin C, and beta carotene. Theoretical considerations also suggested other possible therapeutic agents such as those known to elevate neuronal (intracellular) glutathione levels for protection from ischemic injury.

iii. Phospholipase activation has been implicated as a significant source of injury in both cold and warm ischemia. The phospholipase inhibitor quinacrine has reduced cold ischemic injury in an organ preservation model as well as myocardial reperfusion injury.

iv. Inhibition of the inflammatory cascade and the adhesion and degranulation of polymorphonuclear lymphocytes by both drug treatment and by their removal via filtration have been shown to lessen reperfusion injury in the heart.

2.9. INTERNATIONAL STATUS ON CVD

CVD is a leading cause of mortality and is responsible for one third of global death. The majority of the estimated 32 million heart attacks strokes that occur every year are caused by one or more CVD risk factors such as hypertension, diabetes smoking, high levels of blood lipid and physical inactivity. In China, the corresponding figure is 35% an estimated 16.7 million or 29.2% of total global death result from the various form of cardiovascular disease. More than 50% of death and disability from heart disease and strokes which together kill more than 12 million people per year. An estimated 16.7 million of 29.2% of total global deaths result from the various forms of cardiovascular disease (CVD).

Cardiovascular disease (CVD) has been the dominant cause of death in Australia for many decades, with coronary heart disease (CHD) and stroke ranking high among leading causes of death. CVD accounts for more than 46,000 deaths annually in Australia, and 3.7 million Australians have a long-term cardiovascular disease (AIHW 2008). There is some evidence from the United Kingdom and the United States that CHD mortality rate declines have accelerated in some older age groups but slowed in some younger age groups in the recent past (Allender *et al.*, 2008, Ford & Capewell 2007 and O'Flaherty *et al.*, 2008). An estimated 17.5 million people died from cardiovascular disease in 2005, representing 30% of all global deaths. Of these deaths, 7.6 million were due to heart attacks and 5.7 million due to stroke. About 80% of these death occurred in low and middle income countries. If current trends are allowed to continue, by 2015 an estimated 20 million people will die from cardiovascular disease (WHO, 2005)

2.10. NATIONAL STATUS ON CVD

CVD is the number one cause of mortality in India today. Previously, CVD was considered to be a result of an urban lifestyle; however, many recently published studies have indicated that CVD is also on the rise in rural areas. (Patil *et al.*, 2004; Goel *et al.*, 2003) The prevalence of CVD is four-fold higher in urban India and two-fold higher in rural India than

in the United States (Goel *et al.,* 2003). Apart from current lifestyle factors, there is an additional 3–4-fold risk from genetic and ethnic factors for heart disease. Certain ethnic groups in India, like the Jains, Marwaris, Banias and other communities belonging to the states of Rajasthan, Gujarat, Haryana and Punjab, are genetically predisposed to central obesity. (Dwivedi, 2004) They are at a higher risk of developing CVD. The picture is even gloomier for migrant Indians. (Hoogeveen *et al.,* 2001)

A new research publication in *The Lancet 2008* finds that India will bear 60% of the world's heart disease burden in the next two years. In addition, researchers have determined that compared to people in other developed countries, the average age of patients with heart disease is lower among Indian people and Indians are more likely to have types of heart disease that lead to worse outcomes. The leading cause of death in the world is ischemic heart disease, a condition characterized by reduced blood supply to the heart that is usually due to coronary artery disease. In 2001 alone, some 7.1 million deaths were attributed to ischemic heart disease, 80% of which were in relatively poor countries. Medical and public health professionals expect that in developing countries, there will be a 137% and 120% increase in the disease for males and females, respectively, whereas these predictions lie in the 30% to 60% range for developed countries.

As per WHO report the morbidity and mortality due to cardiovascular diseases in India will reach an epidemic proportion by the middle of this century. In India, approximately 53% of CVD deaths are in people younger than 70 years of age. The prevalence of CAD in urban North India varies from 7% to 10% compared to 3% in USA and <1% in Japan. The CAD rates in South India are two folds higher than in North India, with Kerala reporting 14% in urban and 7% in rural Cardiovascular diseases particularly IHD, have become a worldwide health problem affecting all economic groups of the society and are responsible for 35% of all deaths in the world. The mortality due to CVD ranges from 16% to 50% in the developing and developed countries. Since 78% of all deaths of the world occur in the developing countries, 85% of the global death and disease burden from CVD is borne by low and middle income countries. In India app 53% of CVD deaths are in people younger than 70years of age. By 2020 CVD will be the leading cause of death in developing countries like India.

2.11. PHARMACOLOGY OF PYRAZOLONES

The various pharmacological aspects of pyrazolone derivatives and its role on various mediators which are associated with CVS are described as follows.

2.11.1. Cardioprotective activity

Edaravone (3-methyl-1-phenyl-2-pyrazolone), a strong free radical scavenger **(Fig.6.)** is used for treatment of patients with acute brain infarction. Edaravone has been developed by Mitsubishi-Tokyo Pharmaceuticals Inc (Tokyo, Japan). Edaravone has preventive effects on myocardial injury following ischemia and reperfusion in patients with acute myocardial infarction. Antioxidant actions of edaravone include enhancement of prostacyclin production, inhibition of lipoxygenase metabolism of arachidonic acid by trapping hydroxyl radicals, inhibition of alloxan-induced lipid peroxidation, and quenching of active oxygen, leading to protection of various cells, such as endothelial cells, against damage by reactive oxygen species (ROS). The novel free radical scavenger edaravone may represent a new therapeutic intervention for endothelial dysfunction in the setting of atherosclerosis, chronic heart failure, diabetes mellitus, or hypertension. In addition, edaravone improves endothelial function in smokers through an increase in nitric oxide (NO) bioavailability.

This focused on clinical findings and on putative mechanisms underlying the beneficial effects of the antioxidative agent edaravone on the atherosclerotic process in patients with cardiovascular diseases.

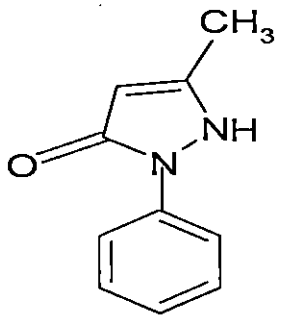

Fig.6. Structure of 3-methyl-1-phenyl-2-pyrazolone (Edarvone)

2.11.2. Benefits of edarvone (pyrazolone) for cardiovascular diseases

It is expected that edaravone has beneficial effects on coronary artery and myocardial cells after ischemic and post-ischemic myocardial injury in patients with ischemic heart diseases, including acute myocardial infarction and angina pectoris. Some animal studies using acute myocardial ischemia-reperfusion models have suggested protective effects of edaravone on myocardial damage. Yanag isawa *et al.,* 1994 showed that intravenous infusion of edaravone at a dose of 3 mg/kg attenuates the loss of myocardial creatine kinase activity from the left ventricular free wall in rats subjected to coronary artery occlusion for 10 minutes followed by reperfusion for 24 hours and reduced infarct size by approximately 50% compared with that in the control vehicle group. Minhaz *et al.,* 1996 reported that edaravone attenuated the myocardial necrotic area by approximately 50% in isolated reperfusion rat heart subjected to coronary artery occlusion.

This beneficial effect was related to reduction in myocardial damage. Also, in rabbit hearts subjected to ischemic reperfusion, a bolus infusion of edaravone reduced the necrotic area (Wu *et al.,* 2000). It has been reported that edaravone at a dose of 15 mmol reduced the death of isolated adult rabbit ventricular cells by approximately 40% compared with that in the control vehicle group (Yamawaki *et al.,* 2004).

Tsujita *et al.,* 2004 investigated the effects of edaravone on left ventricular function and infarct size using a randomized, placebo-controlled, open-label protocol in 80 patients with acute myocardial infarction. Intravenous administration of edaravone at a dose of 30 mg for 10 minutes before myocardial reperfusion decreased serum concentrations of creatine kinase-MB isoenzymes, a surrogate point of infarct size, and improved left ventricular ejection fraction in patients with acute myocardial infarction compared with those in the placebo group (**Fig.7**).

These findings suggested that edaravone has cardioprotective effects. This drug had shown protective effect on post ischemic injury in the coronary vasculature and myocardium in patients with cardiovascular diseases though a decrease in oxidative stress. In other post ischemic reperfusion models, the usefulness of edaravone for organ protection has been reported.

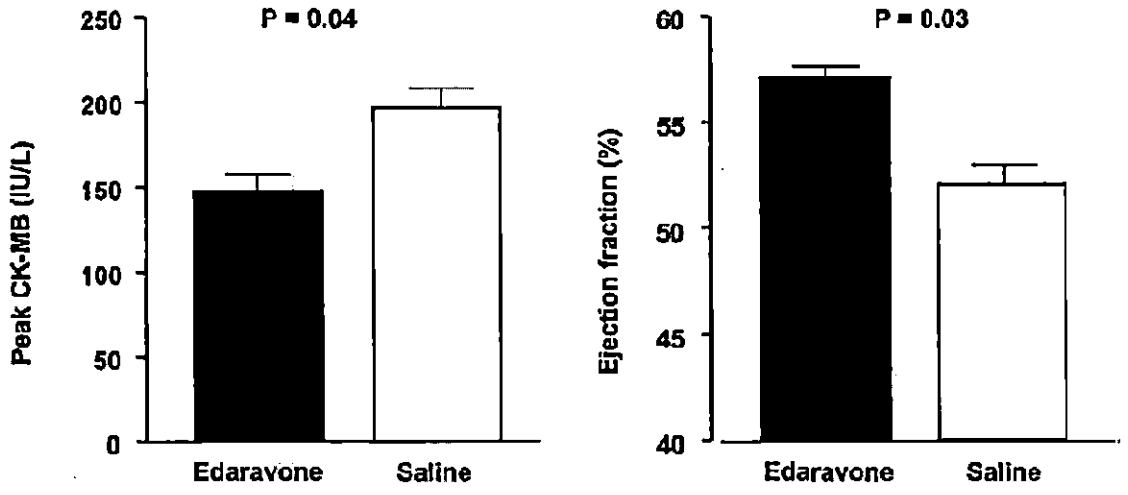

Fig.7. Effects of edaravone on peak CK-MB and ejection fraction in patients with acute myocardial infarction

Edaravone improves gastrocnemius and tibialis anterior muscles injury in a rat ischemic limb model and prevents kidney post ischemic reperfusion injury in rats(Masaki *et al.,* 1996) and lipopolysaccharide-induced liver damage in rats (Kono *et al.,* 2003) . These findings suggest that edaravone may have beneficial effects on ischemia-reperfusion injury in various muscles, vessels, and tissues in different organs.

2.11.3 Putative mechanisms underlying antioxidant action of edaravone

After ischemia-reperfusion, large amounts of ROS are produced from vascular smooth muscle cells, endothelial cells, and mononuclear cells. It has been shown that edaravone reduces or restores the amount of ROS increased by post ischemic reperfusion and prevents impairment of the antioxidant defense system (Tosaka *et al.,* 2002; Yamaguchi *et al.,* 2003). Scavenging ROS by edaravone may play a key role in preventing post ischemic reperfusion injury in various types of cells and tissues. The reported antioxidant actions of edaravone include

i. Enhancement of prostacyclin production,
ii. Inhibition of lipoxygenase metabolism of arachidonic acid by trapping hydroxyl radicals

iii. Inhibition of alloxan-induced lipid peroxidation,

iv. Quenching of reactive oxygen,leading to protection of various cells, such as endothelial cells, against damage by ROS (Kawai *et al.,* 1997).

The putative mechanism underlying the antioxidant action of edaravone is as follows (Yamamoto *et al.,* 1996) **(Fig.8.)**: an electron transfer from an edaravone anion to peroxyl radical yields an edaravone radical and peroxyl anion, and this reaction breaks the chain oxidation of lipids. Then, edaravone peroxyl radical transforms to 4, 5-dione by elimination of a hydrogen atom and one electron. Finally, 2-oxo-3-(phenylhydrazono)- butanoic acid (OBP) is produced by the hydrolysis of 4,5- dione.

It is thought that edaravone exists near the cell membrane or perhaps on the cell membrane. Edaravone has a low molecular weight (MW 174.2), is both lipid-soluble and water-soluble, and has good cell membrane permeability (Yamamoto *et al.,* 1996). It has been confirmed that edaravone has the ability to pass through the blood-brain barrier in dogs. Edaravone directly prevents hydroxyradical-induced injury of cultured bovine aortic endothelial cells. In addition, edaravone stimulates the conversion of arachidonic acid to prostacyclin and inactivates ROS, resulting in protection of endothelial cells.

Interestingly, edaravone induced endothelial NO synthase (eNOS) in the ischemic spinal cord in rabbits, preventing spinal cord damage (Takahashi *et al.,* 2004), and it also restored the reduced expression of eNOS, mRNA and protein in the rabbit artery following irradiation (Zhang *et al.,* 2003).

Yoshida et al 2005 recently reported that edaravone enhances the expression of eNOS and restores the reduction in eNOS by oxidized low-density lipoprotein in endothelial cells. These findings suggest that edaravone prevents the cell damage induced by oxidative stress through not only direct ROS scavenging effect but also restoration of reduced eNOS expression.

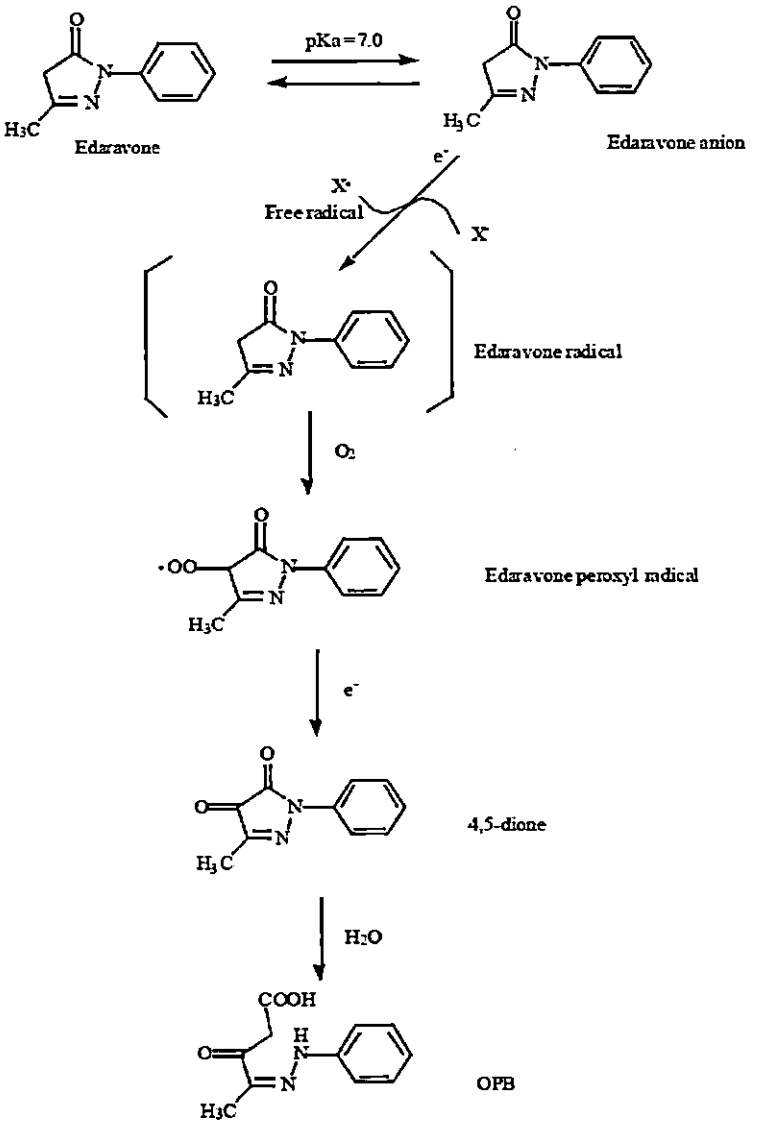

Fig.8. Putative mechanisms of antioxidant actions of edaravone. OPB indicates 2-oxo-3-(phenylhydrazono)-butanoic acid. (Hirokawa, T., Sugawara, K., Tanaka, T.: Japan Patent 04091441A2 (2004)

The novel free radical scavenger edaravone may represent a new therapeutic intervention for endothelial dysfunction in the setting of atherosclerosis, chronic heart failure, diabetes mellitus, or hypertension through its free radical scavenging and antioxidant actions. The role of edaravone in CVD is shown in the **Fig.9.**

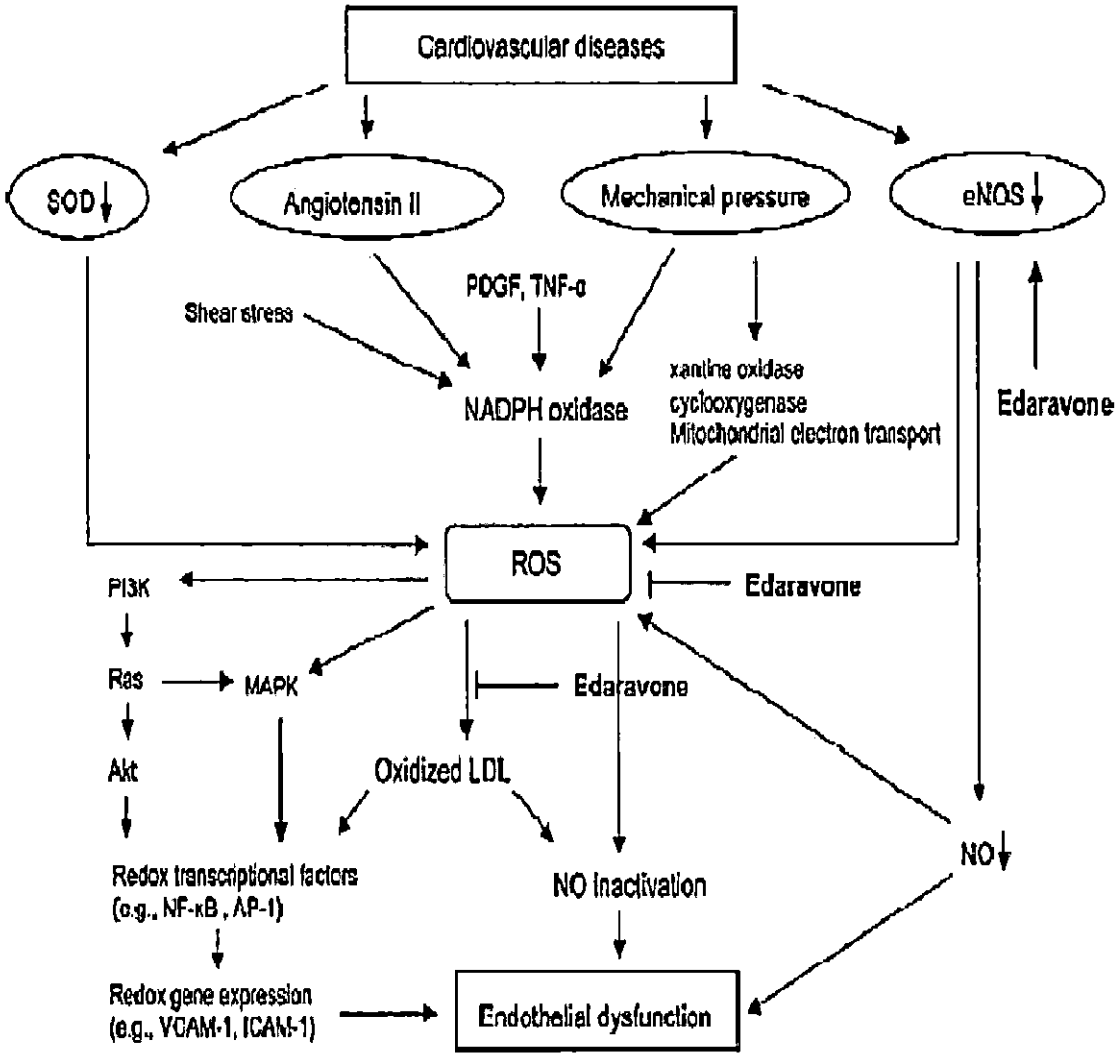

Fig.9. Putative mechanisms of edaravone-induced improvement in endothelial dysfunction in patients with cardiovascular disease

Pyrazolone derivatives reported to possess wide spectrum of activities. Recent research report shows that Pyrazolone could be useful in treatment of cholesterol ester storage disease and the treatment of conditions such as thrombosis and myocardial infarction, vasospastic disorders and bronchospasm or in reperfusion salvage therapy. But the literature and reported research articles have limited information on myocardial ischemic reperfusion injury.

2.11.4. Free radical scavenging activity

Recent research evidences that pyrazolones are reported as free radical scavangers. Pyrazolone derivatives, pertaining to the first groups of compounds such as Dipyrone aminopyrine, isopropylantipyrine and antipyrine used as analgesic, antipyretic, and anti-inflammatory therapeutic drugs (**Fig.10.**).

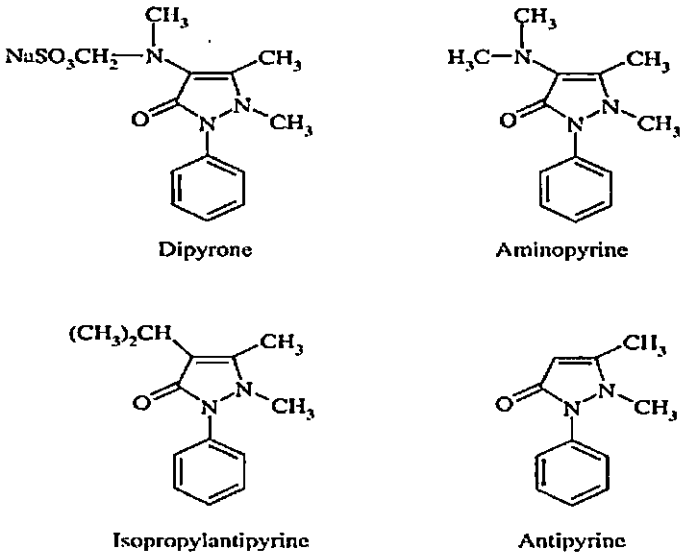

Fig.10. Structure of pyrazolone derivatives

The therapeutic activity of antipyrine was increased by an introduction of an isopropyl group on C-4 to produce isopropyl antipyrine, which improved the antipyretic and analgesic properties, while maintaining the anti-inflammatory activity. The introduction of a dimethylamino group on C-4 of the antipyrine molecule resulted in aminopyrine.

A disadvantage of aminopyrine is its relative insolubility in water. The search for more soluble compounds led to the production of the sodium salt of antipyridinyl methyl aminomethane sulfonic acid (dipyrone) (Brogden, 1986). The pharmacological mechanism of action of pyrazolones is that it involves the inhibition of cycloxygenase isoenzymes, platelet thromboxane synthesis, and prostanoids synthesis. The risk of agranulocytosis associated with the use of pyrazolone drugs at therapeutic doses and for short periods of time has been considered to be very low. However, little or no attention at all has been devoted to the possible hindrance of neutrophil burst and scavenging of neutrophil generated

reactive oxygen species (ROS) by these compounds. The studied pyrazolones was capable of scavenging O^{2-} or H_2O_2, while dipyrone was shown to be the most reactive against ROOS.

2.11.5. Role of pyrazolones for cerebrovascular diseases

Experimental studies have shown beneficial effects of edaravone on post ischemic reperfusion injury (Nakamura *et al.,* 2003; Takashi *et al.,* 2004; Nakajima *et al.,* 2005 ; Otani *et al.,* 2005 ; Ikeda *et al.,* 2002). Edaravone has been shown to ameliorate infarct size and brain edema in embolization and transient focal, global, and hemispheric ischemia models in adult rats (Takashi *et al.,* 2004., Jin *et al.,* 2002; Nakajima *et al.,* 2005) and to attenuate the hypoxic-ischemia encephalopathy in neonatal rats (Ikeda *et al.,* 2002).

In Japan, edaravone was approved in April 2001 for treatment of acute brain infarction and subarachnoid hemorrhage in the acute phase. Several investigators have reported that edaravone has beneficial effects on prevention of brain damage in patients with stroke (Ogasawara et al., 2004). Although the usefulness of edaravone for treatment of mild to moderate stroke in the acute phase has been established, it is unclear whether edaravone is effective against brain damage in patients with severe stroke.

2.11.6. TNF α and cytokine inhibition

The over expression of cytokines such as TNF-α and IL-16, has been implicated in a number of serious inflammatory disorders. Consequently, agents that inhibit the production of TNF- α can decrease levels of these pro-inflammatory cytokines and thereby reduce inflammation and prevent further tissue destruction in diseases such as rheumatoid arthritis (RA), osteoarthritis (OA), and Crohns disease. The efficacy of inhibitors against the overall cascade that leads to TNF-α production and have been identified p38 MAP kinase as one of the critical targets of inhibition.

Hence monocyclic and bicyclic pyrazolone derivatives have been synthesized and screened for cytokine inhibitory action. 4-Aryl-5-pyrimidyl based cytokine synthesis inhibitors that contain a novel monocyclic, pyrazolone heterocyclic core **(Fig.11.)**. One of the compounds was found to be efficacious in the rat iodoacetate (RIA) *in vivo* model of the pyrimidyl based cytokine synthesis inhibitors that contain a novel monocyclic, pyrazolone heterocyclic

core were described (Jennifer *et al.*, 2005) as cytokine synthesis inhibitors. Many other small molecules TNF- α production inhibitors have been reported containing a common 4-aryl- 5-pyrimidinyl based motif fused to a 5- or 6-membered heterocyclic core. A prototypical pyridyl imidazole-based inhibitor, although numerous structural classes, for example, pyrroles, pyrimidines, pyridines, pyrimidones, indoles, heteroindoles, ureas and various fused bicyclic heterocycles containing a variety of functionality have been reported to inhibit cytokine activity.

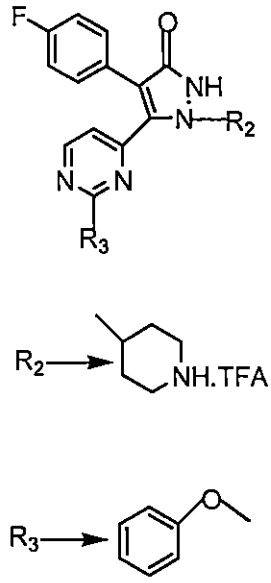

Fig.11. Structure of pyrimidine fused pyrazolones

2.11.7. Map kinase inhibition

Researchers from Merck reported studies on pyrazolones and other heterocycles as inhibitors of MAP p38 kinase (**Fig.12.**). Two monocyclic pyrazolones showed with good p38a-kinase inhibition, but weak whole cell assay activities. The alkyl substitution of both pyrazolone ring nitrogen atoms could facilitate cell membrane permeability and improve cellular cytokine synthesis inhibition profile.

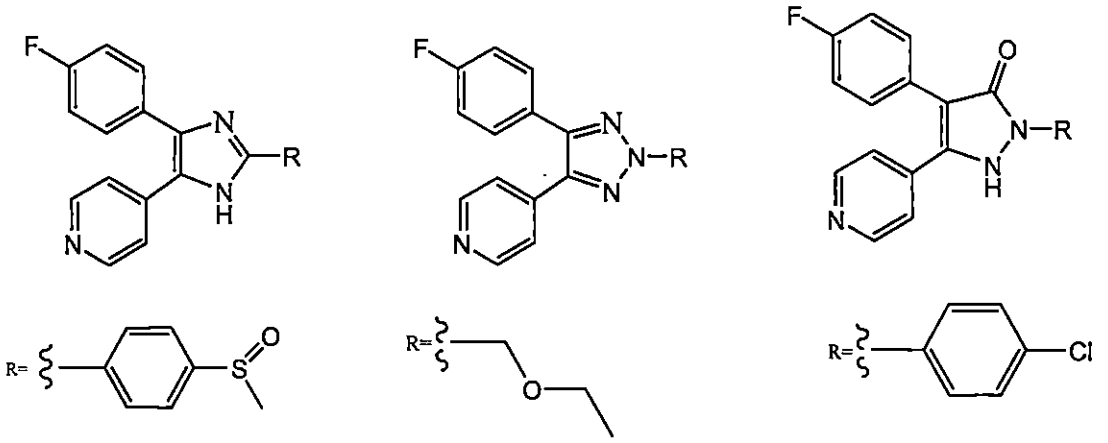

Fig.12. p38a-kinase/TNF -α synthesis inhibitors

All compounds were tested for the inhibition of TNF- α production using lipopolysaccharide stimulated human monocyclic cells based on a monocyclic pyrazolone scaffold at nanomolar activity in whole cell assay and reported to inhibit TNF- α.

2.12. CURRENT AND FUTURE DEVELOPMENTS ON CVS

The treatment of IHD involves expensive and chronic drug therapy or equally expensive interventional procedure such thrombolytic therapy and surgical recannulation, which has its own drawbacks in the form of reperfusion injury. The etiopathogenesis of this phenomenon is complex and multi-factorial of which oxygen free radical (OFR) has been identified as the major contributor. Efforts to control OFR induced damage, using modern pharmacological agent have met with little success. More importantly, the results of such will provide us with better cost effective and socially acceptable therapeutic options for a disease, which threatens to be the number one killer disease in this century.

The various drugs, which have so far been tried out are calcium antagonists, beta blockers and free radical scavenger. Although these drugs provide significant benefit in acute conditions and in secondary prevention, they are not advisable or acceptable for chronic use as primary preventive measures in large number of patients, who possess a high level of risk of having acute ischemic episodes, later in life. Moreover, chronic use of various drugs in the treatment of IHD showed major limitations due to various side effects. Under such circumstances, other options need to be explored which will help in circumventing this problem.

In this literature review, we indicated the possibility that pyrazolone has beneficial effects on not only myocardial and vascular injury following ischemia and reperfusion in patients with acute myocardial infarction, but also in atherosclerosis in the chronic phase. Due to the lack of clinical studies using pyrazolone it remains unclear whether pyrazolone treatment is beneficial for patients who have excess oxidative stress and whether pyrazolone reduces the mortality rate of these patients. It is expected that pyrazolone will be useful for treatment of various diseases in which oxidative stress may be involved in the pathogenesis. Awareness of the rising incidence of ischemic heart disease (IHD) in India, coupled with prohibitive cost of treatment, particularly for developing country generated urgency for the rapid development of an novel synthetic molecule with less side effects in the amelioration of IHD, hence the present study is aimed at evaluate the cardio protective effect of pyrazolone derivatives.

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CHAPTER 3

AIMS AND OBJECTIVES

3. AIMS AND OBJECTIVES

Cardiovascular disease (CVD) results in approximately 7 million deaths per annum world-wide. Many of these result from sequelae following myocardial ischemia / reperfusion (I/R) injury. In recent years, more than 6 million people died of ischemic heart disease (IHD). It is predicted to be the leading cause of deaths in the world over in the near future, although treatments for ischemic heart disease such as recanalization therapy have progressed, reperfusion treatment often makes way for myocardial injury by increasing inflammatory responses. There is a growing body of evidence that inflammation is one of the major contributors to myocardial infarction and to ischemic reperfusion injury (IRI). Research findings are unveiling the potential role of inflammatory mediators in ischemic-reperfusion injury.

Recent developments in immunology and cell biology have demonstrated the importance of inflammation in IRI. Since available treatment for IHD is minimally effective, substantial efforts are being directed towards the discovery of new drugs for IHD. In this context, pyrazolone derivatives have been developed and proved as a strong novel free radical scavenger. Moreover, it has been shown that pyrazolone has preventive effects on myocardial injury following ischemia and reperfusion in the rat heart and in patients with acute myocardial infarction. As the literature revealed that 3-methyl pyrazol-5-one is a versatile lead molecule for synthesizing newer drug candidates, the main object of the project is designed as follows.

3.1. Synthesis of novel 3-methyl pyrazol-5-one derivatives as heterocyclic scaffold

3.2. Characterization of synthesized compounds by modern physio chemical methods

viz Melting Point , TLC and by spectroscopic methods such as UV-Visible Spectroscopy, FTIR Spectroscopy, NMR Spectroscopy, Mass Spectroscopy and elemental analysis.

3.3 Evaluation of cardio protective activities by chemical methods.

3.3.1. Estimation of Plasma lipid profile

Plasma total cholesterol (TC), total protein (TP), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoproteins (VLDL).

3.3.2. Estimation of Plasma cardiac specific injury markers

Activity levels of creatine phospokinase (CPK) lactate dehydrogenase (LDH), alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphate (ALP) in plasma.

3.3.3. Estimation of Cardiac endogenous antioxidant

Superoxide dismutase (SOD); Catalase (CAT) and reduced glutathione (GSH).

3.4. Evaluation of cardio protective activities by using Langendorff's apparatus

3.4.1. Estimation of Myocardial tissue injury markers

Malonaldehyde (MDA) and 4- Hydroxy- 2- Noneal (4-HNE).

3.5. Histopathology

Light microscopic Haematoxylin and Eosin stained sections are studied.

3.6. Analgesic and anti inflammatory activity studies

Study of analgesic and anti inflammatory activity.

3.7. Statistical analysis to verify the significance of the results

Data were analysed for statistical significance using one way analysis of variance (ANOVA) followed by Dunnets test and results are expressed as mean±S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.

CHAPTER 4

MATERIALS AND METHODS

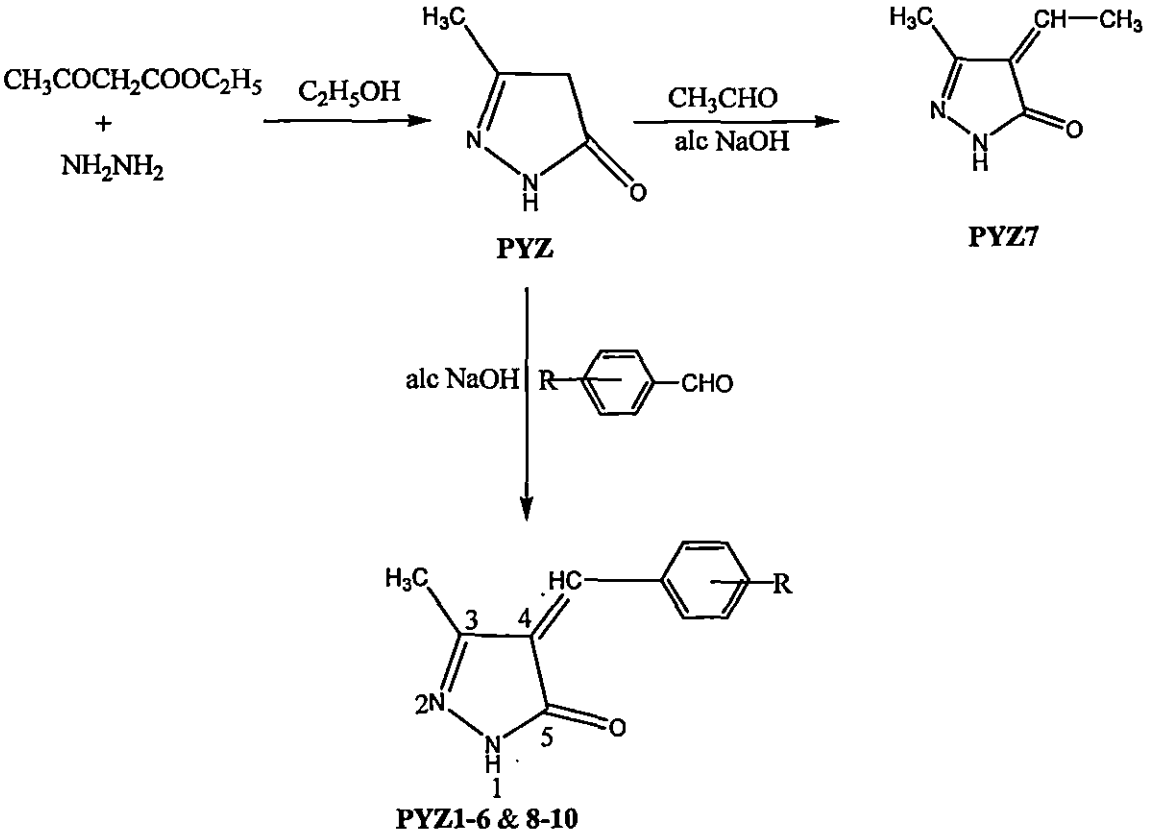
4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals used for synthesis

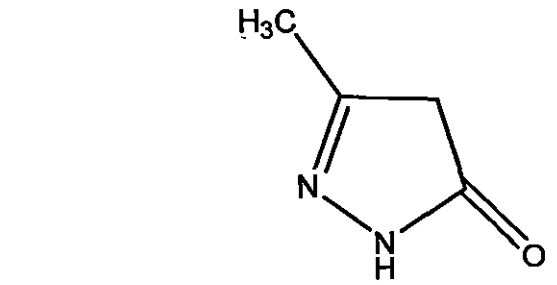
Ehyl acetoacetate, hydrazine hydrate, absolute ethanol, sodium hydroxide and various substituted aromatic aldehyde, were obtained from S. D Fine Chemicals, Mumbai, India.

4.2 Scheme of synthesis

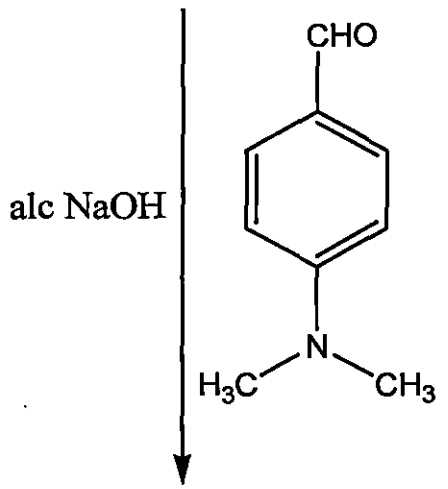


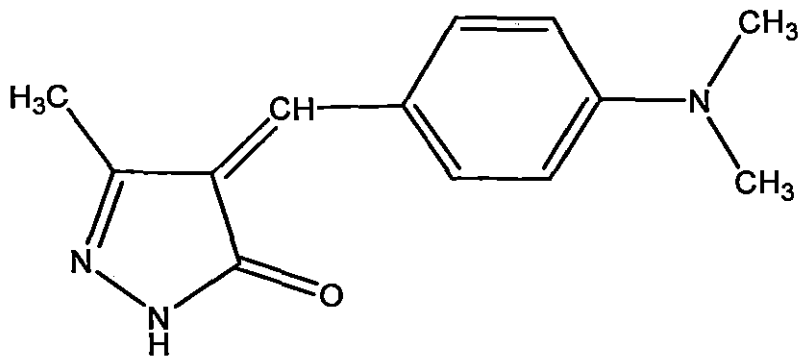
Compounds	R	Compounds	R
PYZ1	$-N(CH_3)_2$	PYZ 6	H
PYZ 2	$C_6H_5CH=CH-$	PYZ 8	4—OMe
PYZ 3	$-OCH_3(_{2',3})$	PYZ 9	3-OCH_3
PYZ 4	3-NO_2	PYZ 10	2-OH
PYZ 5	3-Cl		

4.2.1. Scheme of synthesis of 4-[4-N dimethyl aminobenzylidine] -3-methyl pyrazolin-5(4H) - one (PYZ1)



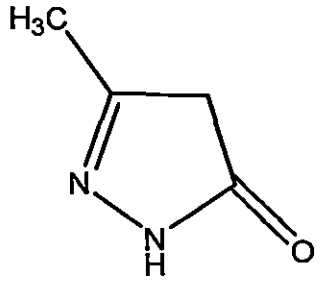
PYZ



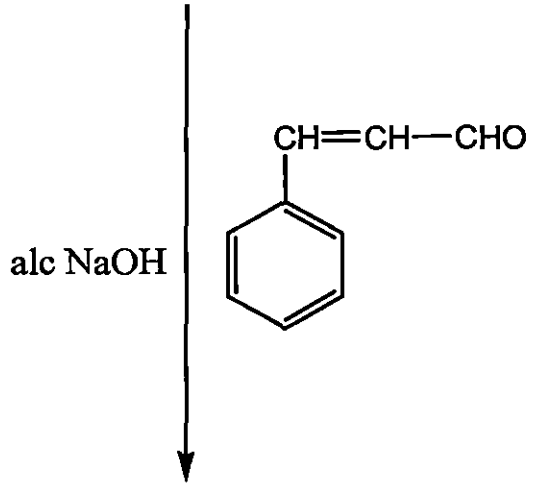


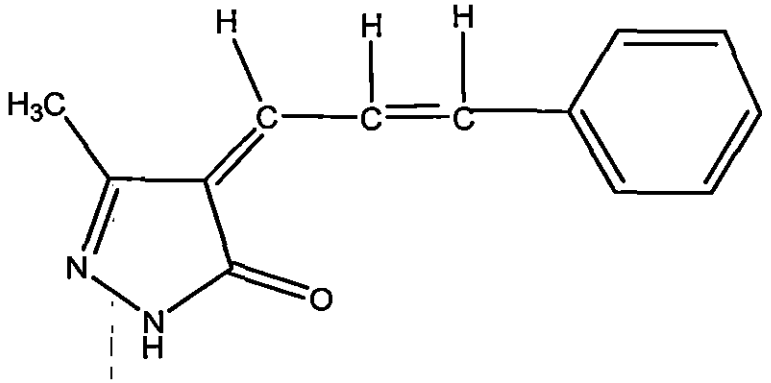
PYZ1

4.2.2 Scheme of synthesis of 4-[phenyl allylidene]-3-methyl pyrazolin-5(4H) – one (PYZ2)



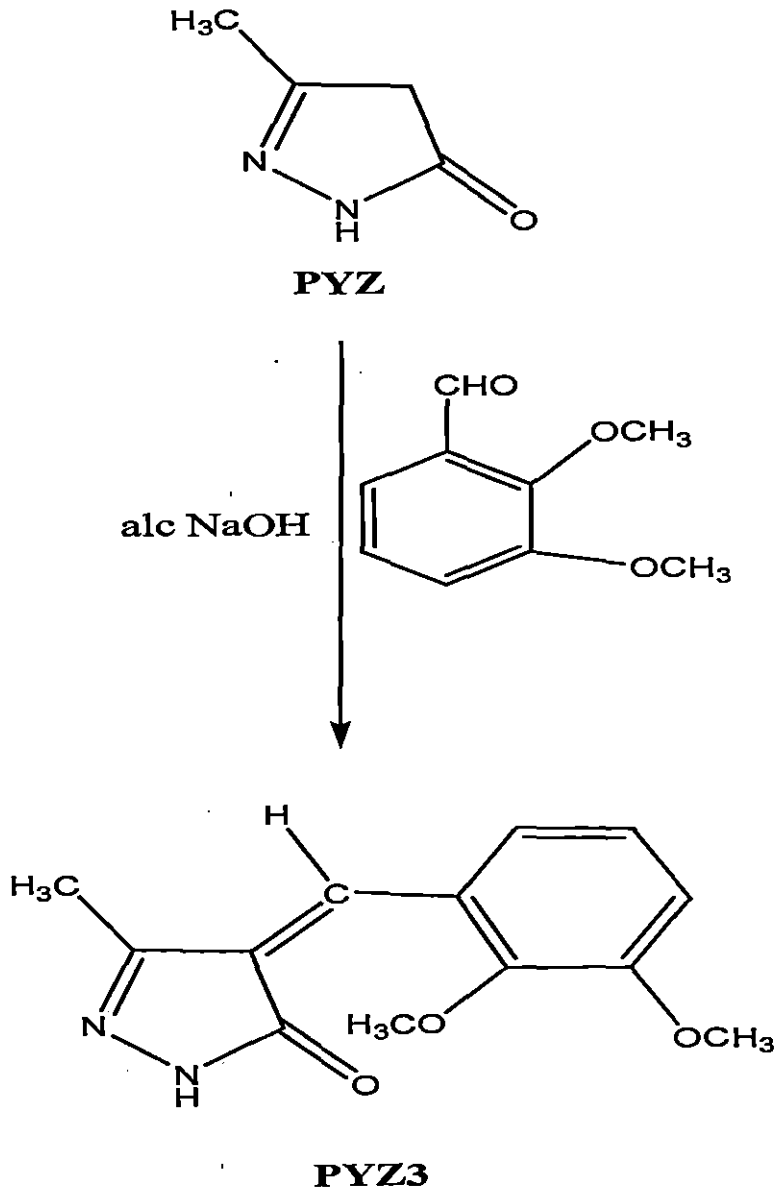
PYZ





PYZ2

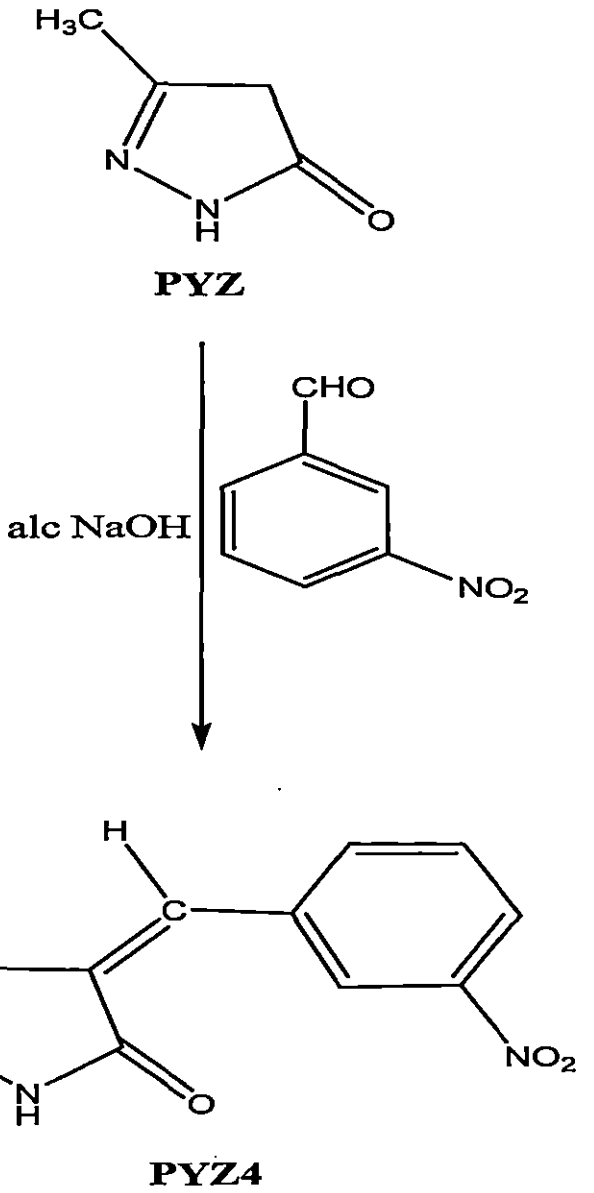
4.2.3. Scheme of synthesis of 4[2, 3- dimethoxy benzylidine]-3-methyl pyrazolin-5(4H) - one (PYZ3)



PYZ

PYZ3

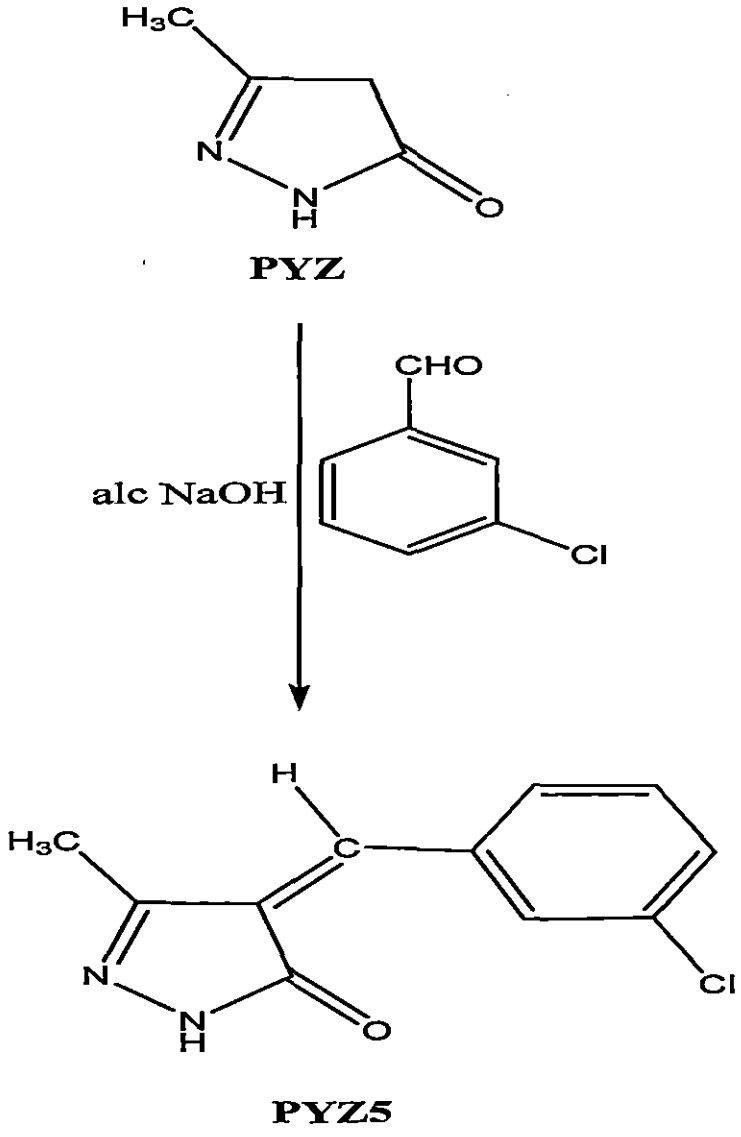
4.2.4. Scheme of synthesis of 4-[3-nitro benzylidine]-3-methyl pyrazolin-5(4H) – one (PYZ4)



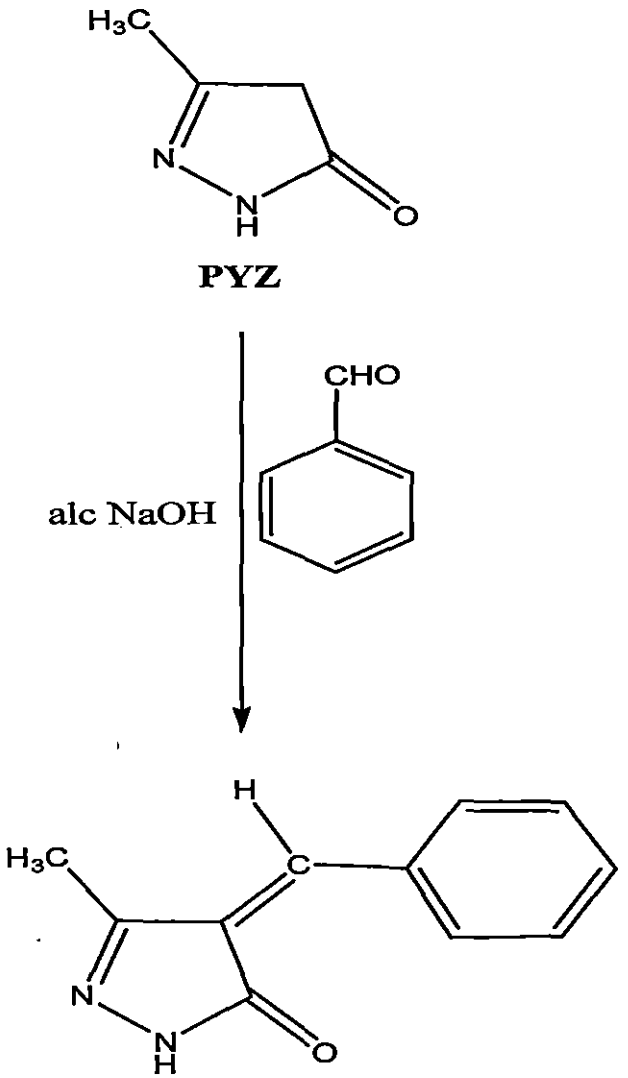
PYZ

PYZ4

4.2.5. Scheme of synthesis of 4-[3-chloro benzylidine]-3-methyl pyrazolin-5(4H) – one (PYZ5)



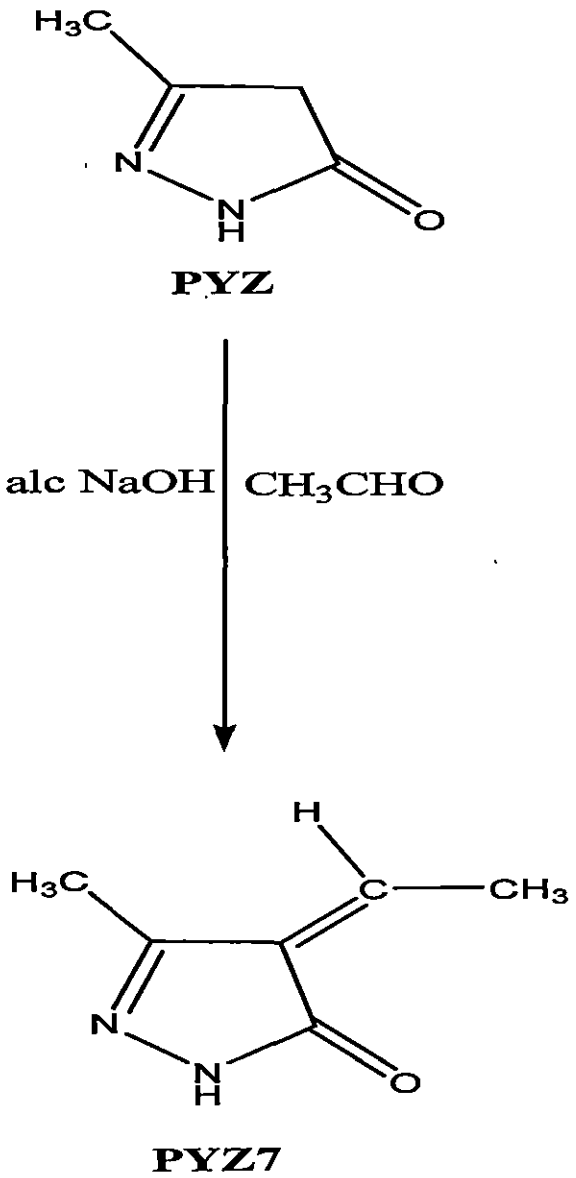
4.2.6. Scheme of synthesis of 4-[benzylidine]-3-methyl pyrazolin-5(4H)-one (PYZ6)



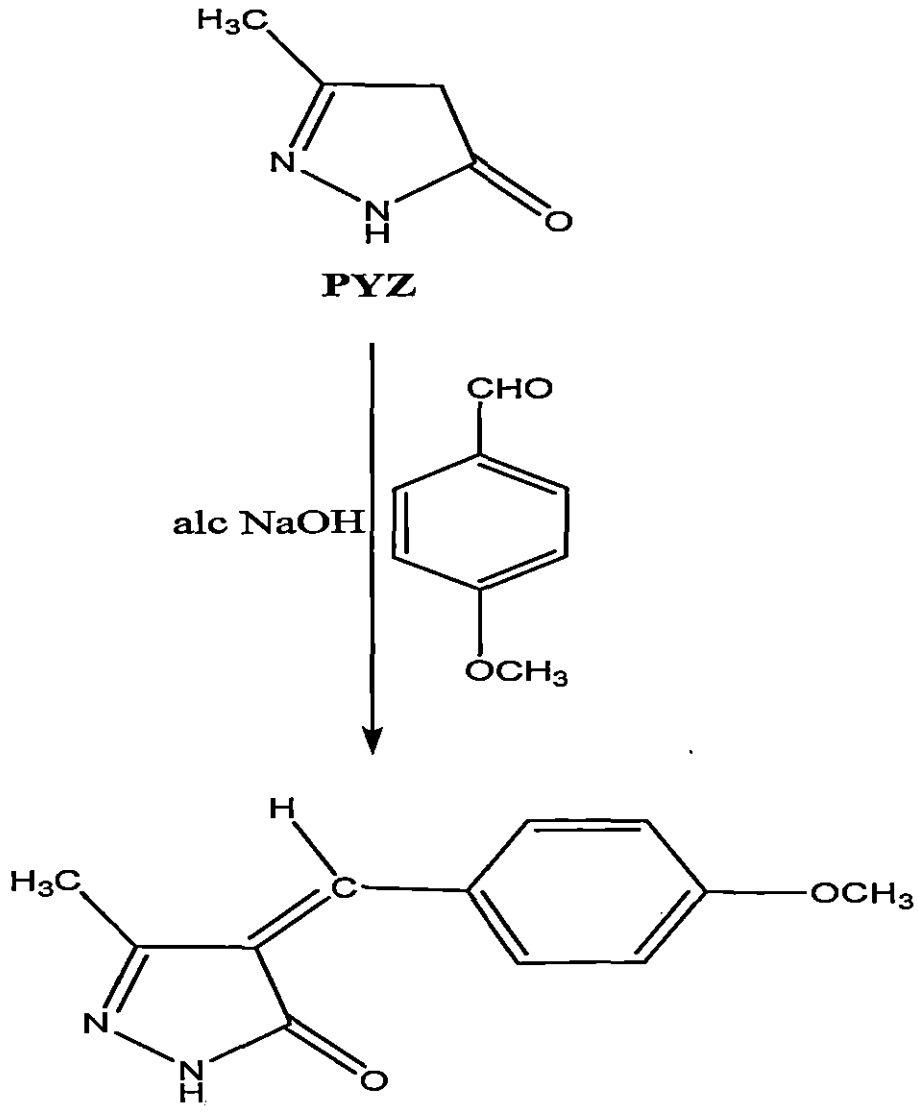
PYZ

PYZ6

4.2.7. Scheme of synthesis of 4-[acetylidine]-3-methyl pyrazolin-5(4H)- one (PYZ7)



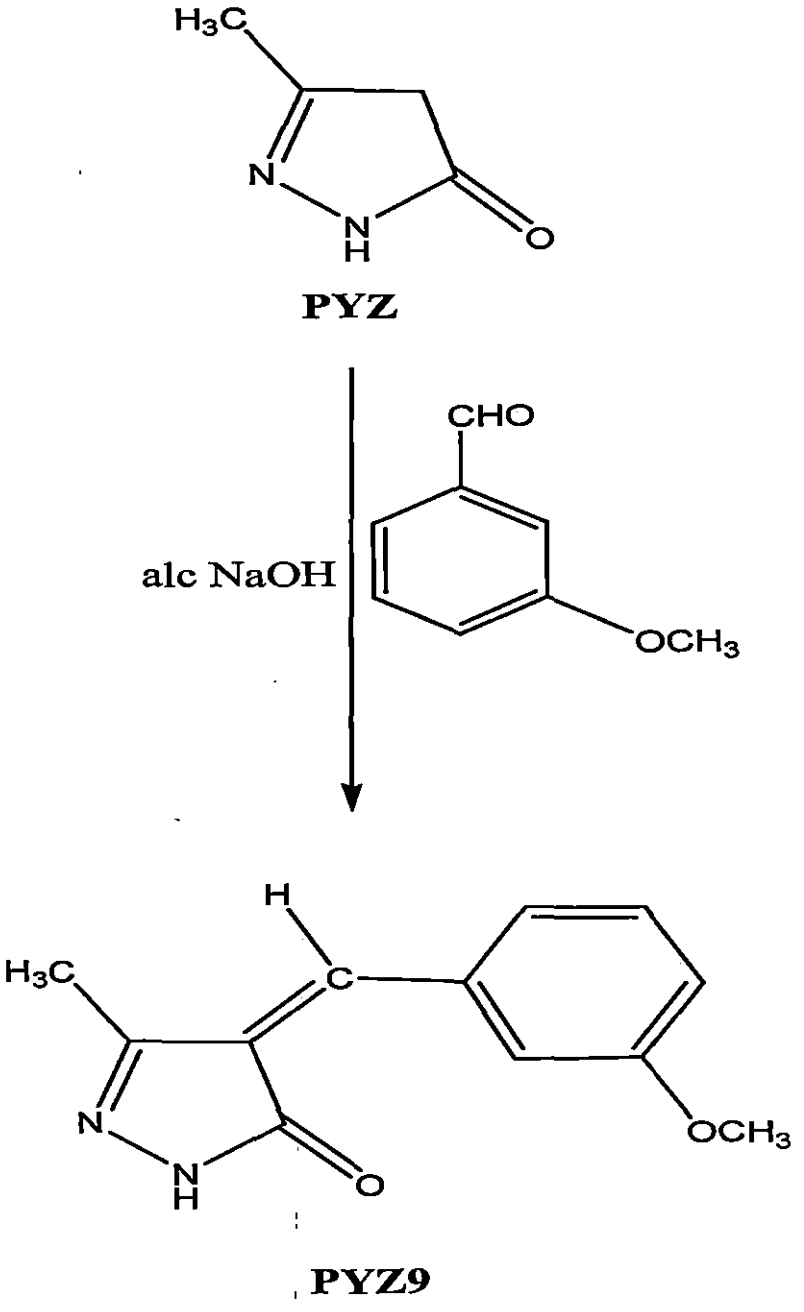
4.2.8. Scheme of synthesis of 4-[4-methoxy benzylidine]-3-methyl pyrazolin-5(4H) -one (PYZ8)



PYZ

PYZ8

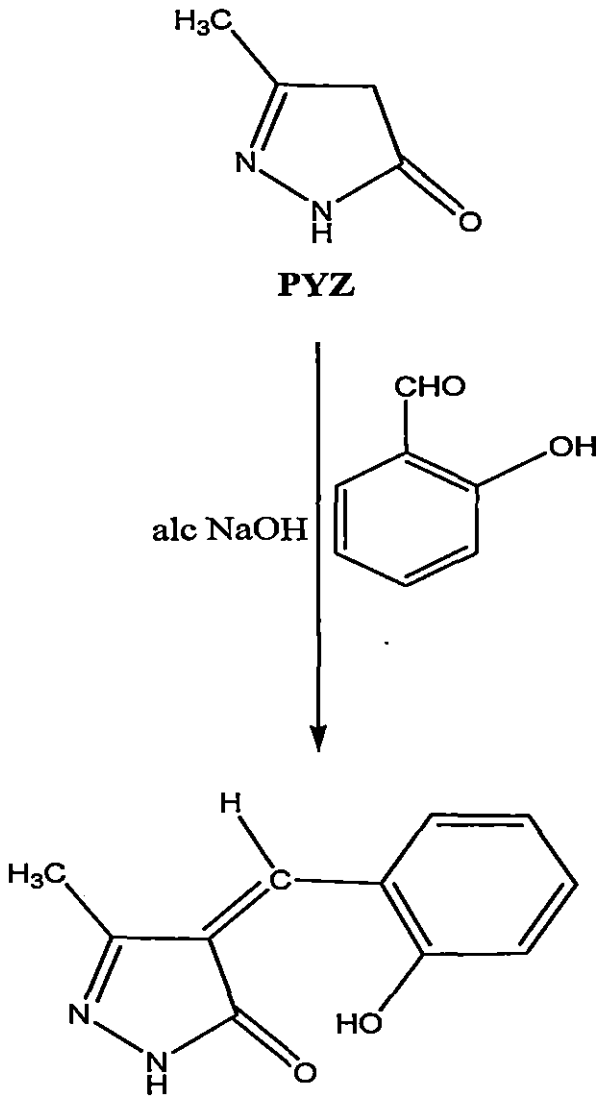
4.2.9. Scheme of synthesis of 4-[3-methoxy benzylidine]-3-methyl pyrazolin-5(4H) -one (PYZ9)



PYZ

PYZ9

4.2.10 .Scheme of synthesis of 4-[2-hydroxy benzylidine]-3-methyl pyrazolin-5(4H) – one (PYZ10)



PYZ

PYZ10

The compound code, various substituted group and I.U.P.A.C nomenclature are summarised in the following table.

Table.4.2.11. Substituted groups in the synthesized compounds

Sl No	Compound code	-R	I.U.P.A.C.name
1	PYZ1	$N(CH_3)_2$	4-[4-N dimethyl amino benzylidine]-3-methyl pyrazolin-5(4H)- one
2	PYZ2	phenyl allyl	4-[phenyl allylidene]-3-methyl pyrazolin-5(4H)- one
3	PYZ3	2-OCH_3 3- OCH_3	4-[2,3- dimethoxy benzylidine]-3-methyl pyrazolin-5(4H)- one
4	PYZ4	3-NO_2	4-[3-nitro benzylidine]-3-methyl pyrazolin-5(4H)- one
5	PYZ5	3-Cl	4-[3-chloro benzylidine]-3-methyl pyrazolin-5(4H)- one
6	PYZ6	-H	4-[benzylidine]-3-methyl pyrazolin-5(4H)- one
7	PYZ7	-CH_3	4-[acetylidine]-3-methyl pyrazolin-5(4H)- one
8	PYZ8	4-OCH_3	4-[4-methoxy benzylidine]-3-methyl pyrazolin-5(4H)- one
9	PYZ9	3-OCH_3	4-[3-methoxy benzylidine]-3-methyl pyrazolin-5(4H)- one
10	PYZ10	2-OH	4-[2-hydroxy benzylidine]-3-methyl pyrazoline-5(4H)- one

4.3. Methods

4.3.1. Synthetic procedure

4.3.1.1. Preparation of 3-methyl pyrazol-5-one (PYZ)

65g (0.5mol) of freshly distilled ethylacetoacetate was taken in a 250ml two necked round bottomed flask fitted with reflux condenser and was stirred with magnetic stirrer. 25g (0.5mol) of hydrazine hydrate in 40ml absolute ethanol was added drop wise from a dropping funnel. The temperature of the reaction mixture was maintained at about 60°C. A crystalline deposit was separated after stirring for 1 hour and then the reaction mixture was cooled in an ice bath to complete the crystallization. The crystals were separated by filtration under suction pump and dried in air. The crystals were recrystallised from alcohol. m.p 222°C, yield 42gm (89%).

4.3.1.2. Synthesis of 4-[4-N,N dimethyl aminobenzylidine]-3-methyl pyrazolin-5-one (PYZ1)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of N, N-dimethyl amino benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Dark Brick Red powder , mp 236-238 °C, %yield 65%, TLC (Ethanol & Water 1:1) R_f 0.91, λmax(nm) ethanol 466 IR (KBr, cm^{-1}): 3147(N-H), 1666(C=O), 1616(C=C), 2829[C–H(CH_3)], 1367 (C=N), 974, 819, 769, 516 (C–H Ar) ^{1}H NMR ($CDCl_3$): δ 8.25 (s, 1H, NH), 6.80-8.61 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.11 (s, 6H, $N(CH_3)_2$), 1.93 (s, 3H, CH_3). MS : *m/z 229,* , $[M]^{+\cdot}$, *230* $[M+1]^{+\cdot}$, *231* $[M+2]^+$ Anal Calcd for $C_{13}H_{15}N_3O$: C, 68.12 ;H, 6.55 ;N, 18.34 ;Found: C68.01, ;H6.25, ;N 18.00.

4.3.1.3. Synthesis of 4-[phenyl allylidene]-3-methyl pyrazolin-5- one (PYZ2)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of cinnamaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Dark yellow powder, mp 168-170°C, % yield 72 % , TLC (Ethanol & Water 1:1) R_f 0.85, λmax (nm) ethanol 268 IR (KBr, cm^{-1}): 2953 (N-H str.), 3070 (C-H str., Ar-H), 2833 (C-H str., CH_3), 1600 (C=O str.), 1364 (C=N str.); ^1H NMR ($CDCl_3$): δ 8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, $N(CH_3)_2$), 1.93 (s, 3H, CH_3). MS : *m/z* 212 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 213 Anal Calcd for $C_{13}H_{12}N_2O$: C, 73.58 ;H, 5.66 ;N, 13.20 ;Found: C 73.05, ;H 5.01, ;N 13.21,.

4.3.1.4. Synthesis of 4-[2,3-dimethoxy benzylidine]-3-methyl pyrazolin-5-one (PYZ3)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of 3, 4 dimethoxy benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

White crystalline powder, mp 208-210°C, % yield 68%, TLC (Ethanol& Water 1:1) R_f 0.92, λmax (nm) ethanol 231 IR (KBr, cm^{-1}): 3196 (N-H str.), 3070 (C-H str., Ar-H), 2937 (C-H str., CH_3), 1585 (C=O str.), 1363 (C=N str.); ^1H NMR ($CDCl_3$): δ 8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, $N(CH_3)_2$), 1.93 (s, 3H,

CH_3). MS : *m/z* 246 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 247.83 Anal Calcd for $C_{13}H_{14}N_2O_3$: C, 63.41; H, 5.69; N, 11.38; Found: C, 62.98; H 5.56, N 11.23.

4.3.1.5. Synthesis of 4-[3-nitro benzylidine]-3-methyl pyrazolin-5-one (PYZ4)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of 3- nitro benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Pale yellow coloured powder, mp 180-182°C, % yield 56 % , TLC (Ethanol & Water 1:1) R_f 0.94, λmax (nm) ethanol 253 : IR (KBr, cm^{-1}): 2831 (N-H str.), 3070 (C-H str., Ar-H), 2715 (C-H str., CH_3), 1631 (C=O str.), 1635 (C=N str.); ^1H NMR ($CDCl_3$): δ 8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, $N(CH_3)_2$), 1.93 (s, 3H, CH_3). MS : *m/z* 231 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 232 Anal Calcd for $C_{11}H_9N_3O_3$: C, 57.14; H, 3.89; N, 18.18; Found: C 56.91, ;H 3.65, ;N 18.00,

4.3.1.6. Synthesis of 4-[3-chloro benzylidine]-3-methyl pyrazolin-5-one (PYZ5)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of 3- chloro benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Pale pinkish white powder, mp 216-218°C, % yield 84 % , TLC (Ethanol & Water 1:1) R_f 0.87, λmax (nm) ethanol 300: IR (KBr, cm^{-1}): 3070 (N-H str.), (C-H str., Ar-H), 2715 (C-H str., CH_3), 1697 (C=O str.), 1363 (C=N str.); ^1H NMR ($CDCl_3$): δ 8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, $N(CH_3)_2$), 1.93 (s, 3H, CH_3). MS : *m/z* 221 $[M]^+$ Anal Calcd for $C_{11}H_9N_2OCl$: C, 59.73; H, 4.07; N, 12.66; Found: C 59.38; H, 4.57 ;N, 12.98.

4.3.1.7. Synthesis of 4-[benzylidine]-3-methyl pyrazolin-5- one (PYZ6)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Pale yellow white powder, mp 240-242°C, % yield 61 % , TLC (Ethanol & Water 1:1) R_f 0.89, λmax (nm) ethanol 249: IR (KBr, cm^{-1}): 3176 (N-H str.), 3070 (C-H str., Ar-H), 2831 (C-H str., CH_3), 1604 (C=O str.), 1491 (C=N str.); ^{13}C NMR ($CDCl_3$): δ 18.36 (CH_3), 48.4-48.99 (C3 of pyrazolone), 49.27-49.84 (C4 of pyrazolone), 58.32 (C5), 143 (olefinic C), 126-129 (C Ar). MS : *m/z* 186 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 187, $[M+2]^{+\cdot}$, 188, Anal Calcd for $C_{11}H_{10}N_2O$: C, 70.96; H, 5.37; N, 15.04; Found: C 70.86, ;H 5.65, ;N 15.14,

4.3.1.8. Synthesis of 4-[acetylidine]-3-methyl pyrazolin-5-one (PYZ7)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of acetaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during

which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

white powder, mp 258-260°C, % yield 75 % ,TLC (Ethanol &Water 1:1) R_f 0.88, λmax (nm) ethanol 437: IR (KBr, cm^{-1}): 3352 (N-H str.), 3070 (C-H str., Ar-H), 2715 (C-H str., CH_3), 1610 (C=O str.), 1452 (C=N str.); ^{13}C NMR ($CDCl_3$): δ 48.14 (CH_3), 48.42 (C3 of pyrazolone), 48.76 (C4 of pyrazolone), 48.99 (C5), 49.27(olefinic C), 49.56-49.84 (C6 of pyrazolone). MS : *m/z* 124 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 125, $[M+2]^{+\cdot}$, 126, Anal Calcd for $C_6H_8N_2O$: C, 56.25; H, 6.25; N, 15.05; Found: C 56.04, ;H 6.12, ;N 15.68,

4.3.1.9. Synthesis of 4-[4-methoxy benzylidine]-3-methyl pyrazolin-5- one (PYZ8)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of 4-methoxy benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Yellow powder, mp 258-260°C, % yield 66 %, TLC (Ethanol &Water 1:1) R_f 0.96, λmax (nm) ethanol 284: IR (KBr, cm^{-1}): 3070 (N-H str.), 2883 (C-H str., Ar-H), 2713 (C-H str., CH_3), 1680 (C=O str.), 1510 (C=N str.). ^{13}C NMR ($CDCl_3$): δ 10.63 (CH_3), 48.5-48.71 (C3 of pyrazolone), 49.00-49.57 (C4 of pyrazolone), 33.41 (C5), 128-133 (olefinic C), 55.62-56.20 (OMe), 144-145 (C Ar). MS : *m/z* 216 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 217 Anal Calcd for $C_{12}H_{12}N_2O_2$: C, 66.65; H, 5.55; N, 12.96; Found: C 66.10, ;H 5.14, ;N 12.46.

4.3.1.10. Synthesis of 4-[3-methoxy benzylidine]-3-methyl pyrazolin-5-one (PYZ9)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02

moles of 3- methoxy benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Pale orange powder, mp 170-172°C, % yield 57 % , TLC (Ethanol & Water 1:1) R_f 0.83, λmax (nm) ethanol 314: IR (KBr, cm^{-1}): 3070 (N-H str.), 2883 (C-H str., Ar-H), 2715 (C-H str., CH_3), 1600 (C=O str.), 1489(C=N str.); ^{13}C NMR ($CDCl_3$): δ 10.36 (CH_3), 48.15-48.72 (C3 of pyrazolone), 49.00-49.57 (C4 of pyrazolone), 34.17 (C5), 142.83-145.16 (olefinic C), 55.51-55.60 (OMe), 112-130 (C Ar). MS : *m/z* 216 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 217, $[M+2]^{+\cdot}$, 218. Anal Calcd for $C_{12}H_{12}N_2O_2$: C, 66.65; H, 5.55; N, 12.96; Found: C 66.47, ;H 5.68, ;N 12.86,

4.3.1.11. Synthesis of 4-[2-hydroxy benzylidine]-3-methyl pyrazolin-5-one (PYZ10)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of 2-hydroxy benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

orange powder, mp 212-214°C, % yield 79 %, TLC (Ethanol & Water (1:1) R_f 0.82, λmax (nm) ethanol 249: IR (KBr, cm^{-1}): 3070 (N-H str.), 2831 (C-H str., Ar-H), 2362 (C-H str., CH_3), 1511 (C=O str.), 1604 (C=N str.); ^{13}C NMR ($CDCl_3$): δ 10.84 (CH_3), 48.15-48.71 (C3 of pyrazolone), 49.00-49.48 (C4 of pyrazolone), 28.90 (C5), 105.79 (olefinic C), 115.60-130.83 (C Ar). MS : *m/z* 202 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 203, $[M+2]^{+\cdot}$, 204 Anal Calcd for $C_{11}H_{10}N_2O_2$: C, 65.34; H, 4.95; N, 13.85; Found: C 64.90; H, 4.48; N, 13.44.

4.4. Pharmacological studies

4.4.1. Animals

Swiss albino mice (CF-1 strain or Swiss (18-25 g) and rats (Sprague-Dawley, 100-150 g) were used as an experimental animals. They were housed under standard conditions of temperature (24±1°C), relative humidity (65±10%) and 12 light/dark cycle environment. During the study period, guidelines of CPCSEA (IAEC) were followed for the maintenance of animals and the experimental protocol was approved by Institutional Animals Ethics Committee (IAEC) No: HPI/09/60/IAEC/0075.

4.4.2. Chemicals and drugs

Isoproterenol hydrochloride (ISO) was purchased from (Sigma Chemical Co. St. Louis, MO, USA). Sodium chloride, magnesium chloride, adenosine triphosphate and trichloroacetic acid (TCA) were obtained from (Himedia Laboratories Private Ltd. Mumbai, India). Nitroblue tetrazolium (NBT), phenazine methosulphate and nicotinamide adenine dinucleotide (NAD) were purchased from Sisco Research Laboratories Ltd. Mumbai, India. Thiobarbituric acid (TBA), 2, 4-dinitrophenyl hydrazine (DNPH), 1, 1, 3, 3-tetramethoxy propane (TMP), 5, 5 dithiobis 2-nitrobenzoic acid (DTNB) and 4-HNE (4- Hydroxy- 2- Nonenal) were obtained from Sigma Chemicals, USA. The other chemicals used in this study were of analytical grade unless otherwise stated.

4.4.3. Test compounds

Pyrazolone derivatives investigated in the present study were synthesized and characterized in Department of Pharmaceutical Chemistry, Himalayan Pharmacy Institute, Sikkim, India.

4.4.4. Biochemical diagnostic Kits

The diagnostics kit of Total Cholesterol, Total Protein, HDL, VLDL, LDL, TG, AST (SGOT), and ALT (SGPT) and were purchased from Span Diagnostics Ltd., Surat, India.

4.4.5. Acute toxicity study (Determination of LD_{50})

It is conducted to determine the median lethal dose (LD_{50}) i.e. the dose which will kill 50% of the animals of a particular species. In addition, such studies may also indicate the probable target organ of the chemical and its specific toxic effect. It provides guidance on the doses to be used in more prolonged studies.

Up and Down Method by Organization for Economic Co-operation and Development (OECD) guidelines for the testing of chemicals are periodically reviewed in the light of scientific progress or changing assessment practices. The concept of up and down testing approach was first described by Dixon and Mood. In 1985; Bruce proposed to use Up and Down procedure (UDP) for the determination of acute toxicity of chemicals. There exist several variations of the Up and Down experimental design for estimating an LD_{50}.The method permits estimation of LD_{50} with a confidence interval and the result allow a substance to be ranked and classified according to the Globally Harmonised system for the classification of chemicals, which cause acute toxicity. The method is easiest to apply to materials that produce death within one or two days. The method would not be practical to use when considerably delayed death (five days or more) can be expected. A limit test can be used efficiently to identify chemicals that are likely to have low toxicity.

The limit test is a sequential test that uses a maximum of 5 animals. A test dose of up to 2000, or exceptionally 5000mg/kg may be used. The procedure for testing at 2000 and 5000mg/kg are slightly different. The main test consists of single ordered dose progression in which animals are dosed, one at a time, at 48 hour intervals. The first animal receives a dose a step below the level of the best estimate of the LD_{50}. If the animal survives, the dose for the next animal is increased to a factor of 3/2 of original dose; if it dies, the dose for the next animal is decreased by a similar dose progression. Each animal should be observed carefully for up to 48 hours before making decision on whether and how much to dose the next animal. Dosing is stopped when one of these criteria is satisfied, at which time an estimate of LD_{50} and a confidence interval are calculated for the test based on the status of all animals at termination.

4.4.6. Experimental Animals

Fifty (50) adult male albino rats (10 groups, 5 in each) 9 - 10 weeks old, weighing between 84 and 200 g were procured from the laboratory animal house of the Himalayan Pharmacy Institute. The rats were kept in metal cage, fed with commercial standard growers feed and water *ad libitum* and maintained under standard laboratory condition prior to the procedure. During the study period, guidelines of CPCSEA (IAEC) were followed for the maintenance of animals and the experimental protocol was approved by Institutional Animals Ethics Committee (IAEC) No: HPI/09/60/IAEC/0075.

4.4.7. Method of Acute toxicity study

The limit dose test, up and down procedure as revised by Dixon (1965, 1991) was used to evaluate the acute oral toxicity of Pyrazolone derivatives in adult male rats. Five (5) adult male rats were randomly selected for the experiment. They were marked and housed individually in cages in the laboratory for 7 days to allow for acclimatization to the laboratory conditions. The rats were fasted overnight but allowed free access to water prior to dosing on each occasion. A rat from Group I was picked, weighed and dosed orally with a limit dose 2000 mg/kg body weight of the freshly prepared pyrazolone derivative (PYZ1). Another animal from the same group was given the same dose of the pyrazolone derivative (PYZ1) until all the animals in the group were fed with the same dose of PYZ1.

Each animal was observed each time for instant death and then watched for the successive 24 h for the short-term outcome and finally for the next 24 hours for any sign toxic effects. Similarly all the groups were tested with rest of the pyrazolone derivatives. But after 48 hours, there was no death on rats due to any derivative. Hence the same procedure was repeated with 5000 mg/kg b.w. of all the pyrazolone derivatives. All the animals were observed for next 48 hours for any sign of toxic effects.

4.4.9. Experimental Procedure for Cardio Protective Study by Chemical Methods

Seventy two male rats were completely randomized into twelve groups of six animals in each group. Group 1: normal control (distilled water p.o.), Group 2: ISO-treated (5.5 mg and 8.5 mg/ kg, s.c.) at an interval of 24 h for two days. Groups 3 to 12 were administered with 10 mg/kg body weight/ day p.o. of pyrazolone derivatives (PYZ1-PYZ10) for 5 days followed by ISO treatment at an interval of 24 h for two days. 24 hours after the second injection of ISO, the rats were sacrificed by ether anaesthetization and the heart was dissected out. The neck area was quickly cleared of fur to expose the jugular vein. The vein, after being slightly displaced, was sharply cut with sterile surgical blade and an aliquot (5 ml) of the blood was collected and centrifuged at 10000 rpm for 5 minutes. The serum was carefully aspirated with a Pasteur pipette into sample bottles for biochemical analysis.

Blood (2ml) was aspirated from the left ventricle, collected in a heparinised vial, centrifuged at 1000 rpm for 30 min and the plasma stored at -20 °C for estimation of plasma LDH, total cholesterol, triglycerides, total protein and creatinine kinase using the

ready to use kits supplied by Span Diagnostics Ltd., Kolkata, India. The heart was removed and stored in liquid nitrogen for biochemical estimation. The heart was washed with ice-cold saline, and the homogenate prepared in 0.1M Tris-HCl buffer (pH 7.4). The homogenate was centrifuged and the supernatant was used for the assay of superoxide dismutase (SOD) and catalase (CAT).

4.4.9.1. Estimation of reduced glutathione (GSH)

Reduced glutathione (GSH) was determined by the method of (Ellman, 1959). To 0.1ml of tissue homogenate 2.4 ml of 0.02M EDTA solution was added and kept on ice bath for 10 min. Then 2 ml of distilled water and 0.5 ml of 50% TCA were added. This mixture was kept on ice for 10-15 min and then centrifuged at 3000 rpm for 15 min. 1 ml of supernatant was taken and 2 ml of Tris-HCl buffer was added. Then 0.05 ml of 5, 5 dithiobis 2-nitrobenzoic acid (DTNB) solution (Ellman's reagent) was added and vortexed thoroughly. The absorbance was read within 2-3min after the addition of DTNB at 412 nm against a reagent blank. The Absorbance values were compared with standard curve, generated from known GSH. The amount of GSH was expressed as µg/mg wet tissue.

4.4.9.2. Estimation of superoxide dismutase (SOD)

SOD activity was estimated by the method described by (Kakkar et al., 1984). The reaction mixture contains 0.1ml of supernatant, 1.2ml of sodium pyrophosphate buffer(pH 8.3, 0.052M), 0.1ml of phenazine methosulphate (186µM), 0.3ml of nitro blue tetrazolium, 300µM, 0.2ml of NADH(Nicotinamide adenine dinucleotide reduced disodium salt, 750µM). The reaction was started by addition of NADH. After incubation at 30^0C for 90 sec, the reaction was stopped by the addition of 0.1ml of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0ml of n-butanol. The mixture was allowed to stand for 10 min, centrifuged and the butanol layer was separated. The colour intensity of the chromogen in the butanol was measured at 560nm by spectrophotometrically and the concentration of SOD was expressed as units/mg of protein.

4.4.9. 3. Estimation of catalase (CAT)

Catalase activity was assayed by the method of Beers and Sizer 1952. The catalase activity was assayed by adding 0.1ml of homogenates to 1.4ml of freshly prepared 13.2 mM H_2O_2 IN 0.05 M K_2HPO_4 (pH 7.0)(0.15 ml of 30% H_2O_2 per 100 ml). Procedures in which the H_2O_2 was added last to initiate the reaction caused a loss of enzyme activity. The solution was mixed, and a loss of absorbance was determined at 240 nm by using UV-spectrophotometer for 1 to 3 min. The units of catalase were calculated as IU/min/mg of tissue.

4.4.9. 5. Estimation of total protein

Protein concentration was estimated according to the method of Lowery *et al.*, 1951. Standard dilution of bovine serum albumin of 25, 50, 75 and 100µg/ml were prepared and appropriate serial dilution of the serum sample to be measured were also prepared. 1.0ml of each of the above was placed in to separate tubes. 100µl of 72% w/v trichloroacetic acid was added to each tube. The tubes were centrifuged for 15 minutes at 3000 rpm and the supernatant were discarded. Then 1ml of water was added to each tube to dissolve the pellet. 1.0ml of water was also added to a new tube (including blank), vortexed and allowed to set for 10min. After that, 500µl Folin-ciocalteu was added to each tube, vortexed and allowed to set for 30 minutes and read spectrophotometrically at 750 nm. Data were expressed as mg/ml.

4.4.9. 6. Estimation of lactate dehydrogenase (LDH)

Lactate dehydrogenase activity was estimated in serum by the method of Tietz, 1977. The method uses NADH and pyruvate as substrates. To 10µL of the sample, 1000µl of the reagent containing substrate was added and the coloured complex was measured spectrophotometrically at 340nm. Data were expressed as IU/L.

4.4.9. 7. Estimation of total cholesterol

Total cholesterol was estimated by the method of Wybenga *et al.*, 1970. To 10µL of the serum sample, 1000µL of the reagent containing ferric perchlorate, ethyl acetate and sulphuric acid was added incubated for 10 min at 37^0C to form a colored complex which was measured spectrophotometric ally at 540nm. Data were expressed as mg/dL.

4.4.9.8. Estimation of total triglycerides

Total triglycerides were estimated by the method of Trinder, 1969. To 10µl of the serum sample, 1000µL of the working reagent containing the enzyme was added and incubated for 10 min to form a coloured complex which was measured spectrophotometrically at 505 nm. Data were expressed as mg/dL.

4.4.9.9. Estimation of creatine kinase

Creatine kinase was estimated by the method of Szasz, 1978. To 50 µL of the serum sample, 1050 µL of the working reagent containing the antibody was added. The rate of NADH formation was measured at 340 nm. CK activity was expressed as U/L.

4.4.9.10. Estimation of SGPT and SGOT

Serum transaminase (SGPT and SGOT) was estimated by the method of Reitman and Frankel, 1957. Each Substrate (0.5 ml) [either α L-alanine (200mM) or L-aspartate (200mM)] with 2mM α-ketoglutarate] was incubated for 5 min at 37°C in a water bath. Serum (0.1 ml) was then added and the volume was adjusted to 1ml with sodium phosphate buffer (pH 7.4). The reaction mixture was incubated for exactly 30 min and 60 min for SGPT and SGOT, respectively. Then to the reaction mixture, 0.5 ml 2, 4- dinitro phenyl hydrazine 1 mM was added and left for another 30 min at room temperature. Finally the colour developed by addition of 5ml NaOH (0.4N) and the colour complex formed was read at 505 nm. Data were expressed as U/L.

4.4.9. 11. Estimation of ALT

Serum alkaline phosphatase (ALT) was determined by the method of Kind and King, 1954. The buffered substrate pH 10 (reagent1) was prepared by the following method: 1.09 g of disodium phenyl phosphate was dissolved in water and the volume was made up to 500 ml. It was heated up to the point of boiling and cooled. Little amount of chloroform was added and kept in refrigerator. This was designated as solution I.

8.18 g of anhydrous sodium carbonate and 1.69 mg of sodium bicarbonate were dissolved in water and volume made up to 500ml .This was designated as solution II.

Finally equal volume of solution I and II were mixed and pH adjusted to 10 to get reagent 1. Four test tubes were taken for control, blank, standard and test samples. In each of the test tube, 0.5ml of reagent 1 was taken. In blank test tube 1.55ml distilled water was added and in rest of the tubes 1.5 ml of water was added, shaken well for

uniform mixing and incubated for 3 min at 37°C. Then 0.05 ml serum was added to the experimental test tube and 0.05 ml of phenol standard 10% mg (reagent 3) was added to the standard test tube. These were mixed well and incubated for 15 min at 37°C.Then 1ml p-Nitrophenyl phosphate 10 µM (reagent-2) was added to each of the test tubes. Lastly, 0.05 ml of serum was added to the control tube. The content of the tubes were mixed well and optical density of the samples were read at 510nm. Data were expressed as U/L.

4.4.10. *In vitro* antioxidant activity of pyrazolone derivatives

4.4.10.1. *In vitro* myocardial ischemic-reperfusion injury by using Langendorff's apparatus

Seventy two male rats were completely randomized into twelve groups of six animals in each group. Group 1: normal control (K-H buffer only), Group 2: Ischemia induced control. Groups 3 to 12 were administered with 10 mg/kg body weight/ subjected to 5 min flow + 9 min. no-flow + 12 min. reperfusion. After 48 hours the rats were anaesthetized with ether, the chest opened and the heart along with one cm of ascending aorta attached was quickly removed and dipped in ice-cold saline. The hearts were then mounted on Langendorff's apparatus and perfused with Krebs's Hensleit(K-H) buffer (NaCl (120 mM), $NaHCO_3$(25 mM), $MgSO_4$(1.2 mM), KH_2PO_4 (1.2 mM), $CaCl_2$(1.2 mM), and glucose (11 mM) at a constant pressure of 60-70mm Hg at 37 °C, and aerated with a mixture of O_2 (95%) and CO_2 (5%). Following an initial period of 5 min of stabilization, the flow was stopped for 9 min (ischemia) followed by perfusion with K-H buffer for 12 minutes (reperfusion). Then the heart was removed from the apparatus and subjected for the biochemical estimation of TBARS and 4-HNE.

4.4.10.2. Estimation of Thiobarbituric acid reactive substances (TBARS)

TBARS activity in the myocardium was determined by a modified version of the method described by Okhawa *et.al.* 1979. Hearts were homogenized in 10% trichloroacetic acid at 4°C. 0.2 ml homogenate was pippeted in to a test tube followed by the addition of 0.2 ml of 8.1% sodiumdodecyl sulphate (SDS), 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA. All tubes were boiled for 60min at 90°C and then cooled on ice. 1.0 ml double distilled water and 5ml of n-Butanol: pyridine (15:1v/v) mixture was added to the tubes and centrifuged at 4000 rpm for 10 minutes. The absorbance of developed colour

in organic layer was measured at 532 nm. TBARS activity was determined, from the standard curve of TBA adduct formation when various concentration of commercially available 1, 1, 3, 3-tetramethoxypropane. The concentration of malonaldehyde (MDA) was expressed in nM.

4.4.10.3. Estimation of 4- Hydroxy- 2- Nonenal (4-HNE) (Esterbauer et al., 1941)

The heart homogenate was prepared as per the procedure mentioned above. 2ml of filtrate was taken in a 13x100mm glass tube with cap. 1ml of DNPH was added to all the tubes containing heart homogenate, mixed thoroughly and set aside for 1h to react with 4-HNE. The formed adduct of 4-HNE and DNPH was extracted by hexane, which was evaporated under argon at 40°C. After cooling, 2ml of methanol was added to all the samples and the absorbance was measured at 350 nm in the spectrophotometer. The quantity of 4-HNE was calculated by linear regression analysis. The concentration of 4-HNE present in myocardial tissues was expressed in nM.

4.4.10. 4. Analgesic activity study

It was measured (D'Amour and Smith, 1941). The tips of tail of animals were individually placed on radiant heat source at constant temperature 55±1°C and the reaction of the animals, like flicking of the tail was noted. Male albino rats of 12 groups (6 in each group) were taken for study. First group was kept as control, second as standard and rest as test groups for different synthesized compounds. Test drugs **1-10** at a dose of 100 mg/kg p.o were administered. Diclofenac 10 mg/kg p.o was used as standard drug. The tail-flick latency was assessed by analgesiometer (Techno, India). Basal reaction time to radiant heat was taken for rat. The rat which responded within 2-3 seconds only considered for studies. After administration, the reaction time was noted at 1h, 2h, 3h and 4 h time interval of the above mentioned groups. The cut-off reaction time was fixed at 10 sec to avoid tissue damage. The observations were made and data obtained were statistically analyzed and results are summarized in table 4.9.

4.4.10.5. Anti-inflammatory activity study

It was studied by inducing paw edema (Winter et al., 1962). Male albino rats of 12 groups (6 in each group) were taken for study. Group one was kept as control, group two was treated with standard drug aspirin 100mg/kg p.o and the remaining groups were administered with test drugs **(1-10)** at a dose of 100 mg/kg p.o. A mark was made on left

paws just beyond tibio-tarsal junction (knee joint) of each animal of all groups, so that each time the paw was dipped in the water column of digital paw edema meter (520-R, IITC Life Science, USA) up to the fixed mark made on left paws to ensure constant paw volume. Carrageenan (1%, 0.1 mL) (Sigma-Aldrich, Milan, Italy) was injected subcutaneously into the plantar surface of the rat hind paw 1hr after the oral administration of the test compound. After the administration of carrageenan solution, the paw volume of control, standard and test groups were noted at 1h, 2h, 3h and 4h time interval. The percentage of inhibition was calculated by applying New bould formula (New bould, 1963).

4.4.10. 6.Antipyretic activity study

This activity was performed on rabbits of either sex according to the reported method described by Lu et al. (2004). An aliquot of 3ml/kg of 10% Brewer's yeast suspension was subcutaneously injected into the rabbit back. The temperature was monitored by means of thermometer inserted at least 10 cm into the rectum and recorded by calibrated thermometers. An animal was excluded from the study if the baseline temperature was not within the range of 39.2 °C to 39.8°C. After 5 hour, animals showing at least an increase of 1°C of rectal temperature were selected for the experiment. The animals were administered with standard paracetamol (100mg/kg p.o) and all pyrazolone derivatives (400mg/kg) orally. The rectal temperature was measured at 0, 1, 2 and 3 hour after treatment.

4.4.11. Histopathological studies

Histopathological processing of liver and heart was carried out after a proper wash with cold isotonic saline solution and slices were prepared. The liver and heart sections were fixed in10% (v/v) formalin solution. After dehydration pieces of liver and heart were embedded in paraffin wax, cut into 4–6 mm thick sections, and stained using haematoxylin and eosin. They were then examined under a microscope for histo architecture changes. The following morphological criteria (Zingarelli *et al.*, 1998) were used to assess the histopathological damage: interstitial edema and focal necrosis; diffuse myocardial cell swelling and necrosis; necrosis with the presence of contraction bands, neutrophil infiltration and the capillaries were compressed; and wide spread necrosis

with the presence of contraction bands, neutrophil infiltration, capillaries compressing and hemorrhage. The observed histopathological changes were shown in the figure 4.60.

4.4.12. Statistical analysis

The data were expressed as mean ± standard error of mean (S.E.M.). The statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Dunnett's test with the help of GraphPad Prism 3 statistical software. P-values <0.05 were considered as significant.

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CHAPTER 5

RESULTS

5. RESULTS

3-methyl pyrazol-5-one derivatives were synthesized by reacting ethyl acetoacetate with hydrazine hydrate in presence of absolute ethyl alcohol. It was then treated with different substituted aldehyde in presence of alcoholic sodium hydroxide to produce pyrazolone derivatives. The synthesized compounds were characterized by physico chemical and spectral analysis.

All the synthesized compounds were characterized on the basis of

- Physical parameters
- Melting point measurement
- Thin layer chromatography
- Determination of λ_{max} by UV-Visible spectrophotometer
- FTIR spectrum analysis
- Nuclear Magnetic Resonance (NMR) spectrometry analysis
- Mass spectra analysis
- CHN analysis (Elemental analysis)

5.1. Physical parameters

The following physical parameters were noted for the preliminary identification of the compounds.

1. Physical state (solid, liquid, color, odor and solubility etc)

5.2. Melting point measurement

The melting point of the synthesized compounds was determined in open end capillary tube and are uncorrected (within the range of 0.1 to 0.2°C)

5.3. Thin layer chromatography

Thin layer chromatography was used to assess the completion of reaction and purity of the synthesized compounds using Silica gel G450 glass plate and ethanol: water (1:1) as the mobile phase. The spots were developed in TLC chamber and visualized in an iodine chamber.

5.4. Determination of λ_{max} by UV-Visible spectrophotometer

The compounds exhibit the chromophore C=C-C=O which is responsible for maximum absorbance. Ultra violet visible spectroscopic analysis was carried out in UV-Pharma Spec 1700 (SHIMADZU) UV-Visible spectrophotometer using the concentration of 0.01% of the synthesized compounds in ethanol. The UV Visible spectra of all the ecompounds are given below.**(Fig.5.1-Fig 5.10)**

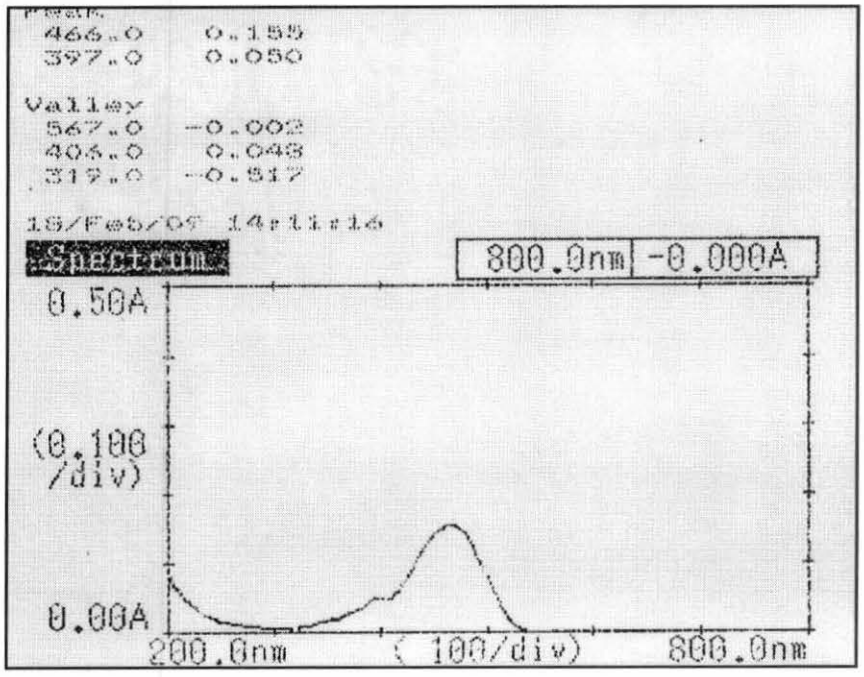


Fig: 5.1. Ultraviolet spectrum of compound PYZ1

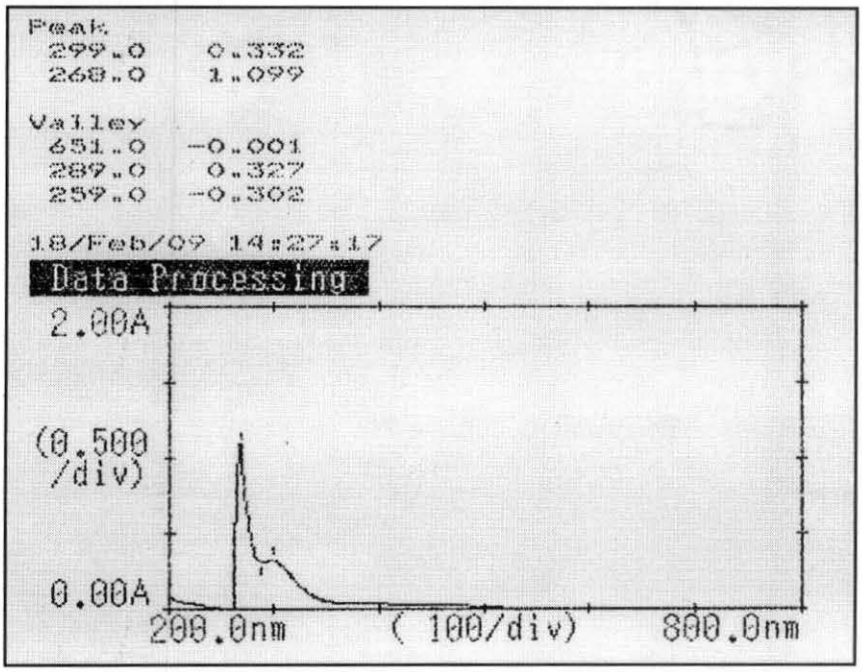


Fig: 5.2. Ultraviolet spectrum of compound PYZ2

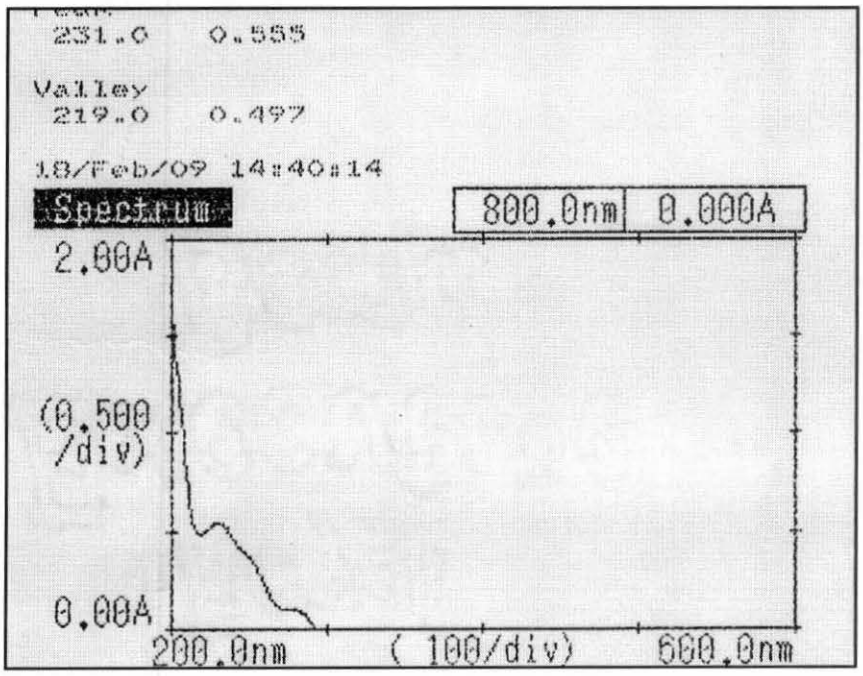


Fig: 5.3. Ultraviolet spectrum of compound PYZ3

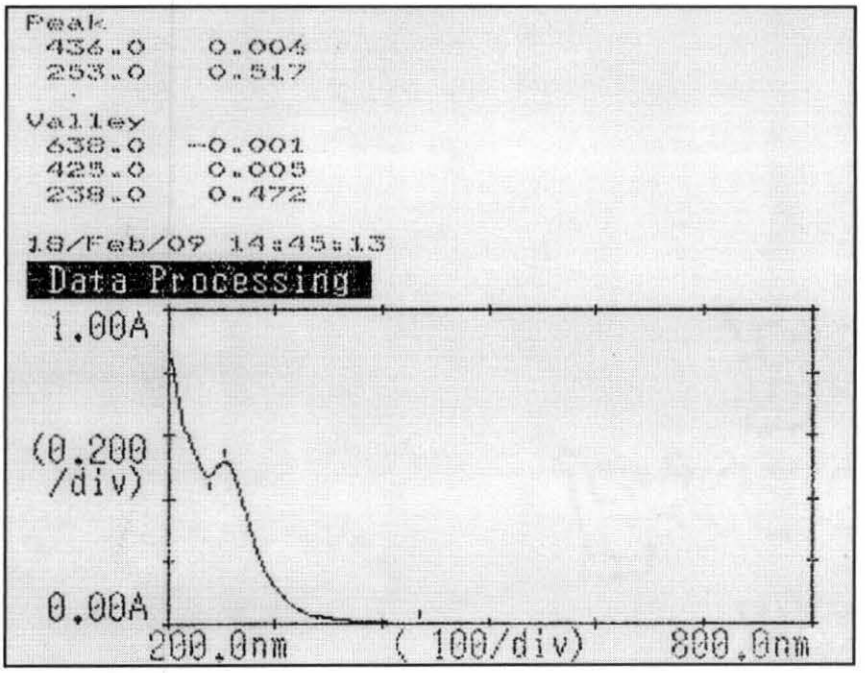


Fig: 5.4. Ultraviolet spectrum of compound PYZ4

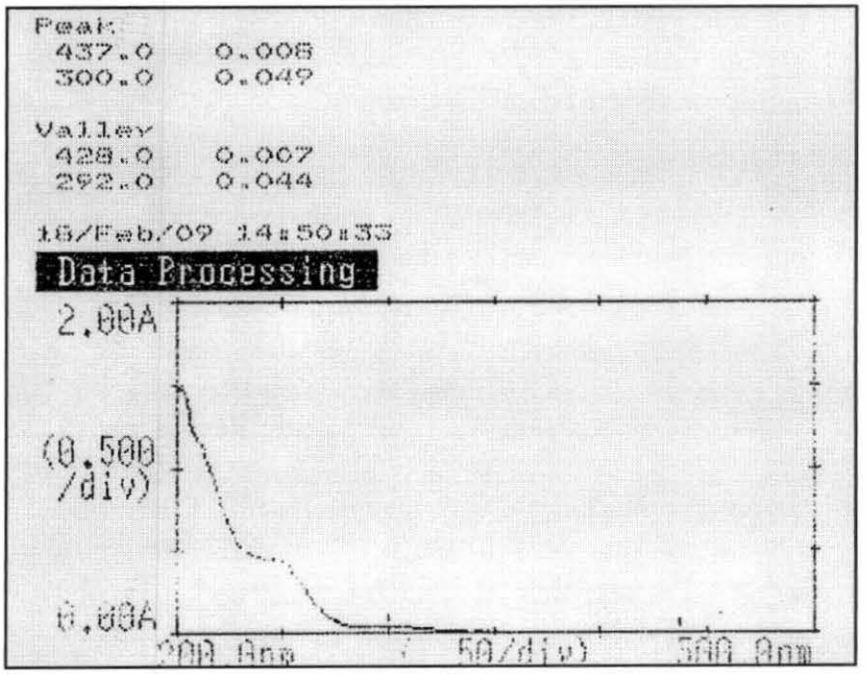


Fig: 5.5. Ultraviolet spectrum of compound PYZ5

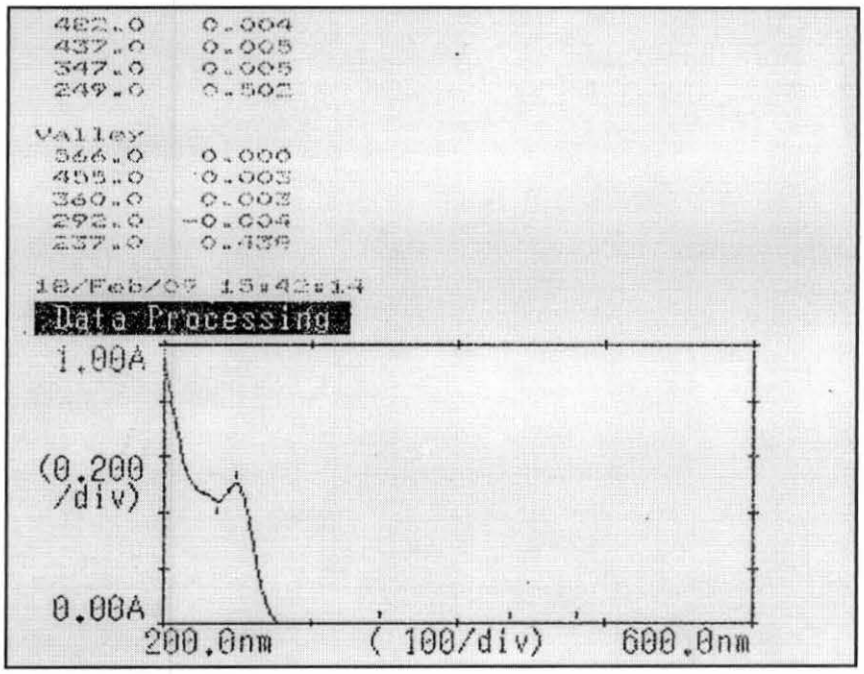


Fig: 5.6. Ultraviolet spectrum of compound PYZ6

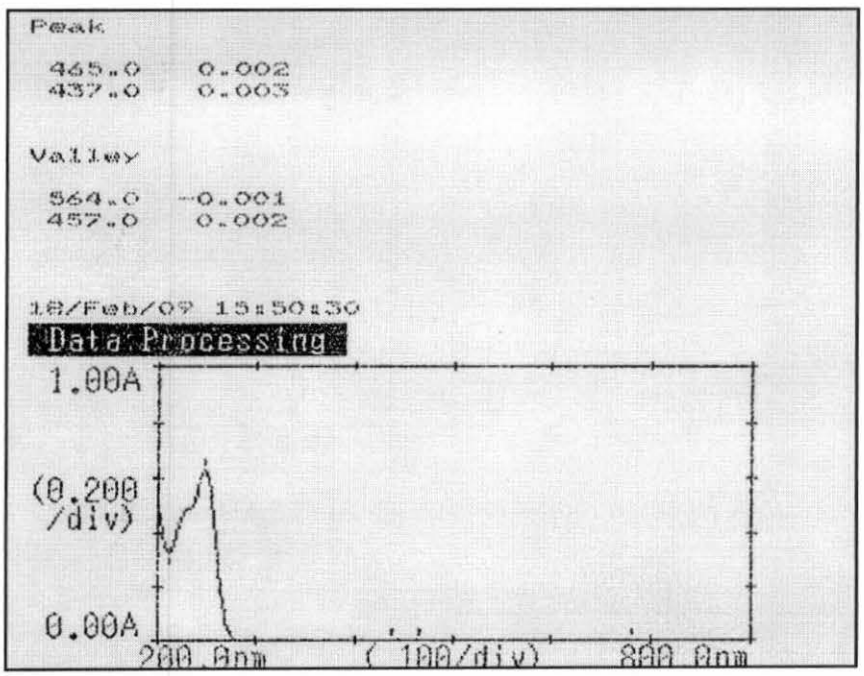


Fig: 5.7. Ultraviolet spectrum of compound PYZ7

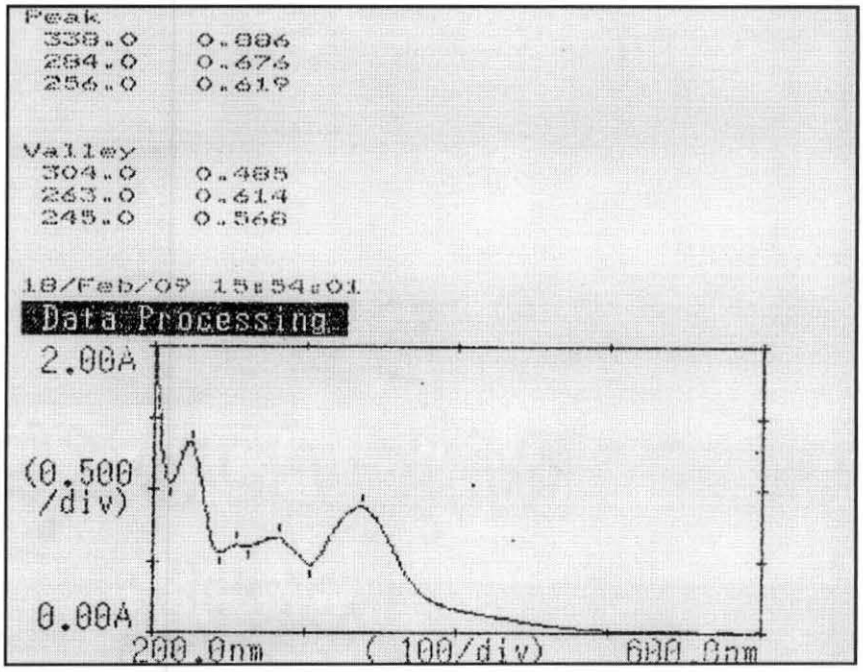


Fig: 5.8. Ultraviolet spectrum of compound PYZ8

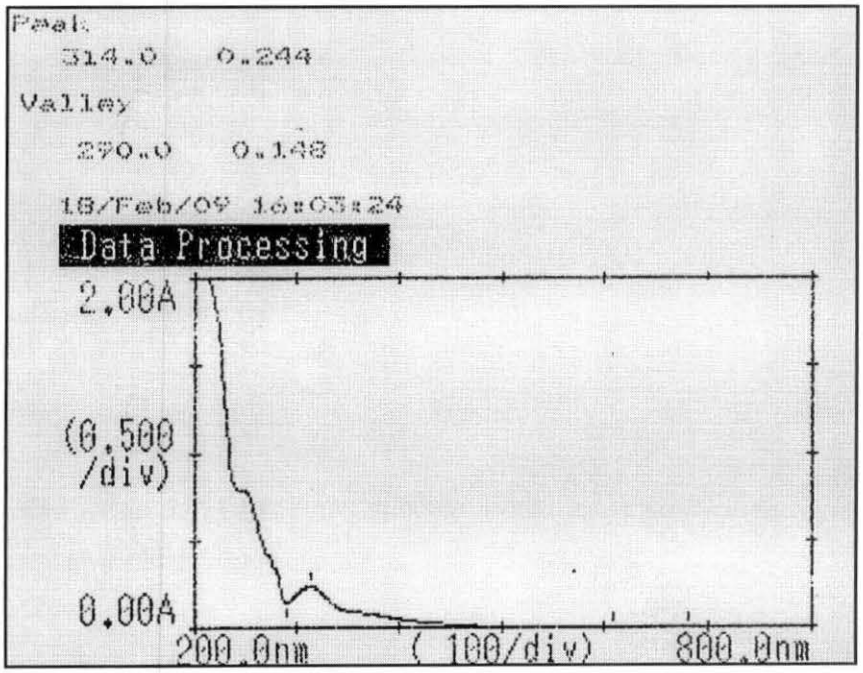


Fig: 5.9.Ultraviolet spectrum of compound PYZ9

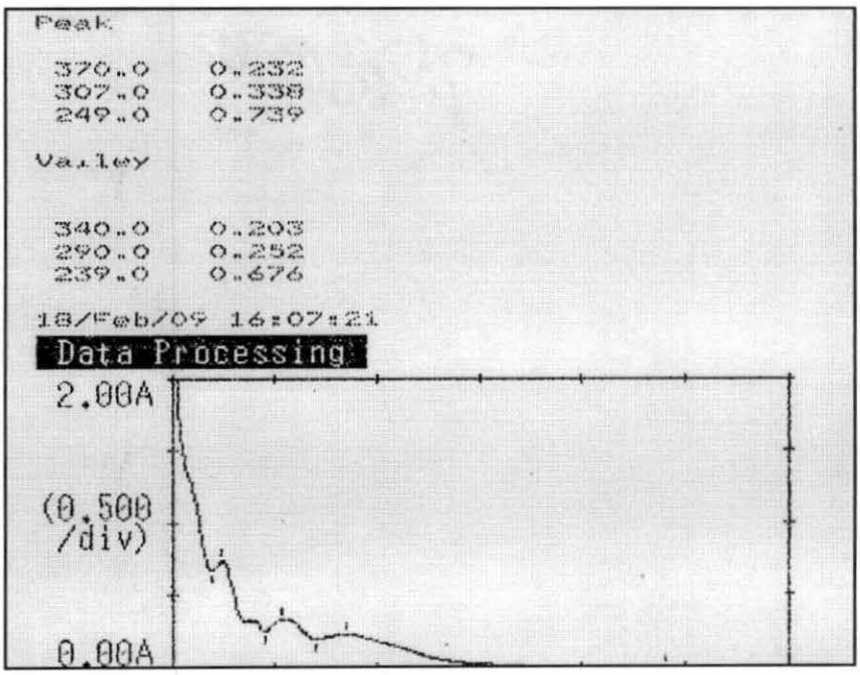


Fig: 5.10. Ultraviolet spectrum of compound PYZ10

5.5. FTIR spectrum analysis

IR spectroscopy deals with the infrared region of the electromagnetic spectrum. The infrared portion of the electromagnetic spectrum is usually divided into three regions; the near, mid and far infrared. The mid-infrared, approximately 4000–400 cm^{-1} (30–2.5 μm) is used to study the fundamental vibrations and associated with the structure of various functional groups. The presence of the different functional groups in the synthesized compounds was identified by FTIR spectra.

The characteristic functional groups present in the synthesized pyrazolone derivatives are NH, =C-H, C=O, C=N, Aromatic C=C and alkyl C-H. IR spectra showed the appearance of peak at 2900-3100 cm^{-1} indicated the presence of N-H group in pyrazolone ring in all the compounds (PYZ1-PYZ10). The presence of peak at 1500-1700 cm^{-1} confirmed the C=O group in pyrazolone hetero cycle. The peaks at 1300-1500 cm^{-1} ascertain the presence of C=N group and C=C of aromatic ring. Apart from these compounds 1, 3, 8, 9 &10 displayed characteristic peaks at 2829, 2717, 2359, 2360 & 2955 cm^{-1} confirmed the presence of NMe_2, OMe,(2,3) 4-OMe, 3-OMe and OH respectively. The IR spectra of all the compounds are given below. **(Fig.5.11-Fig. 5.20)**

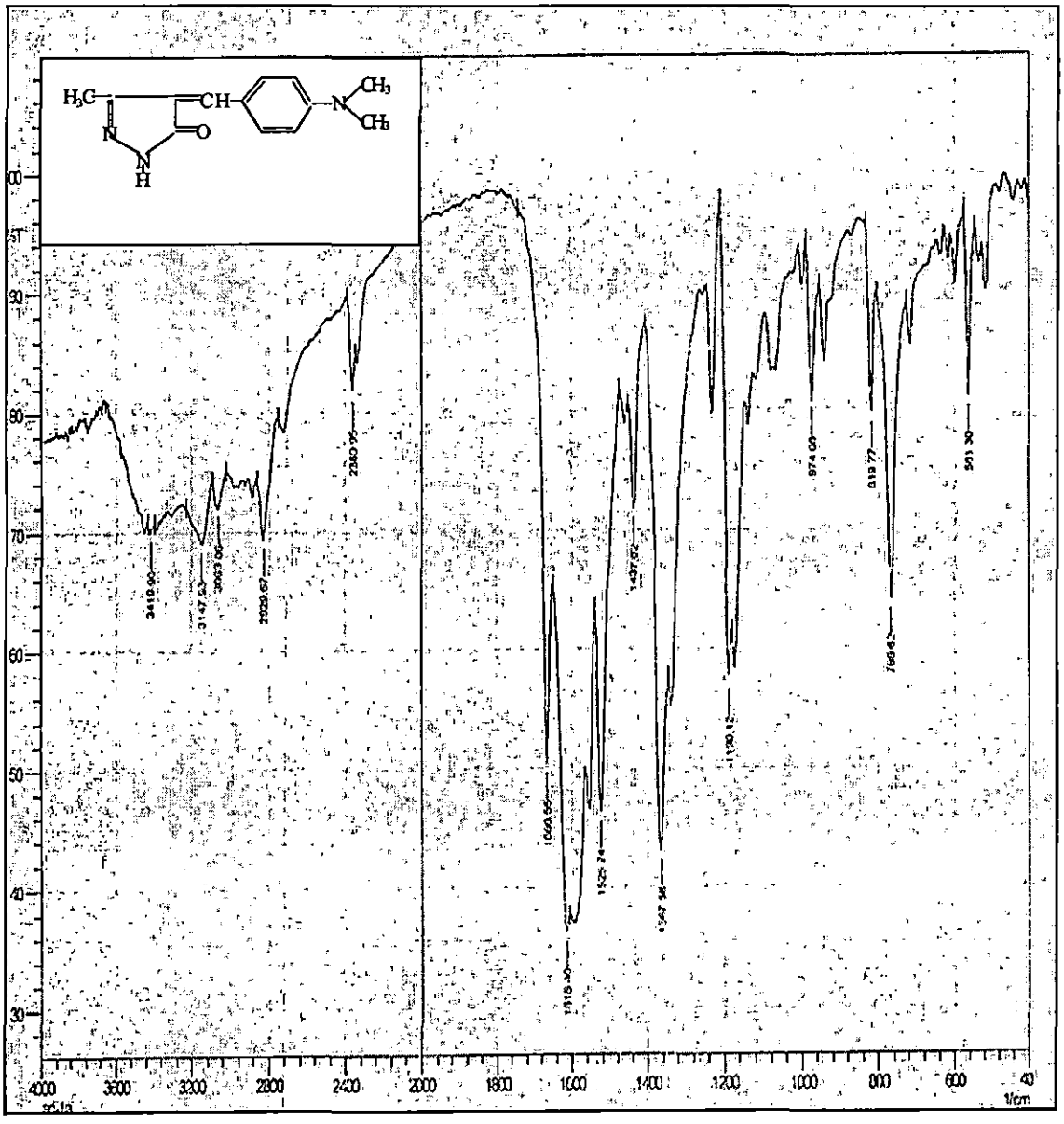


Fig: 5.11. Infrared spectrum of compound PYZ1

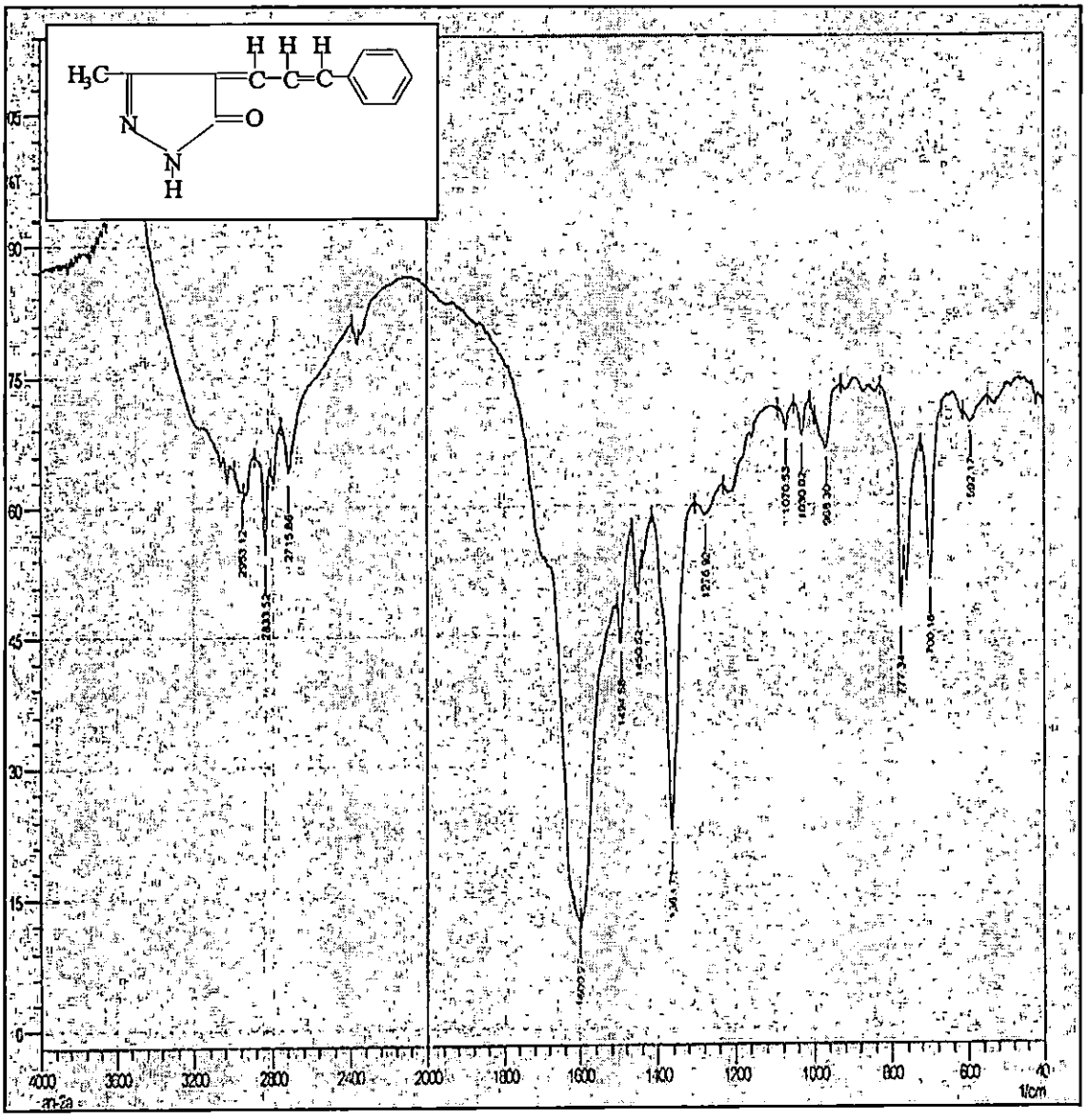


Fig: 5.12. Infrared spectrum of compound PYZ2

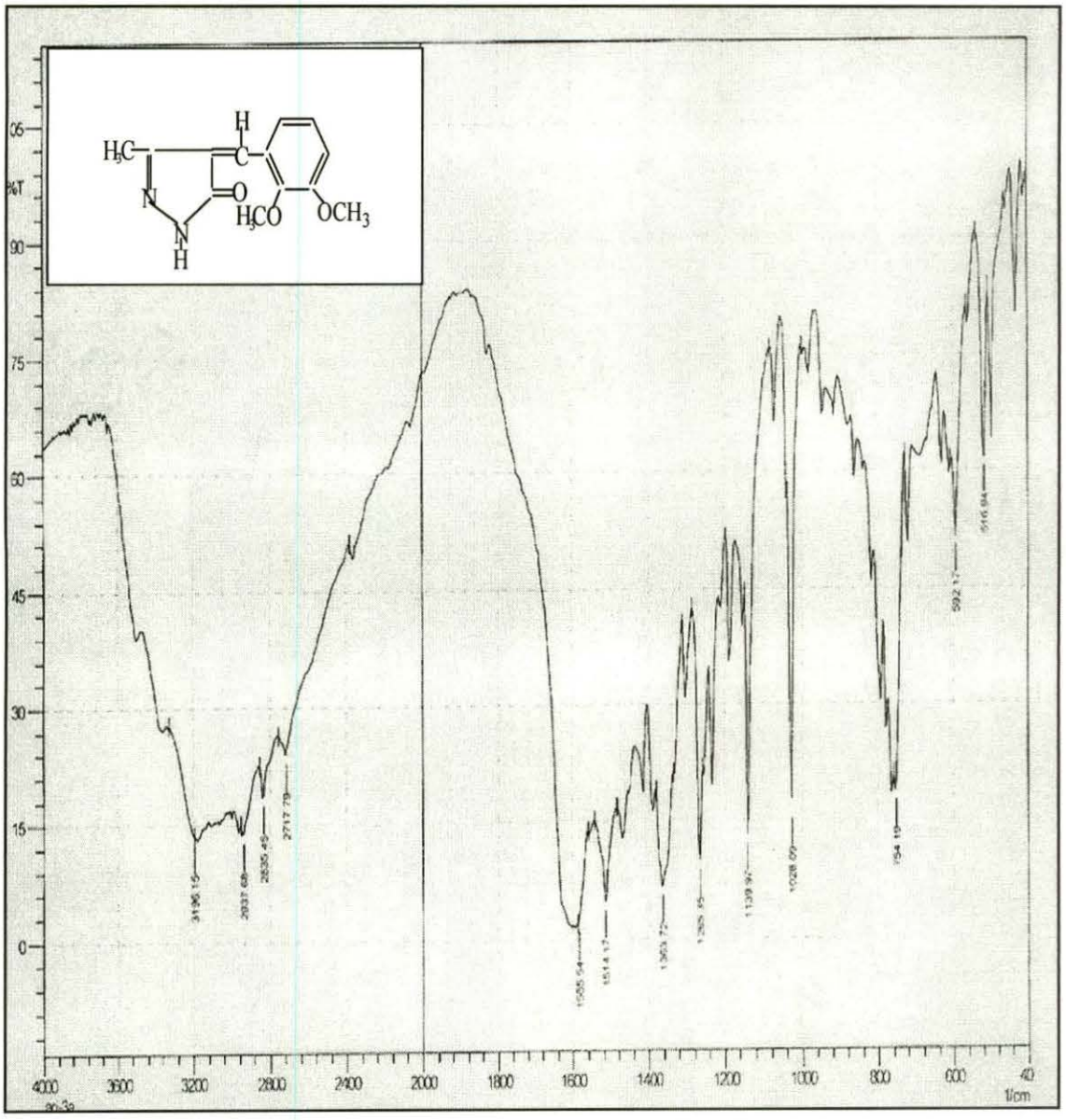


Fig: 5.13. Infrared spectrum of compound PYZ3

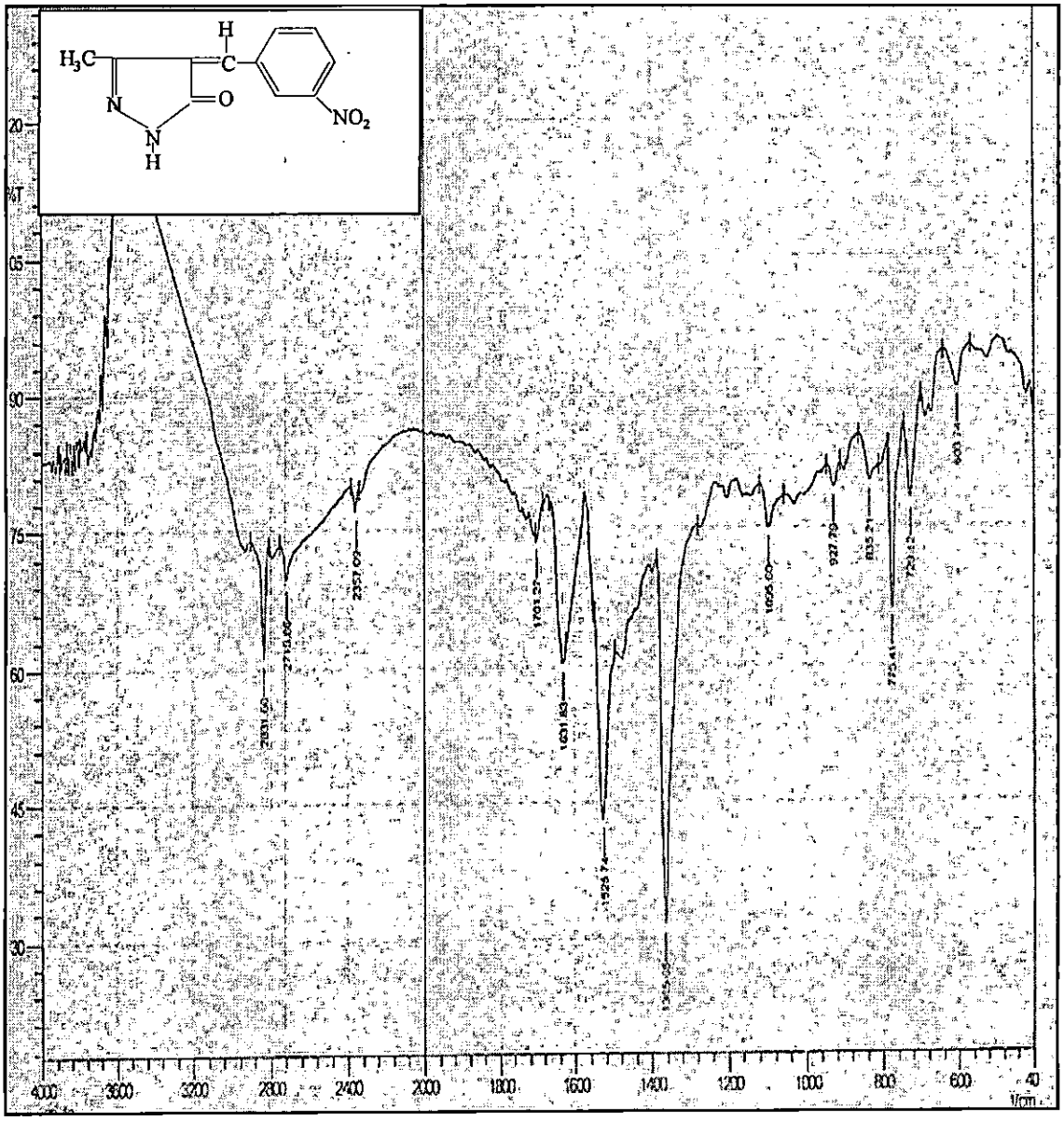


Fig: 5.14. Infrared spectrum of compound PYZ4

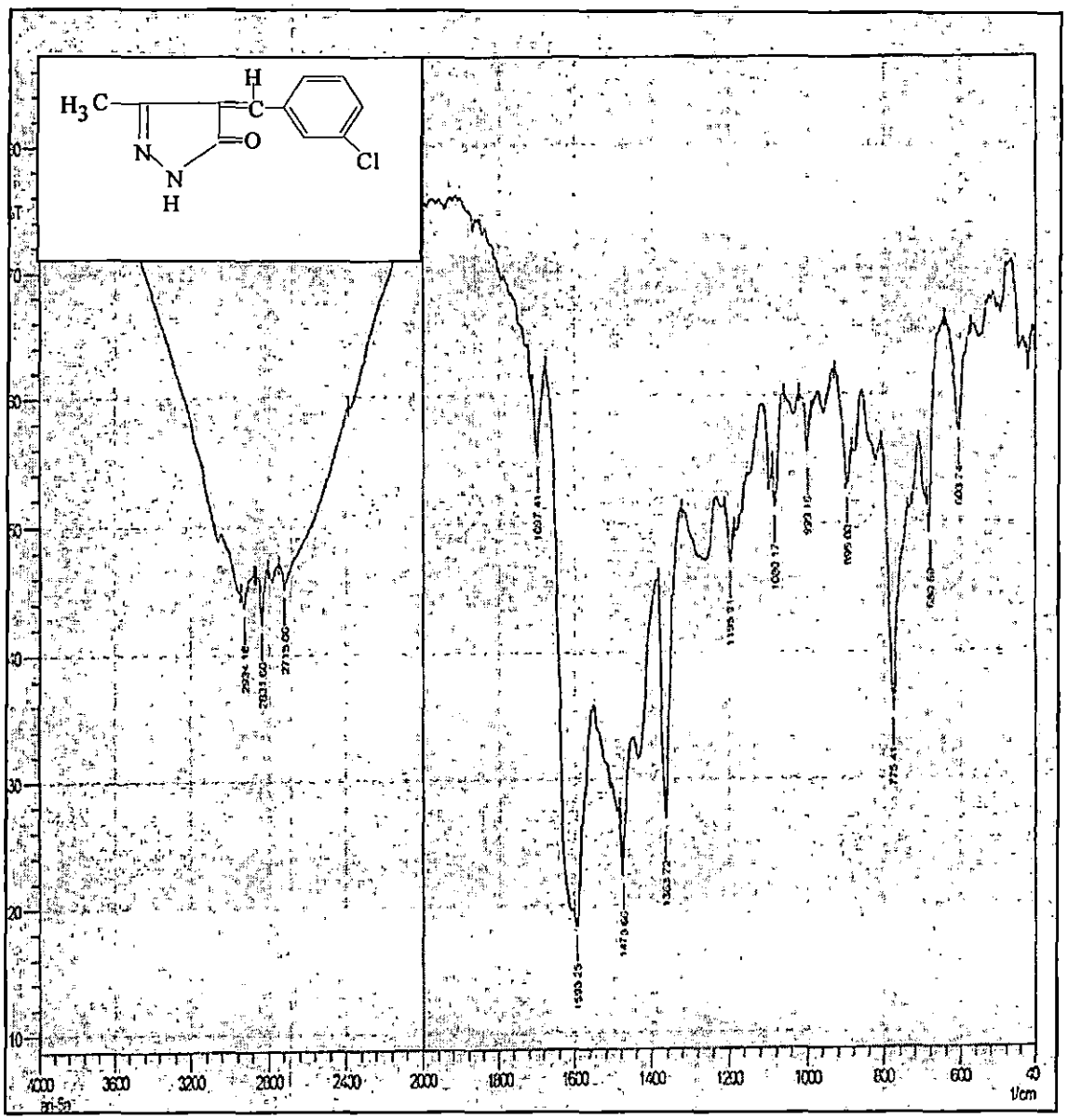


Fig: 5.15. Infrared spectrum of compound PYZ5

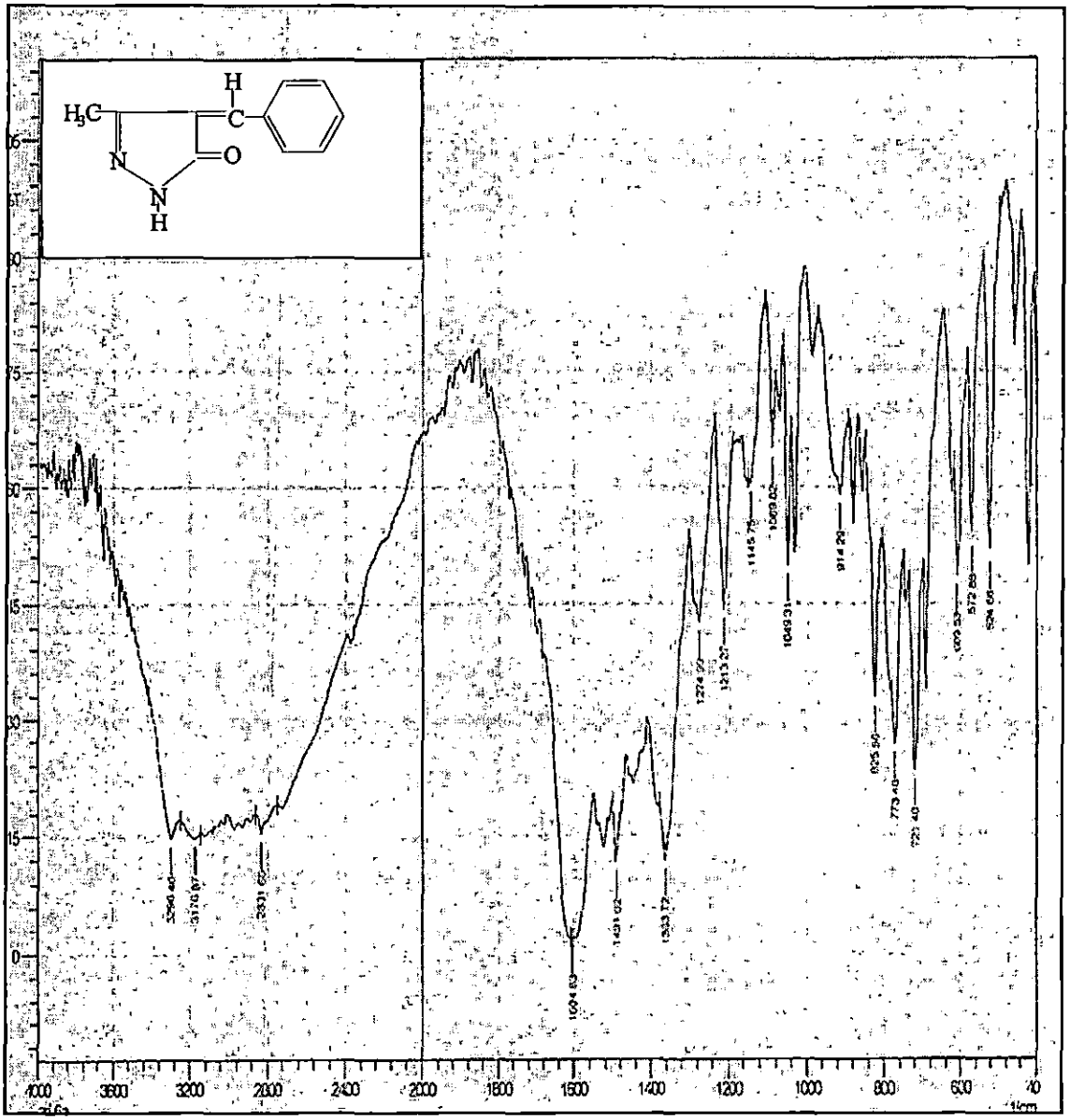


Fig: 5.16. Infrared spectrum of compound PYZ6

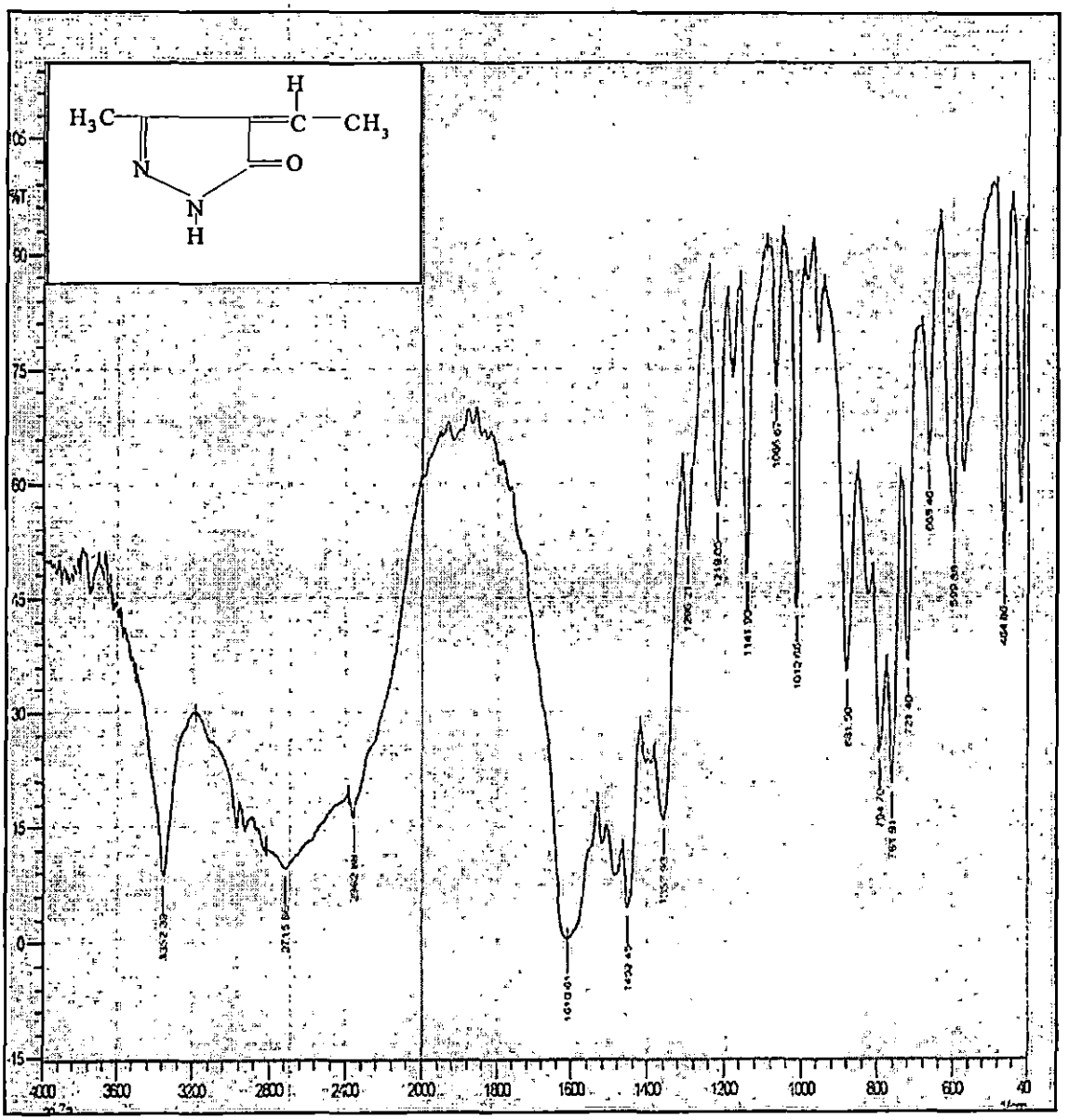


Fig: 5.17. Infrared spectrum of compound PYZ7

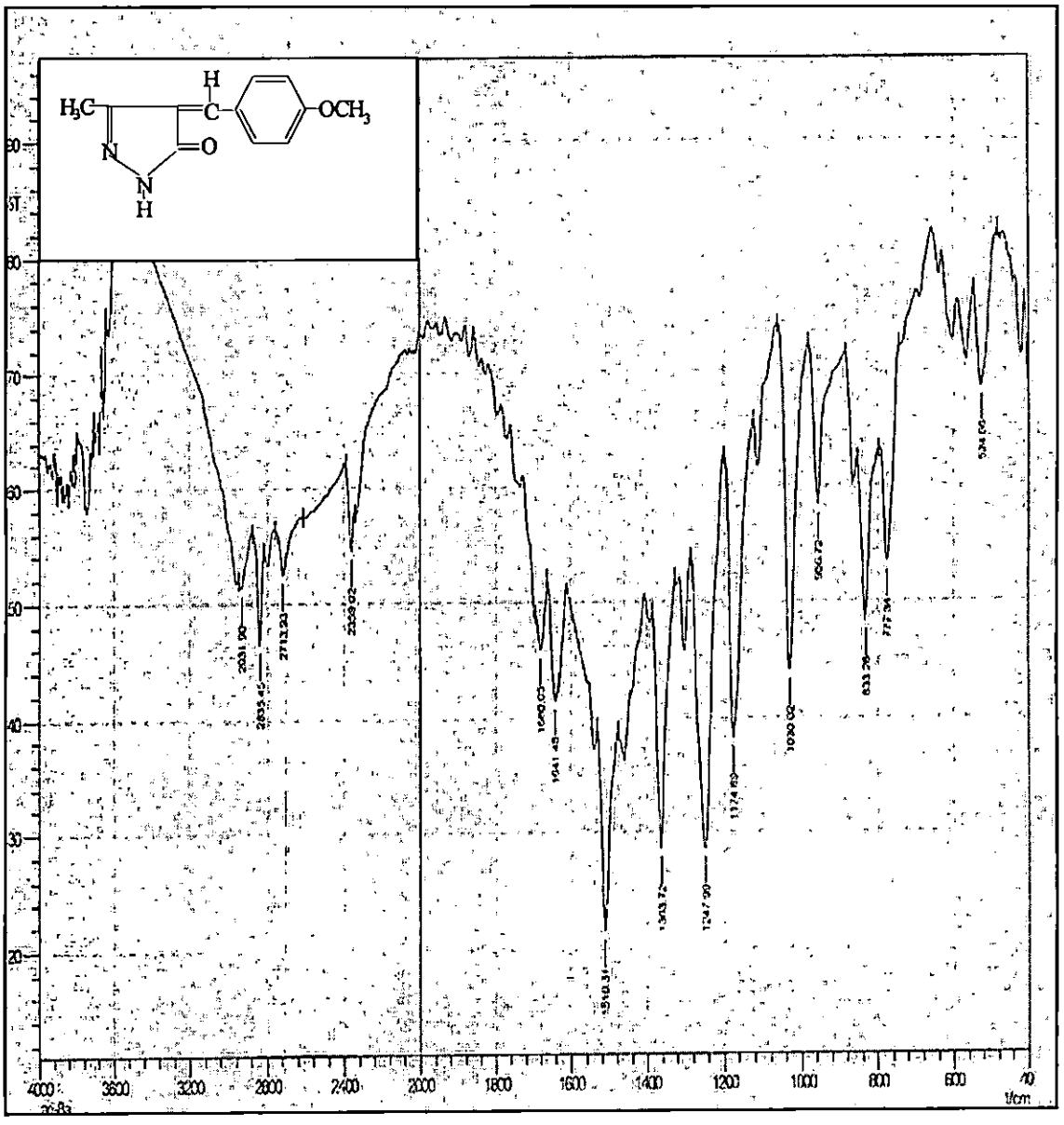


Fig: 5.18. Infrared spectrum of compound PYZ8

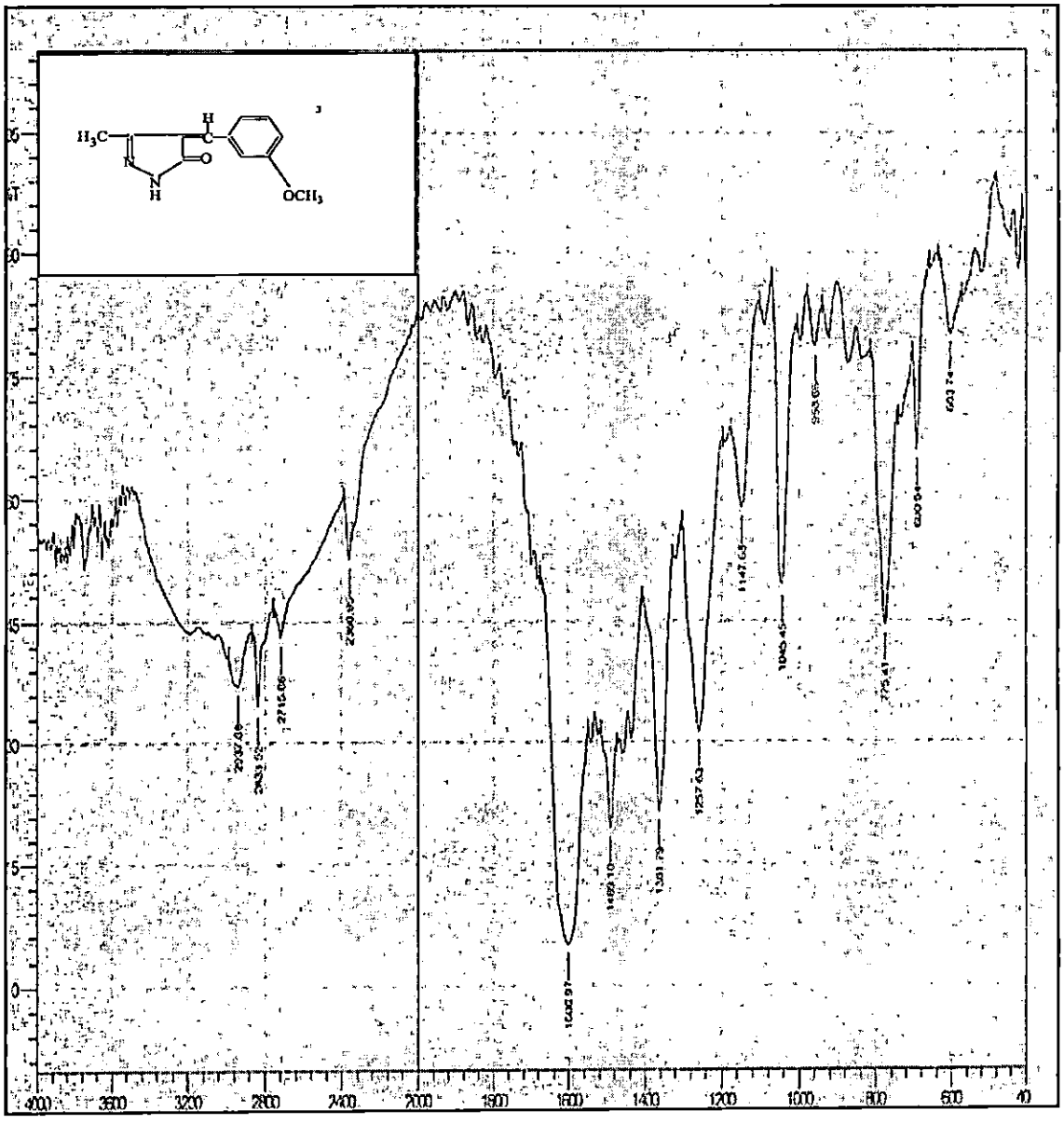


Fig: 5.19. Infrared spectrum of compound PYZ9

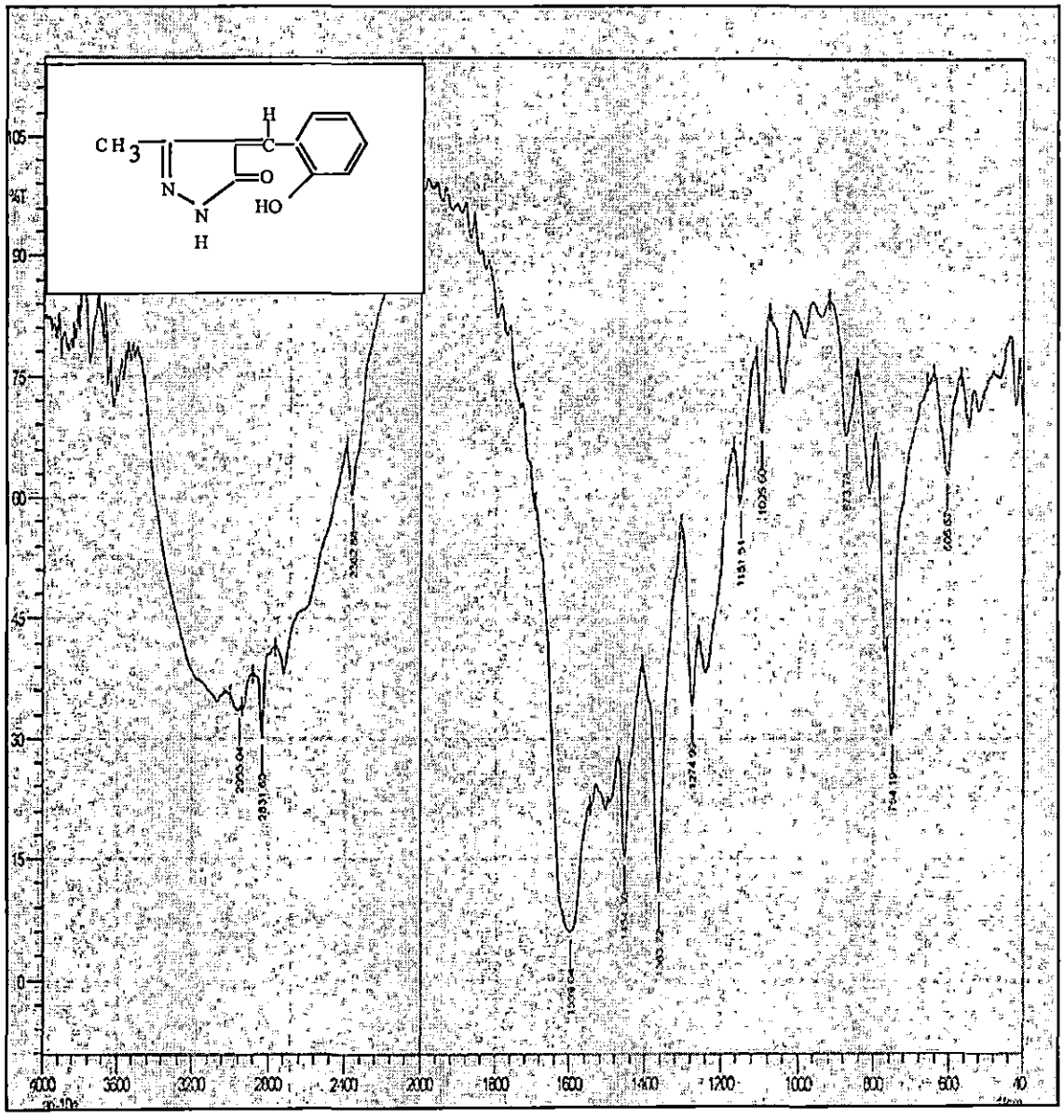


Fig: 5.20. Infrared spectrum of compound PYZ10

5.6. NMR Spectral Analysis

5.6.1. ^{1}H NMR ANALYSIS and ^{13}C NMR ANALYSIS

^{1}H NMR spectra of compounds (PYZ1-PYZ5) shows an intense signal at 6.83- 7.86 due to the presence of olefinic proton all the compounds. The 3- methyl group of pyrazolone ring leads to a sharp singlet at 3- 3.32 ppm which signifies 3 protons. In all the compounds a sharp singlet at 6.83- 8.36 ppm indicates the proton of NH groups. The proton present in aromatic ring was confirmed by multiplet at 6-8 ppm. In addition these common groups, the proton of N $(CH_3)_2$, OCH_3 were also identified by the signal at 3-4 ppm. The ^{1}H NMR spectra of all the ecompounds are given as below.**(Fig.5.21-Fig. 5.25)**

In ^{13}C NMR spectra of compounds (PYZ6-PYZ10) the signal at 10-18 ppm indicated presence of carbon in methyl group attached to pyrazolone ring. The signal at 30-50 ppm indicates presence of carbon at C3, C4 & C5 of pyrazolone heterocycle. A sharp singlet at 105-145 ppm confirmed the olefinic carbon. The signal at 120-145 ppm ascertained the presence of aromatic carbon. Interestingly the compound does not show any signal for aromatic carbon since it was synthesized by aliphatic aldehyde. The ^{13}C NMR spectra of all the ecompounds are given as below.**(Fig.5.26-Fig. 5.30)**

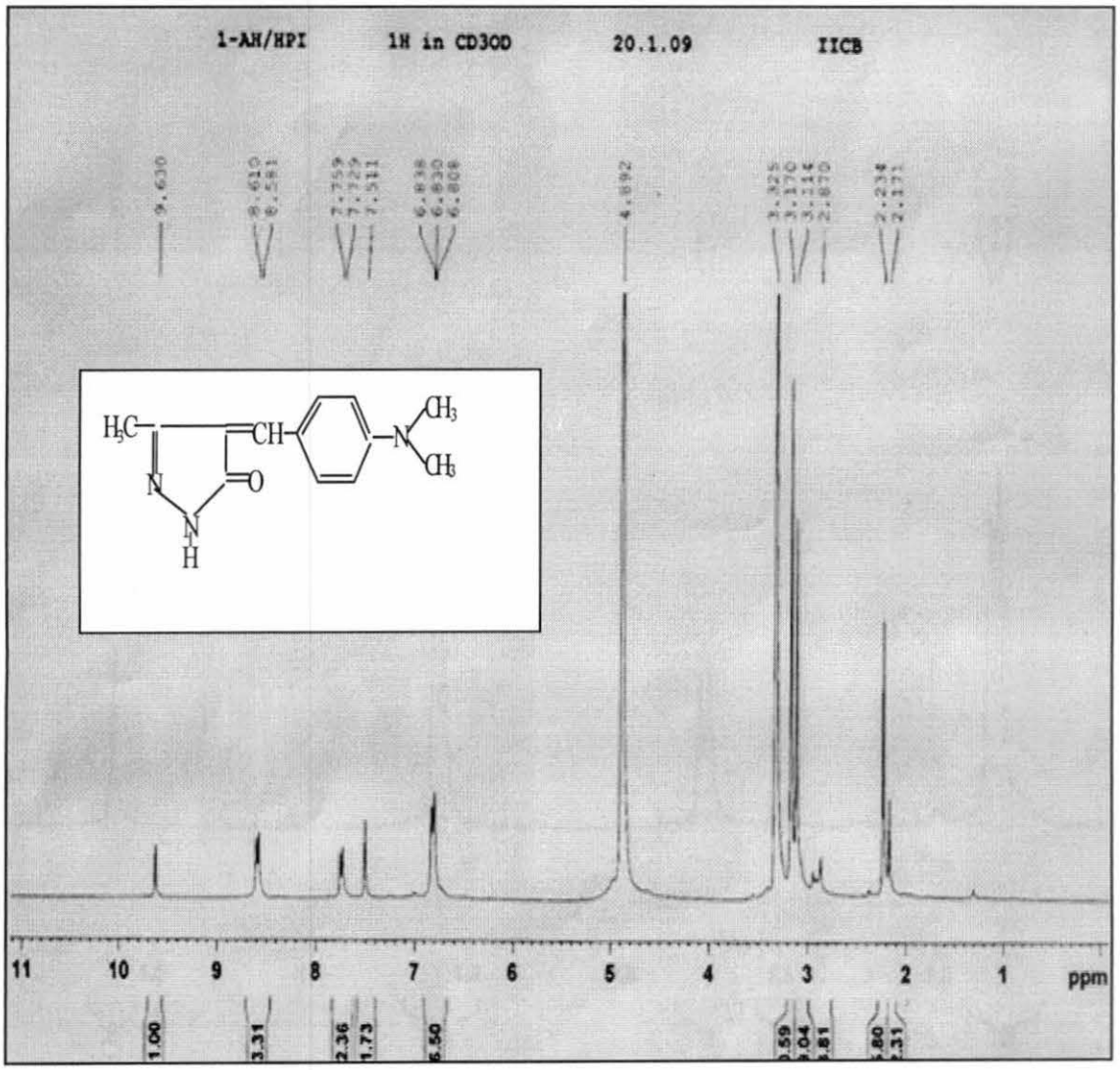


Fig: 5.21. ^{1}H NMR spectrum of PYZ1

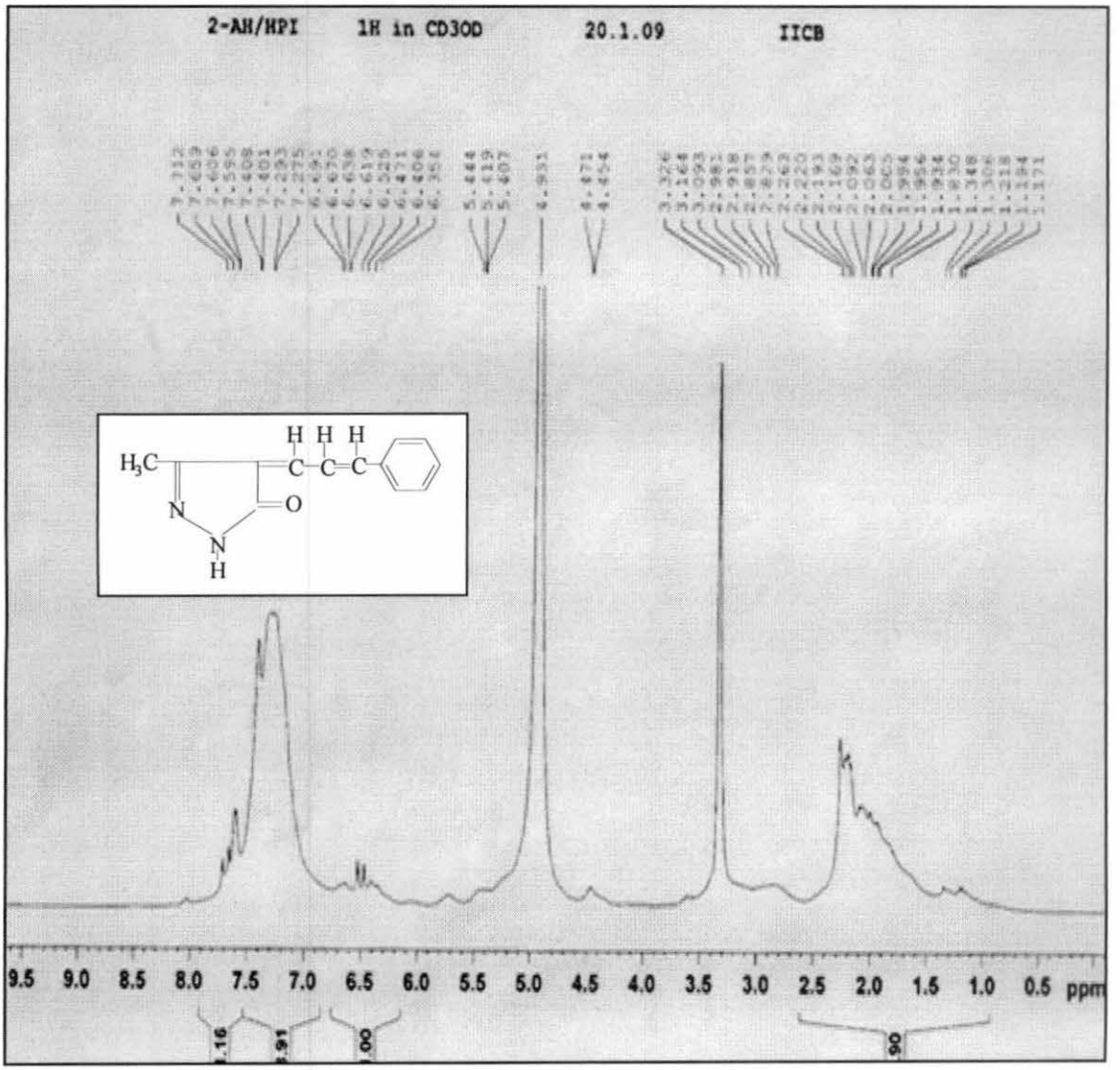


Fig: 5.22. ^{1}H NMR spectrum of PYZ2

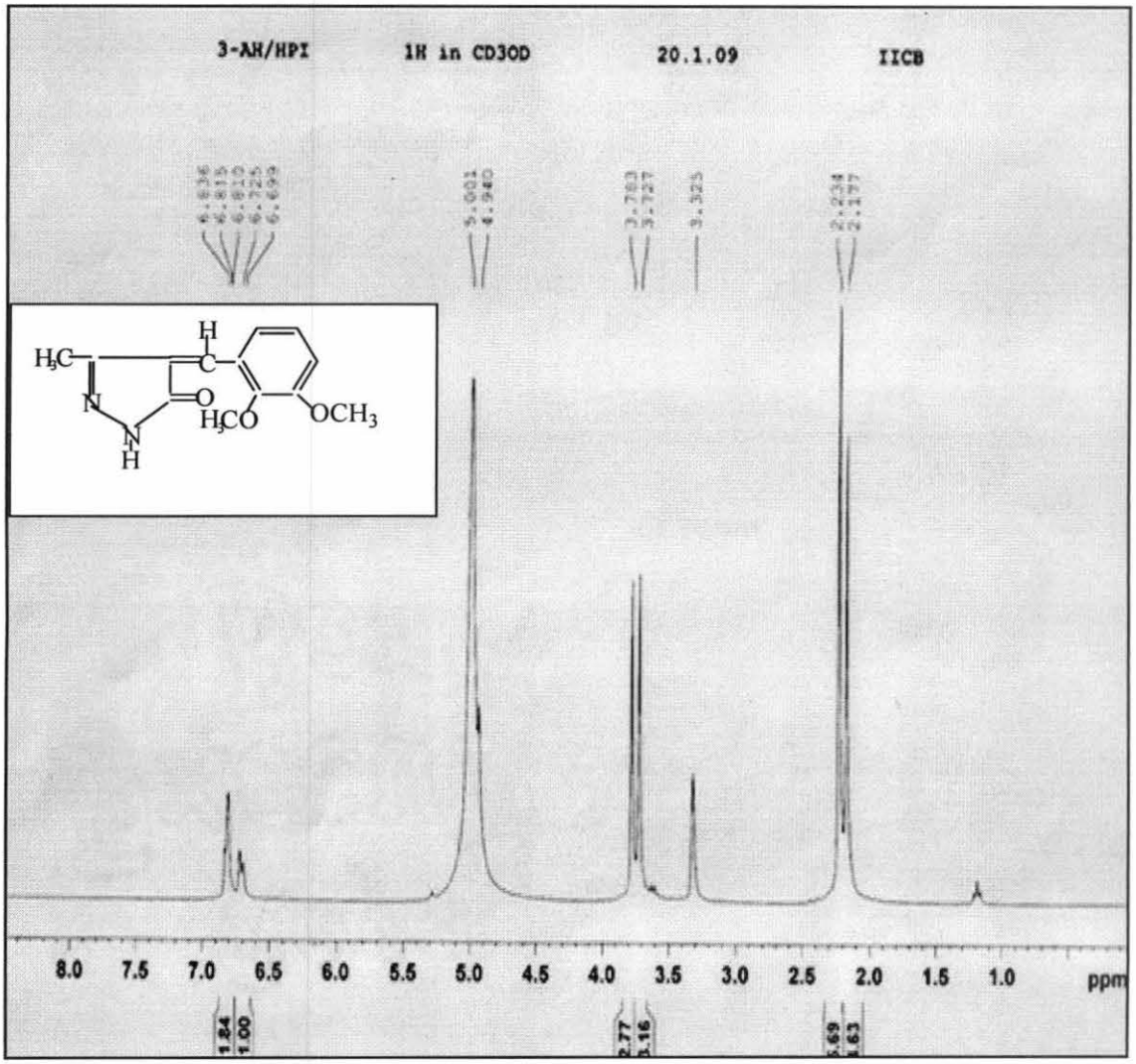


Fig: 5.23. ^{1}H NMR spectrum of PYZ3

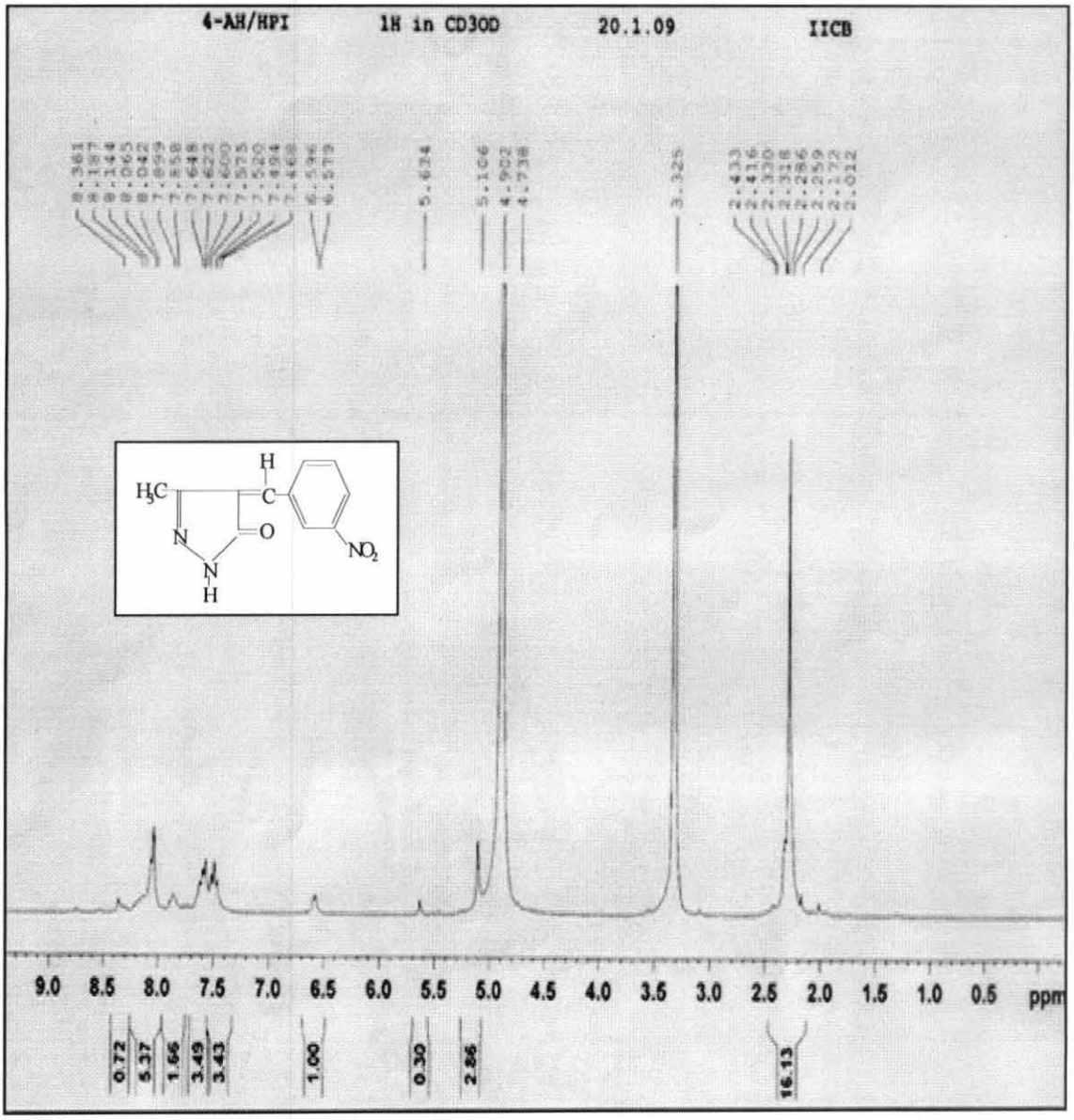


Fig: 5.24. ^{1}H NMR spectrum of PYZ4

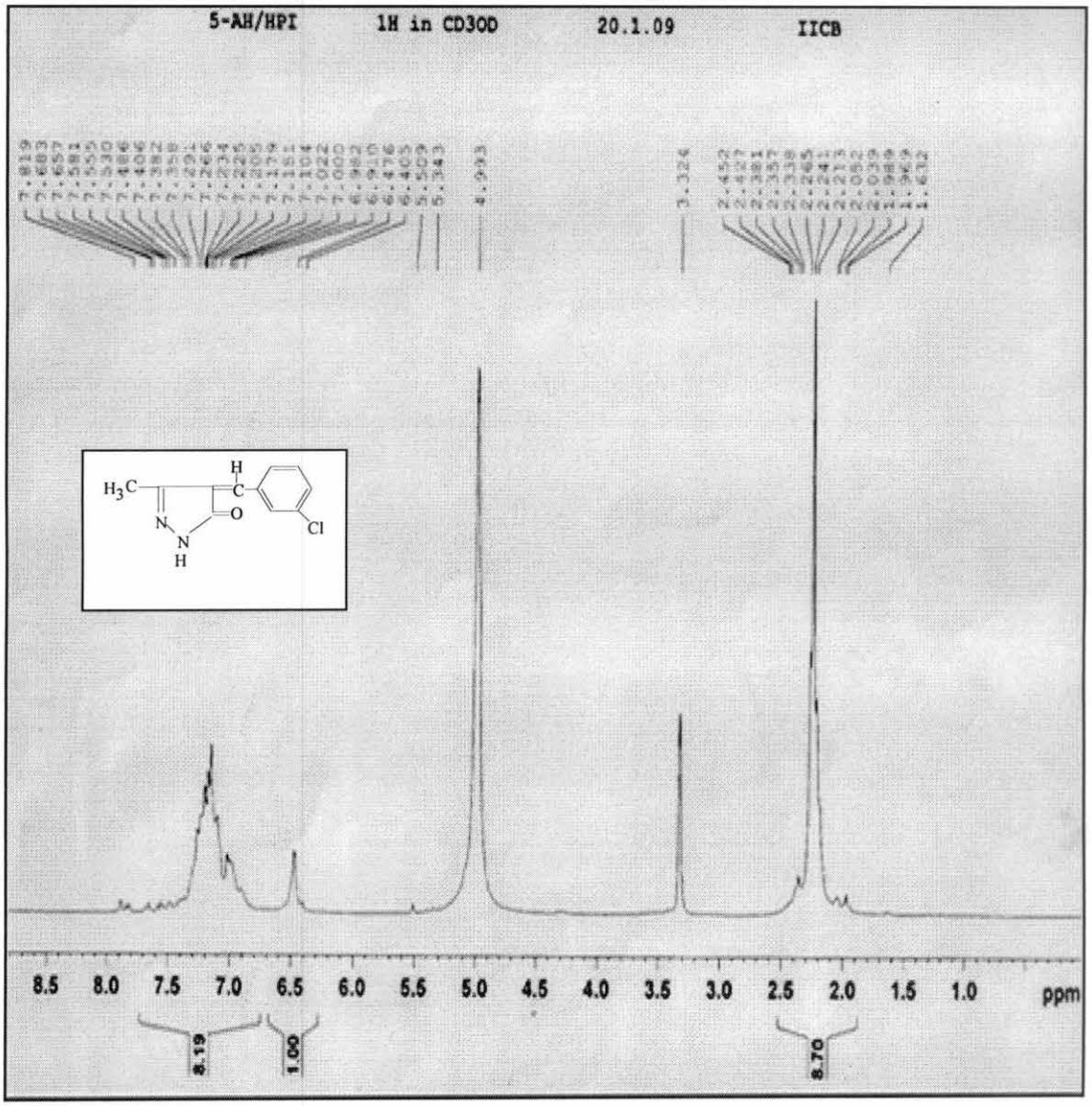


Fig: 5.25. ^{1}H NMR spectrum of PYZ5

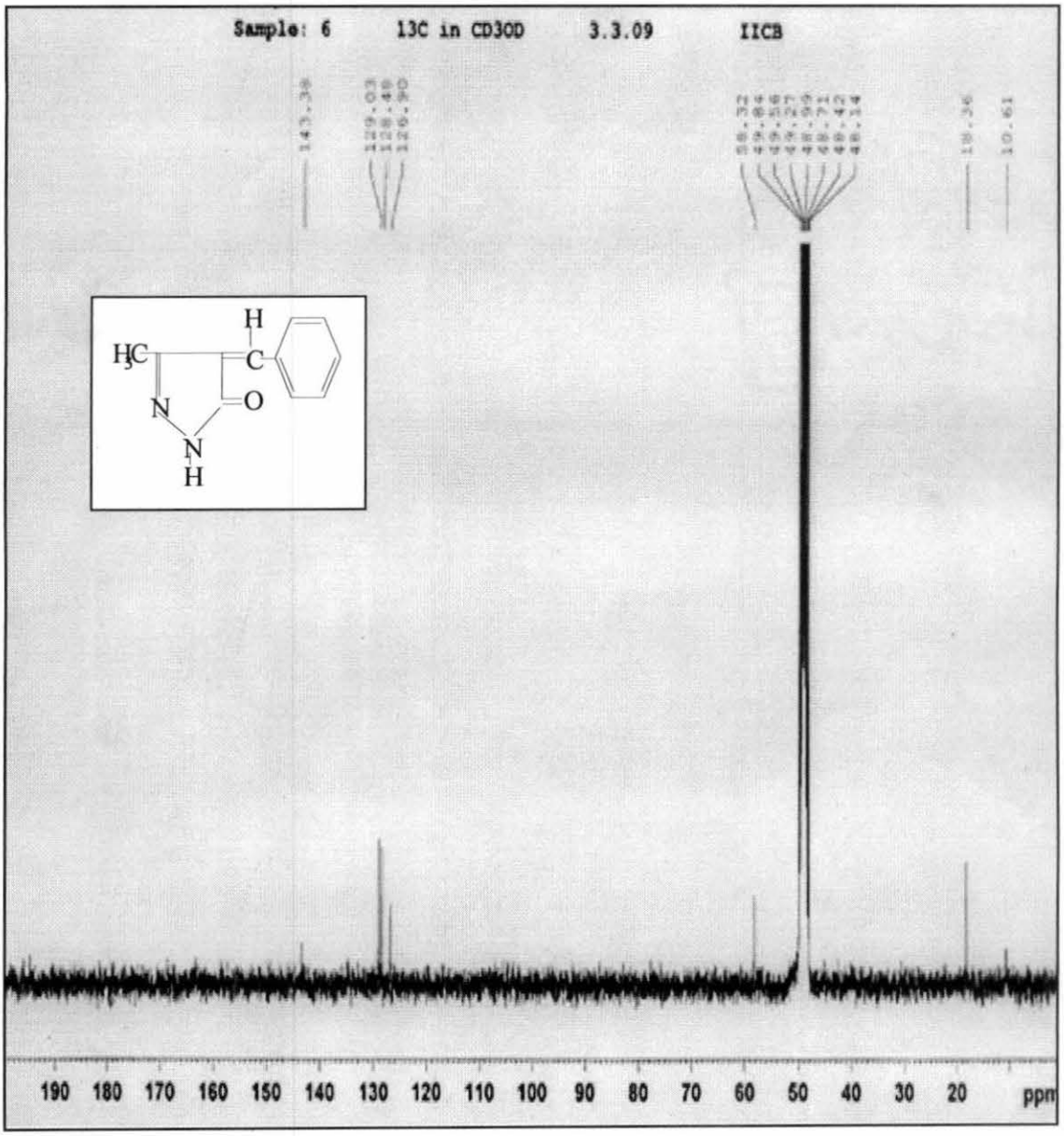


Fig: 5.26. ^{13}C NMR spectrum of PYZ6

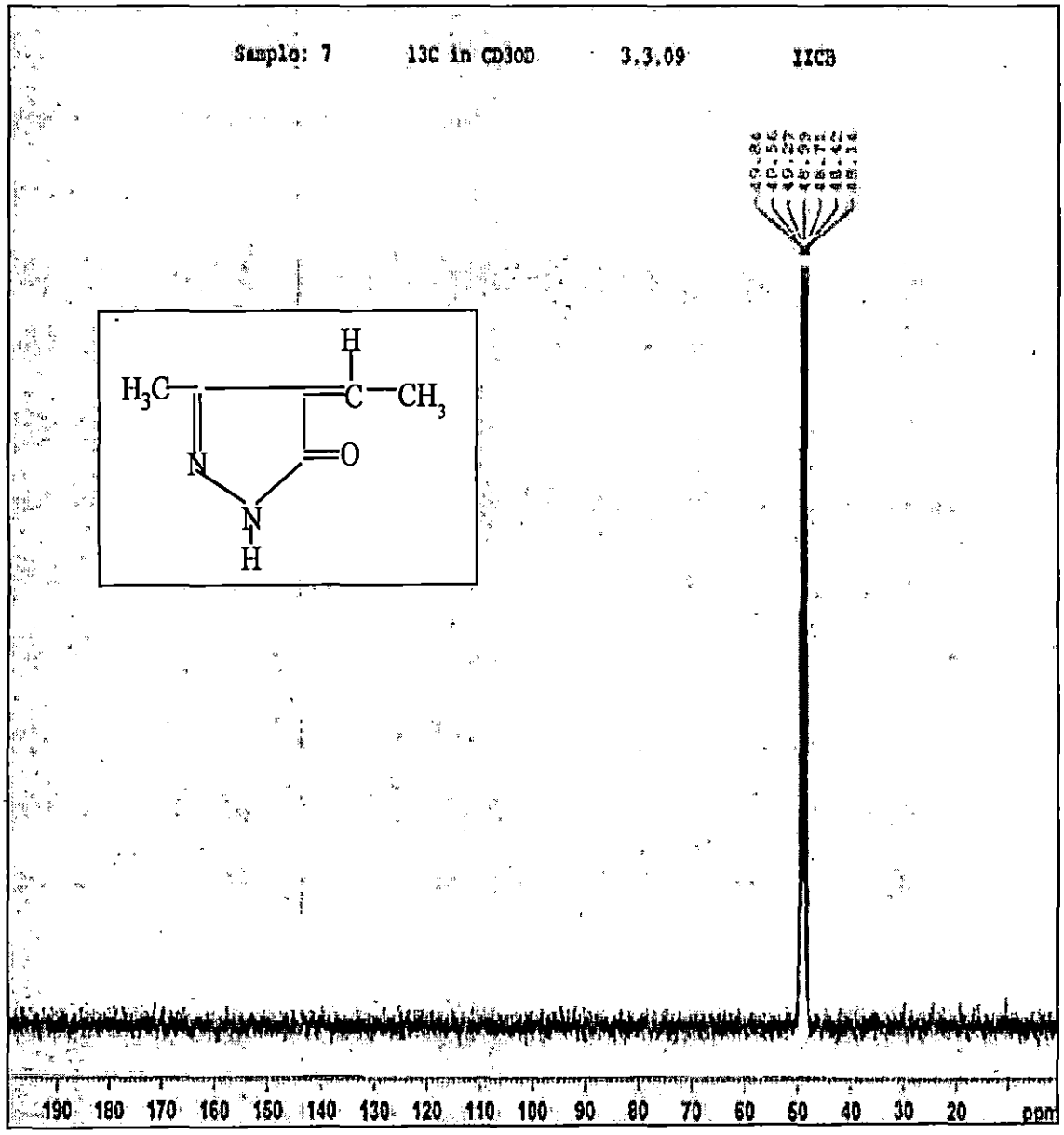


Fig: 5.27. ^{13}C NMR spectrum of PYZ7

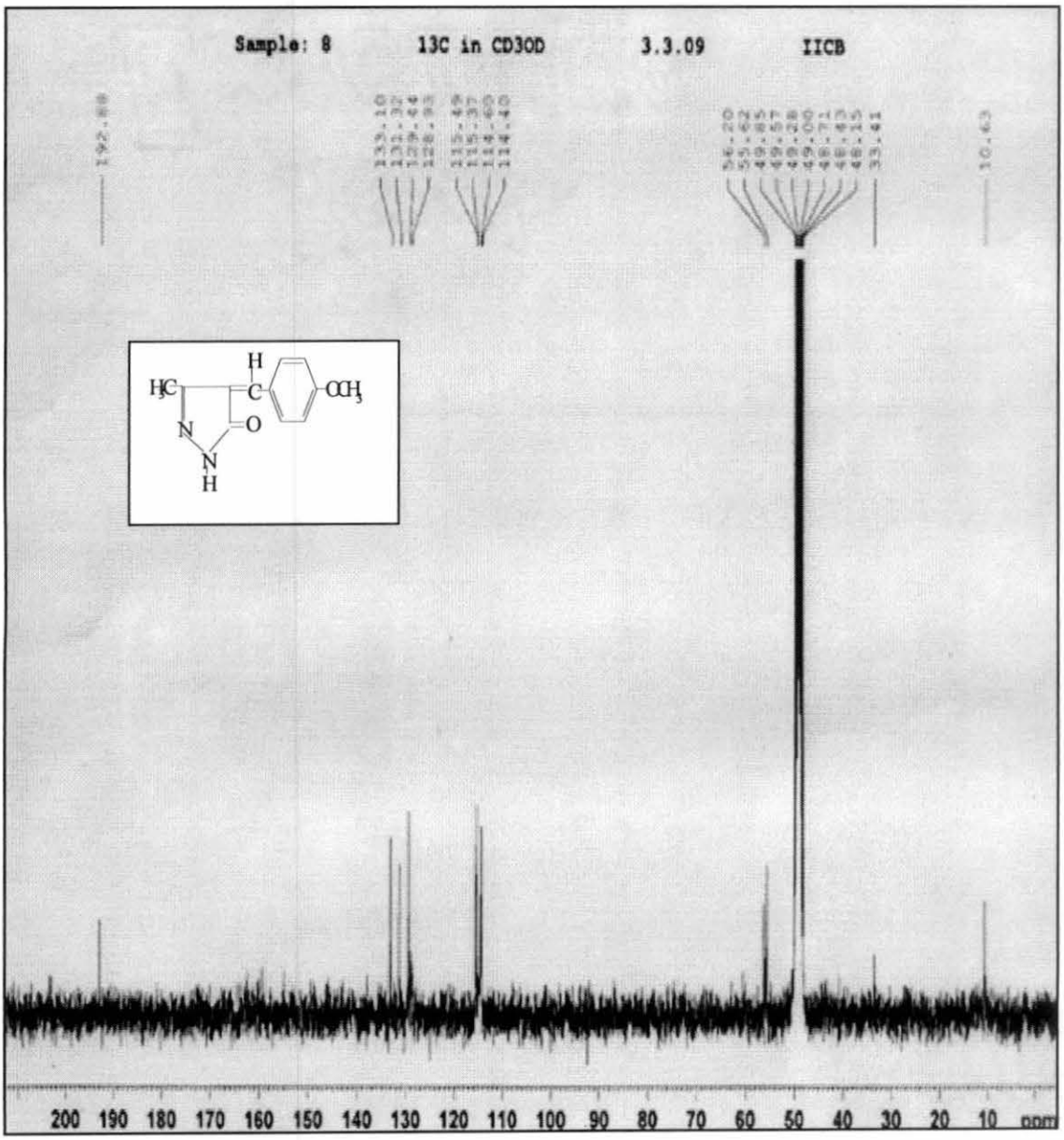


Fig: 5.28. ^{13}C NMR spectrum of PYZ8

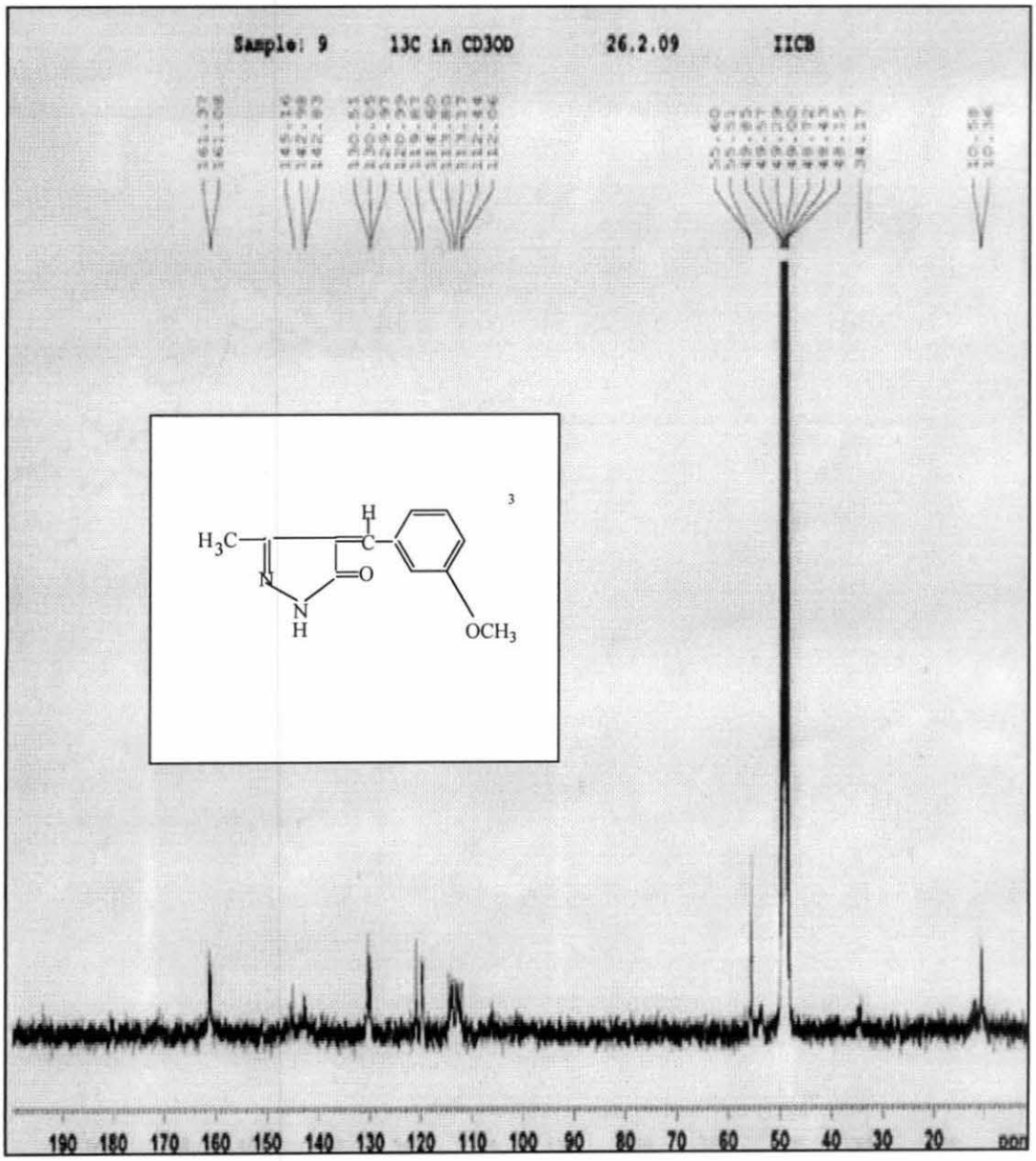


Fig: 5.29. ^{13}C NMR spectrum of PYZ9

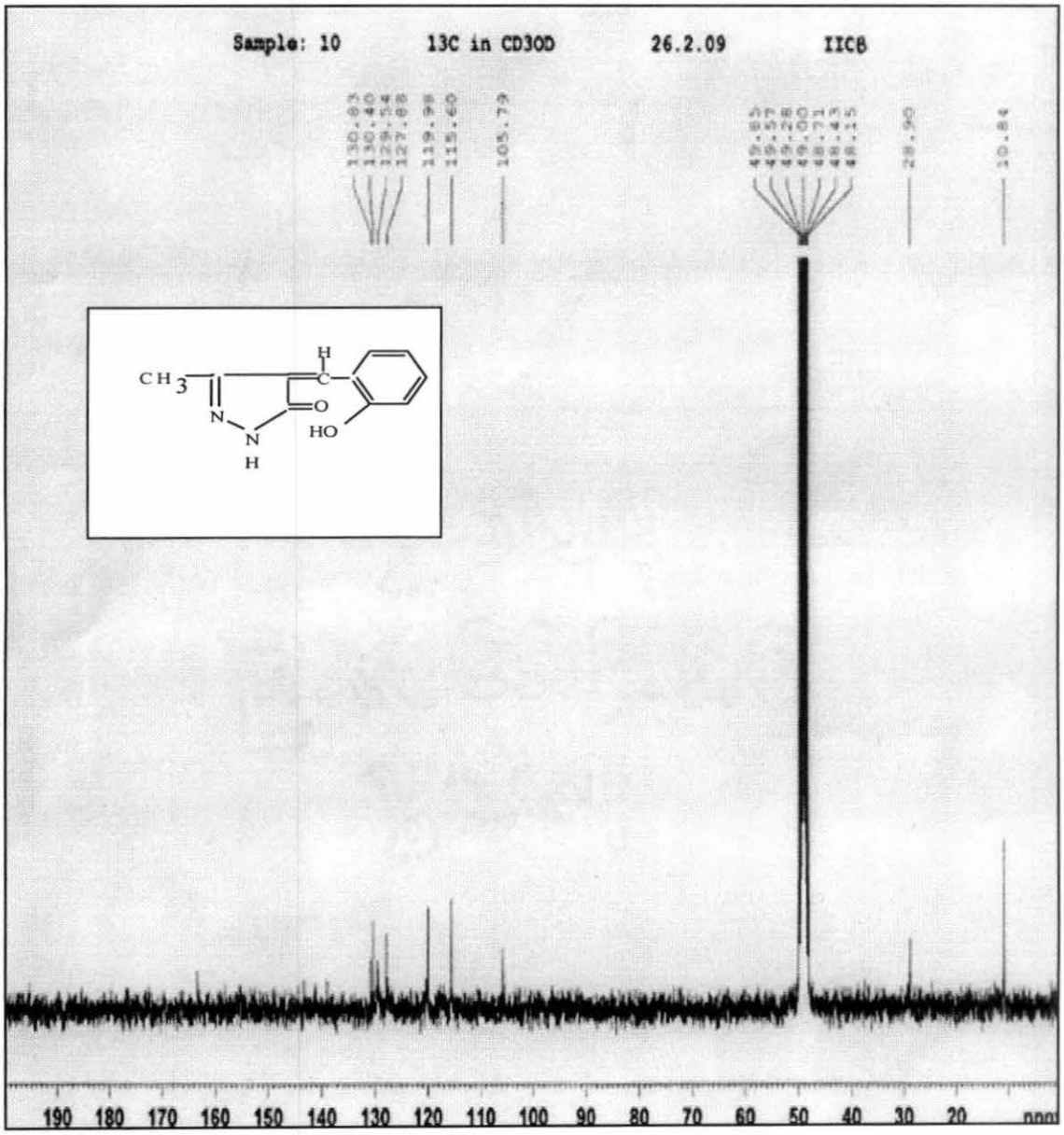


Fig: 5.30. ^{13}C NMR spectrum of PYZ10

5.7. Mass Spectrophotometry

The mass spectra of the synthesized compound were recorded on Micro mass Q-TOF and Shimadzu LCMS 2010A Mass spectrometer. The mass spectra of the synthesized compounds are given in **Fig: 5.31-5.40**

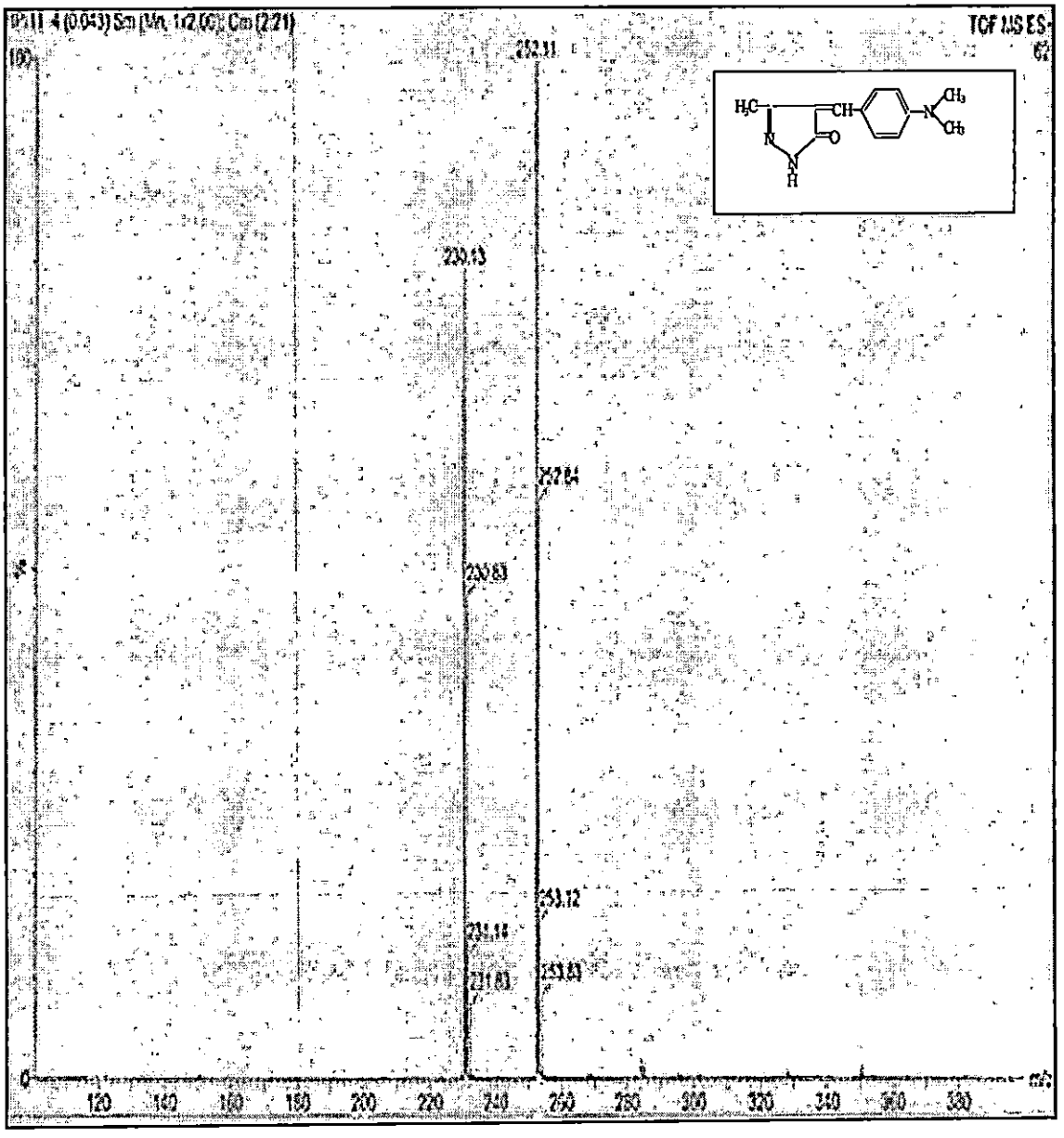


Fig: 5.31. Mass spectrum of Compound PYZ1

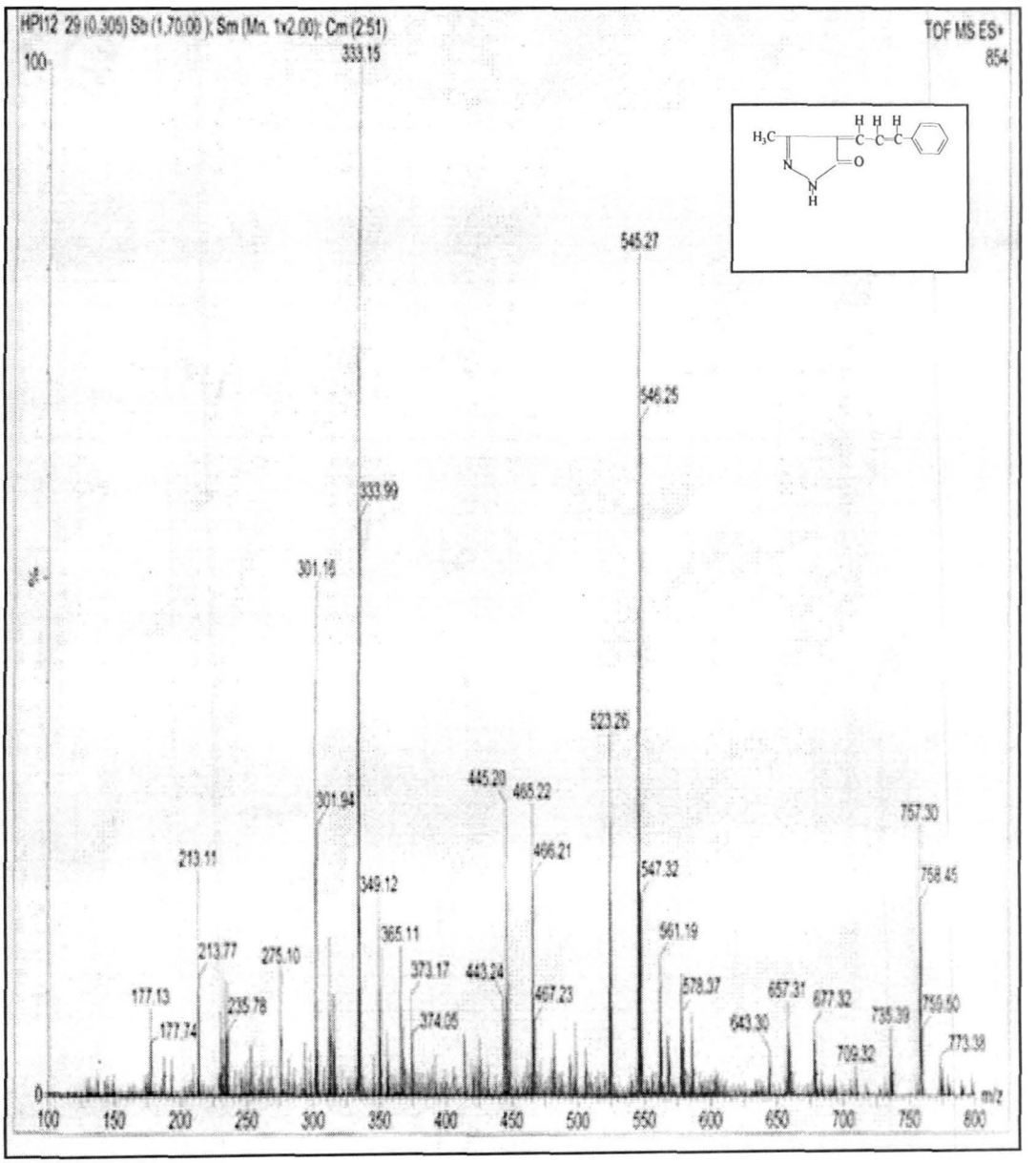


Fig: 5.32.Mass spectrum of Compound PYZ2

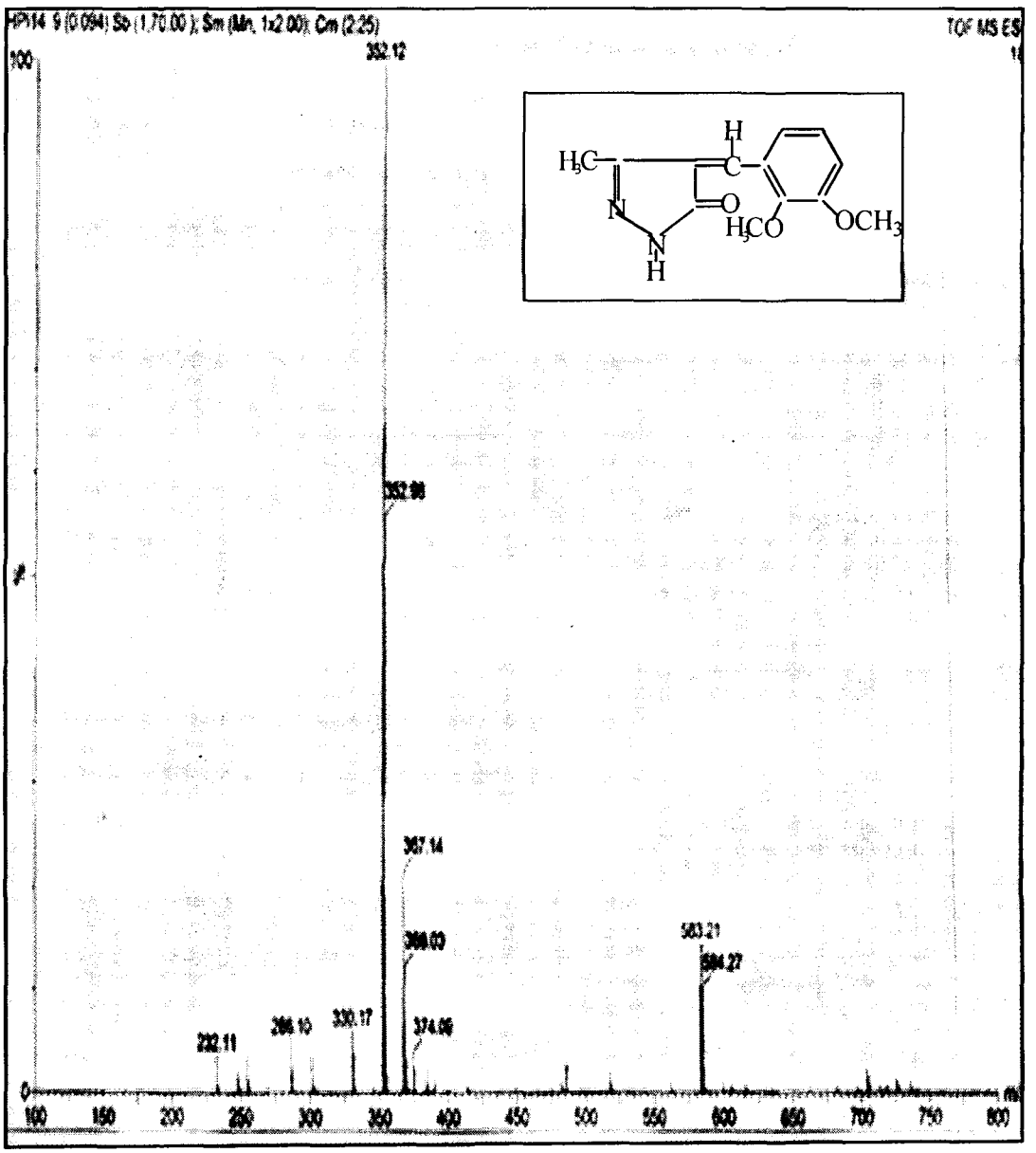


Fig: 5.33.Mass spectrum of Compound PYZ3

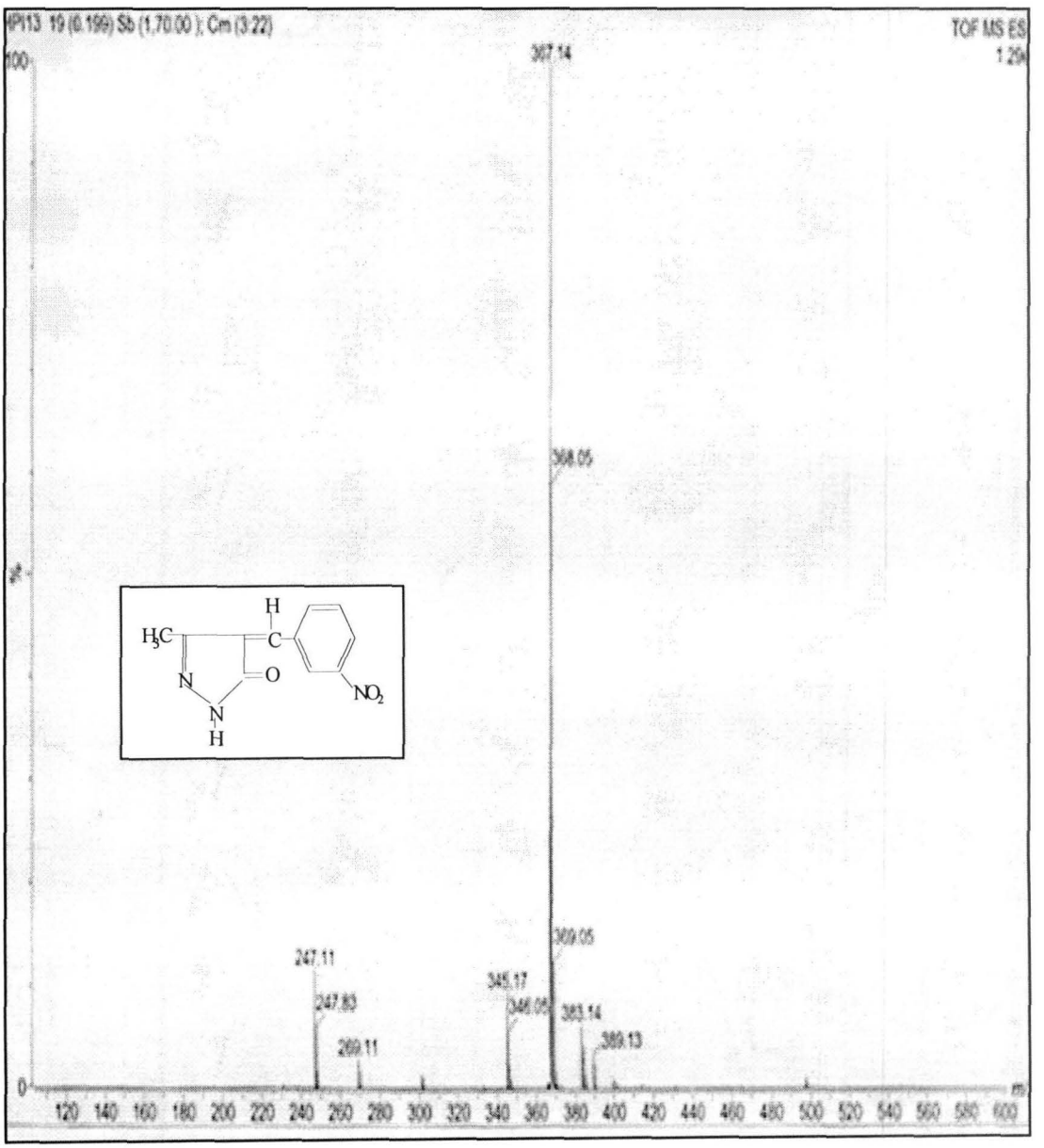


Fig: 5.34.Mass spectrum of Compound PYZ4

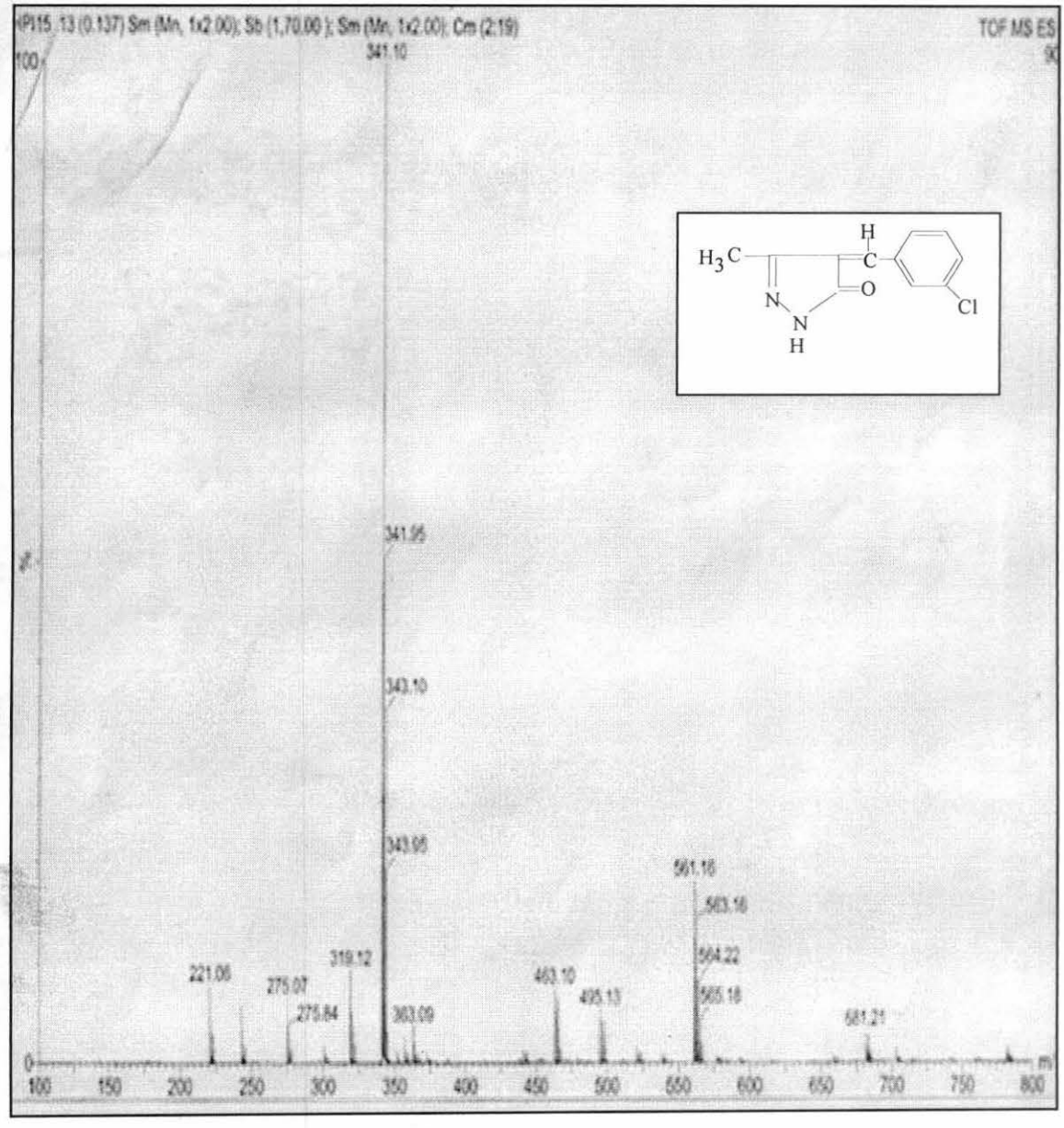


Fig: 5.35.Mass spectrum of Compound PYZ5

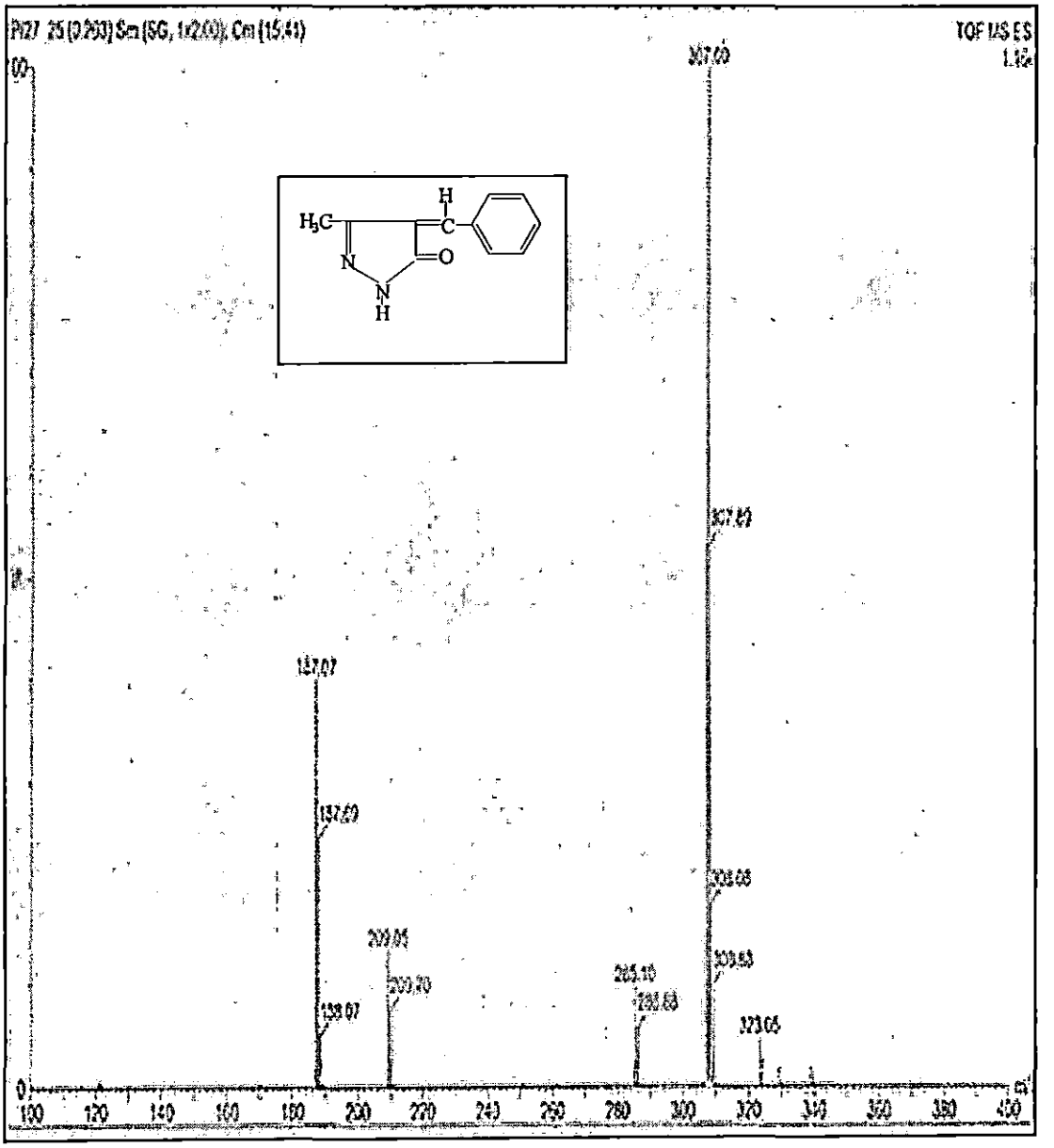


Fig: 5.36. Mass spectrum of Compound PYZ6

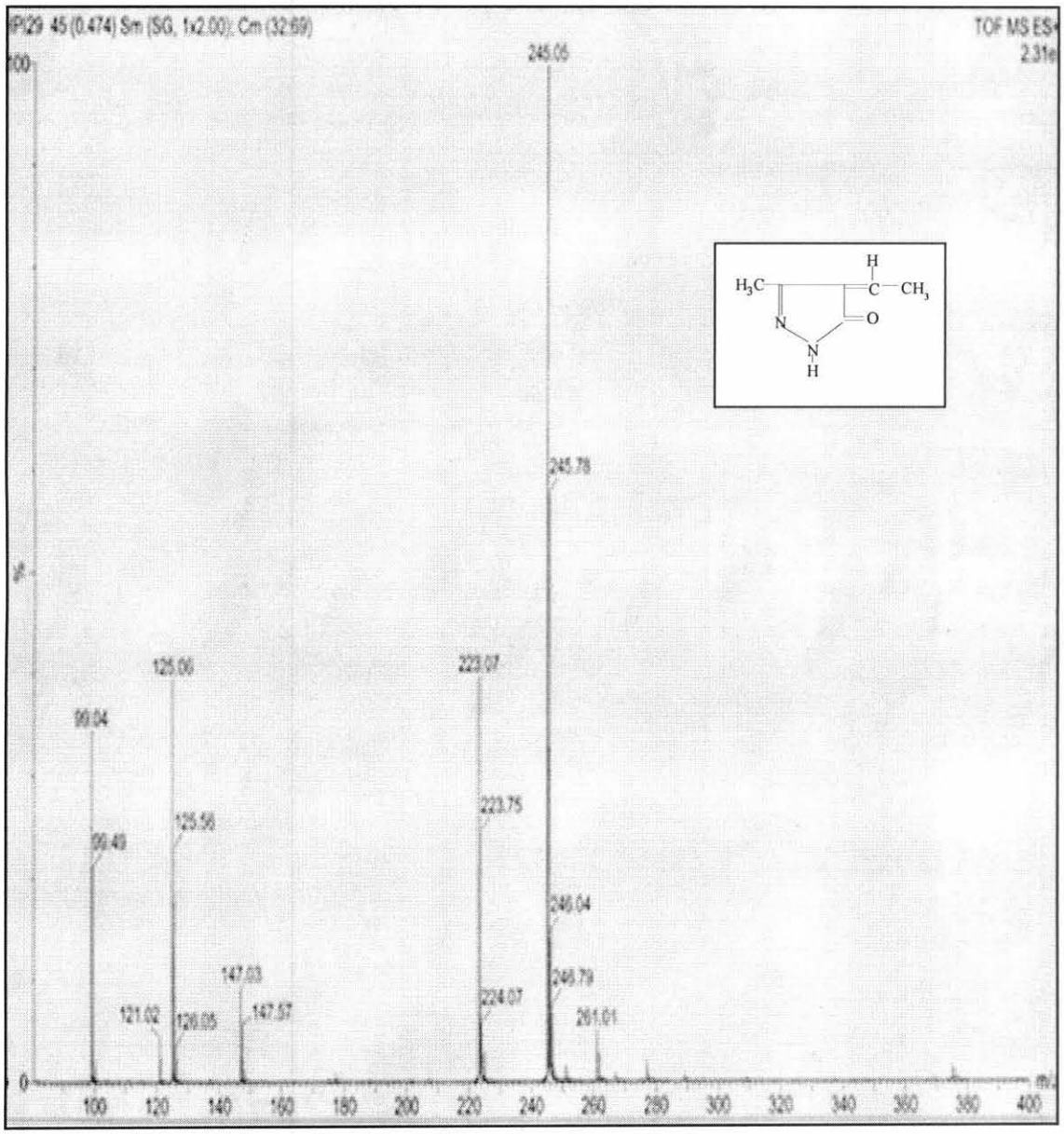


Fig: 5.37.Mass spectrum of Compound PYZ7

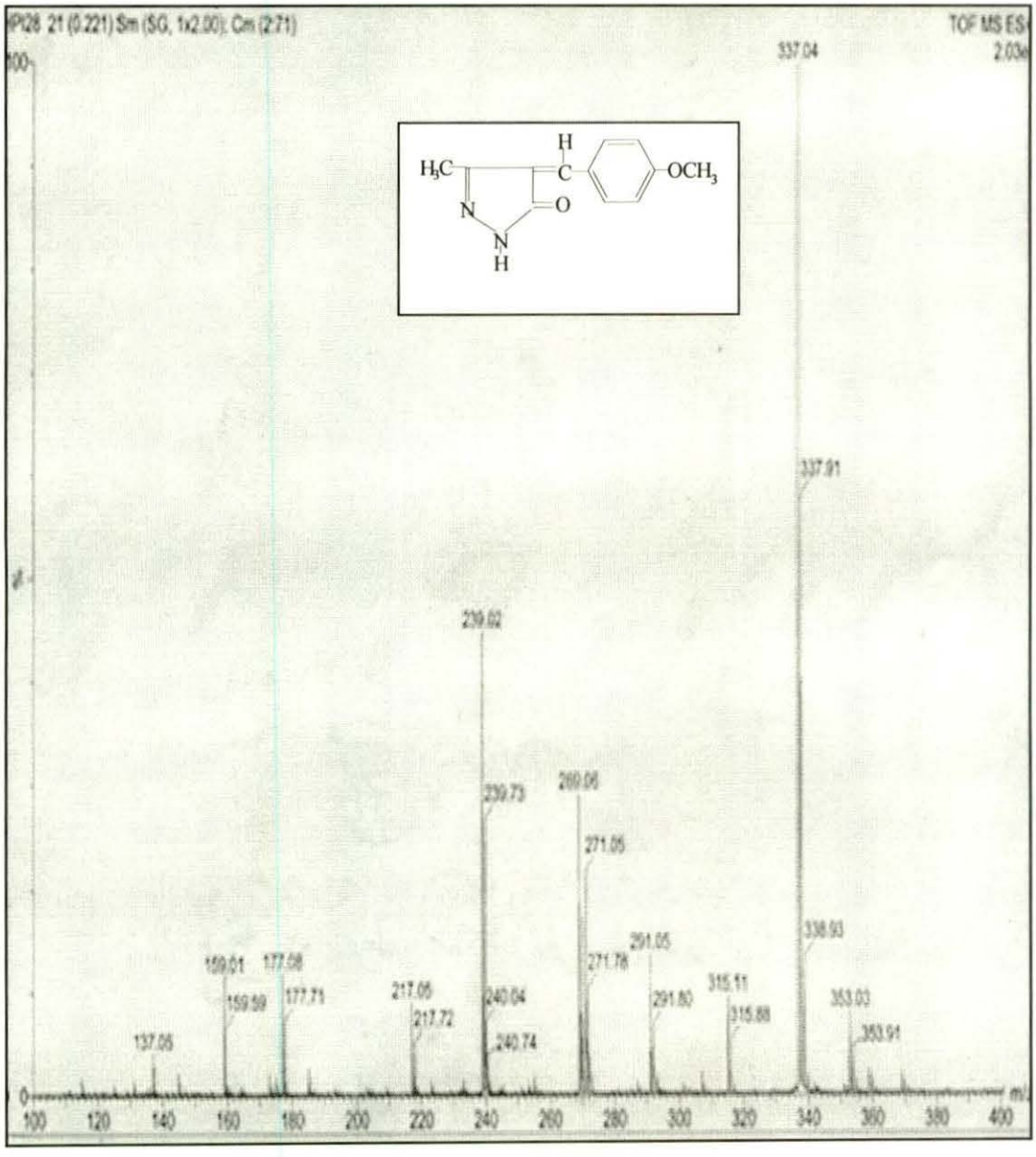


Fig: 5.38. Mass spectrum of Compound PYZ8

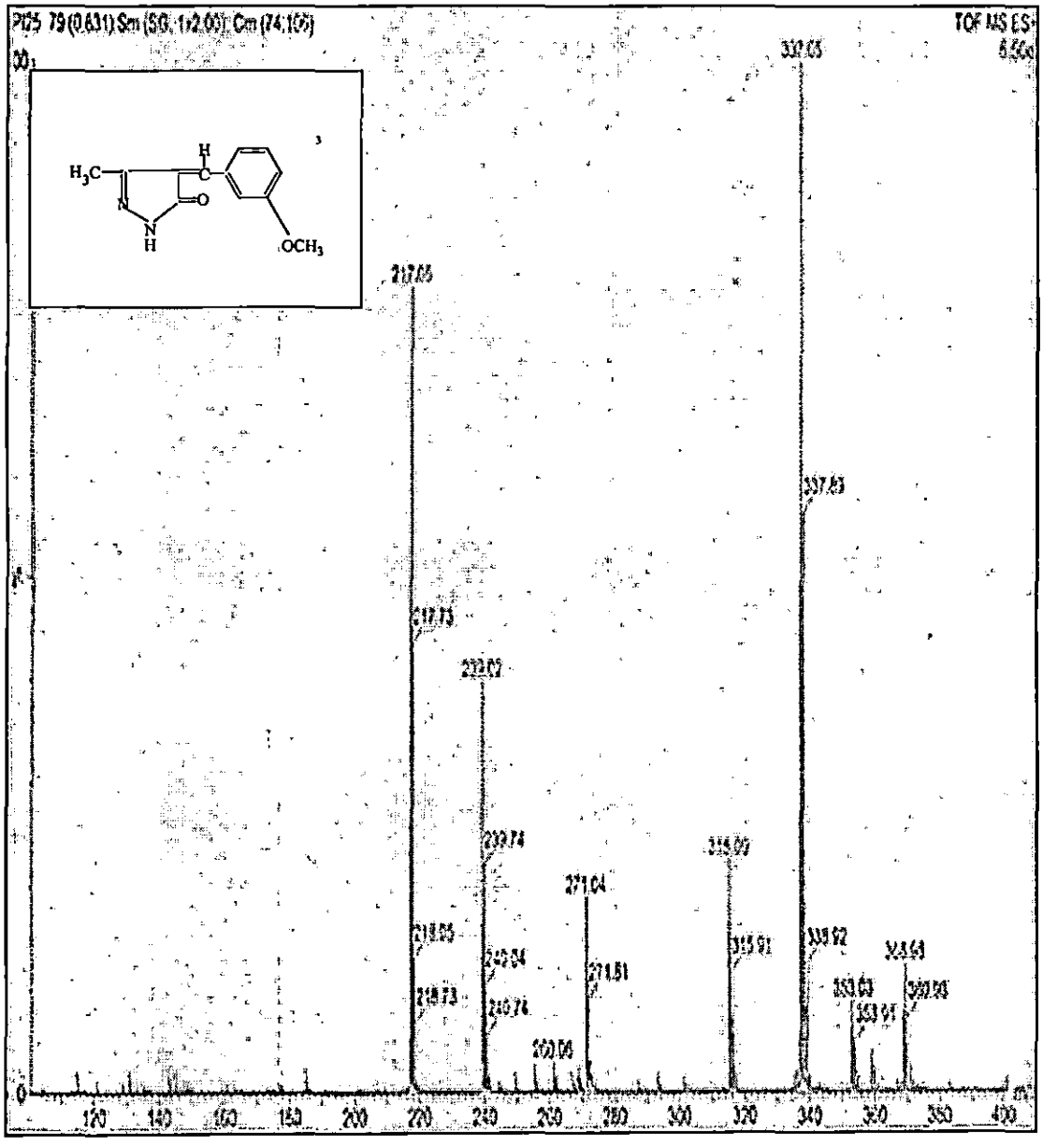


Fig: 5.39.Mass spectrum of Compound PYZ9

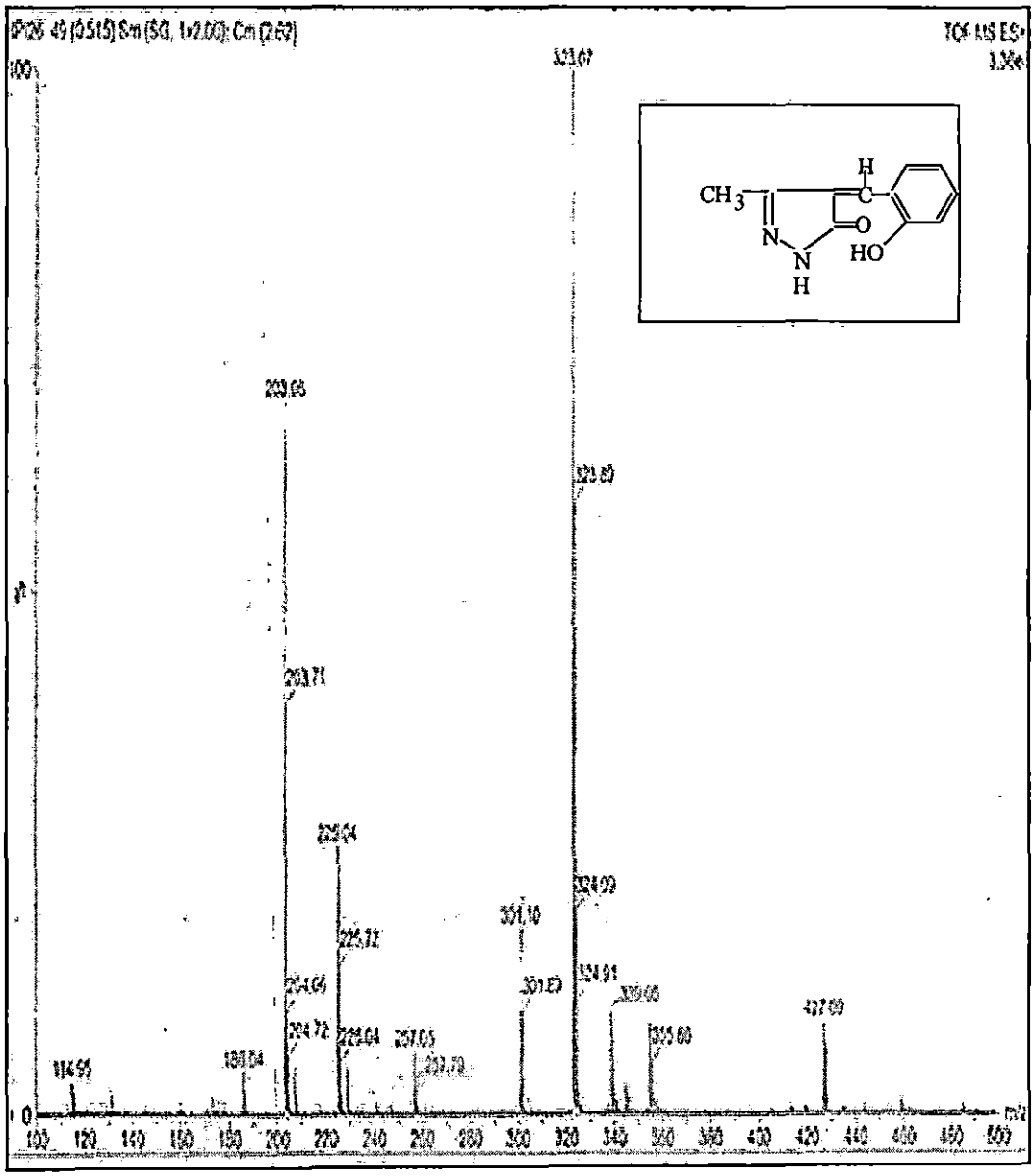


Fig: 5.40.Mass spectrum of Compound PYZ10

5.8. BIOLOGICAL EVALUATION

5.8.1 Acute toxicity study:-

Pyrazolone compounds were studied for acute toxicity as per OECD guidelines Up and Down procedure- 405. There was no death of rats dosed up to 5000 mg/kg bodyweight of the pyrazolone derivatives within the short outcome of the limit dose test of Up and Down method (Table **5.1.**). The LD50 was calculated to be greater than 5000 mg/kg body weight /orally

Table: 5.1. Result of Limit Dose Test of Pyrazolone Derivatives (PYZ1-10)

Compound code	No of animals	Dose(mg/kg)	Short term result (48hrs)
PYZ1	5	5000	Survived
PYZ2	5	5000	Survived
PYZ3	5	5000	Survived
PYZ4	5	5000	Survived
PYZ5	5	5000	Survived
PYZ6	5	5000	Survived
PYZ7	5	5000	Survived
PYZ8	5	5000	Survived
PYZ9	5	5000	Survived
PYZ10	5	5000	Survived

Table: 5.2. Effect of pyrazolone derivatives on marker enzymes in ISO induced myocardial injury in rat model

Compd code	SGOT (IU/ml)	SGPT (IU/ml)	ALP (IU/ml)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
Control	152.56±0.15	40.56±0.31	141.81±.71	43.87±.01	99.56±.11	8.05±1.25
ISO (8.5mg/kg)	203.26±.02	81.23±0.43	213.89±0.81	20.58±1.2	186.87±.13	30.22±.01
PYZ1	191.52±0.61^{b}	79.01±0.26^{b}	210.56±0.42^{ns}	23.54±1.65^{a}	165.25±.56^{b}	23.33±.11^{b}
PYZ2	190.12±0.51^{b}	79.99±0.11^{ns}	208.20±0.34^{b}	23.99±.11^{a}	165.66±.23^{b}	23.01±.51^{b}
PYZ3	181.84±0.63^{b}	76.44±0.13^{b}	207.32±0.62^{b}	24.89±.31^{b}	154.84±.29^{b}	20.38±.65^{b}
PYZ4	184.91±0.71^{b}	76.00±0.01^{b}	200.89±.10^{b}	25.63±.84^{b}	153.22±.55^{b}	19.99±2.63^{b}
PYZ5	182.38±0.02^{b}	74.55±0.71^{b}	198.36±0.52^{b}	27.00±.67^{b}	146.40±.10^{b}	18.85±1.85^{b}
PYZ6	180.01±0.41^{b}	72.36±0.10^{b}	195.39±1.20^{b}	28.56±.24^{b}	136.55±.61^{b}	15.84±1.95^{b}
PYZ7	179.85±0.53^{b}	70.85±0.63^{b}	193.95±1.90^{b}	29.66±.01^{b}	134.66±.01^{b}	14.55±2.55^{b}
PYZ8	164.22±0.87^{b}	61.02±0.45^{b}	176.95±.97^{b}	31.11±.03^{b}	130.58±.33^{b}	12.86±.01^{b}
PYZ9	162.00±0.72^{b}	55.00±.06^{b}	175.00±.15^{b}	32.33±.21^{b}	125.98±.78^{b}	10.62±.11^{b}
PYZ10	158.01±1.01^{b}	54.62±0.14^{b}	172.83±1.57^{b}	36.98±.02^{b}	120.88±.91^{b}	10.01±.02^{b}

Values are expressed as mean±SEM, (N=6); ns non significant

$^{a}P < 0.05$, $^{b}P < 0.01$ compared with ISO control (ANOVA followed by Dunnett's test). All the test compound were administered at 10 mg/kg

Table: 5.3. Effect of pyrazolone derivatives on lipids profile in ISO induced myocardial injury in rat model

Compd code	Total Protein (g/L)	Triglycerides (mg/dL)	Cholesterol (mg/dL)	Creatinine (mg/dL)	Lactose Dehydrogenase (IU/L)
Control	39.80±0.98	79.04±3.97	117.15±2.02	2.26±0.04	120.07±0.54
ISO(8.5 mg/kg)	86.86±0.25	186.47±0.34	201.65±7.56	3.93±0.08	198.71±0.29
PYZ1	56.46±.050[b]	135.43±3.18[b]	168.61±0.63[b]	2.81±0.007[b]	134.25±0.58[b]
PYZ2	66.33±0.28[b]	122.32±0.70[b]	162.75±0.74[b]	2.92±0.01[b]	142.70±0.51[b]
PYZ3	53.00±4.70[b]	180.58±4.44[b]	153.95±0.81[b]	2.65±0.18[b]	148.45±0.41[b]
PYZ4	40.43±0.85[b]	152.94±4.29[b]	133.46±0.58[b]	2.39±0.03[b]	158.39±0.47[b]
PYZ5	39.23±0.92[b]	158.33±3.74[b]	132.14±0.73[b]	2.65±0.01[b]	138.55±0.52[b]
PYZ6	58.07±0.86[b]	123.33±0.61[b]	118.04±0.37[b]	2.67±0.04[b]	128.83±0.71[b]
PYZ7	38.08±0.79[b]	124.66±0.94[b]	120.59±0.53[b]	2.67±0.07[b]	123.68±0.95[b]
PYZ8	48.36±0.66[b]	80.07±0.37[b]	121.01±0.73[b]	2.78±0.07[b]	121.80±0.50[b]
PYZ9	52.20±0.77[b]	81.20±0.35[b]	122.89±0.45[b]	2.56±0.0[b]	127.76±0.59[b]
PYZ10	53.06±0.79[b]	82.95±0.75[b]	123.82±0.64[b]	2.45±0.10[b]	127.25±1.8[b]

Values are expressed as mean±SEM, (N=6);

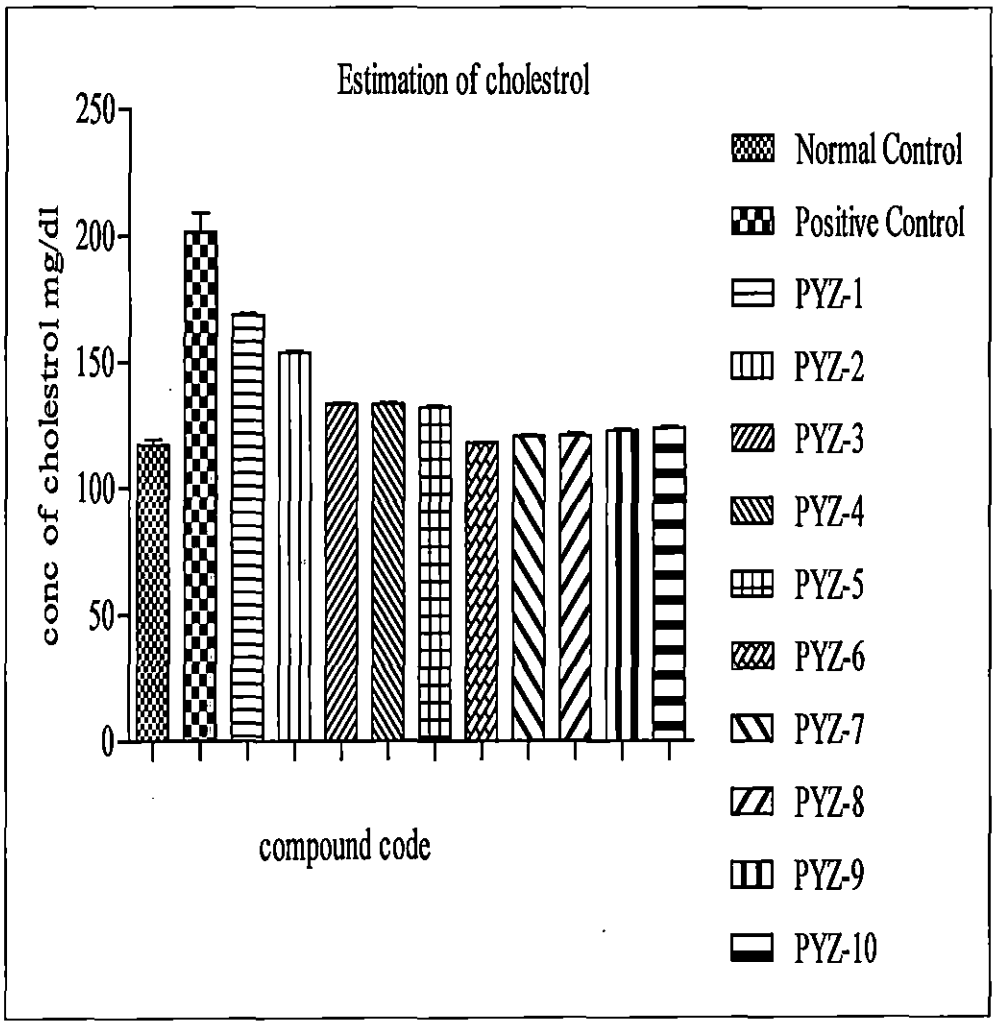
[b]P<0.01 compared with ISO control (ANOVA followed by Dunnett's test). The entire test compounds were administered at 10 mg/kg.

Table: 5.4. Effect of pyrazolone derivatives on endogeneous antioxidant enzymes in ISO induced myocardial injury in rat model

Compound code	SOD U/mg of protein	CAT IU/mg of tissue	GSH µg /mg wet tissue	CPK IU/ml
Control	5.38±0.21	73.05±.02	5.63±.02	110.25±0.01
ISO(8.5 mg/kg)	1.99±0.01	40.11±.10	1.69±0.12	194.45±0.25
PYZ1	2.71±0.02[a]	43.25±.21[a]	2.01±0.11[ns]	190.85±0.74[a]
PYZ2	2.91±0.14[b]	43.00±.06[a]	2.65±0.21[ns]	188.65±0.46[b]
PYZ3	3.01±0.04[b]	44.55±.11[b]	2.97±0.14[b]	184.29±1.01[b]
PYZ4	3.51±0.13[b]	46.88±.56[b]	3.86±0.18[b]	180.11±0.08[b]
PYZ5	3.80±0.02[b]	49.57±.32[b]	3.88±0.19[b]	179.86±0.87[b]
PYZ6	3.91±0.14[b]	50.22±.55[b]	3.15±0.25[b]	175.97±1.56[b]
PYZ7	3.99±0.31[b]	50.00±1.21[b]	3.6±0.3[b]	168.52±1.50[b]
PYZ8	4.01±0.42[b]	53.85±1.38[b]	3.55±0.15[b]	160.28±0.05[b]
PYZ9	4.49±0.02[b]	54.92±.07[b]	4.02±0.49[b]	158.87±0.24[b]
PYZ10	4.99±0.11[b]	58.87±1.02[b]	4.09±0.28[b]	158.77±0.08[b]

Values are expressed as mean±SEM, (N=6); ns. Non significant

[a]P<0.05, [b]P<0.01 compared with ISO control (ANOVA followed by Dunnett's test). The entire test compound was administered at 10 mg/kg.



`Fig: 5.41. Estimation of cholesterol

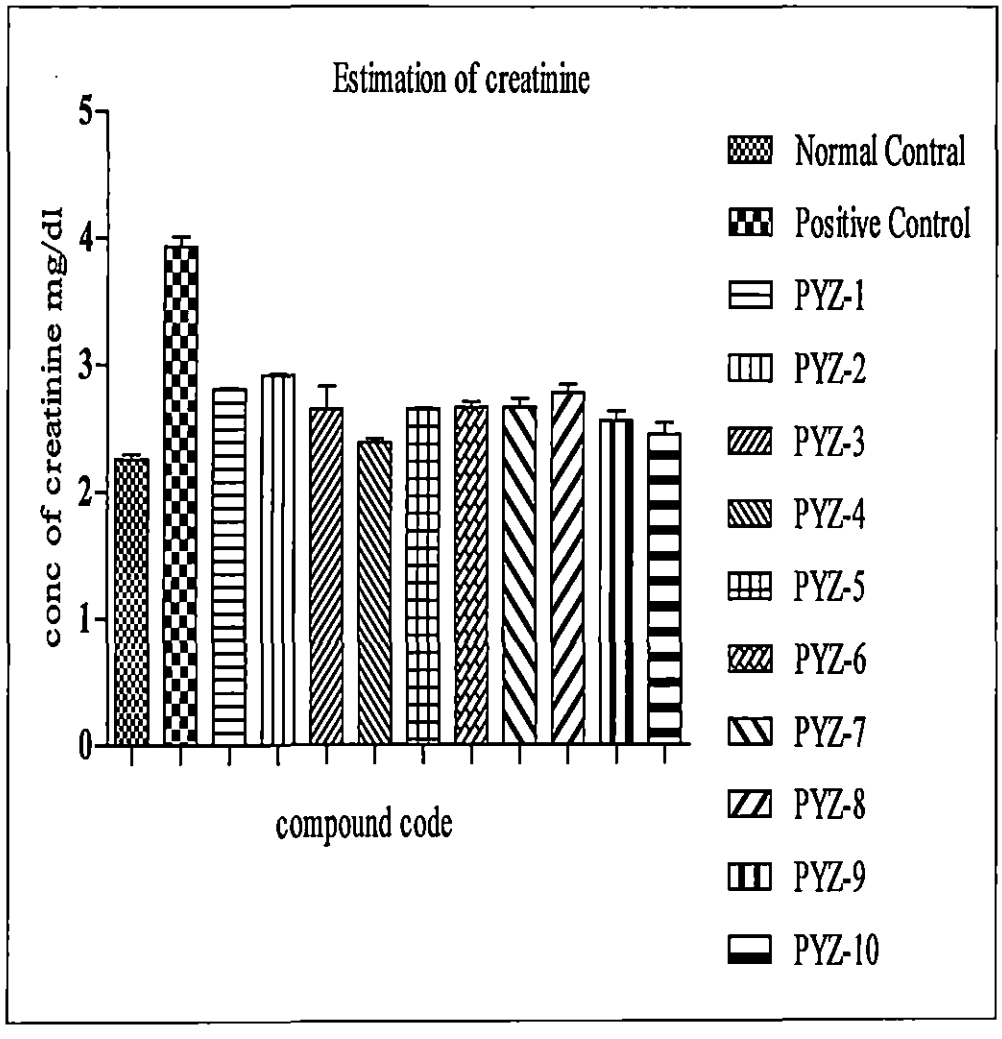


Fig: 5.42. Estimation of creatinine

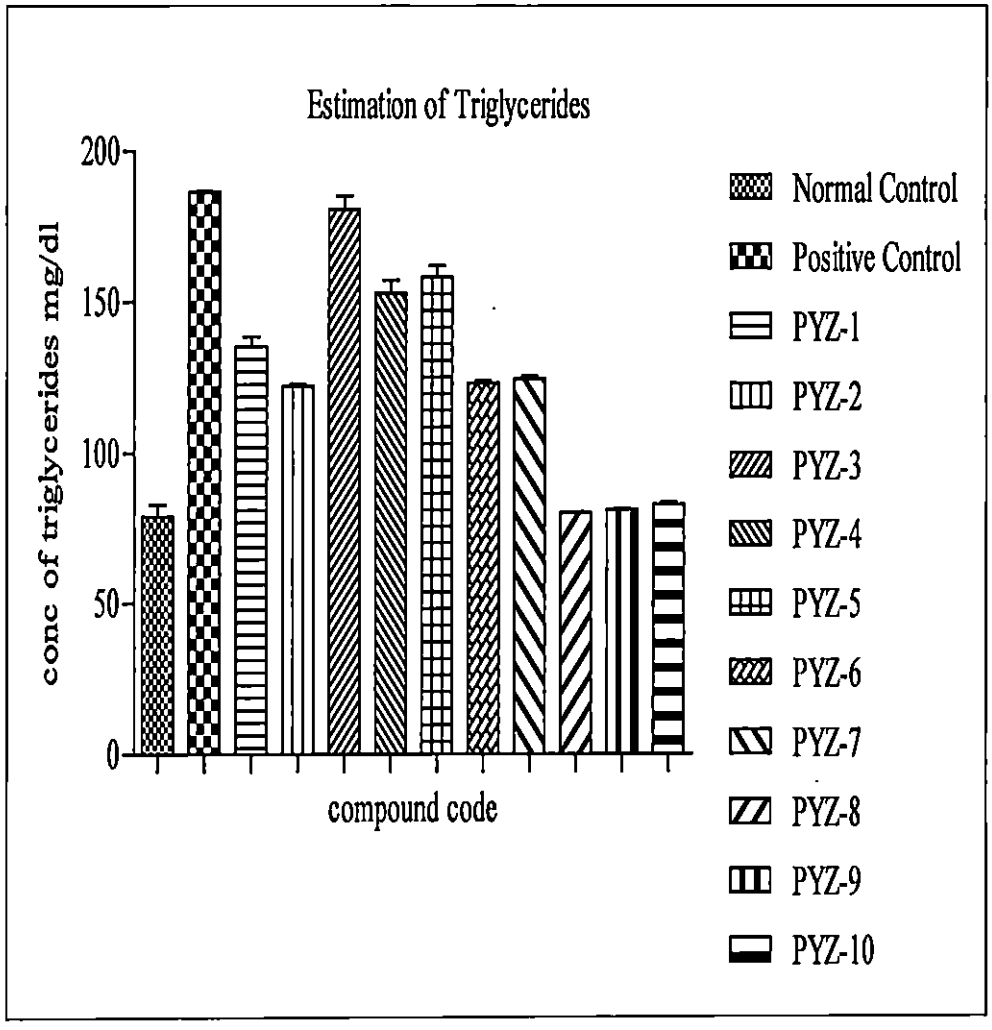


Fig: 5.43. Estimation of triglycerides

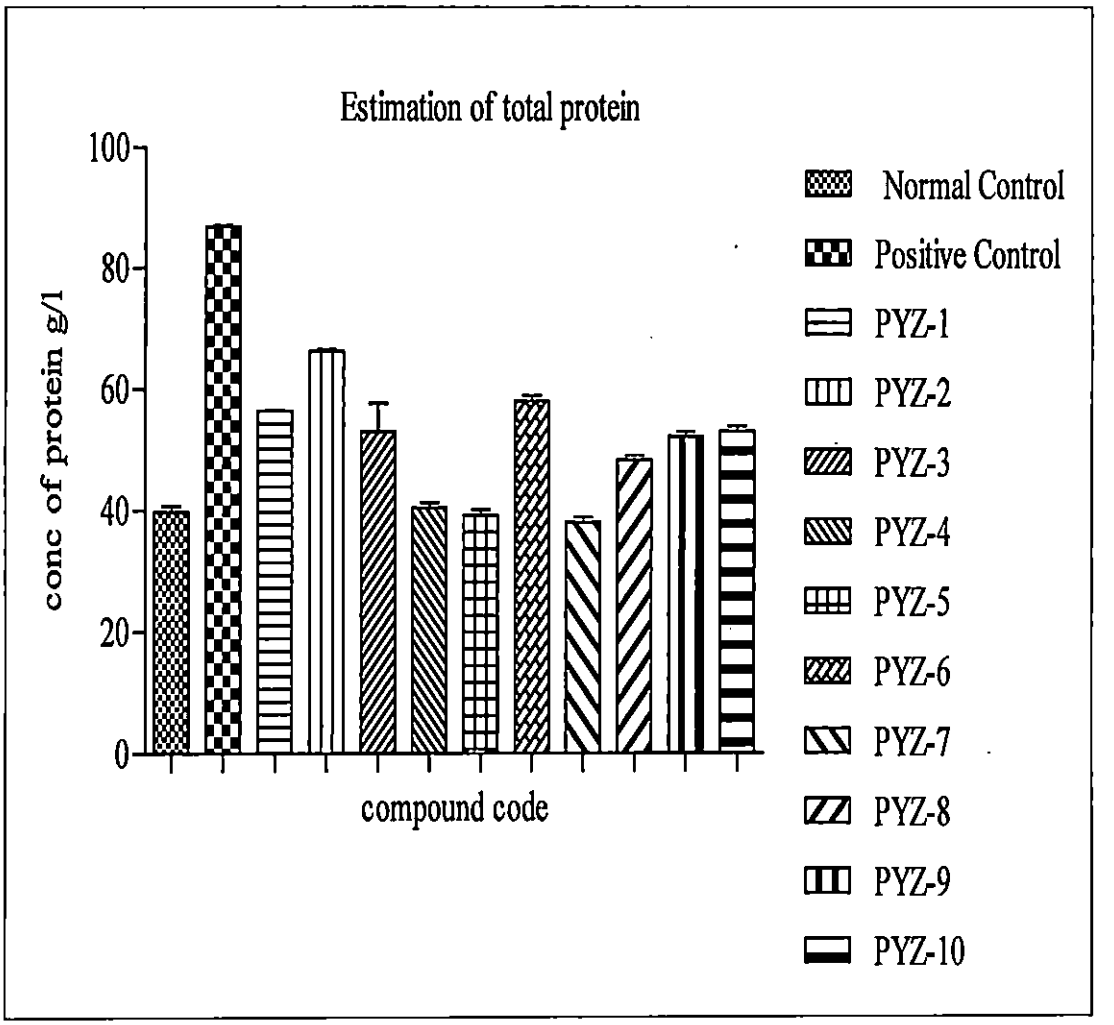


Fig: 5.44. Estimation of total protein

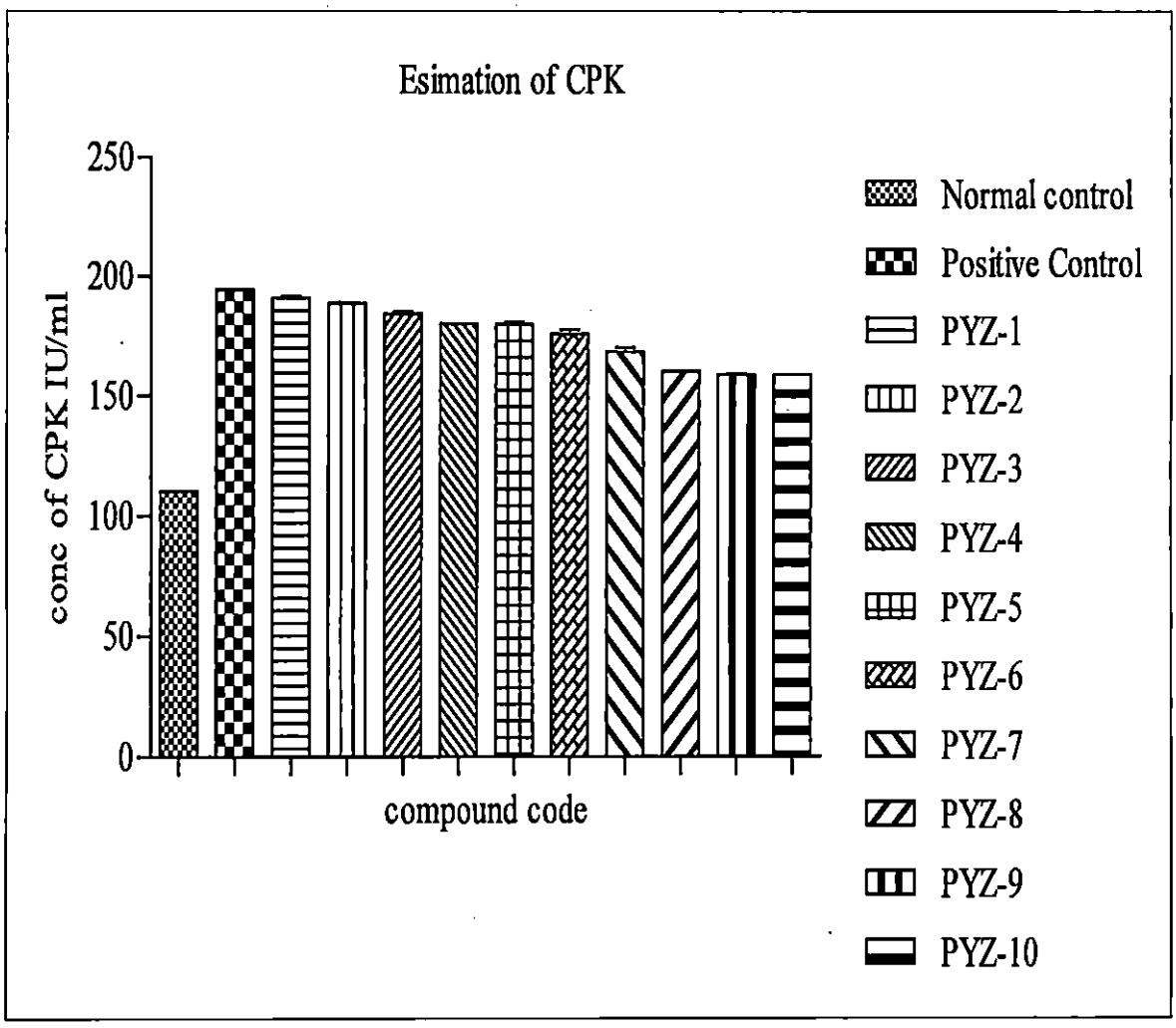


Fig: 5.45. Estimation of CPK

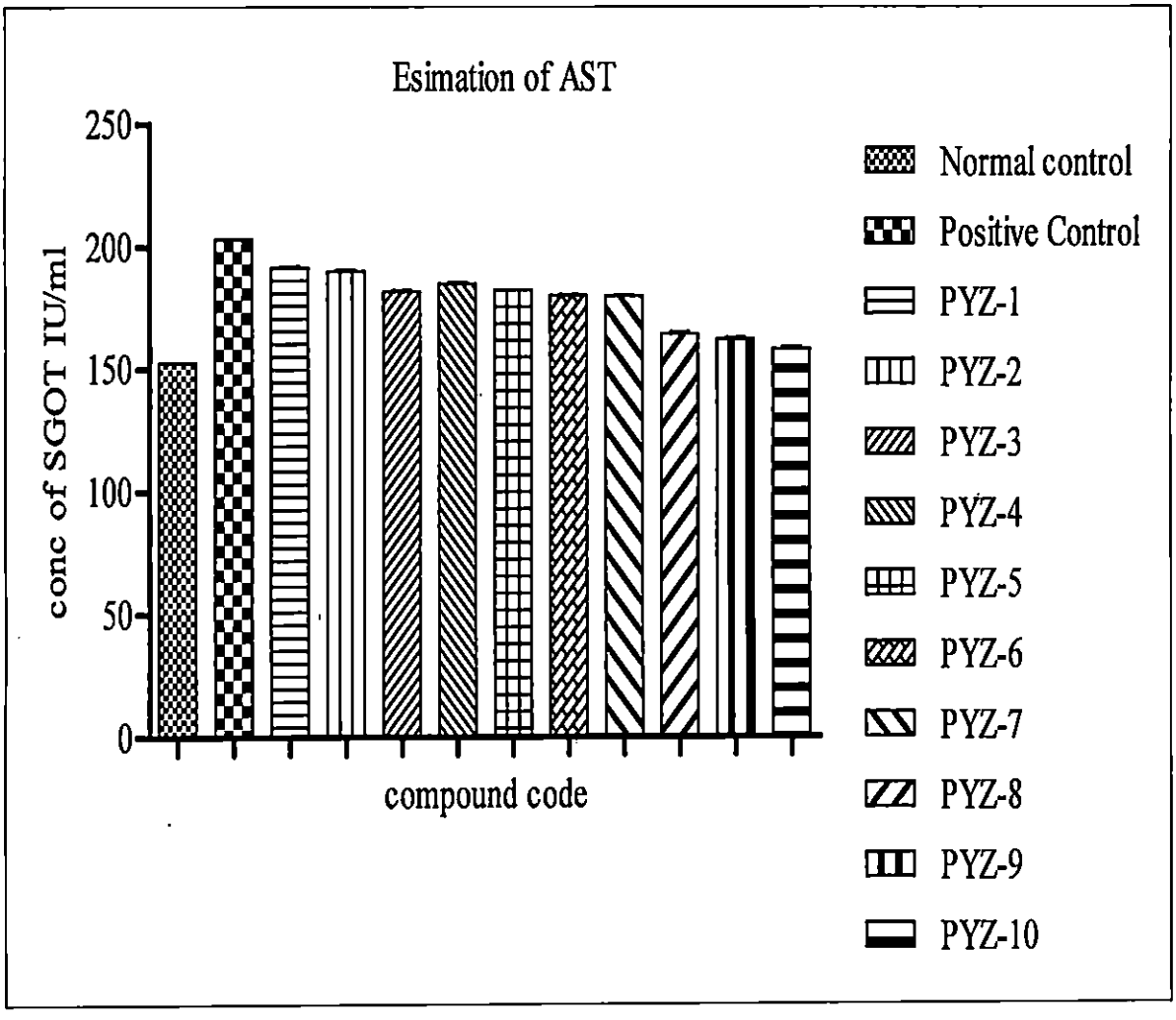


Fig: 5.46. Estimation of AST (SGOT)

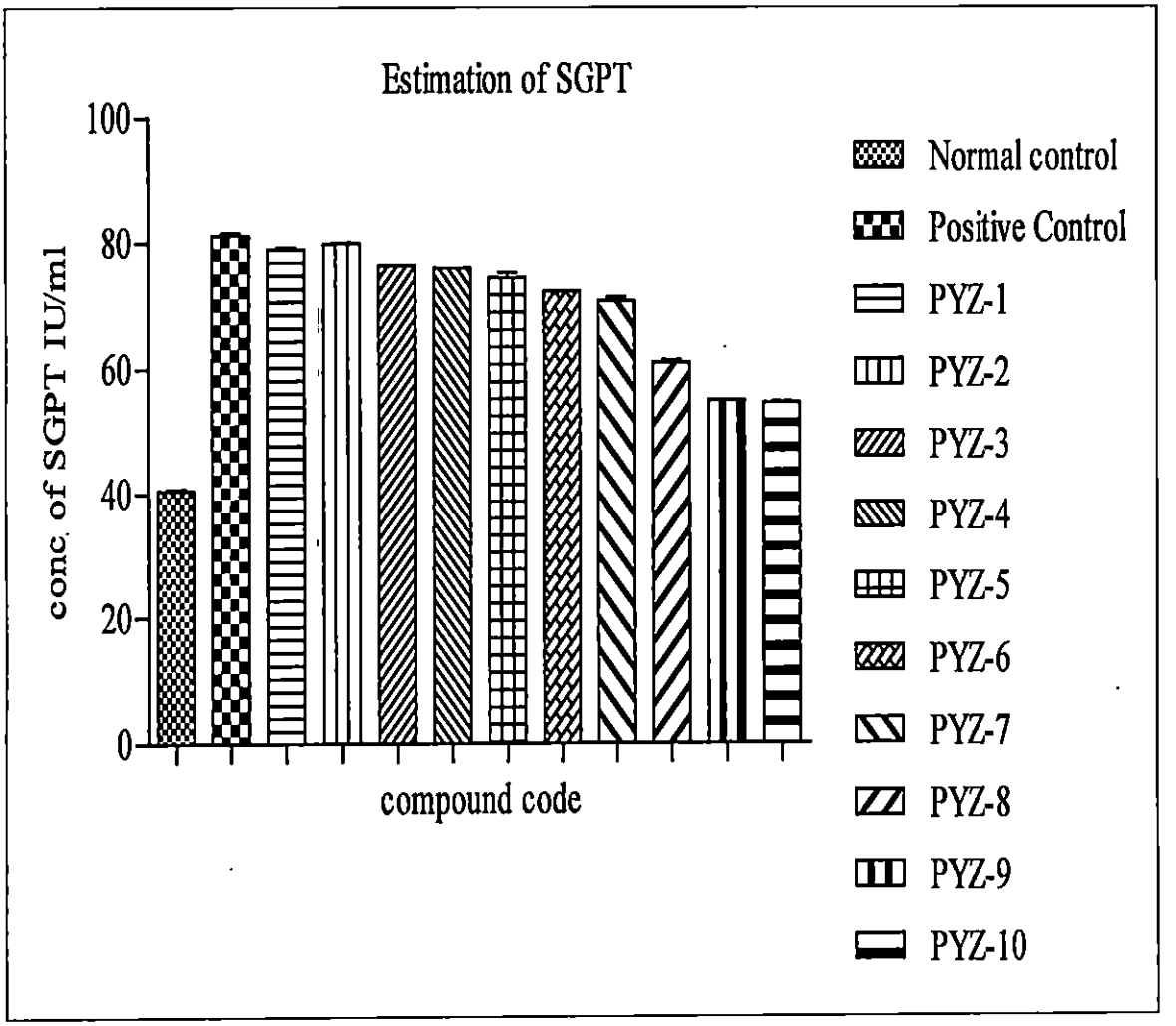


Fig: 5.47. Estimation of SGPT

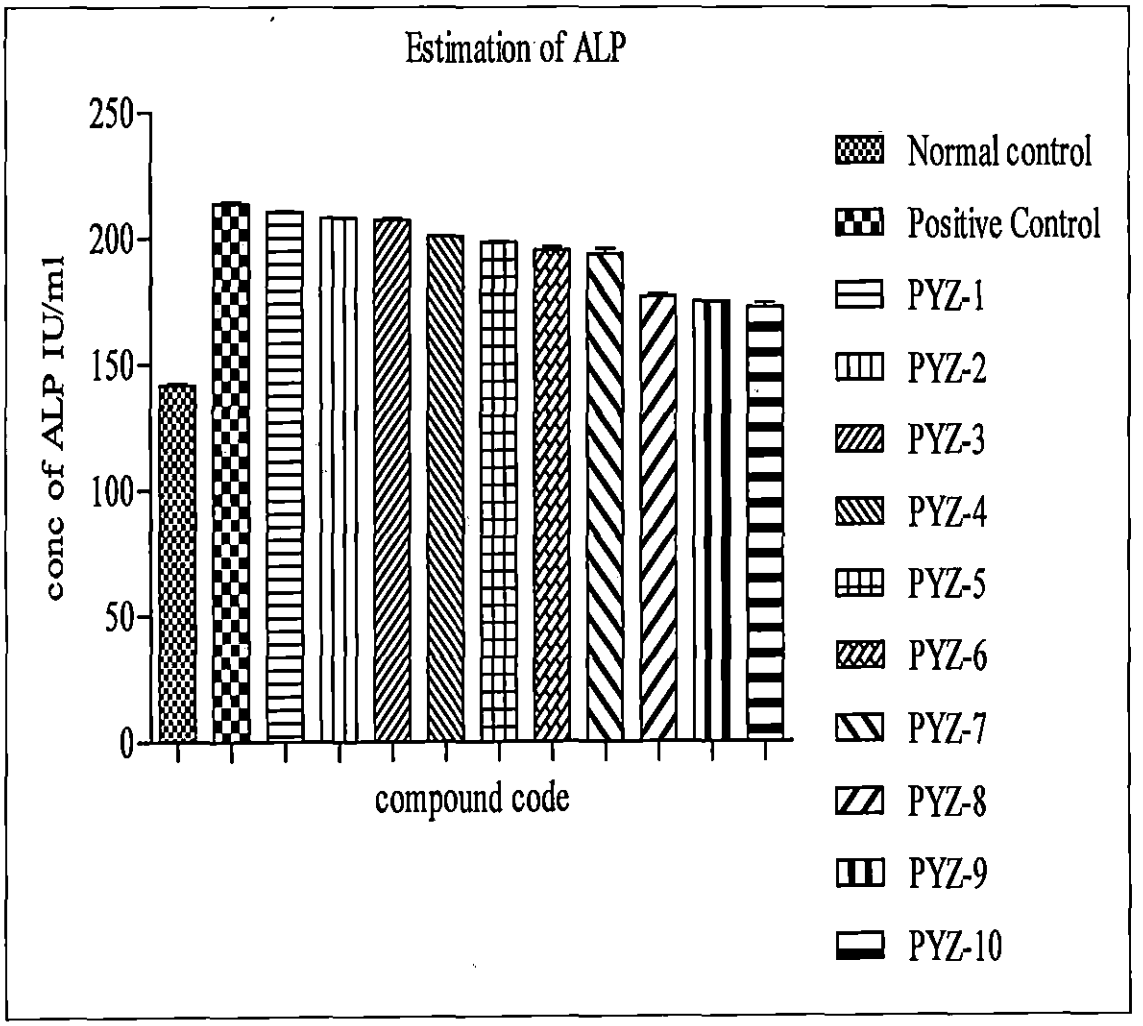


Fig: 5.48. Estimation of ALP

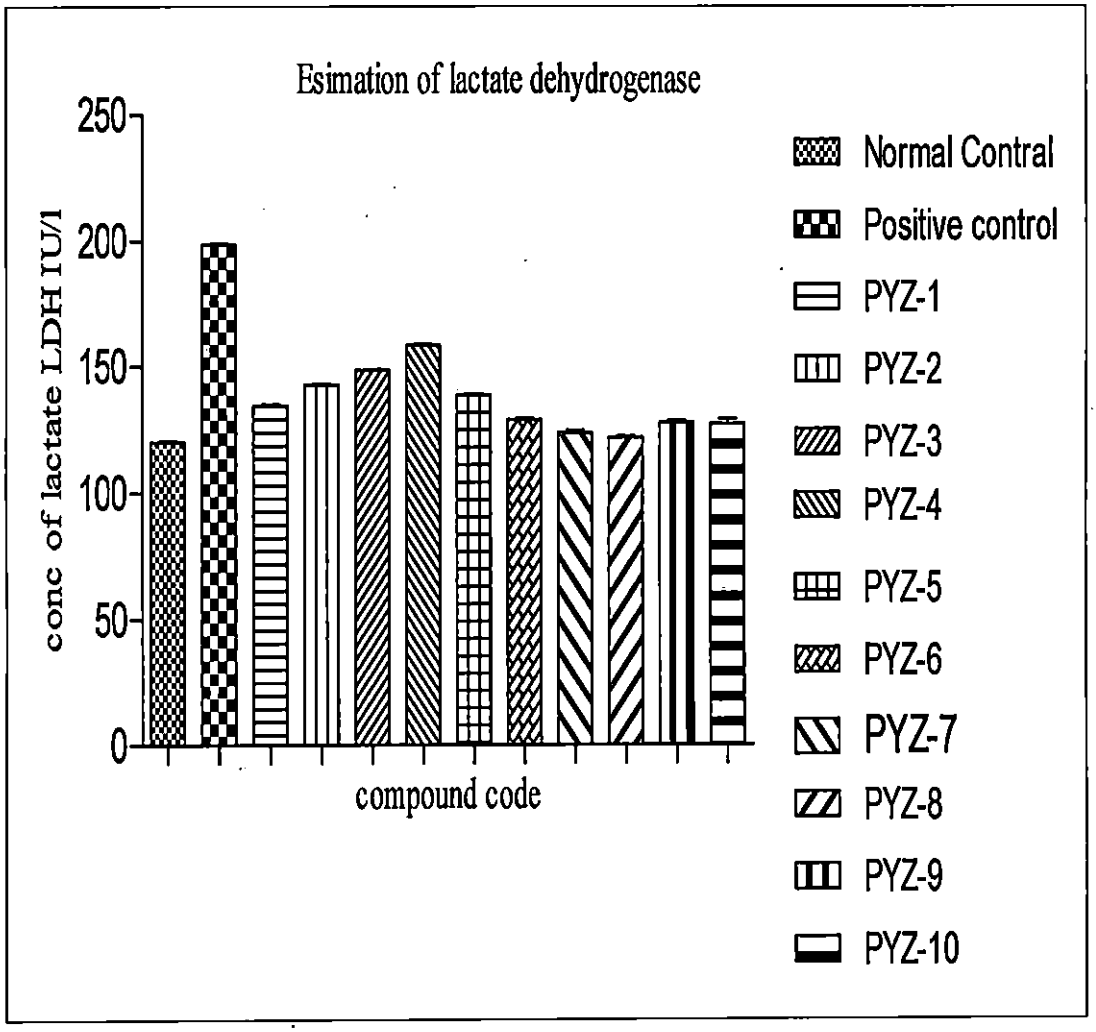


Fig: 5.49. Estimation of lactate dehydrogenase

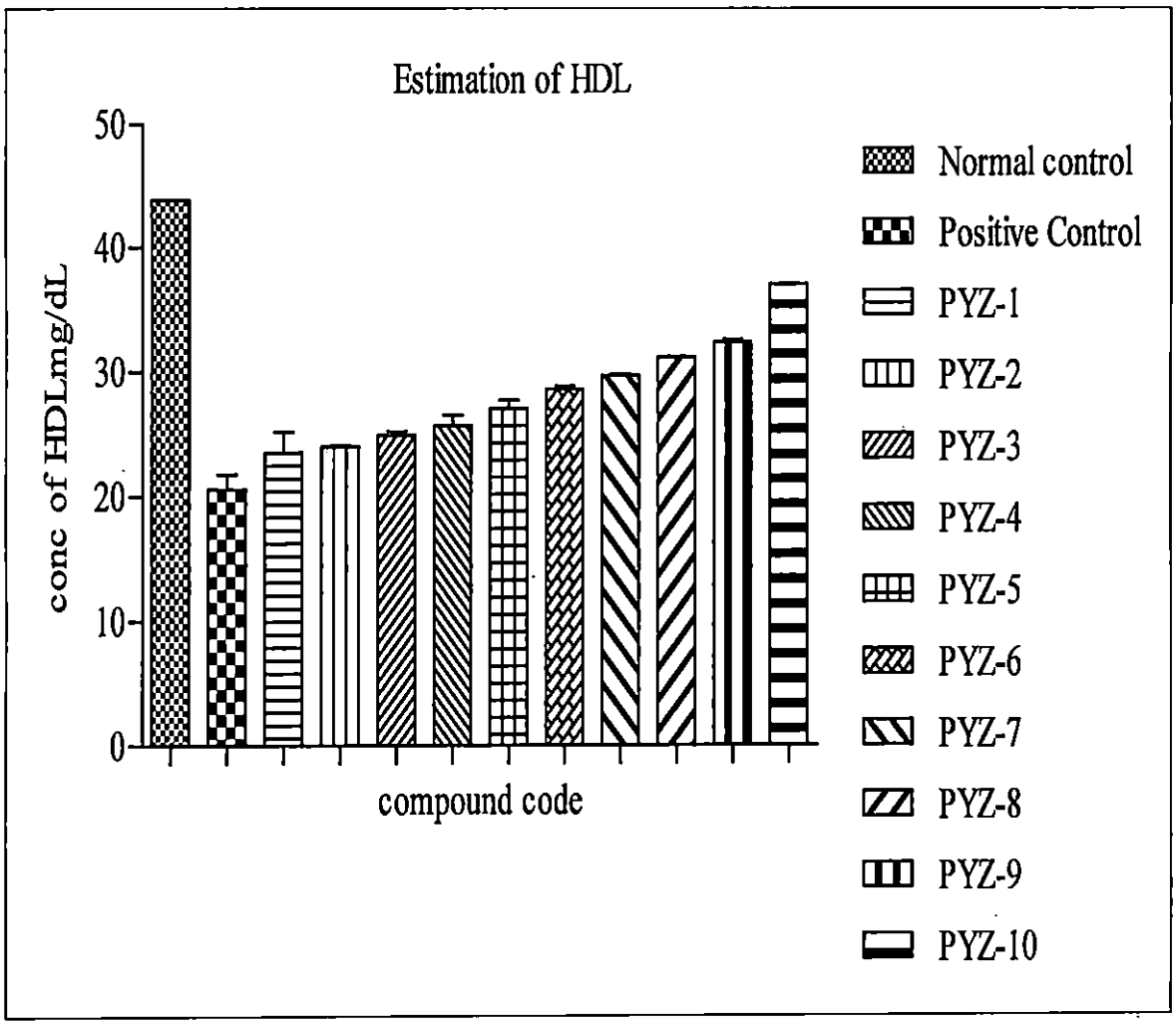


Fig: 5.50. Estimation of HDL

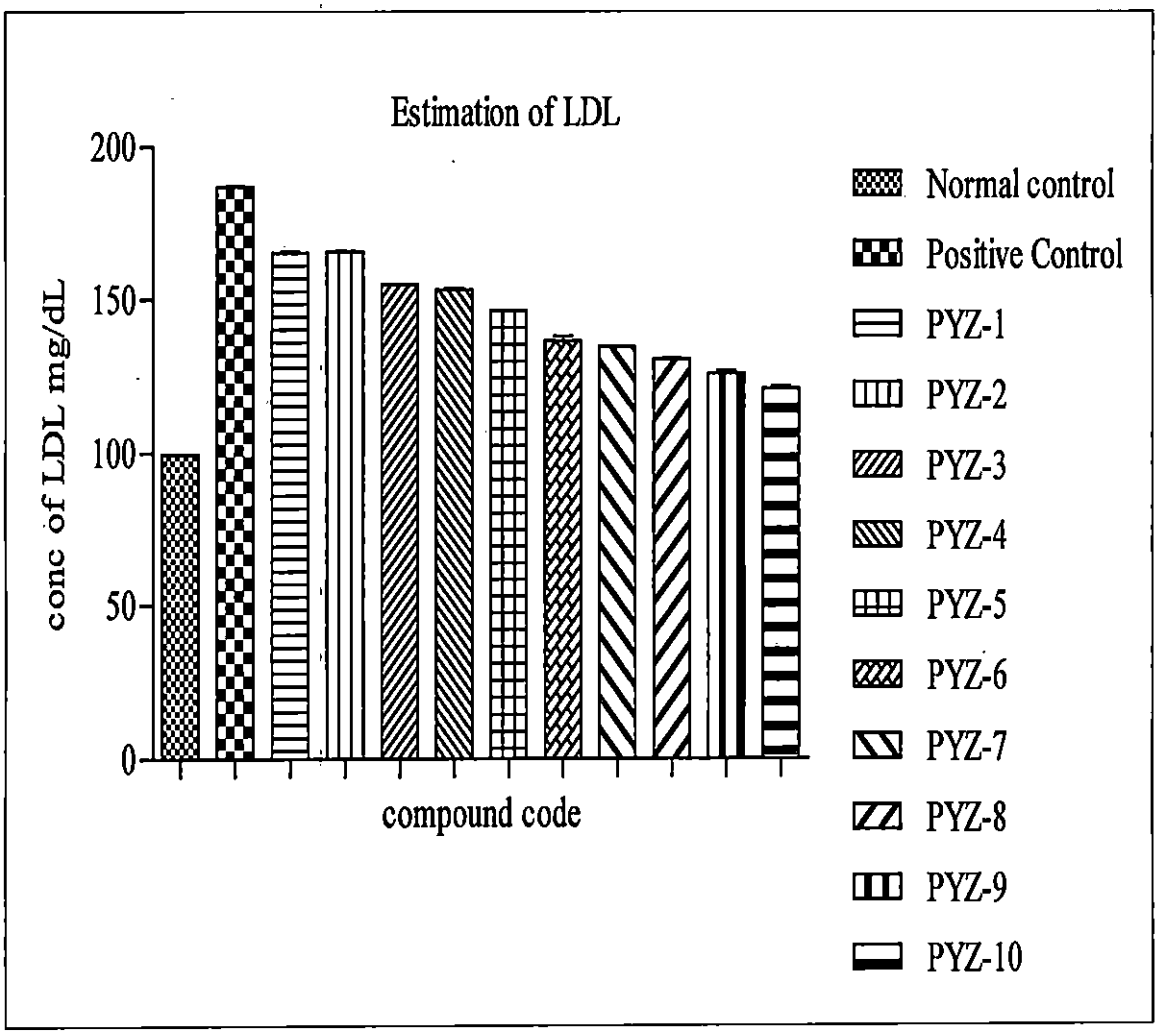


Fig: 5.51. Estimation of LDL

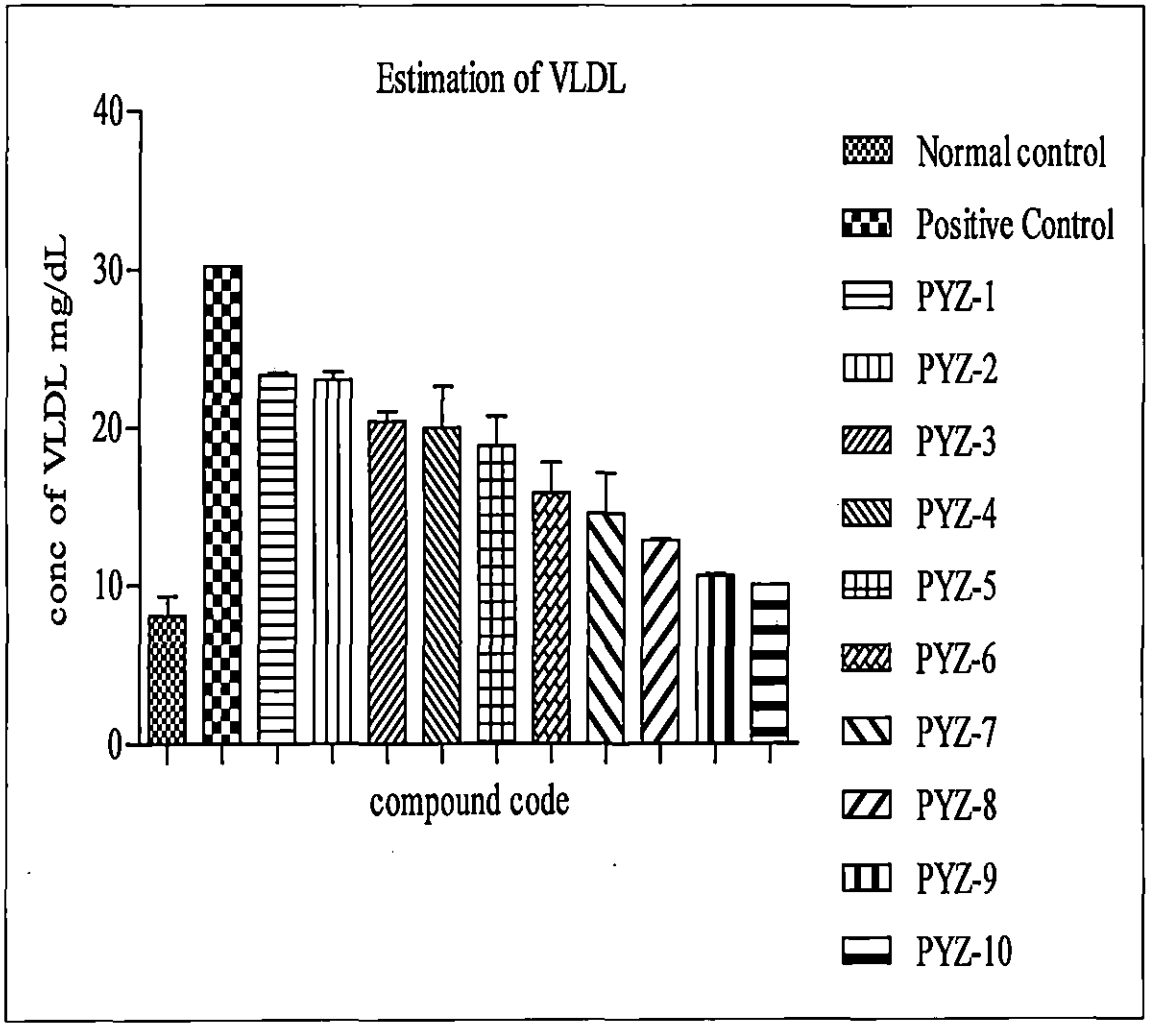


Fig: 5.52. Estimation of VLDL

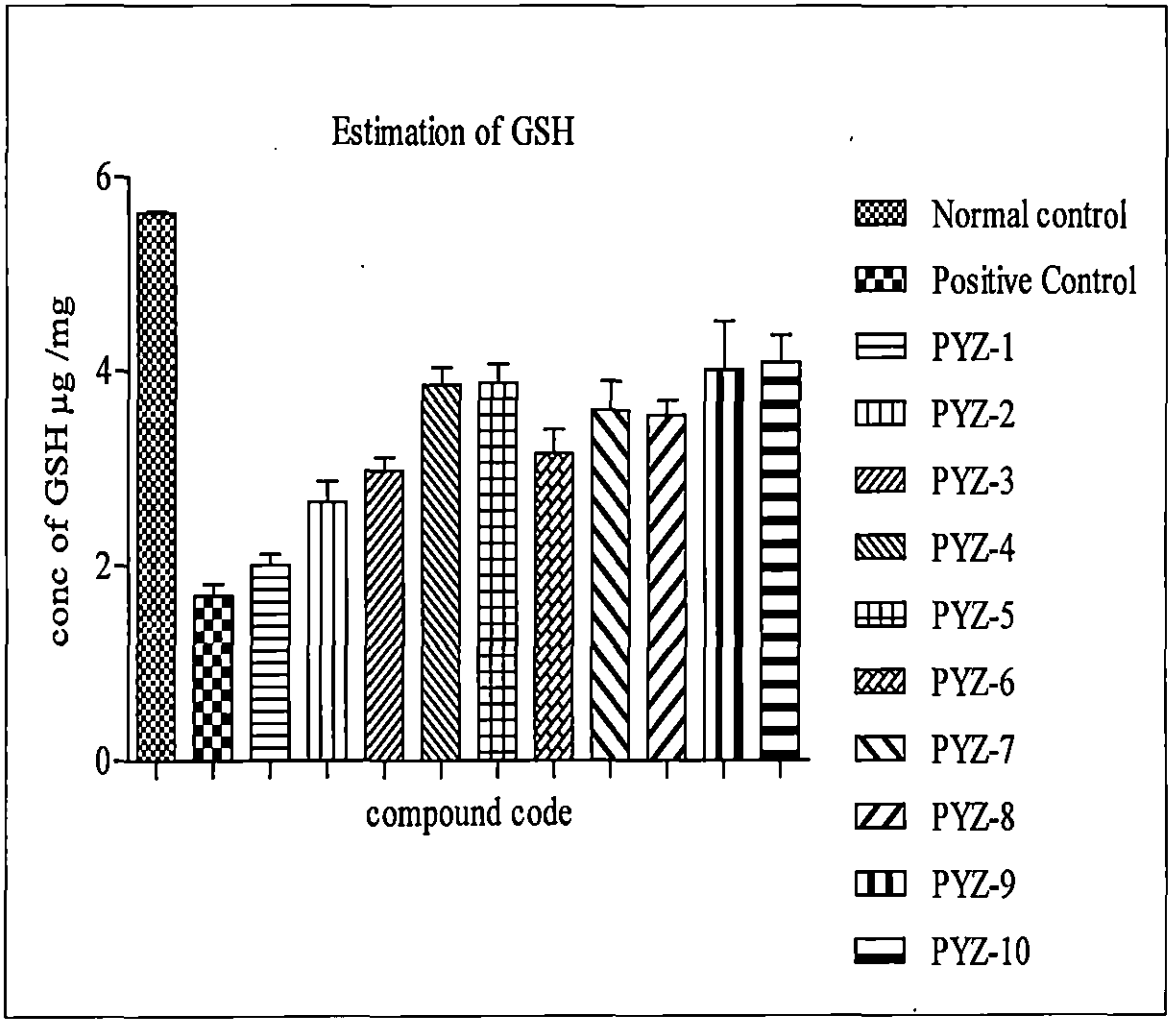


Fig: 5.53. Estimation of GSH

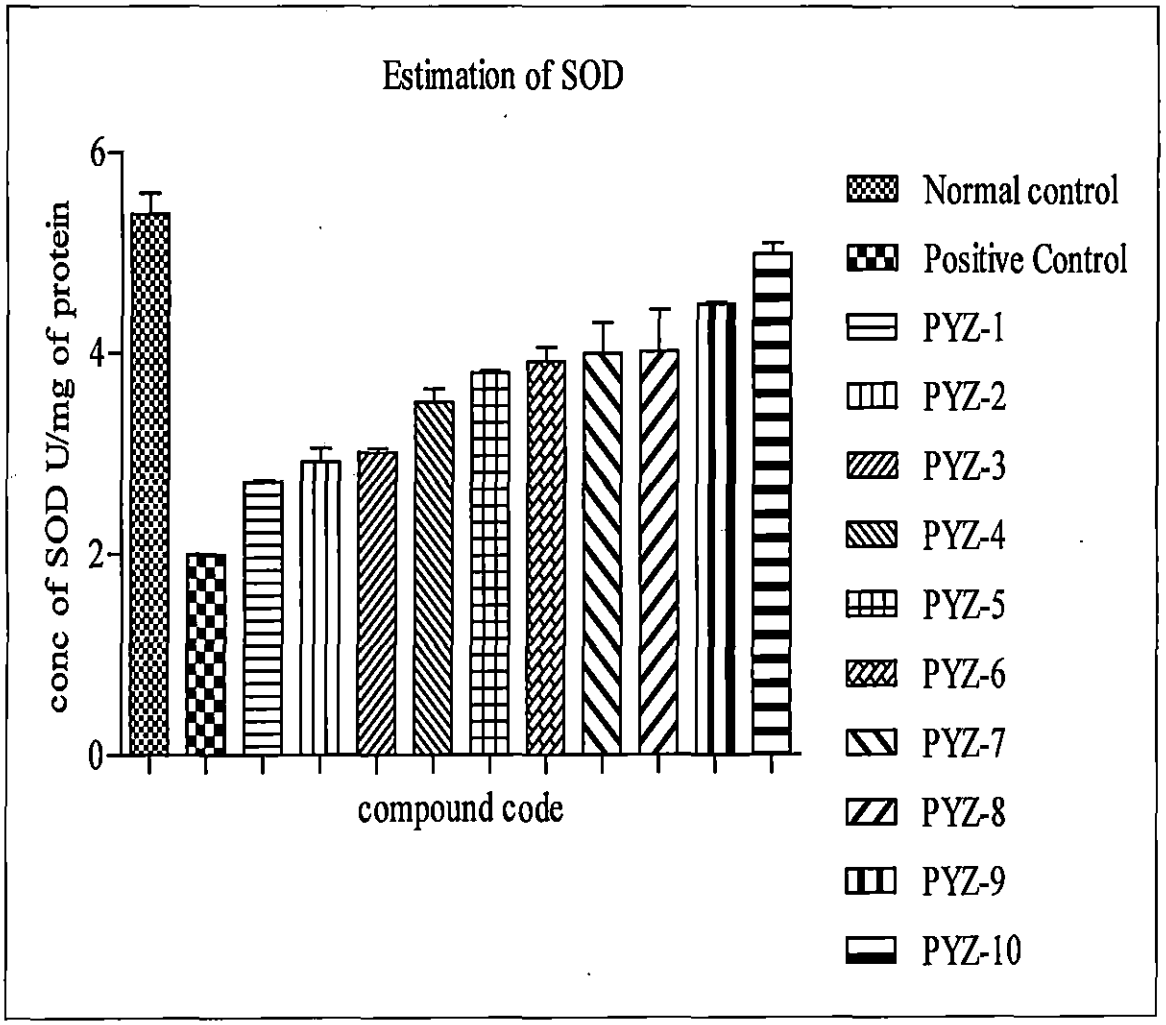


Fig: 5.54. Estimation of S0D

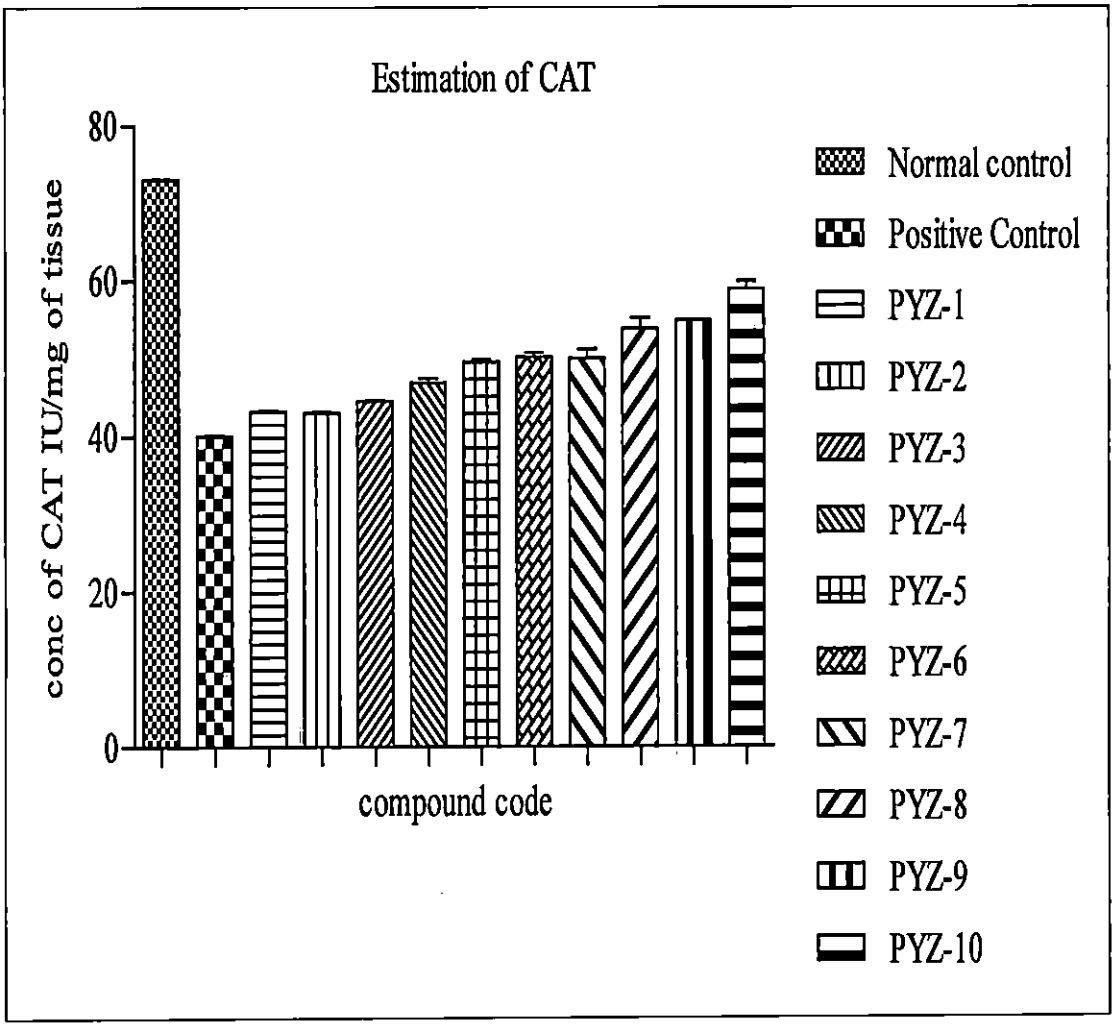


Fig: 5.55. Estimation of CAT

Table: 5.5. *In vitro* antioxidant activity of pyrazolone derivatives by MDA and 4-HNE model

Compounds code	Dose (100mg/kg)	MDA (nM) ($\bar{x}$ ± S.E)	4-HNE(nM) ($\bar{x}$ ± S.E)
Control	-	18.87±0.18	97.59±0.11
PYZ1	100	16.27±0.21	95.21±0.18*
PYZ2	100	15.27±0.19	94.21±0.21
PYZ3	100	15.38±0.18	90.56±0.19
PYZ4	100	16.28±0.20	79.18±0.10
PYZ5	100	17.28±0.22	80.28±0.21*
PYZ6	100	17.01±0.21	80.18±0.11
PYZ7	100	15.01±0.19	79.23±0.20
PYZ8	100	14.29±0.22*	77.19±0.14
PYZ9	100	14.78±0.18*	76.28±0.16*
PYZ10	100	14.23±0.21*	76.23±0.23

$\bar{x}$ = Mean concentration S.E. = Standard Error

* The values are significant at $P < 0.05$

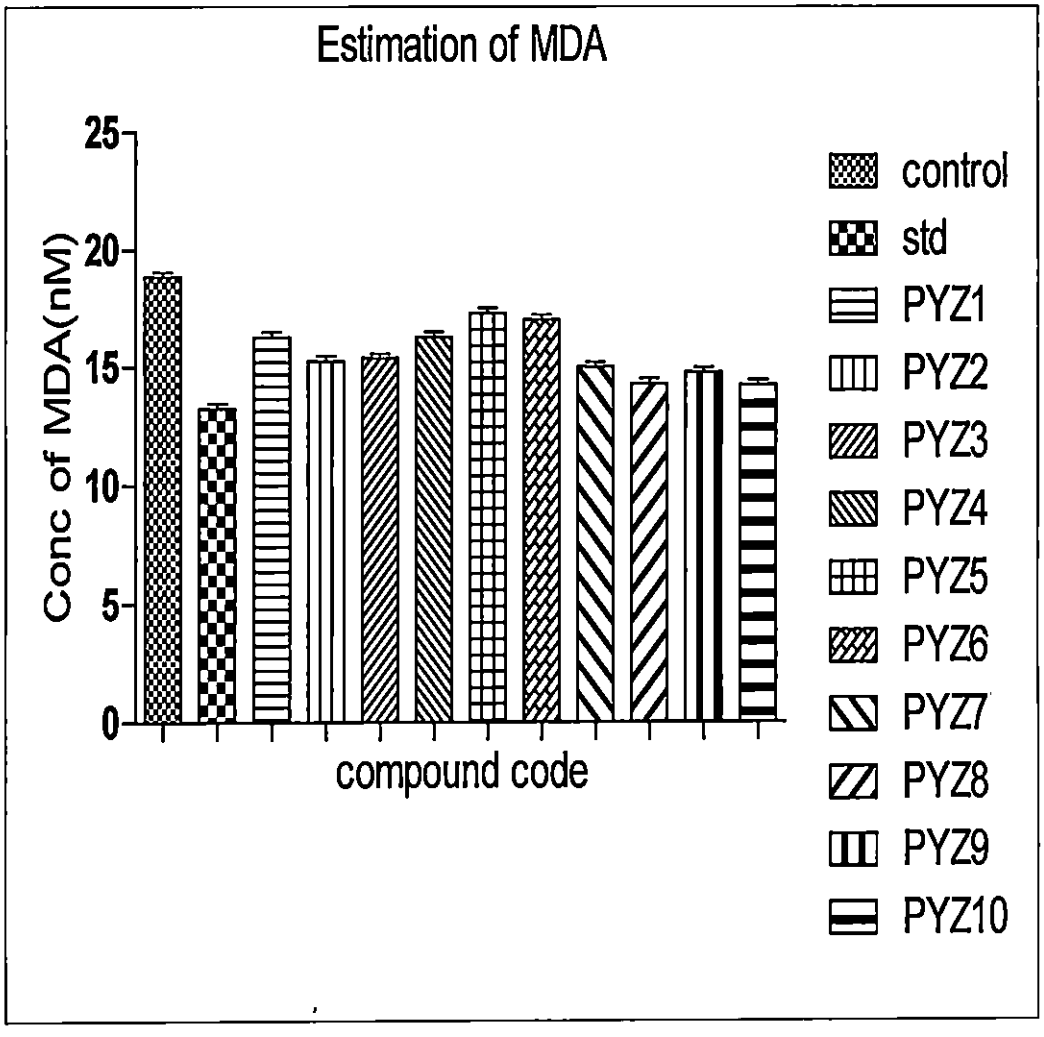


Fig: 5.56. Antioxidant activity of Pyrazolone derivatives on MDA suppression model

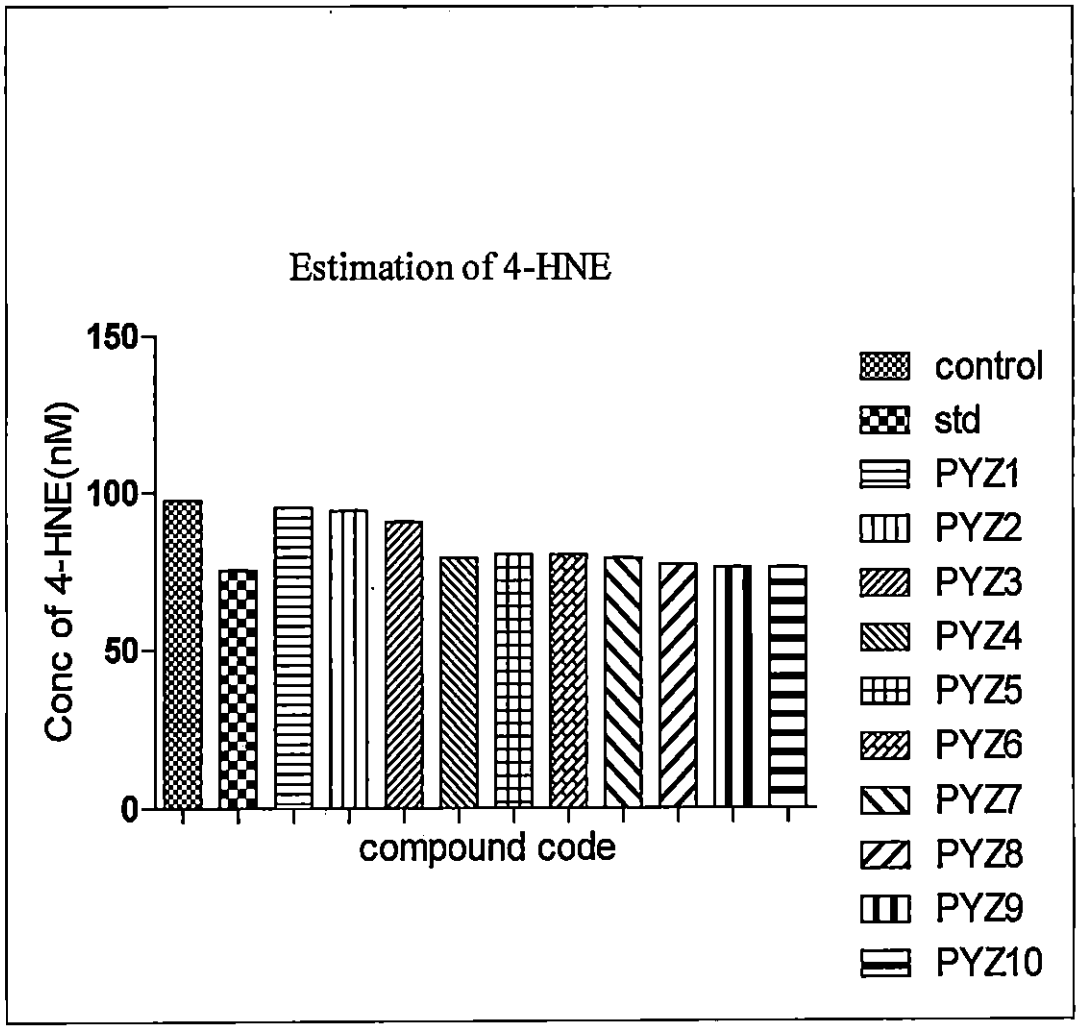


Fig: 5.57. Antioxidant activity of Pyrazolone derivatives on 4-HNE suppression model

Table: 5.6. Anti inflammatory activity of pyrazolone compounds on carrageenan-induced paw edema in rat

Compds 100mg/po	Paw volume in ml, Mean ± SEM (% inhibition of paw edema)			
	1h	2 h	3 h	4 h
Control	0.97 ± 0.016	0.93 ± 0.017	0.91 ± 0.013	0.89 ± 0.013
Aspirin	0.61±0.008(37.11)**	0.53±0.011(42.85)**	0.41±0.008(54.5)**	0.3±0.006(89.99) **
PYZ1	0.84±0.007(13.79) *	0.65± 0.007 (30) *	0.55±0.004 (39.23)*	0.42±0.003(52.59) *
PYZ2	0.83±0.005(14.82) *	0.67±0.008(28.21)*	0.63±0.005 (41.78)*	0.43±0.003 (51.29) *
PYZ3	0.88±0.009 (9.36) *	0.68±0.009(26.78)*	0.50 ±0.023(44.01)*	0.411±0.004(54.32)*
PYZ4	0.88±0.009 (9.19) *	0.68±0.008(27.21)*	0.51 ±0.007(43.79)*	0.40±0.004 (54.81)*
PYZ5	0.65±0.009(33.22)**	0.55±0.005(40.35)**	0.4 ±0.006(54.56)**	0.30±0.003(65.74)**
PYZ6	0.69±0.007(29.13)**	0.56±0.006(39.35)**	0.46±0.007(49.08)**	0.31±0.004(64.99)**
PYZ7	0.65±0.006(33.56)**	0.56±0.008(39.28)**	0.46±0.003(48.89)**	0.38 ± 0.003(57.4)**
PYZ8	0.73 ± 0.010 (25) *	0.61±0.011 (34.5) *	0.51 ± 0.004(44.01)*	0.46±0.005 (48.18) *
PYZ9	0.75±0.006 (23.33) *	0.66±0.010 (28.57) *	0.53 ± 0.005(45.25)*	0.5 ± 0.006 (44.44)*
PYZ10	0.63±0.010(35.26)**	0.56±0.015(39.28)**	0.43±0.006(52.55)**	0.36±0.004(59.25)**

**P<0.01vs Control, * p<0.05vs Control (n=6)

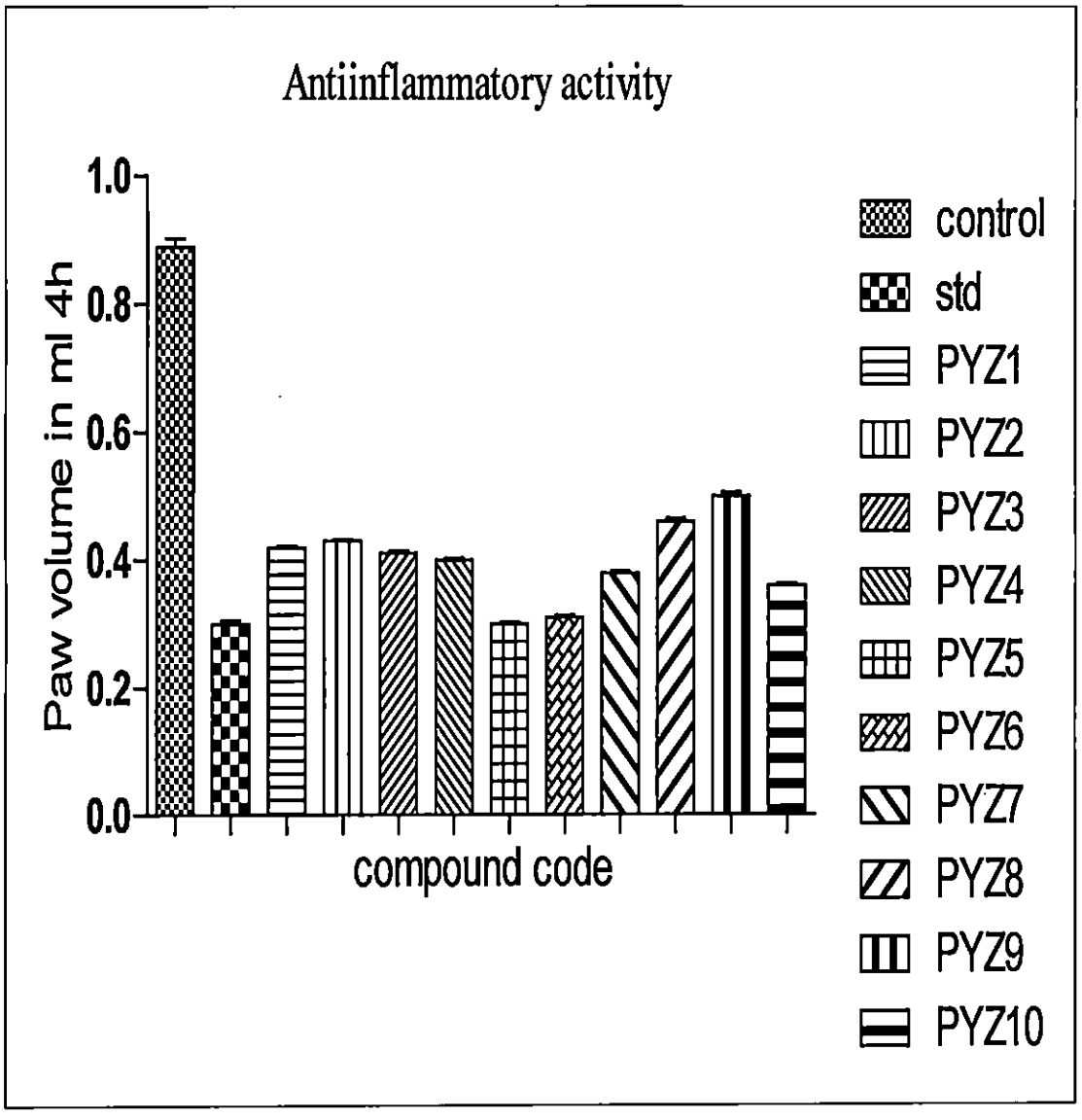


Fig: 5.58. Anti inflammatory activity of pyrazolone derivatives (4h)

Table: 5.7 Analgesic activities of pyrazolone derivatives in rat by tail flick method

Compound Dose (100mg/kg p.o)	Reaction time in second (Mean ±SEM)			
	1h	2h	3h	4h
Control	2.97±0.160	2.97±0.165	2.97±0.165	2.98±0.148
Diclofenac (10mg/kg p.o)	6.93±0.139**	7.65±0.166**	8.25±0.042**	9.03±0.108**
PYZ1	4.91±0.127**	5.82±0.143**	6.4±0.050**	7.03±0.108**
PYZ2	4.19±0.034*	5.07±0.110*	5.9±0.022*	6.22±0.028*
PYZ3	4.19±0.036*	5.06±0.110*	5.79±0.140*	6.91±0.129*
PYZ4	4.72±0.128**	5.30±0.132**	6.17±0.027**	7.03±0.104**
PYZ5	4.29±0.026**	5.23±0.028**	5.96±0.136**	6.76±0.181**
PYZ6	5.43 ±0.024*	5.31±0.030*	6.21±0.019*	7.16±0.017*
PYZ7	4.43±0.019*	5.17±0.017*	6.12 ±0.025*	7.07±0.026*
PYZ8	4.07±0.021**	5.81±0.136**	6.52±0.023**	7.60±0.098**
PYZ9	4.30±0.030*	5.12±0.119*	5.93 ±0.137*	6.89±0.126*
PYZ10	4.49±0.029**	5.47±0.030**	6.40±0.029**	7.21±0.024**

**P<0.01vs Control, *p<0.05vs Control (n=6)

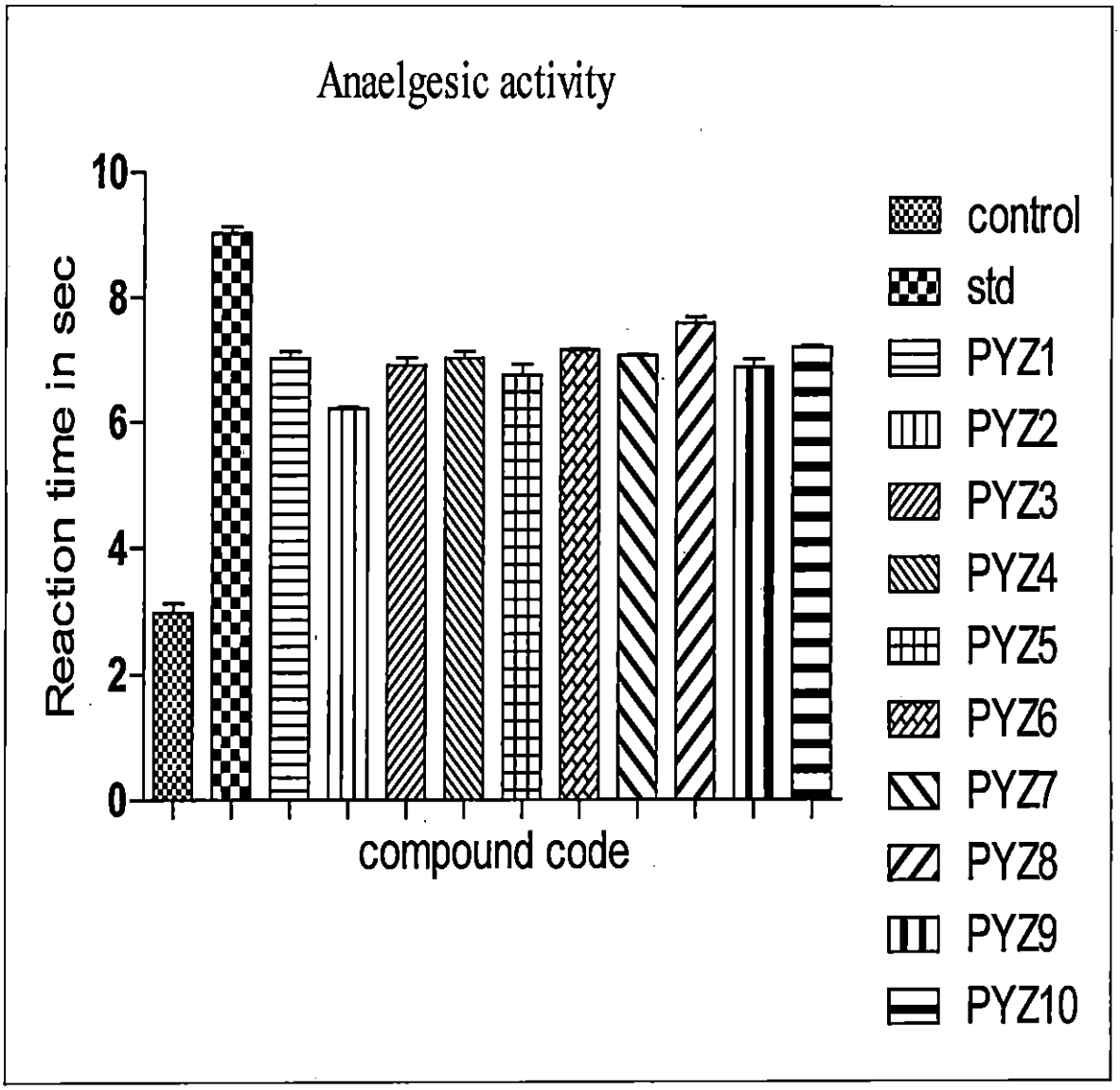


Fig: 5.59. Analgesic activity of pyrazolone derivatives (4h)

Table: 5.8. Effect of pyrazolone derivatives on yeast induced pyrexia in rabbits

Treatment (mg/kg, p.o)	Rectal temperature in ^{0}C Time in Hrs.			
	0	1	2	3
Control	41.03±0.16	41.08±0.13	40.86±0.21	40.86±0.21
STD (100) Paracetamol	40.96±0.26	40.76±0.28	37.93±0.24**	37.46±0.15**
PYZ 1	41.98±0.12	41.46±0.12	40.76±0.21*	39.76±0.16**
PYZ 2	41.6±0.38	41.48±0.07	39.65±0.37**	38.8±0.16**
PYZ 3	41.85±0.28	41.51±0.21	40.71±0.29*	39.13±0.24**
PYZ 4	41.88±0.12	41.56±0.12	40.96±0.21*	39.86±0.16**
PYZ 5	41.6±0.38	41.48±0.07	39.65±0.37**	38.9±0.16**
PYZ 6	41.99±0.28	41.65±0.21	40.87±0.29*	39.18±0.24**
PYZ 7	41.3±0.38	41.24±0.07	39.32±0.37**	38.4±0.18**
PYZ 8	40.85±0.28	40.51±0.21	39.71±0.29*	38.13±0.24**
PYZ 9	40.6±0.38	40.48±0.07	38.65±0.37**	37.8±0.16**
PYZ 10	41.85±0.24	41.61±0.21	40.41±0.23*	39.1±0.22**

Values are expressed as mean±SEM, (N=6); *P<0.05,

**P<0.01 compared with vehicle control (ANOVA followed by Dunnett's test)

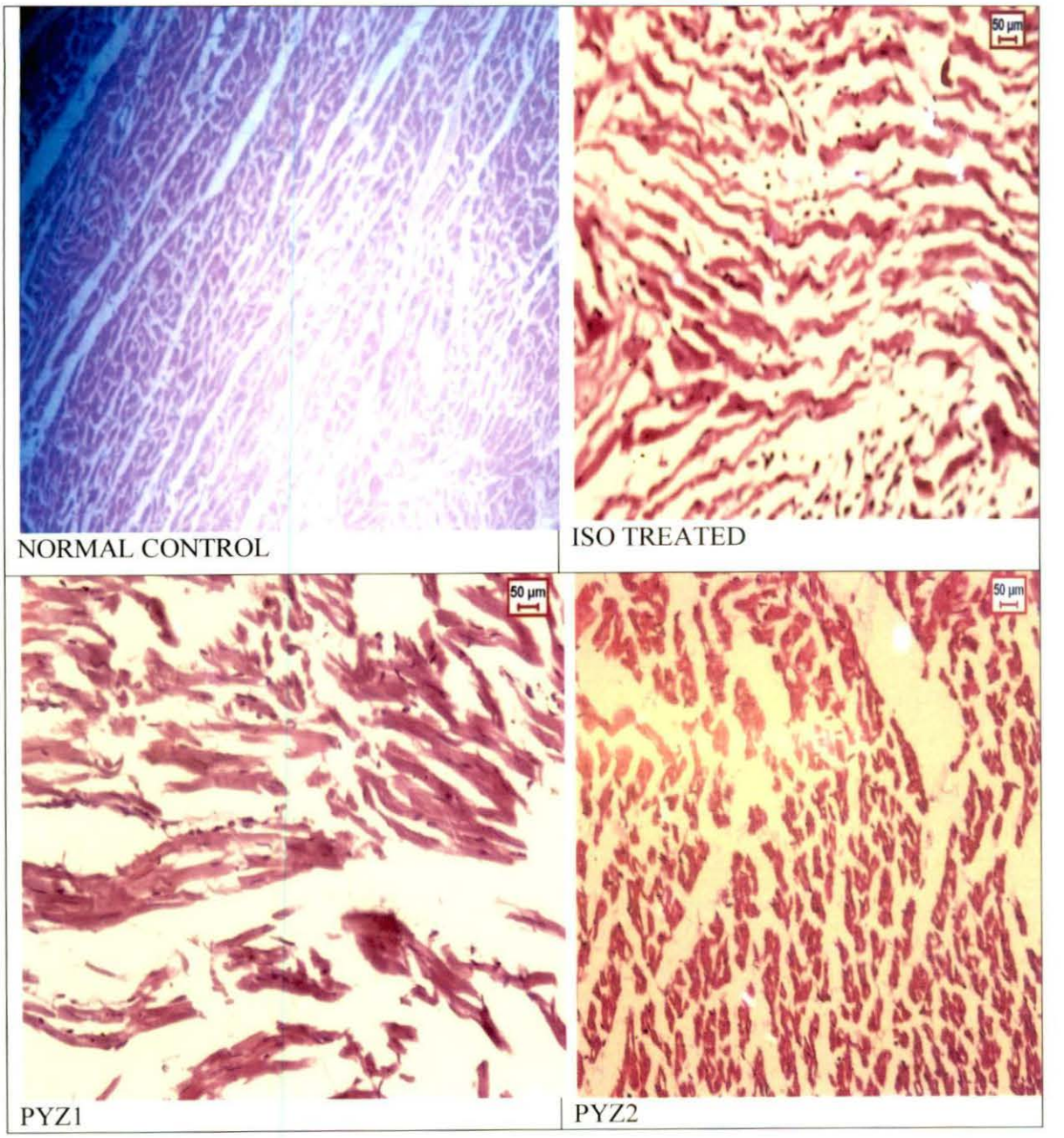


Fig: 5.60.Photomicrograph of the heart of control, ISO treated and pyrazolone treated

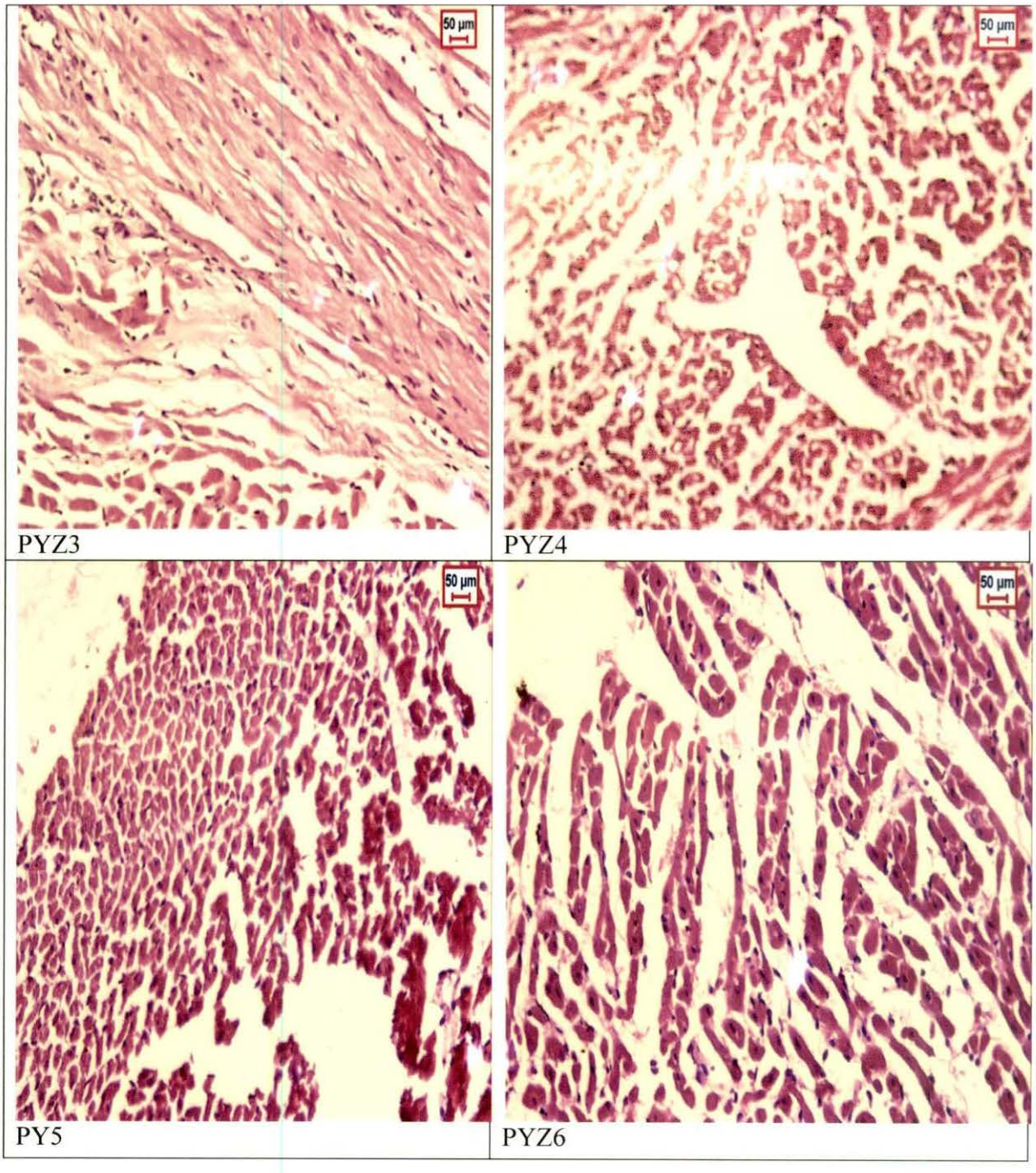


Fig: 5.61.Photomicrograph of the heart of control, ISO treated and pyrazolone treated

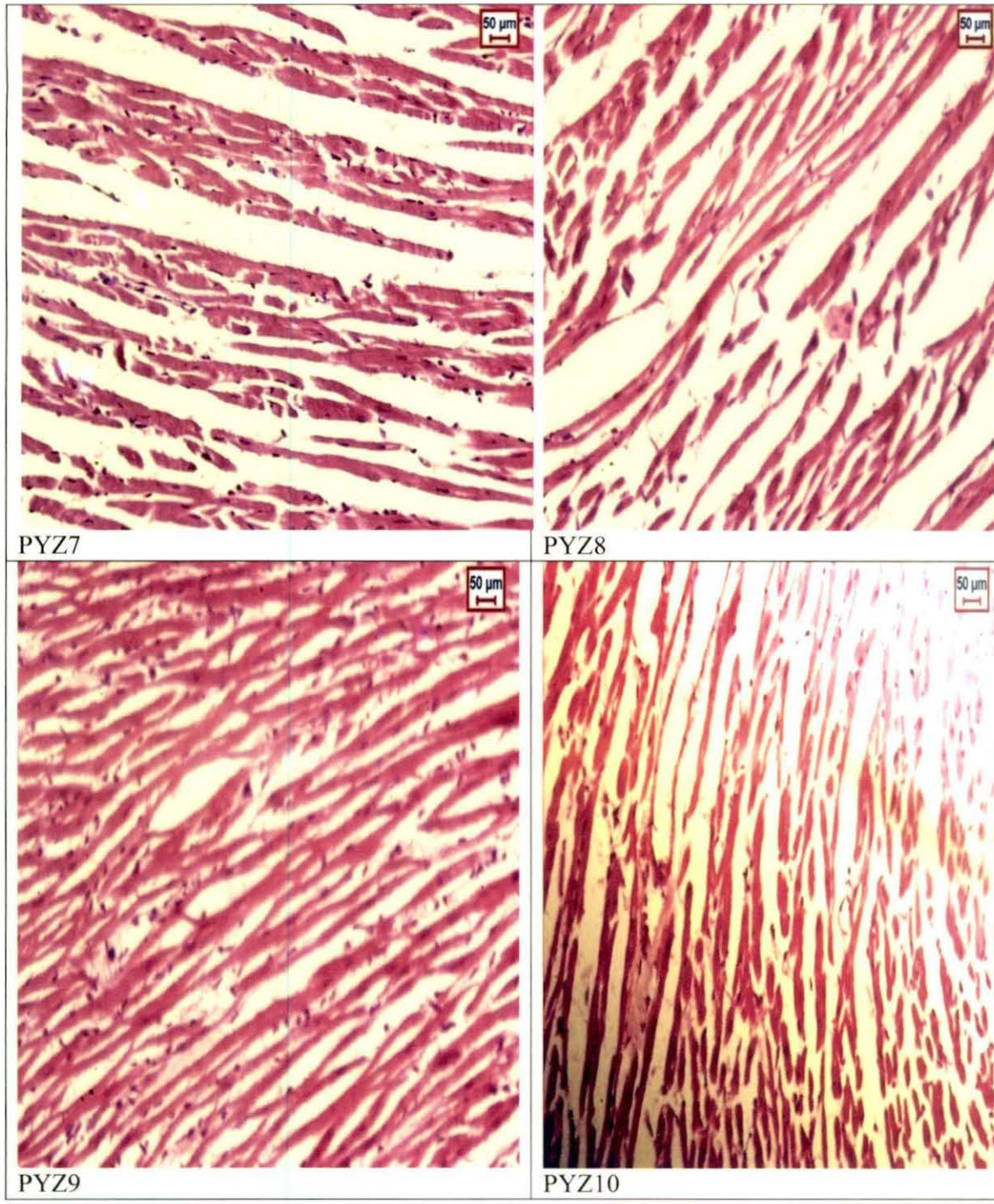
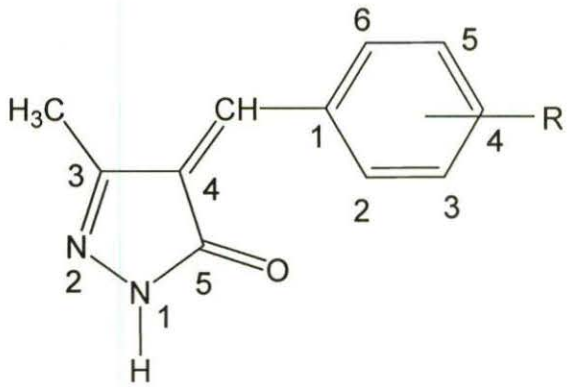


Fig: 5.62. Photomicrograph of the heart of control, ISO treated and pyrazolone treated

STRUCTURE ACTIVITY RELATIONSHIP OF PYRAZOLONE DERIVATIVES



Compd	R
PYZ1	$N(CH_3)_2$
PYZ2	-
PYZ3	$OCH_{3(2,3)}$
PYZ4	3-NO_2
PYZ5	3-Cl
PYZ6	H
PYZ7	-
PYZ8	4-OCH_3
PYZ9	3-OCH_3
PYZ10	2-OH

1. The basic moiety 3-methyl pyrazol-5-one is crucial for basic pharmacologic activities.
2. All the derivatives had shown significant cardioprotective, analgesic and anti inflammatory activities.
3. Amongst all, PYZ8, PYZ9 and PYZ10 had shown promising cardio protective properties.
4. All the compounds were observed to possess remarkable anti oxidant properties. It also has been proved from the literature that phenolic compounds are potent antioxidants. Therefore, it can be assumed that compounds which generate the aromatic hydroxyl group by keto-enol tautomerization would show radical-scavenging and antioxidant activity.
5. Preferable functional groups and the position of the substituent on the phenyl ring were investigated.
6. The cardioprotective activity of meta and para substituted compounds [PYZ 8, 9, 10] largely increased except for a N,N di methyl amino analogue [PYZ 1]
7. The electron donating substituent [PYZ 8, PYZ 9 and PYZ 10] increases the cardio protective activity as compared to electron withdrawing substituent.

8. A disubstituted alkoxy analogue [PYZ 3] showed decreased activity compared with mono substituted analogues. This may be restriction of keto enol tautomerism due to steric hindrance offered by the alkoxy group attached in ortho position.
9. No significant difference in activity was observed between substituted compounds [PYZ7 and PYZ8, PYZ9, PYZ10]
10. This shows that the pyrazolones derived from aliphatic aldehyde and aromatic aldehyde does not have much variation in biological response.

CHAPTER 6

DISCUSSION

DISCUSSION

3-Methyl-4-substituted benzylidene-pyrazol-5-ones (**PYZ1- PYZ10**) were synthesized by the condensation of 3-methyl-pyrazol-5-one with substituted aliphatic and aromatic aldehydes. Their structures have been elucidated by sophisticated instrumental analysis such as UV, IR, ^{1}H NMR, Mass spectral data and elemental analysis. **(Fig: 5.1 - 5.40)**

Toxicological studies of the test compounds (as suspension in 0.5%w/v carboxy methyl cellulose) were carried out by Up and Down method in oral dose of 2000 to 5000 mg/kg body weight in albino rat. The rats were continuously observed for 8hr for any signs of acute toxicity such as increased/decreased motor activity, ataxia, tremors, convulsions, sedation, lacrimation etc. After 24hrs the rats were sacrificed, then stomach, intestine and liver were examined under the magnifying lens for any ulcer-hemorrhagic spots. Since no such spots were detected, it is concluded that the drugs are nontoxic up to that dose limit. Acute toxicity and gross behavior studies revealed that the entire series of compounds in the present investigation were found to be nontoxic up to 5000 mg/kg body weight.**(Table: 5.1.)**

Lipid metabolism plays an important role in myocardial injury produced by ischemia by deposition of cholesterol ion coronary arteries (Mathew et al., 1981). Abnormalities in lipid profile (LDL, VLDL, HDL TG, TC, creatinine and TP) are associated with increased risk of myocardial infarction. High level of circulating cholesterol and its accumulation in the heart tissue is usually accompanied by cardiovascular damage (Mediene-Benchekor et al., 2001). According to Patsch, 1993 and Ng et al., 1997, patients with cardiovascular diseases experience markedly elevated triglycerides and reduced HDL levels. It can be attributed that metabolism of triglyceride-rich lipoproteins present in HDL, particularly the sub-fraction HDL2 which has a negative association with cardiovascular risk. Hence the estimation of lipid profile can be directly correlated with the intensity of myocardial injury.

The Isoproterenol elevated the levels of total cholesterol, total protein, LDL, VLDL, triglycerides; and decreased HDL in serum. It causes hyperlipidemia and it increases the LDL cholesterol in the blood, which in turn leads to harmful deposits in the arteries

thus favoring coronary heart diseases (CHD) (Rajdurai and Prince, 2006). In the present study also, isoproterenol administration caused a significant rise in the serum lipids thereby increases lipid biosynthesis and lipid peroxidation. Rats treated with pyrazolone derivatives showed decreased concentration of serum total cholesterol, triglycerides, LDL cholesterol indicates the beneficial effects of pyrazolone derivatives in reducing hyperlipidemia caused by Isoproterenol. All the pyrazolone derivatives (10mg/kg b.w) have controlled the elevated lipid profile to near normal level. Amongst the entire compounds, **PYZ8, PYZ9, PYZ10** had shown profound effect to lower the various lipids. **(Table: 5.2 and Fig 5.41-Fig: 5.52.)** The results are statistically significant at $p < 0.01$ level.

The administration of Isoproterenol in rats resulted significant increase in the serum levels of cardiac marker enzymes such as LDH, SGOT, SGPT and ALP. Isoproterenol induced myocardial injury has been reported to alter membrane permeability (Mathew *et al.*, 1981) and to cause leakage of marker enzymes of cardiac damage (LDH, CPK, AST, ALT and ALP) into the blood stream(Khalid and Ashraf, 1993; Mohanty *et al.*, 2004). All the synthesized compounds (10 mg/kg b.w) brought back the elevated myocardial injury markers to near normal level. **(Table.5.3).**Comparatively, the compounds **PYZ8, PYZ9** and **PYZ10** had shown profound effect to lower the myocardial injury markers such as LDH, SGOT, SGPT and ALP. The results are statistically significant at $p < 0.01$ level.

Pyrazolone derivatives pre-treatment improves cardiac antioxidant status in isoproterenol induced myocardial injury by effective scavenging of free radicals generated during oxidation of lipids thus collectively contributing to its overall antioxidant and anti ischemic activity. The significantly decreased activities of endogenous enzymatic antioxidants (CAT,GSH and SOD) observed in the heart of ISO-treated rats were improved following pretreatment with pyrazolone derivatives for 5 days. All the test compounds had shown significant activity to elevate reduced level of enzymatic antioxidant. **(Table: 5.4 and Fig: 5.53 - Fig 5.55.)** Amongst them, **PYZ8, PYZ9, PYZ10** had shown to improve the level of endogenous enzymatic antioxidants effectively. The results are statistically significant at $p < 0.01$ level.

The present study has clearly demonstrated that the pyrazolone derivatives have antioxidant activity which could prevent the occurrence of heart related diseases. Significantly elevated activities levels of SOD and CAT recorded in pyrazolone treated group could be due to its potent free radical scavenging ability. GSH scavenges singlet oxygen, superoxide and peroxy radicals to form oxidised glutathione and other disulfides (Meister, 1984). Also, antioxidant compounds have been shown to increase glutathione reductase activity that maintains GSH in a reduced state (Mohanty *et al.*, 2004). The elevated GSH content observed in pyrazolone treated groups may be due to its enhanced synthesis. ISO treatment is also known to create an imbalance between enzymatic as well as non enzymatic antioxidant defense system leading to production of free radicals that induce myocardial injury and lipid peroxidation.(Ojha *et al.*, 2008). The significant decrement in lipid peroxidation in pyrazolone derivatives treated group further justifies the role of pyrazolone derivatives as a potent antioxidant and free radical scavenger. These results are in conformity with reports that have been demonstrated the modulation of cellular antioxidant activities by treatment with pyrazolone derivatives (David et al., 2006).

Pyrazolone pre-treatment to isoproterenol treated rats provide cardio protection by inhibiting the formation of free radicals generated during oxidation of lipids thus inhibiting peroxidation of membrane lipids and preventing subsequent leakage of soluble enzymes. Pyrazolone derivatives pre-treatment appears to improve the status of enzymatic antioxidants that further contributes to its overall cardioprotective property. Hence, it can be concluded that pyrazolone derivatives pretreatment provides cardio protection myocardial injury via multiple mechanisms. It was assessed by estimation of Malonaldehyde (MDA) and 4-Hydroxyl-2-noneal (4-HNE) as lipid peroxidation markers in myocardial ischemic reperfusion injury by Langendorff isolated rat heart model. The quantification of MDA and 4-HNE can be directly correlated with the lipid peroxidation inhibition capacity of the pyrazolone derivatives. The toxic radical's quantification is also an indicator to monitor the overall progress of lipid peroxidation which is associated with myocardial ischemic reperfusion injury.

The antioxidant activity of pyrazolone derivatives was compared with standard antioxidant (ascorbic acid). Comparatively, all the compounds have shown significant antioxidant effect where as **PYZ7, PYZ8, PYZ9** and **PYZ10** having effective role to control both MDA and 4-HNE generation. **(Table: 5.5, Fig: 5.56 and Fig 5.57.)** All the experimental data were statistically significant at $p<0.05$ level.

Histopathological effects of **(Fig: 5. 60, 5.61 and 5.62.)** show the light micrograph of control heart showing normal architecture and integrity of myocardial cell membrane. Severe degenerations of the myofibrils, with focal necrosis, vacuolated cytoplasm , lymphocytic infiltration in sub endocardial region indicative of infarct like lesions as reported in various studies (Teerlink et al., 1994; Grimm et al., 1998). Isoproterenol intoxication also induced eosinophilic cytoplasm, focal hemorrhage and with inflammatory cell infiltrations. Scrutiny of cardiac tissue of isoproterenol + pyrazolone group revealed that there was minimum damage to the myocardium with much reduced myonecrosis and lymphocyte infiltration than ISO treated group. The animals pretreated with PYZ1-PYZ10 showed better-preserved appearance of cardiac muscle fibers with slight degeneration and some leukocyte infiltration.

Myocardial injury is associated with inflammatory response (Entman et al., 1991). Endothelial injury plays a critical role in the pathogenesis of myocardial ischemia-reperfusion (I/R) injury by setting the stage for adherence of neutrophils to the vascular endothelium and subsequent development of inflammatory component of the I/R. The suppression of polymorph nuclear leukocytes (PMNs) infiltration and inhibiting NF-kappa B (NF-kB) activation diminishes I/R damage and potentially offers myocardial protection (Kim et al., 2009). The high mobility group box 1 protein (HMGB1) maintains the nucleosome structure and regulates gene transcription, can be released by necrotic cell or activated innate immune cells (Lotze and Tracey, 2005). Preconditioning with HMGB1 protects against myocardial I/R injury. It is binding to its receptors activates intracellular signaling pathways, such as the NF-kB pathway, which induces downstream cytokine release (Fiuza et al., 2003).

Based on the above facts it can be assessed that PYZ1-PYZ10 derivatives could protect the heart from the injury induced by ischemia and inflammation on rat myocardial I/R injury . All the synthesized compounds were screened for anti-inflammatory activity against Carrageenan- induced paw edema in rats. When compared with the control, all the compounds showed reduction in edema volume with prominent percentage inhibition to the inflammatory response ranging from 44% to 65% at 4^{th} hour of observation. Compounds **PYZ 5, PYZ 6, PYZ 7** and **PYZ 10** were found to have a potent anti-inflammatory response at $p<0.01$ levels **(Table: 5.6 Fig 5.58)**

Carrageenan-induced edema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1-2 h) carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissues surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorph nuclear cells and prostaglandins produced by tissues macrophages (Brito and Antonio, 1998; Gupta et al., 2006). The pyrazolone derivatives reduced the carrageenan induced paw edema in rats and all the compounds are active. It may be due to inhibition of cyclooxygenase enzyme followed by prevention of inflammatory mediator's release. There is increasing evidence that lysosomal enzymes play an important role in the development of acute and chronic inflammation. (Anderson *et al.* 1971; Jannoff and Zweifach, 1964).

Most of anti-inflammatory drugs exert their beneficial effect by inhibiting either release of lysosomal enzymes or by stabilizing lysosomal membrane which is one of the major events responsible for the inflammatory process. The experimental findings of pharmacological parameters suggest that pyrazolone derivatives are the promising non steroidal anti-inflammatory agents.

The underlying mechanism by which pyrazolone derivatives improved myocardial function in I/R rats might be partially associated with decrease of PMNs infiltration and the infiltrating PMNs is activated by an ischemic insult, directly contact and injure neighboring myocyte via the release of inflammatory substances (Vinten , 2004). PMNs activation in reperfusion areas has detrimental consequences on cardiac

function (Shandeyla et al., 1993). It induces post ischemic damage by liberating reactive oxygen metabolites, hydrolytic enzymes, and eicosanoids, which would lead to microvascular injury (Zimmerman and Granger, 1990), leukocyte accumulation was increased (Abe et al., 2008). Pyrazolone reduced the PMNs infiltration in ischemic myocardial tissue. Pyrazolone derivatives prevented I/R-induced myocardial injury and inflammation partially due to inhibition of neutrophils infiltration since the compounds are having significant anti-inflammatory response.

Acetic acid which is used as an inducer for writhing syndrome (Koster et al., 1959) causes analgesia by releasing of endogenous substances, which then excite the pain nerve ending. The abdominal constriction is related to the sensitization of nociceptive receptors to prostaglandins. It is possible that pyrazolone exert an analgesic effect probably by inhibiting prostaglandin synthesis. Perusal of the results on their analgesic activity by tail-flick method revealed that almost all of them exert significant activity. Amongst them, compounds **PYZ1, PYZ 4, PYZ 5, PYZ 8** and **PYZ 10** were found to have an effective analgesic response at $P < 0.01$ level **(Table: 5.7.and Fig 5.59.).**

The perusal of results of antipyretic study revealed that **PYZ9** significantly reversed hyperthermia similar to standard drug paracetamol (100 mg/kg, p.o) as shown in **Table: 5.8.** Fever results due to generation of mediators such as IL-1β, IL-6, interferons and TNF-α cytokines increase the synthesis of prostaglandin which elevates the body temperature. From the results of antipyretics study, it can be suggested that pyrazolone derivatives produce the antipyretic action by inhibiting the prostaglandin synthesis by blocking cycloxygenase isoenzymes, platelet thromboxane synthesis and prostanoids synthesis (Graham and Scott, 2003; Bentur and Cohen, 2004).

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CHAPTER 7

SUMMARY AND CONCLUSION

The World Health Organization estimates that 60 per cent of the world's cardiac patients will be Indian by 2020. Nearly 50 per cent of CVD related deaths in India occur below the age of 70, compared with just 22 per cent in the West. In addition, researchers have determined that compared to people in other developed countries, the average age of patients with heart disease is lower among Indian people and Indians are more likely to have types of heart disease that lead to worse outcomes.

Despite significant advances in prevention and treatment, cardiovascular disease remains the leading cause of death in economically advanced countries. The developing world has also begun to experience a disconcerting growth in prevalence, where the insurgence of CVD has exacerbated the risk factors associated with indigenous communicable diseases. The overriding challenge for industrial players is to keep pace with epidemiological change in both the developed and developing world. This involves successfully integrating into localized healthcare systems and reimbursement structures, in addition to remaining aligned with product demand. In the field of novel drug research area, this challenge is being met with a focus on new molecules that reduce adverse effects, improve patient outcomes and extend the range of formulations for existing indications.

The high cost of tertiary level management of IHD is unaffordable for a large section of the population as well as for the state health care system. Therefore, there is a global approach for primary prevention by altering life style and diet on the one hand and research to identify newer and cheaper agents which can prevent or delay the occurrence of and/or complications of IHD on the other hand.

The various drugs, which have so far been tried out are calcium antagonists, beta blockers and free radical scavenger. Although these drugs provide significant benefit in acute conditions and in secondary prevention, they are not advisable or acceptable for chronic use as primary preventive measures in large number of patients, who possess a high level of risk of having acute ischemic episodes, later in life. Moreover, chronic use of various drugs in the treatment of IHD showed major limitations due to various side effects. Under such circumstances, other options need to be explored which will help in circumventing this problem. After an extensive literature survey, pyrazolone basic moiety was undertaken for

the novel drug development since it has been proved as cardiovascular agents by Mitsubishi-Tokyo Pharmaceuticals, Japan. In chapter 1, the physiology, pathology of myocardial ischemic reperfusion injury and its prevention by pyrazolone derivatives have been dealt as well.

Chapter 2 dealt the extensive literature survey on pyrazolone and its role in various cardio vascular diseases. **A review article regarding various pharmacological actions of pyrazolone has been published in Journal of Pharmacy Research.** In chapter 3, the aims and objective of the present work has been furnished.

In Chapter 4, the synthesis and structural characterization of novel analogues of the pyrazolone derivatives by UV, IR, NMR, Mass and CHN analysis have been presented. By maintaining the basic 3-methyl pyrazolone as substitution pattern, the derivatives were synthesized with a variety of different atoms and functional groups as substituent. This was achieved with the aim of emulating or surpassing the biological potency of the parent compound 3-methyl pyrazolone. Edaravone (pyrazolone derivative) has preventive effects on myocardial injury following ischemia and reperfusion in patients with acute myocardial infarction. Bearing this in mind, ten contrasting series of pyrazolone analogues were synthesized. **A research paper regarding synthesis and bioactivity evaluation has been published in Indian Journal of Chemistry Section B.**

Acute toxicity and gross behavior studies revealed that the entire series of compounds in present investigation were found to be nontoxic even up to 5000 mg/kg b.w. Amongst all, PYZ2 was studied for its acute and sub acute toxicity profile. **A research paper regarding analgesic, anti-inflammatory, antipyretic and toxicological evaluation of pyrazolone derivatives has been published in Saudi Pharmaceutical Journal.**

In chapter 4, the cardioprotective effect of pyrazolone derivatives on plasma lipid profile, serum marker enzymes, endogenous enzymatic and non-enzymatic antioxidants in cardiac tissues against isoproterenol (ISO) induced myocardial ischemic injury in rats has been described systematically. The result of the study has been published in **Pharmacology online 2, 986-994(2010), Italy.** The present study explored the cardioprotective effect of pyrazolone derivatives against ISO induced myocardial

ischemic injury in rats. The existing experimental evidence suggests that ISO induced the generation of free radicals in heart tissue. The generated reactive oxygen species such as superoxide radicals are potential to cause damage to various intracellular components. Heart tissue is particularly susceptible to free radical injury, because it contains low levels of detoxifying enzymes/molecules like SOD, GSH and CAT. Further ISO has high affinity towards cardiac myocyte leading to accumulation of ISO in heart. The ISO induce myocardial injury is critical to the heart because it would presumably have extremely adverse effect on heart. Hence the experimental protocol was designed in such a method that it would initiate ISO induced myocardial damage followed by pyrazolone derivatives intervention was used to explore the extent of control of progressive myocardial tissue damage.

Pretreatment of pyrazolone derivatives was able to reduce the ISO induced cardio toxic manifestations in multiple ways: Increase in the level of plasma triglycerides, total cholesterol and low density lipoproteins in ISO treated groups indicate that ISO may be interfering the metabolism or bio synthesis of lipids. Pretreatment of pyrazolone derivatives showed reduction of blood lipid profile with concomitant increase in HDL cholesterol was observed. The lipid lowering effect of pyrazolone derivatives may be due to inhibition of hepatic cholesterol biosynthesis, increased fecal bile acid secretion and stimulation of receptor mediated catabolism of LDL cholesterol and increase in the uptake of LDL from the blood by liver. Myocardial injury induced by ISO in rats was indicated by elevated level of the marker enzymes such as serum LDH and CPK.

Pyrazolone derivatives were found to inhibit ISO induced LDH and CPK in the serum of rat. It is widely reported that ISO induced free radical generation triggers the membrane peroxidation and disruption of cardiac myocyte which can lead to increased release of LDH and CPK in the serum. Pyrazolone derivatives pretreatment led to inhibition of LDH and CPK release which resulted in either complete reversal or considerable recovery of the serum enzyme activities. The cardio protective activity was further supported by increased level of myocardial anti oxidant enzymes like SOD, GSH and CAT in pyrazolone pretreated groups.

The anti oxidant potential of pyrazolone derivatives further supported by decreased lipid peroxidation because it is known to cause cellular damage and primarily responsible for reactive oxygen species induced heart damage. The increased levels of MDA and 4-HNE were observed in ischemic heart tissue. Pretreatment of pyrazolone derivatives efficiently counter acted reperfusion induced myocardial ischemic injury by significant decrease level of MDA and 4-HNE. The evaluation of anti oxidant potential of pyrazolone derivatives has been furnished by a publication in **Journal of Advanced Pharmaceutical Technology and Research.**

In chapter 5, the results of all the studies viz synthesis, characterization and cardioprotective studies have been described adequately. In chapter 6, the results were discussed with relevant references to draw the possible mechanism of pyrazolone derivatives. The results obtained for the synthesized pyrazolone derivatives were in conformity with reported literature.

Comprehensively, it is summarized that reperfusion of coronary flow is necessary to resuscitate the ischemic or hypoxic myocardium. The reperfusion injury induced ischemic myocardium may be protected by administration of exogenous cardioprotective agents or by classical ischemic preconditioning. Although protection provided by ischemic preconditioning appears to be robust, a drawback of the majority of preconditioning studies is that protection is not absolute when ischemia is severe and prolonged, even those preconditioned areas of myocardium will go further to develop complete infarction. Therefore, possibility of preconditioning the heart with an exogenous agent (e.g pyrazolone derivatives) was used in the present study to provide new avenues to induce preconditioning for cardio-protection and by an external pharmacological intervention. Administration of pyrazolone derivatives to preconditioned heart before the insult of ischemia produced a significant improvement to ischemic heart by reducing the elevated levels of lipid profiles, cardiac injury markers to near normal level in rat heart model.

Finally it can be concluded that, the pyrazolone derivatives were found to possess significant cardio protective property in animal models. Further structural modification and clinical studies will unveil the therapeutic efficacy of the pyrazolone pharmacophore in the field of new drug discovery on cardio vascular disease.

LIST OF PUBLICATIONS

List of Publications

1. **G.Mariappan**, Saha BP, Sutharson L & Haldar A., Synthesis and bioactivity evaluation of pyrazolone derivatives. *Indian Journal of Chemistry, Sec-B*, Vol 49B, Dec 2010, 1671-1674.
2. **G.Mariappan**, B. P Saha, N.R. Bhuyan, P.R. Bharti, D. Kumar., Evaluation of anti oxidant potential of pyrazolone derivatives, *Journal of Advanced Pharmaceutical Technology & Research Vol* 1 (2), Apr-Jun, 2010, 260-267.
3. **G Mariappan**, B P Saha, P K Haldar, R B Suresh Kumar, Lipika Pandey, Deepak Kumar., Cardio protective properties of pyrazolone derivatives against isoproterenol Induced Myocardial Ischemic Injury *Pharmacology online* 2 ,2010,986-994.
4. **G Mariappan**, B.P Saha, L. Sutharson, Ankit, S. Garg, Lipika Pandey, Deepak kumar., The diverse pharmacological importance of Pyrazolone derivatives: A Review *Journal of Pharmacy Research.* 201, 3(12) 2856-2859.
5. **G Mariappan**, B P Saha, L Sutharson, Ankit, S. Garg, L. Pandey, Deepak Kumar Analgesic, anti-inflammatory, antipyretic and toxicological evaluation of some newer 3-methyl pyrazolone derivatives *Saudi Pharmaceutical Journal* 2011,19, 115-122.

Abstracts Published/Presented

G.Mariappan, B.P Saha, sutharson L, Lipika Pandey Antipyretic activity of some Pyrazolone derivatives 62ND Indian Pharmaceutical Congress, Manipal University, Karnataka, Dec-17-19, 2010.

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he diverse pharmacological importance of Pyrazolone Derivatives : A Review

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ABSTRACT

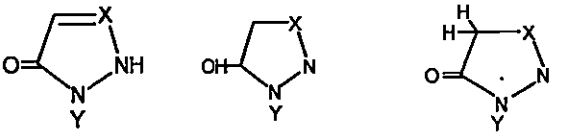
:entific research programs and reports are continuously pouring in with respect to improvised synthetic techniques to prepare numerous pyrazolone derivatives and :h regard to their diverse biological, pharmacological and chemical applications. When pyrazolones were discovered, they were only known as NSAID but in recent ıes, they are known to exhibit antioxidant, anticancer, antibacterial and several other pharmacological actions. These derivatives were withdrawn from the market :ause of their adverse effects such as agranulocytosis, skin rashes and blood dyscrasis etc, but recently they are again finding their place in the market and are being :ensively used in cerebral ischemia and cardio vascular diseases. Since its introduction into medicine, there have been more than 1000 compounds made in an effort find others with more potent analgesic action combined with less toxicity. Keeping in view the increasing importance of these derivatives, a need for the review is t. This review deals with up to-date literature on biological and pharmacological properties of pyrazolone derivatives.

:ywords: Pyrazolones, Edaravone, cardio protective, Antioxidant, Anticancer.

ITRODUCTION

: late 19th century gave rise to the discovery of the three prototypes of modern non-oid antipyretic and analgesics such as acetaminophen (acetanilide), aspirin and sali-ic acid and phenazone[1]. The Chemistry of pyrazolone began in 1883 when Knorr orted the first pyrazolone derivative. The reaction of phenyl hydrazine and ethylacetoacetate ulted in novel structure identified in 1887 as 1-phenyl-3-methyl-5-pyrazolone[2]. The orr pyrazole synthesis is the reaction of hydrazines with 1, 3 dicarbonyl compounds to ıvide the pyrazole or pyrazolone ring system. Pyrazolone is a five membered lactum g containing two nitrogen and a ketone group in its ring. The prototype molecule, ipyrine was synthesized for clinical use in 1883. The methylated nitrogen derivative inopyrine was introduced in 1897 and taken off from the market in the 1970s because its property to form nitrosamines. Dipyrone had been in clinical use since 1922. tipyrine was the first pyrazolone derivative as a drug introduced in 1887 and as the ne implies it was the first agent to reduce fever and used in the treatment of arthritis, sculoskeletal and joint disorder. These derivatives were widely used in medical prac-e viz antipyrine, aminopyrine, analgin etc. This discovery initiated the beginnings of great German drug industry that dominated the field for about 40 years. The Com-unds like 3-Alkyl-4- arylmethylpyrazol-5-ones are reported to exhibit potent ihyperglycemic activity, while 1-phenyl-3-tetrafluoroethylpyrazol- 5-one is an anxiolytic. us, the biological activities of pyrazol-5-ones depend upon the nature of the substitu-s[3]. 3- methyl -1- phenyl- 2- pyrazolin- 5- one (Edaravone); a strong novel free radical venger is used for the treatment of patients with acute brain infarction[4]. Demethylated ipyrine is a novel potent free radical scavenger that has been clinically used to reduce neuronal damage following ischemic stroke. Demethylated antipyrine exerts roprotective effects by inhibiting endothelial injury and by ameliorating neuronal damage in brain ischemia[5].The pharmacological spectrum of pyrazolone compounds are very similar to that of aspirin and some other (NSAID) nonsteroidal anti-inflammatory agents.The drugs containing pyrazolone nucleus are known to display diverse pharmacological activities such as antibacterial, antifungal, anti-inflammatory, analgesic, and antipyretic.

The pyrazolone nucleus has been known to exist in three tautomeric structures.

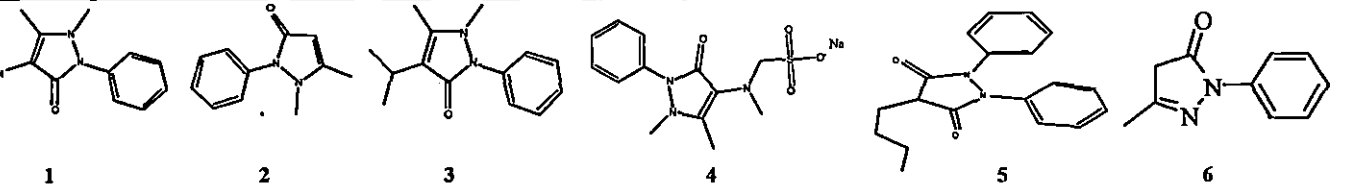


I II III

Strucutre I is present in several substituted pyrazolones which are widely known and used as antipyretic agents. All these compounds are characterized by the presence of a phenyl group attached to nitrogen atom in the 1- position and a methyl group in 3-position. Phenyl group in 1- position and a methyl group in 3-position seem to be essential for antipyretic activity. Several 4, 4-dimethyl derivatives, as well as Pyrazole Blue and Tartrazine, are derived from formula II whereas from structure III several pyrazolone dyes have been derived[6]

ble 1. Pyrazolone derivatives available in the market

ne	Structure	IUPAC Name	Brand Name	Uses
ipyrine	1	1,2 dihydro-1,5- dimethyl-2-phenyl-3H-pyrazol-3-one	Phenzone Analgesine	Analgesic,Antipyretic
inophenazone	2	4-dimethylamino-1,5-dimethyl-2-phenylpyrazol-3-one	Aminopyrin	Analgesic,Antiinflammatory
pyphenazone	3	1,5-dimethyl-2-phenyl-4-propan-2yl pyrazol-3-one	Pyramidone Anodymin	Analgesic,Antiinflammatory,in rheumatism,in cardiovascular disorder
amizole	4	Sod.[(2,3-dihydro-1,5 dimethyl-3-oxo-2-phenyl-1H-pyrazol-4-yl) methylamino] Methanesulfonate	Novalgin Dipyrone Analgin Algozone	Analgesic,Antipyretic,Antiinflammatory
nylbutazone	5	4-butyl-1,2-diphenyl-pyrazolidine-3,5 dione	Atropan Azdid Butazolidin Phanyzone	AnalgesicAntipyretic,Antiinflammatory,in rheumatism, in cardiovascular disorder
ravone	6	3-methyl-1-phenyl-2-pyrazolin-5-one	Edaravone MCI-186	As antioxidant,In cerebral ischemia,in rheumatism, in cardiovascular disorder



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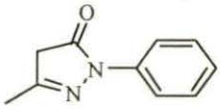
Diverse Pharmacological Properties of Pyrazolones

Aanalgesic anti-inflammatory and antipyretic activity

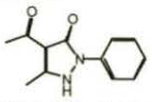
Phenylbutazone, a pyrazolone drug is useful in the treatment of acute gout, rheumatoid arthritis and allied disorders.4-acetyl-1-phenyl-3-methyl pyrazolone

AP) and 4-trifluroacetyl-1-phenyl-3-methyl azolone (HTFP) significantly reduces the car eenan induced inflammation in rats[7].

: selective inhibition of Phospholipase A_2 is cial in the search of a more efficient anti-in-nmatory drug with fewer side effects. Dipyrone ell known pyrazolone inhibitor having anti-ammatory activity is strongly found to be as-iated to PLA_2s through three hydrogen bonds ere as 1-phenyl-3-methyl-5-pyrazolone pre-ts an interamolecular hydrogen bond that makes ficult the formation of more efficient interac-ns with PLA_2[8].



3-methyl-1-phenyl-2-pyrazolin-5-one



4-acetyl-1-phenyl-3-methylpyrazolone

e radicals have some roles in inflammation and systemic and local tissue uries. Intrathecally administered edaravone, a free radical scavenger, had lgesic effects on inflammatory-induced acute and facilitated pain[9]. Oral yrone has been shown to be more effective than an equal dose of aspirin or acetamol in alleviating postoperative pain, and intravenous dipyrone 2.5g s similar in efficacy to pethidine 50 mg. In patients with acute ureteral or ary colic, dipyrone 2.5g intravenously was similar in efficacy to indometha-50 mg or pethidine 50 mg[10]. pyrazolones exert analgesic effect by inhib-g prostaglandin synthesis. The early phase (1-2 h) inflammation is mainly diated by histamine, serotonin and increased synthesis of prostaglandins in damaged tissues surroundings. The late phase is sustained by prostaglandin ease and mediated by bradykinin, leukotrienes, polymorph nuclear cells and staglandins produced by tissues macrophages. Fever results due to genera-n of mediators such as IL-1ß, IL-6, interferons and TNF-α cytokines rease the synthesis of prostaglandin which elevates the body temperature. m the results of antipyretics study, it can be suggested that pyrazolone ivatives produce the antipyretic action by inhibiting the prostaglandin thesis by blocking cycloxygenase isoenzymes, platelet thromboxane syn-sis, and prostanoids synthesis[11, 13]. There is increasing evidence that lysoso-enzymes play an important role in the development of acute and chronic ammation[14, 15]. Most of anti-inflammatory drugs exert their beneficial ef-by inhibiting either release of lysosomal enzymes or by stabilizing lysoso-memberane which is one of the major events responsible for the inflam-tory process.

tioxidant/Free radical scavenging activity

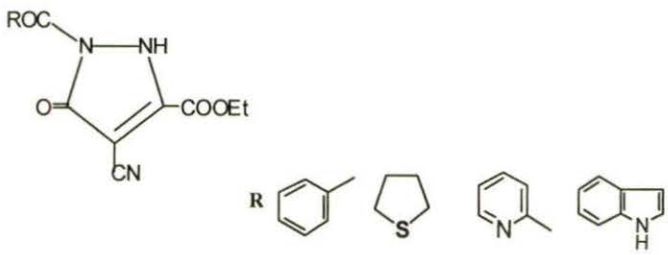
as been proved that the pyrazolone derivatives have significant antioxidant vity. The quantification of MDA and 4-HNE can be directly correlated with lipid peroxidation inhibition capacity of the pyrazolone derivatives. The ic radicals' quantification is also an indicator to monitor the overallprogress ipid peroxidation which isassociated with myocardial ischemic reperfusion ury[16]. Dipyrone and aminopyrine prevent phorbol-12-myristate-13-acetate-uced neutrophil burst with high efficiency and are highly potent scavengers HO and HOCl. Mitsubishi-Tokyo Pharmaceuticals Inc (Tokyo, Japan), de-oped 3-methyl-1-phenyl-2-pyrazolin-5-one (Edaravone) which is a strong el free radical scavenger. It has been shown that Edaravone reduces or ores the amount of ROS increased by postischemic reperfusion and prevents airment of the antioxidant defense system[17]. The putative mechanism un-ying the antioxidant action of edaravone is as follows- an electron transfer n an edaravone anion to peroxyl radical yields an edaravone radical and oxyl anion, and this reaction breaks the chain oxidation of lipids. Then, ravone peroxyl radical transforms to 4, 5-dione by elimination of a hydro-atom and one electron. Finally, 2-oxo-3- (phenylhydrazono) - butanoic (OBP) is produced by the hydrolysis of 4, 5-dione[18].

ucture Activity Relationship (SAR)

- Sterically small substituents such as hydrogen and methyl group did not show any activity, substituents containing carbocyclic moieties such as cyclohexyl, naphthyl, and benzyl maintained or increased the *in vitro* lipid peroxidation-inhibitory activity.
- The activity of 2-substituted compounds largely decreases except for a phenolic hydroxyl analogue.
- The activity is increased by the lipophilic substituents such as alkyl and halogen. Longer alkyl and alkoxy chains show increase in activity.
- A disubstituted halogen analogue increases activity as compared with monosubstituted halogen analogues.
- The introduction of hydrophilic substituents significantly decreased the activity. A phenyl analogue showed excellent activity which was far better than that of a 2-furyl analogue having the lipophilic aromatic group[19].
- The isobutyl group showed increased activity in contrast to the 2-hydroxyethyl group which showed almost no inhibitory activity.
- 4, 4-Disubstituted compounds showed no inhibitory activity, which supports hypothesis that compounds which generate the aromatic hydroxyl group by the keto–enol tautomerization have lipid peroxidation-inhibitory activity.

Anticonvulsant activity and Antidepressant activity

Some 4,4 disubstituted pyrazolone compounds exhibit anticonvulsant activity[20]. For instance, Dipyrone was found to have anticonvulsant activity in three experimental epilepsy models. At a dose of 300 mg/kg i.p., dipyrone blocked the maximal hind limb extension in the electroshock model in Wistar rats, the tonic-clonic component of acute sound-induced seizures and the limbic component of audiogenic kindling in genetically susceptible wistar rats. In the electroshock model higher doses (400 and 500 mg/kg) were also effective but lower doses (100 and 200 mg/kg) were not[21].

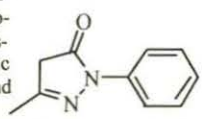


Antihyperglycemic activity

A group of 4-(arylmethyl and heteroarylmethyl)-5-substituted-3-pyrazolone derivatives have been found to have antihyperglycemic activity which is useful in non insulin dependent diabetes mellitus[25].1,2-dihydro-4-[[4-(methylthio)phenyl]methyl]-5-(trifluoromethyl)-3*H*-pyrazol-3-one in oral and subcutaneous glucose tolerance tests, indicated that unlike the renal and intestinal glucose absorption inhibitor phlorizin, it does not effectively block intestinal glucose absorption. Substitution of 4-methylthio, methylsulfinyl, or ethyl to a benzyl group at C4, in combination with trifluoromethyl at C5 of pyrazol-3-one, generated potent antihyperglycemic agents in obese, diabetic db/db mice (16-30% reduction in plasma glucose at 2 mg/kg).5-alkyl-4-(arylmethyl)pyrazol-3-ones (hydroxyl tautomers) have been discovered as potential new oral antidiabetic agents, based on their ability to lower plasma glucose when administered orally to obese, diabetic mice[26, 27].

Role of Pyrazolone in Cardiovascular Disease

Yoshida *et al.* in 2005 reported that Edaravone enhances the expression of eNOS and restores the reduction in eNOS by oxidized low-density lipoprotein in endothelial cells. It shows it prevent cell damage induced by oxidative stress through not only direct ROS scavenging effect but also restoration of reduced eNOS expression[28]. According to French patent application 2529786 a group of 3-phenyl or pyridyl - 5 - pyrazolone derivatives have been discovered which is useful in improving cardiac contractability[31]. It is expected that edaravone has beneficial effects on coronary artery and myocardial cells after ischemic and postischemic myocardial injury in patients with ischemic heart diseases, including acute myocardial infarction and angina pectoris[29, 30].In 1994, Yanagisawa *et al* showed that intravenous infusion of 3methyl-1-phenyl-2-pyrazolin-5-one derivative at a dose of 3 mg/kg attenuates the loss of myocardial creatine kinase activity from the left ventricular free wall in rats subjected to coronary artery occlusion for 10 minutes followed by reperfusion for 24 hours and reduced infarct size by approximately 50% compared with that in the control vehicle group[31]. Minhaz *et al* reported that 3methyl-1-phenyl-2-pyrazolin-5-one derivative attenuated



myocardial necrotic area by approximately 50% in isolated reperfusion rat rt subjected to coronary artery occlusion. Tsujita *et al.* investigated the :cts of edaravone on left ventricular function and infarct size using a ran-nized, placebo-controlled, open-label protocol in 80 patients with acute ocardial infarction. Intravenous administration of edaravone at a dose of 30 for 10 minutes before myocardial reperfusion decreased serum concentra-is of creatine kinase-MB isoenzymes, a surrogate point of infarct size, and proved left ventricular ejection fraction in patients with acute myocardial arction compared with those in the placebo group[30].

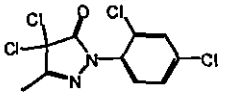
timicrobial activity

iew series of 4-arylhydrazono-2-pyrazoline-5-ones were tested in vitro .inst one Gram-positive and two Gram-negative bacterial strains, two my-acterial strains and a fungus, *Candida albicans*. Compounds were found to more active against *Staphylococcus aureus* than the other compounds at a icentration of 15.6 µg/mL[32]. The synthesis of Cu (II) complexes derived m Schiff base ligands obtained by the condensation of 2-lroxybenzaldehyde or terephtalic aldehyde with 4-aminoantipyrine (4-ino-2, 3-dimethyl-1-phenyl-3-pyrazolin-5-one) was prepared and screened antimicrobial activity, the qualitative and quantitative antimicrobial ac-ity test results proved that all the prepared complexes are very active, ecially against samples of *Ps. aeruginosa, A. Boumanii, E. coli* and *S. eus*[33]. Various 1-isonicotinyl-3-methyl-4-(substituted phenyl hydrazono)-yrazolin-5-one compounds were synthesized and evaluated for their anti-terial activity by Amir et al.

titumor activity

c25A and B are potential oncogenes and it is due to their overexpression t in various human carcinomas (breast, lung, colorectal, gastric, prostate, d and neck, ovary, lymphomas, and melanomas) and tumor cell lines oc-s[34]. In 2004 Korea Research Institute of Chemical Technology synthesized eral 3- methyl-4-oximinopyrazolin-5-one scaffold which were found to be c25B inhibitor, out of which 3-Methyl-4-(*O*-methyl-oximino)-1-enylpyrazolin-5-one and 1,3-dimethyl-4-(*O*-propargyloximino)- pyrazolin-one were found to be most potent. The activity decreases when phenyl up at 1-position was modified to bigger aromatic groups[35,36].

new derivative of 1-phenyl-3-methyl-5-pyrazolone,4,4-dichloro-1-(2,4-hloro phenyl)-3-methyl-5-pyrazolone, ned TELIN, was chemically synthesized d identified as a potent inhibitor of hu-n telomerase in the cell-free telomeric eat amplification protocol. It inhibits the omerase activity at submicromolar level h IC50 of 0.3µM. Kinetic studies showed t binding to telomerase protein, and the de of inhibition by this substance was npetitive–noncompetitive mixed-type with respect to the TS primer, ereas it was uncompetitive or noncompetitive – uncompetitive mixed-e with respect to the three deoxyribonucleosides. TELIN is a specific ent catalytic blocker of telomerase, and is considered to be a valuable stance for medical treatment of cancer and related diseases[37].



4,4-dichloro-1-(2,4-dichlorophenyl)-3-methylpyrazolin-5-one

new class of VEGFR(vascular endothelial growth factor receptor)-2/KDR ase inhibitors bearing heterocyclic substituted pyrazolones was designed as R kinase inhibition is considered to play an important role in regulating iogenesis, which is vital for the survival and proliferation of tumor cells. e thiadiazole series of pyrazolones are potent VEGFR-2/KDR kinase in-itors[38].

tithrombotic activity

perimental studies have shown beneficial effects of 3methyl-1-phenyl-2-azolin-5-one derivative (Edaravone) on postischemic reperfusion injury[39]. t has been found to ameliorate infarct size and brain edema in embolization l transient focal, global, and hemispheric ischemia models in adult rats and ttenuate the hypoxic-ischemia encephalopathy in neonatal rats[41]. In Ja-, edaravone was approved in April 2001 for treatment of acute brain rction and subarachnoid hemorrhage in the acute phase. Several investiga-s have reported that edaravone has beneficial effects on prevention of in damage in patients with stroke[42].

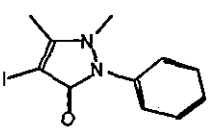
fazatrom, a pyrazolone derivative has dual arachidonate enzyme inhibi-n. It exhibits antithrombotic and thrombolytic action by inhibiting 5-lypoxigenase catabolism of arachidonate. This drug reduces the myocardial infarct size after experimental coronary artery occlusion and reperfusion.

Radioprotective effect

Analgin, antipyrine, and aminopyrine, if administered to mice in large doses 3 h before irradiation (800 R), increases the survival rate and prolonges the life of the dying animals. In combination with cystamine, these compounds increases the chances of survival of the mice after the period of acute intestinal death following irradiation in a dose of 1050 R. Experiments have shown that pyrazolone derivatives considerably increase the resistance to hypoxia of both healthy mice and irradiated mice at various periods of acute radiation sickness[43].

Antiviral activity

In addition to anti-inflammatory and analgesic activity, pyrazolones are known to possess antiviral activity.Saratikov et al discovered that antipyrine and related molecules can possess antiviral activity against a wide range of viruses. Iodoantipyrine or 4-iodo-1, 5-dimethyl-2-phenyl-pyrazol-3-one is an iodinated form of antipyrine. The anti-inflammatory action of Iodoantipyrine produces several effects such as reduction of degranulation of the mast cells; suppression of prostaglandins and arachidonic acid synthesis; membrane stabilizing activity; normalization of liver damage associated enzymes such as ALT and AST; lower intensity of oxidation and phosphorylation processes. This derivative displays antiviral activity against wide range of microorganisms including tick-borne encephalitis virus; hantavirus; influenza type A virus; herpes viruses; hepatitis B and C (HBV and HCV) viruses; Coxsackie A and B enteroviruses; papilloma virus; Venezuelan equine encephalomyelitis (VEE) virus; Rift Valley fever virus; poxviruses; and chlamydia.[44] This compound have been approved by Russia and neighboring countries for prevention and treatment of tick-borne encephalitis (TBE), hemorrhagic fever with renal syndrome (HFRS), and seasonal flu.



4-iodo-1-5-dimethyl-2-phenyl-pyrazol-3-one

Neuroprotective effects

Parkinson's disease is a neurological disorder characterized by the degeneration of nigrostriatal dopaminergic systems. *In vitro* study showed that edaravone significantly ameliorated the survival of TH-positive neurons in a dose-responsive manner. The number of apoptotic cells and HEt-positive cells significantly decreased, thus indicating that the neuroprotective effects of edaravone might be mediated by anti-apoptotic effects through the suppression of free radicals by edaravone. *In vivo* study demonstrated that edaravone-administration at 30 minutes after 6-OHDA (hydroxydopamine) lesion reduced the number of amphetamine-induced rotations significantly than edaravone administration at 24 hours[45].

Hepatoprotective activity

Fulminant hepatic failure is a serious disease that has a poor cure rate unless liver transplantation is performed. 3-methyl-1-phenyl-2-pyrazolim-5-one (edaravone) has the ability to prevent Fas-induced acute liver failure in mice. Edaravone reduces the number of apoptotic hepatocytes and also preventes cytochrome c release and caspase 3 activities, recognized as markers of apoptosis after mitochondrial disruption. Thus it protects hepatocytes from Fas-induced mitochondria-dependent apoptosis by regulating mitochondrial Bcl-xL and Bax[46].These results suggest that edaravone has a marked preventive effect on oxidative stress-induced acute liver injury. The same derivative prevents endotoxin-induced liver injury after partial hepatectomy not only by attenuating oxidative damage, but also by reducing the production of inflammatory cytokines, CINC and iNOS, in part through the inhibition of NF-κB activation.

Spasmolytic effect on smooth muscles

Dipyrone showed a spasmolytic effect on precontracted smooth muscle in vitro model. In a case reported by Hady, it was reported that premedication with dipyrone allowed the bronchoscope to pass through the bronchus more easily and increased the gas exchange in the lungs[47]. Dipyrone was also found to increase the gas exchange in the lungs when given as an analgesic for postoperative pain relief.Resta et al also reported on 2 asthma patients whose airway obstructions improved with dipyrone[48]. The mechanism by which dipyrone relieves bronchospasm is not clearly understood. Although anti-inflammatory properties by way of cyclooxygenase (COX) enzyme and thus prostaglandin synthesis inhibition by NSAIDs is thought to be responsible for

spasmolytic effect of some NSAIDs, as dipyrone has no or minimal anti-ammatory effect.

ıicity and adverse effects

ı most frequently reported side effects of the pyrazolone derivatives are ı rashes. Gastrointestinal side effects are rare. Blood dyscrasias, mostly ɔciated with aminopyrine[49]. Side effects, including acute renal failure, liver function, acute allergic reaction, disseminated intravascular coagulation, ɔmbocytopenia, leukocytopenia and renal dysfunction.Edaravone should ɔarefully used in elderly patients and patients with liver disease, renal dis-e, hematologic disease, or dehydration. Therapeutic usefulness of ylbutazone is limited because it possesses toxic side effects which include ɪtic ulcer with hemorrhage or perforation, hypersensitivity reactions of the ım sickness type, hepatitis, nephritis, aplastic anemia, leucopenia, agranu-ytosis and thrombocytopenia[50].Hence it is necessary to modify the struc-ɔ of pyrazolones to minimize the side effect and to improve its therapeutic ılication.

ɔNCLUSION

azolone derivatives are gaining importance through their diverse biologi- and pharmacological properties. In this review, we have described that azolone derivatives are not only having NSAID action but they also pos-s other pharmacological activities such as antioxidant, antihyperglycemic, itumour, anticonvulsant, hepatoprotective, neuroprotective, antiviral, ithrombotic, antimicrobial and radioprotective effects. These derivatives e beneficial effects on myocardial and vascular injury following ischemia l reperfuion in patients with acute myocardial infarction and also in athero-ɔrosis in the chronic phase .This review article may enlighten the medicinal mists who are aspiring to discover a versatile drug candidate for the benefit nankind.

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Note

Synthesis and bioactivity evaluation of pyrazolone derivatives

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3-Methyl-4-substituted benzylidene-pyrazol-5-ones **1-10** are synthesized by the condensation of 3-methyl-pyrazol-5-one with substituted aliphatic and aromatic aldehydes. Their structures have been elucidated from UV-Vis, IR, ^{1}H NMR and mass spectral data. Among the synthesized derivatives **5, 6, 7** and **10** are found to have a potent anti-inflammatory response whereas compounds **1, 4, 5, 8** and **10** have an effective analgesic response. There is no remarkable difference in bioactivity of pyrazolones derived from aliphatic and aromatic aldehydes. All the experimental data are statistically significant at $p<0.05$.

Keywords: Pyrazolone, analgesic, anti-inflammatory, protein kinase C inhibitor

Pyrazolone is a key structure in numerous compounds of therapeutic importance. Pyrazolone derivatives have been used as analgesics[1], antimicrobial agents[2], fungicides[3], and hypoglycemic agents[4]. Some of them have been tested as potential cardiovascular drugs[5]. They inhibit the production of TNF-α and decrease levels of pro-inflammatory cytokines and thereby reduce inflammation and prevent further tissue destruction in diseases such as Rheumatoid Arthritis (RA), Osteoarthritis (OA), and Crohns disease[6-8]. Researchers from Merck proved that pyrazolones are inhibitors of p38 kinase[9]. Recently their new derivative, named TELIN, was chemically synthesized and identified as a potent inhibitor of human telomerase. Another effective pyrazolone derivative, Nafazatrom, has dual arachidonate enzyme inhibition property. It exhibits antithrombotic and thrombolytic action. It also reduces the myocardial infarct size after experimental coronary artery occlusion and reperfusion. The above mentioned literature is worthwhile to prompt synthesis of some novel molecules by taking pyrazolone as heterocyclic key pharmacophore.

Results and Discussion

The present study reports the synthesis (**Scheme I**), analgesic and anti-inflammatory activity of pyrazolone derivatives. Perusal of the results on their analgesic activity by tail-flick method revealed that almost all of them to exert significant activity. Among them, compounds **1, 4, 5, 8** and **10** were found to have an effective analgesic response at $P < 0.01$ (**Table I**). All the synthesized compounds were screened for anti-inflammatory activity against Carrageenan-induced paw edema in rats. When compared with the control, all the compounds showed reduction in edema volume with prominent percentage inhibition to the inflammatory response ranging from 44% to 65% at 4th hour of observation. Compounds **5, 6, 7** and **10** were found to have a potent anti-inflammatory response at $p < 0.01$ level (**Table II**).

Experimental Section

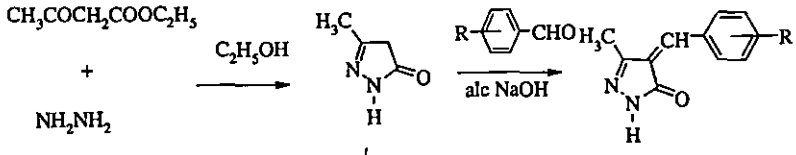
Melting points were determined in open capillary tubes and are uncorrected. The lambda max of the compounds were measured by UV-Vis spectrophotometer (UV–Pharma Spec 1700 Shimadzu). The IR spectra were recorded on Perkin-Elmer IR spectrometer 8400s using KBr disc. The ^{1}H NMR spectra were obtained on a Bruker DRX-600 MHz spectrometer in $CDCl_3$ using TMS as internal standard and chemical shifts are expressed in δ scale. The mass spectra were recorded on a Jeol SX-102 (FAB) Spectrometer.

Synthesis of 3-methyl pyrazol-5-one[10]

65 g (0.5 mole) of ethyl acetoacetate was taken in a 250 mL conical flask and stirred magnetically during slow drop wise addition of a solution of 25 g (0.5 mole) of hydrazine hydrate in 40 mL absolute ethanol. The temperature of the reaction mixture increased during reaction so that temperature was regulated at 60°C. A crystalline deposit was separated after stirring for 1 hr at 60°C. The reaction mixture was cooled in an ice bath to complete the crystallization. After standing for some time for completion of crystallization, it was filtered and the solid was washed with cold alcohol, dried and used for further step.

Synthesis of 3-methyl pyrazol-5-one derivatives 1-10

Pyrazolone (0.01 mole) was taken in a 100 mL round bottom flask, and then 50 mL of freshly prepared 20% sodium hydroxide alcoholic solution



Compd	R	Compd	R
1	$-N(CH_3)_2$	6	H
2	$C_6H_5CH=CH-$	8	4–OMe
3	$-OCH_3(_{2+3})$	9	3-OCH_3
4	3-NO_2	10	OH
5	3-Cl		

Scheme I — Synthesis of pyrazolone derivatives

Table I — Analgesic activity of pyrazolone derivatives in rat by tail flick method

Compd Dose (100 mg/kg p.o)	Reaction time in sec (Mean ±SEM)			
	After 1 hr	After 2 hr	After 3 hr	After 4 hr
Control	2.97±0.160	2.97±0.165	2.97±0.165	2.98±0.148
Diclofenac (10 mg/kg p.o)	6.93±0.139**	7.65±0.166**	8.25±0.042**	9.03±0.108**
1	4.91±0.127**	5.82±0.143**	6.4±0.050**	7.03±0.108**
2	4.19±0.034*	5.07±0.110*	5.9±0.022*	6.22±0.028*
3	4.19±0.036*	5.06±0.110*	5.79±0.140*	6.91±0.129*
4	4.72±0.128**	5.30±0.132**	6.17±0.027**	7.03±0.104**
5	4.29±0.026**	5.23±0.028**	5.96±0.136**	6.76±0.181**
6	5.43 ±0.024*	5.31±0.030*	6.21±0.019*	7.16±0.017*
7	4.43±0.019*	5.17±0.017*	6.12 ±0.025*	7.07±0.026*
8	4.07±0.021**	5.81±0.136**	6.52±0.023**	7.60±0.098**
9	4.30±0.030*	5.12±0.119*	5.93 ±0.137*	6.89±0.126*
10	4.49±0.029**	5.47±0.030**	6.40±0.029**	7.21±0.024**

**P<0.01vs Control, *p<0.05vs Control (n=6)

was poured into it. The mixture was stirred with magnetic stirrer for 30 min. After that substituted aromatic and aliphatic aldehyde (0.01 mole) was added to the reaction mixture and kept under stirring for 8 hr. The reaction mixture was transferred into crushed ice and neutralized with dilute hydrochloric acid to precipitate the product. It was filtered, dried and purified by recrystallization from ethanol. Similarly, other compounds were prepared with some change in reflux time and reaction work up. The physiochemical data of the compounds are given in **Table III**. The spectral and analytical data are given in **Table IV**.

Acute Toxicity Studies

Toxicological studies of the test compounds (as suspension in 0.5% w/v carboxy methylcellulose)

Table II — Anti-inflammatory activity of pyrazolone compounds on carrageenan-induced paw oedema in rats

Compd 100 mg/po	Paw volume in mL, Mean ± SEM (% inhibition of paw edema)			
	After 1 hr	After 2 hr	After 3 hr	After 4 hr
Control	0.97 ± 0.016	0.93 ± 0.017	0.91 ± 0.013	0.89 ± 0.013
Aspirin	0.61±0.008(37.11)**	0.53±0.011(42.85)**	0.41±0.008(54.5)**	0.3±0.006(89.99) **
1	0.84±0.007(13.79) *	0.65± 0.007 (30) *	0.55±0.004 (39.23)*	0.42±0.003(52.59) *
2	0.83±0.005(14.82) *	0.67±0.008(28.21)*	0.63±0.005 (41.78)*	0.43±0.003 (51.29) *
3	0.88±0.009 (9.36) *	0.68±0.009(26.78)*	0.50 ±0.023(44.01)*	0.411±0.004(54.32)*
4	0.88±0.009 (9.19) *	0.68±0.008(27.21)*	0.51 ±0.007(43.79)*	0.40±0.004 (54.81)*
5	0.65±0.009(33.22)**	0.55±0.005(40.35)**	0.4 ±0.006(54.56)**	0.30±0.003(65.74)**
6	0.69±0.007(29.13)**	0.56±0.006(39.35)**	0.46±0.007(49.08)**	0.31±0.004(64.99)**
7	0.65±0.006(33.56)**	0.56±0.008(39.28)**	0.46±0.003(48.89)**	0.38 ± 0.003(57.4)**
8	0.73 ± 0.010 (25) *	0.61±0.011 (34.5) *	0.51 ± 0.004(44.01)*	0.46±0.005 (48.18) *
9	0.75±0.006 (23.33) *	0.66±0.010 (28.57) *	0.53 ± 0.005(45.25)*	0.5 ± 0.006 (44.44)*
10	0.63±0.010(35.26)**	0.56±0.015(39.28)**	0.43±0.006(52.55)**	0.36±0.004(59.25)**

**P<0.01*vs* Control, * p<0.05*vs* Control (n=6)

Table III — Physical characterization data of compounds **1-10**

Compd	Mol formula	m.p. (°C)	Yield (%)
1	$C_{13}H_{15}N_3O$	236-38	65
2	$C_{13}H_{12}N_2O$	168-70	72
3	$C_{13}H_{14}N_2O_3$	208-10	68
4	$C_{11}H_9N_3O_3$	180-82	56
5	$C_{11}H_9N_2OCl$	216-18	84
6	$C_{11}H_{10}N_2O$	240-42	61
7	$C_6H_8N_2O$	258-60	75
8	$C_{12}H_{12}N_2O_2$	250-52	66
9	$C_{12}H_{12}N_2O_2$	170-72	57
10	$C_{11}H_{10}N_2O_2$	212-14	79

were carried out by standard method in oral dose of 100 to 1000 mg/kg body weight in albino mice. The mice were continuously observed for 8 hr for any signs of acute toxicity such as increased-decreased motor activity, ataxia, tremors, convulsions, sedation, lacrimation, *etc*. After 24 hr the mice were sacrificed, stomach, intestine, and liver were inspected under the magnifying lenses for any ulcer-haemorrhagic spots. The acute toxicity and gross behavior studies revealed that the entire compounds in the present investigation were nontoxic upto 1000 mg/kg body weight. All the animal experiments were performed by the approval of Institutional Animal Ethics Committee, Himalayan Pharmacy Institute.

Analgesic Activity

It was measured by D'Amour and Smith method[11]. The tips of tail of animals were individually placed on radiant heat source at constant temperature of 55±1°C and the reaction of the animals, like flicking of the tail was noted. Male albino rats of 12 groups (6 no's in each) were taken for study. First group was kept as control, second as standard and rest as test groups for different synthesized compounds. Test compounds **1-10** at the dose of 100 mg/kg p.o were administered. Diclofenac 10 mg/kg p.o was used as standard drug. The tail-flick latency was assessed by analgesiometer (Techno, India). Basal reaction time to radiant heat was taken for rat, the rat which responds within 2-3 sec only considered for studies. After administration, the reaction time was noted at 1 hr, 2 hr, 3 hr and 4 hr time interval of the above mentioned groups. The cut-off reaction time was fixed at 10 sec to avoid tissue damage. The observations were made and data obtained were statistically analyzed.

Anti-inflammatory Activity

It was studied by inducing paw edema according to Winter's method[12]. Male albino rats of 12 groups (6 no's in each) were taken for study. Group one was kept as control, group two was treated with standard drug aspirin 100 mg/kg p.o and the remaining groups were administered with test compounds **1-10** at the dose of 100 mg/kg p.o. A mark was made on left paws just beyond tibio-tarsal junction (knee joint) of each animal of all groups, so that each time the paw was dipped in the water column of digital paw edema meter (520-R,

Table IV — Spectral characterization data of compounds 1-10

Compd	Physical state	UV-Vis (nm)	^{1}H NMR (δ, ppm)	MS (*m/z*)
1	Dark brick red powder	482	δ8.25(s,1H,NH),6.80-8.61(m,4H,Ar-H),6.11 (s,1H,=CH-Ar),3.11(s, 6H,N(CH_3)$_2$),1.93(s,3H, CH_3)	229, 230, 231
2	Dark yellow powder	457	8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, N(CH_3)$_2$), 1.93 (s, 3H, CH_3)	212, 213
3	White crystalline powder	397	8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, N(CH_3)$_2$), 1.93 (s, 3H, CH_3)	246 ,247
4	Pale yellow powder	406	8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, N(CH_3)$_2$), 1.93 (s, 3H, CH_3)	231 ,232
5	Pale pinkish powder	428	8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, N(CH_3)$_2$), 1.93 (s, 3H, CH_3)	221
6	Pale yellow powder	360	8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, N(CH_3)$_2$), 1.93 (s, 3H, CH_3)	186,187,188
7	White powder	370	8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, N(CH_3)$_2$), 1.93 (s, 3H, CH_3)	124,125,126
8	Yellow powder	437	8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, N(CH_3)$_2$), 1.93 (s, 3H, CH_3)	216, 217
9	Pale orange powder	455	8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, N(CH_3)$_2$), 1.93 (s, 3H, CH_3)	216,217,218.
10	Orange powder	465	8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, N(CH_3)$_2$), 1.93 (s, 3H, CH_3)	202,203,204

s-singlet, d-doublet, t-triplet, m-multiplet

IITC Life Science, USA) up to the fixed mark made on left paws to ensure constant paw volume. Carrageenan (1%, 0.1 mL) (Sigma-Aldrich, Milan, Italy) was injected subcutaneously into the plantar surface of the rat hind paw 1 hr after the oral administration of the test compound. After the administration of carrageenan solution, the paw volume of control, standard and test groups were noted at 1 hr, 2 hr, 3 hr and 4 hr time interval. The percentage of inhibition was calculated by applying New bould formula[13].

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CARDIOPROTECTIVE PROPERTIES OF PYRAZOLONE DERIVATIVES AGAINST ISOPROTERENOL INDUCED MYOCARDIAL ISCHEMIC INJURY

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Summary

The present study investigates the cardioprotective effect of pyrazolone derivatives (PYZ1-PYZ10) on plasma lipid profile, serum marker enzymes, endogenous enzymatic and non-enzymatic antioxidants in cardiac tissues against isoproterenol (ISO) induced myocardial ischemic injury in rats. Pretreatment with the pyrazolone derivatives at 10 mg/kg body weight for 5 days prevented the elevation of serum marker enzymes namely lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) in myocardial injured rats. ISO-induced animals exhibited decreased levels of superoxide dismutase (SOD) and glutathione (GSH) in the heart, which were restored to near normal levels following treatment with pyrazolone derivatives. These derivatives also attenuated lipid peroxidation (LPO) in the heart and improved the imbalance in lipid profile (TG, LDL, VLDL, HDL) caused by ISO. These findings revealed the cardioprotective effect of pyrazolone derivatives against isoproterenol induced myocardial injury.

Key words: plasma marker enzymes, cardioprotective, reperfusion injury, pyrazolone

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Introduction

Ischemic heart disease (IHD) is one of the leading causes of deaths worldwide, accounting for 16.7 million deaths per annum [1, 2]. It occurs when blood supply is insufficient to the myocardium as a result, death of myocardial muscle occurs and such condition is known as ischemia. The prolonged ischemia of the myocardium leads to necrosis, which is referred as myocardial infarction [3]. Drugs such as beta-blockers and angiotensin converting enzyme inhibitors (ACEIs) had significantly improved the survival rate of IHD patients. However, attempts are made globally to get complementary and alternative medicines for IHD since no sufficient therapy for this obstinate illness is available. Pyrazolone ring systems represent an important class of compounds not only for their theoretical interest but also for their anti-inflammatory, analgesic, antipyretic [4], hypoglycemic agent [5], fungicide [6], antimicrobial [7] and some of them have been tested as potential cardiovascular drugs [8] including hypertension, hypercholesterolemia, atherosclerosis, myocardial infarction, angina pectoris, and heart failure[9]. Moreover, it has been proved that these molecules have preventive effects on myocardial injury following ischemia and reperfusion in the rat heart [10] and in patients with acute myocardial infarction [11]. Pyrazolone derivatives have been used in patients with acute brain infarction since April 2001 in Japan [12]. These derivatives have been shown to be effective against brain edema after ischemia and reperfusion injury in animal models [13] and in stroke patients [14]. Some animal studies using acute myocardial ischemia-reperfusion models have suggested the protective effects of pyrazolone derivatives on myocardial damage. By taking these research findings as evidence, the present study is focused to explore the cardioprotective properties of pyrazolone derivatives against isoproterenol induced myocardial ischemic injury.

Material and methods

Chemicals and Drugs

Isoproterenol hydrochloride (ISO) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, magnesium chloride, adenosine triphosphate and trichloroacetic acid (TCA) were purchased from Himedia Laboratories Private Ltd. (Mumbai, India). Nitroblue tetrazolium, phenazine methosulphate and nicotinamide adenine dinucleotide (NAD) were purchased from Sisco Research Laboratories Ltd. (Mumbai, India). The chemicals used in this study were of analytical grade.

Test compounds

Pyrazolone derivatives investigated in the present study were synthesized,characterized in Department of PharmaceuticalChemistry, Himalayan Pharmacy Institute, Sikkim. The physiochemical data of the synthesized compounds have been given in Table.1

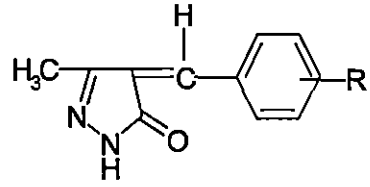
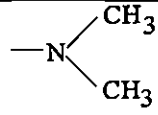


Table 1: Physical data of the synthesized compounds

Compound code	-R	Molecular formula	Mol. weight
PYZ1		$C_{13}H_{15}N_3O$	239
PYZ2	phenyl ethenyl	$C_{13}H_{12}N_2O$	212
PYZ3	$-OCH_{3(2,3)}$	$C_{13}H_{14}N_2O_3$	246
PYZ4	$-NO_2$	$C_{11}H_9N_3O_3$	231
PYZ5	Cl	$C_{11}H_9N_2OCl$	221
PYZ6	H	$C_{11}H_{10}N_2O$	186
PYZ7	$-CH_3$	$C_6H_8N_2O$	128
PYZ8	4-OCH_3	$C_{12}H_{12}N_2O_2$	216
PYZ9	4-OCH_3	$C_{12}H_{12}N_2O_2$	216
PYZ10	- OH	$C_{11}H_{10}N_2O_2$	202

Diagnostic Kits

Total Cholesterol, Total Protein, HDL, VLDL, LDL, TG, AST (SGOT), ALT (SGPT) and Alkaline Phosphatase were purchased from Span Diagnostics Ltd., Surat, India.

Animals

Rats (sprague-dawley or wistar, 100-150 g) were used as an experimental animals. They were housed hygienically under standard conditions of temperature (24±1°c), relative humidity (65±10%) and 12 light/dark cycle environment. During the study period, guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Institutional Animals Ethics Committee (IAEC) were followed for the maintenance of animals. The research work was approved by IAEC No: HPI/09/60/IAEC/0075.

Experimental Procedure

Seventy two male rats were completely randomized into twelve groups of six animals in each group. Group 1: normal control (distilled water p.o.), Group 2: ISO-treated (5.5 mg and 8.5 mg/ kg, s.c.) at an interval of 24 h for two days . Groups 3 to 12 were administered with 10 mg/kg body weight/ day p.o. of Pyrazolone derivatives (PYZ1-PYZ10) for 5 days followed by ISO treatment at an interval of 24 h for two days.

24 hours after the second injection of ISO, the rats were sacrificed by ether anaesthetization and the heart was dissected out. The neck area was quickly cleared of fur to expose the jugular vein. The vein, after being slightly displaced, was sharply cut with sterile surgical blade and an aliquot (5 ml) of the blood was collected and centrifuged at 10000g for 5 mins. The serum was carefully aspirated with a Pasteur pipette into sample bottles for biochemical analysis.

Plasma lipid profile

Plasma total cholesterol (TC), Total Protein, Creatinine, triglycerides (TG) and high density lipoprotein (HDL) were analysed using commercially available kits (Reckon diagnostics, Baroda, India).Very low density lipoproteins (VLDL) and low density lipoprotein (LDL) were calculated as per Friedewald et al. [15].

Plasma cardiac specific injury markers

Activity levels of creatine phospokinase (CPK), lactate dehydrogenase(LDH), alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphate (ALP) in plasma were estimated using commercially available kits (Eve's Diagnostics, Baroda, India).

Estimation of cardiac endogenous antioxidant

Cardiac tissue pieces from control and treated groups were weighed and homogenized (10% w/v) in chilled Tris buffer (10 mM, pH 7.4), centrifuged at 10,000g for 20 min in high speed cooling centrifuge (0 1C). Clear supernatant was used for assaying superoxide dismutase (SOD); [16], catalase (CAT); [17] and reduced glutathione (GSH); [18]

Statistical Analysis

All the results were expressed as mean±SEM. The results were analysed by one way analysis of variance (ANOVA) followed by Dunnett's test through the computer program Graph Pad Instat 3. P value <0.05, $P<0.01$ was considered statistically significant.

Results and discussion

There were alterations in serum lipid profile of the rats administered with pyrazolone derivatives when compared with the Isoproterenol control. The Isoproterenol raised the serum levels of total cholesterol, Total Protein, LDL, VLDL LDL, triglycerides; and decreased HDL level. Pretreatment with the pyrazolone derivatives for 5 days however, restored the lipid profile to near normalcy and improved the cardiac damage caused by isoproterenol (Table 2 and Table 3). Lipid metabolism plays an important role in myocardial injury produced by ischemia [19]. Isoproterenol causes hyperlipidemia and it increases the LDL cholesterol in the blood, which in turn leads to harmful deposits in the arteries thus favoring coronary heart diseases (CHD) [20]. In the present study also, ISO administration caused a significant raise in the serum lipids thereby increases lipid biosynthesis and lipid peroxidation. Rats treated with pyrazolone derivatives showed decreased concentration of serum total cholesterol, triglycerides, LDL cholesterol indicates the beneficial effects of pyrazolone derivatives in reducing hyperlipidemia caused by Isoproterenol.

The administration of Isoproterenol in rats resulted significant increase in the serum levels of heart marker enzymes including LDH, AST, ALT and ATP However, pretreatment with Pyrazolone derivatives reduced the activities of these enzymes to near normal levels (Table 2).

Table 2: Effect of pyrazolone derivatives on marker enzymes in ISO induced myocardial injury in rat model

Compd code	SGOT (IU/ml)	SGPT (IU/ml)	ALP (IU/ml)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
Control	152.56±0.15	40.56±0.31	141.81±.71	43.87±.01	99.56±.11	8.05±1.25
ISO (8.5 mg/kg)	203.26±.02	81.23±0.43	213.89±0.81	20.58±1.2	186.87±.13	30.22±.01
PYZ1	191.52±0.61^{b}	79.01±0.26^{b}	210.56±0.42^{ns}	23.54±1.65^{a}	165.25±.56^{b}	23.33±.11^{b}
PYZ2	190.12±0.51^{b}	79.99±0.11^{ns}	208.20±0.34^{b}	23.99±.11^{a}	165.66±.23^{b}	23.01±.51^{b}
PYZ3	181.84±0.63^{b}	76.44±0.13^{b}	207.32±0.62^{b}	24.89±.31^{b}	154.84±.29^{b}	20.38±.65^{b}
PYZ4	184.91±0.71^{b}	76.00±0.01^{b}	200.89±.10^{b}	25.63±.84^{b}	153.22±.55^{b}	19.99±2.63^{b}
PYZ5	182.38±0.02^{b}	74.55±0.71^{b}	198.36±0.52^{b}	27.00±.67^{b}	146.40±.10^{b}	18.85±1.85^{b}
PYZ6	180.01±0.41^{b}	72.36±0.10^{b}	195.39±1.20^{b}	28.56±.24^{b}	136.55±.61^{b}	15.84±1.95^{b}
PYZ7	179.85±0.53^{b}	70.85±0.63^{b}	193.95±1.90^{b}	29.66±.01^{b}	134.66±.01^{b}	14.55±2.55^{b}
PYZ8	164.22±0.87^{b}	61.02±0.45^{b}	176.95±.97^{b}	31.11±.03^{b}	130.58±.33^{b}	12.86±.01^{b}
PYZ9	162.00±0.72^{b}	55.00±.06^{b}	175.00±.15^{b}	32.33±.21^{b}	125.98±.78^{b}	10.62±.11^{b}
PYZ10	158.01±1.01^{b}	54.62±0.14^{b}	172.83±1.57^{b}	36.98±.02^{b}	120.88±.91^{b}	10.01±.02^{b}

Values are expressed as mean±SEM, (N=6); ns non significant; ^{a}P <0.05, ^{b}P<0.01 compared with ISO control (ANOVA followed by Dunnett's test). All the test compounds were administered at 10 mg/kg.

Table 3: Effect of pyrazolone derivatives on lipids profile in ISO induced myocardial injury in rat model

Compd code	Total Protein (g/L)	Triglycerides (mg/dL)	Cholesterol (mg/dL)	Creatinine (mg/dL)	Lactose Dehydrogenase (IU/L)
Control	39.80±0.98	79.04±3.97	117.15±2.02	2.26±0.04	120.07±0.54
ISO(8.5 mg/kg)	86.86±0.25	186.47±0.34	201.65±7.56	3.93±0.08	198.71±0.29
PYZ1	56.46±.050[b]	135.43±3.18[b]	168.61±0.63[b]	2.81±0.007[b]	134.25±0.58[b]
PYZ2	66.33±0.28[b]	122.32±0.70[b]	162.75±0.74[b]	2.92±0.01[b]	142.70±0.51[b]
PYZ3	53.00±4.70[b]	180.58±4.44[b]	153.95±0.81[b]	2.65±0.18[b]	148.45±0.41[b]
PYZ4	40.43±0.85[b]	152.94±4.29[b]	133.46±0.58[b]	2.39±0.03[b]	158.39±0.47[b]
PYZ5	39.23±0.92[b]	158.33±3.74[b]	132.14±0.73[b]	2.65±0.01[b]	138.55±0.52[b]
PYZ6	58.07±0.86[b]	123.33±0.61[b]	118.04±0.37[b]	2.67±0.04[b]	128.83±0.71[b]
PYZ7	38.08±0.79[b]	124.66±0.94[b]	120.59±0.53[b]	2.67±0.07[b]	123.68±0.95[b]
PYZ8	48.36±0.66[b]	80.07±0.37[b]	121.01±0.73[b]	2.78±0.07[b]	121.80±0.50[b]
PYZ9	52.20±0.77[b]	81.20±0.35[b]	122.89±0.45[b]	2.56±0.0[b]	127.76±0.59[b]
PYZ10	53.06±0.79[b]	82.95±0.75[b]	123.82±0.64[b]	2.45±0.10[b]	127.25±1.8[b]

Values are expressed as mean±SEM, (N=6);
[b]P<0.01 compared with ISO control (ANOVA followed by Dunnett's test). All the test compounds were administered at 10 mg/kg.

Pyrazolone derivatives pre-treatment improves cardiac antioxidant status in ISO induced myocardial injury by effective scavenging of free radicals generated during oxidation of lipids thus collectively contributing to its overall antioxidant and anti ischemic activity. ISO induced myocardial injury has been reported to alter membrane permeability [19] and to cause leakage of marker enzymes of cardiac damage (LDH, CPK, AST, ALT and ALP) into the blood stream[21]. Significantly elevated levels of these marker enzymes have been recorded in ISO induced myocardial damage [22]. However, PYZ treated group controlled the elevation in activity levels of these enzymes suggesting that with PYZ pretreatment ISO induced leakage of marker enzymes can be prevented.

Table 4: Effect of pyrazolone derivatives on endogeneous antioxidant enzymes in ISO induced myocardial injury in rat model

Compound code	SOD U/mg of protein	CAT IU/mg of tissue	GSH µg /mg wet tissue	CPK IU/ml
Control	5.38±0.21	73.05±.02	5.63±.02	110.25±0.01
ISO(8.5 mg/kg)	1.99±0.01	40.11±.10	1.69±0.12	194.45±0.25
PYZ1	2.71±0.02[a]	43.25±.21[a]	2.01±0.11[ns]	190.85±0.74[a]
PYZ2	2.91±0.14[b]	43.00±.06[a]	2.65±0.21[ns]	188.65±0.46[b]
PYZ3	3.01±0.04[b]	44.55±.11[b]	2.97±0.14[b]	184.29±1.01[b]
PYZ4	3.51±0.13[b]	46.88±.56[b]	3.86±0.18[b]	180.11±0.08[b]
PYZ5	3.80±0.02[b]	49.57±.32[b]	3.88±0.19[b]	179.86±0.87[b]
PYZ6	3.91±0.14[b]	50.22±.55[b]	3.15±0.25[b]	175.97±1.56[b]
PYZ7	3.99±0.31[b]	50.00±1.21[b]	3.6±0.3[b]	168.52±1.50[b]
PYZ8	4.01±0.42[b]	53.85±1.38[b]	3.55±0.15[b]	160.28±0.05[b]
PYZ9	4.49±0.02[b]	54.92±.07[b]	4.02±0.49[b]	158.87±0.24[b]
PYZ10	4.99±0.11[b]	58.87±1.02[b]	4.09±0.28[b]	158.77±0.08[b]

Values are expressed as mean±SEM, (N=6); ns. non significant
[a]P<0.05, [b]P<0.01 compared with ISO control (ANOVA followed by Dunnett's test). All the test compounds were administered at 10 mg/kg.

As reported in Table 4, there was significant decrease in GSH levels in the heart of ISO-treated rats. Pretreatment with pyrazolone derivatives resulted in marked improvement in these indices at the end of the experiment and were reverted back to normalcy. In addition, the significantly decreased activities of enzymic antioxidants (CAT,GSH and SOD) observed in the heart of ISO-treated rats were improved following pretreatment with pyrazolone derivatives for 5 days. The present study has clearly demonstrated that the pyrazolone derivatives have antioxidant activity which could prevent the occurrence of heart related diseases. Significantly elevated activities levels of SOD and CAT recorded in PYZ treated group could be due to its potent free radical scavenging ability. GSH scavenges singlet oxygen, superoxide and peroxy radicals to form oxidised glutathione and other disulfides [23]. Also, antioxidant compounds have been shown to increase glutathione reductase activity that maintains GSH in a reduced state [22]. The elevated GSH content observed in PYZ treated groups may be due to its enhanced synthesis.

ISO treatment is also known to create an imbalance between enzymatic as well as non-enzymatic antioxidant defence system leading to production of free radicals that induce myocardial injury and LPO [24]. The significant decrement in LPO in pyrazolone derivatives treated group further justifies the role of pyrazolone derivatives as a potent antioxidant and free radical scavenger. These results are in conformity with reports that have demonstrated modulation of cellular antioxidant by treatment with pyrazolones[25]. It can be summarized that PYZ pre-treatment to ISO treated rats provide cardioprotection by inhibiting the formation of free radicals generated during oxidation of lipids thus inhibiting peroxidation of membrane lipids and preventing subsequent leakage of soluble enzymes. Also, pyrazolone derivatives pre-treatment appears to improve the status of enzymatic antioxidants that further contributes to its overall cardioprotective property. Hence, it can be concluded that pyrazolone derivatives pretreatment provides cardioprotection against ISO induced myocardial injury via multiple mechanisms.

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ORIGINAL ARTICLE

Analgesic, anti-inflammatory, antipyretic and toxicological evaluation of some newer 3-methyl pyrazolone derivatives

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KEYWORDS

Pyrazolone;
Non-steroidal anti-inflammatory;
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Blood dyscrasias

Abstract In this paper, we described the pharmacological and toxicological studies of three pyrazolone derivatives namely **PYZ1**: 4-[4-*N* dimethylamino benzylidine]-3-methyl pyrazolin-5(4*H*)-one, **PYZ2**: 4-[2-chlorobenzylidine]-3-methylpyrazolin-5(4*H*)-one and **PYZ3**: 4-[benzylidine]-3-methylpyrazolin-5(4*H*)-one derivatives. Analgesic, anti-inflammatory and antipyretic studies of 3-methyl pyrazolone derivatives at 400 mg/kg, p.o. have shown significant activity as compared to control. Amongst three pyrazolone derivatives, **PYZ2** was found to be more active. Based on the result of pharmacological studies, **PYZ2** was selected for toxicological studies. Acute toxicity studies revealed that methyl pyrazolone derivatives are non-toxic in rats up to 5000 mg/kg, p.o. The subacute toxicity study of **PYZ2** showed that decrease in Hb content, RBC and WBC count. In biochemical analysis level of blood glucose and bilirubin reduced where as AST, ALT and alkaline phosphatase level elevated. Histopathological studies revealed that there was mild toxicity on liver and kidney at 1000 mg/kg, p.o.

1. Introduction

Pyrazolone ring system represents an important class of compounds not only for their theoretical interest but also for their anti-inflammatory, analgesic, antipyretic (Badaweya et al., 1998), hypoglycemic agent (Das et al., 2008), fungicide (Singh and Singh, 1991), antimicrobial (Sahu et al., 2007) and some of them have been tested as potential cardiovascular drugs (Yukihito et al., 2006). In the recent year, research is focused on existing molecules and their modifications in order to reduce their side effects and to explore their other pharmacological and biological effects. The most frequently reported side effects of the pyrazolone derivatives are skin rashes, agranulocytosis, blood dyscrasias which is mostly associated with

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aminopyrine, have received wide attention in the medical literature, but their true incidence with dipyrone is considerably lower than the often quoted incidence for aminopyrine reported more than 30 years ago (Brogden, 1986). Pyrazolones are an active moiety in the class of NSAIDs and used in the treatment of arthritis, musculoskeletal and joint disorder. Compounds like 3-alkyl-4-aryl methyl pyrazol-5-ones are reported to exhibit potent antihyperglycemic activity, while 1-phenyl-3-tetrafluoroethylpyrazol-5-one is an anxiolytic. Thus, the biological activities of pyrazol-5-ones depend on the nature of the substituents (Sil et al., 2005). Edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one a strong novel free radical scavenger is used for the treatment of patients with acute brain infarction (Yukihito et al., 2006). Demethylated antipyrine is a novel potent free radical scavenger that has been clinically used to reduce the neuronal damage following ischemic stroke. Since few literatures reveal the pharmacological and toxicological studies of pyrazolones, an attempt has been made to explore the pharmacological properties of novel pyrazolone derivatives with their toxic effects.

2. Materials and methods

2.1. Source of compounds

Pyrazolone derivatives **PYZ1, PYZ2** and **PYZ3** (Fig. 1) investigated in the present study were synthesized, characterized in Department of Pharmaceutical Chemistry, Himalayan Pharmacy Institute, Sikkim, India (Mariappan et al., 2010).

2.2. Chemicals and drugs

Carboxy methyl cellulose (CMC), acetic acid, eosin, hematoxylin, dextrene polystyrene xylene, paraffin and xylene were purchased from S.D. Fine, Mumbai, India. Carrageenan, Freund's adjuvant and Brewer's yeast were procured from Sigma, USA. Indomethacin, paracetamol and aspirin were obtained from Cipla Pharmaceuticals, Sikkim, India and pentazocine from Ind-Swift L., Baddi, India as gift sample.

2.3. Biochemical diagnostic kits

Cholesterol, direct HDL-cholesterol, triglycerides, glucose, AST (GOT), ALT (GPT) and alkaline phosphatase kits were purchased from Span Diagnostics Ltd., Surat, India.

2.4. Animals

Male and female albino mice (Swiss, 20–25 g), rats (Sprague–Dawley, 100–150 g) and rabbits (New Zealand strain, 1.5–2 kg) were used as experimental animals. The animals were housed under standard conditions of temperature (24 ± 1 °C), relative humidity (65 ± 10%) and 12 h light/dark cycle environment. During the study period, guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Institutional Animals Ethics Committee (IAEC) were followed for the maintenance of animals and the experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) No.: HPI/09/60/IAEC/0075.

2.5. Experimental procedure

The animals were divided into five groups of six in each. The first group was treated as control and received 1% CMC, second group received the standard drug (indomethacin 10 mg/kg, p.o., pentazocine 3.9 mg/kg, i.p., paracetamol 100 mg/kg, p.o. and aspirin100 mg/kg, p.o.) and rest of the groups were administered test compounds at 400 mg/kg, p.o.

2.5.1. Analgesic activity

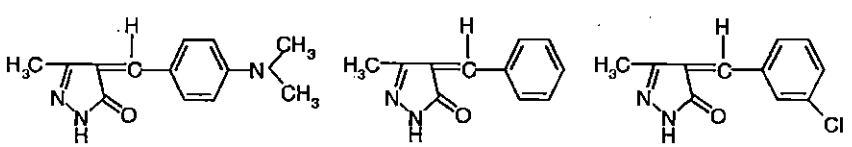
The analgesic activity was evaluated by tail flick and writhing methods using albino mice adopting standard procedure and results are reported in Table 1. Overnight fasted healthy and adult male albino Swiss mice weighing between 20 and 25 g, in group of six each were taken for the investigation.

2.5.1.1. Tail-flick in mice. Tail flick method (D'Armour and Smith, 1941) was used to evaluate the analgesic effect of the pyrazolone compounds. In this method heat is used as a source of pain. The tips of the animals were individually placed on the radiant heat source at constant temperature 55 °C. The tail flick response was measured at 0, 1, 2 and 3 h after treatment of test compounds by digital analgesiometer (INCO, Ambala, India). The cut-off reaction time was fixed at 10 s to avoid tissue damage. The drug pentazocine (3.9 mg/kg, i.p.) was used as standard drug for comparison.

2.5.1.2. Acetic acid-induced writhing test. Acetic acid-induced writhing test was done according to Koster et al. (1959). After 30 min of the administration of the test compounds, all the test group of mice were administered intra-peritoneally the writhing agent, 1% (v/v) aqueous acetic acid, in a dose of 1 ml/100 g b.w. The standard group was administered with indomethacin (10 mg/kg, p.o.). The numbers of writhing episode were counted for 30 min and the number of wriths produced in pyrazolone treated groups was compared with control.

2.5.2. Anti-inflammatory activity

2.5.2.1. Carrageenan-induced rat paw edema. The anti-inflammatory activity of the test compounds was evaluated by carrageenan-induced rat paw edema model described by Win-



4-[4-N dimethyl amino benzylidine]-3-methyl pyrozolin-5(4H)-one **(PYZ1)**

4-[benzylidine]-3-methyl pyrozolin-5(4H)- one**(PYZ2)**

4-[3-chloro benzylidine]-3-methyl pyrozolin-5(4H)- one **(PYZ3)**

Figure 1 Structure and nomenclature pyrazolones.

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Table 1 Analgesic activity of pyrazolone derivatives.

Compound (mg/kg)	Tail flick method				Writhing test
	Average tail withdrawal time (s)				No. of writhings
	0 h	1 h	2 h	3 h	
Control	1.66 ± 0.25	1.66 ± 0.21	1.66 ± 0.21	1.66 ± 0.21	41.7 ± 0.7
Standard (a and b)	1.5 ± 0.30	3.83 ± 0.30**	4.66 ± 0.33**	8.91 ± 0.56**	17.5 ± 0.4**
PYZ1 (400)	1.35 ± 0.21	2.0 ± 0.25	4.16 ± 0.47**	7.16 ± 0.60**	24.5 ± 0.5**
PYZ2 (400)	1.32 ± 0.21	3.16 ± 0.30*	6.33 ± 0.49**	8.5 ± 0.56**	19.4 ± 0.89**
PYZ3 (400)	1.4 ± 0.21	2.0 ± 0.25	4.83 ± 0.60	7.66 ± 0.55	25.4 ± 1.1**

Values are expressed as mean ± SEM ($N = 6$).
a – Pentazocine (3.9 mg/kg, i.p.) used as standard drug in tail flick method; b – indomethacin (10 mg/kg, p.o.) used as standard drug in writhing test.
* $P < 0.05$ compared with vehicle control (ANOVA followed by Dunnett's test).
** $P < 0.01$ compared with vehicle control (ANOVA followed by Dunnett's test).

Table 2 Anti-inflammatory activity of pyrazolone derivatives.

Compound (mg/kg)	Carrageenan-induced paw edema method					Freund's adjuvant method	
	Paw edema volume in ml (% inhibition of paw edema)						
	0 h	1 h	2 h	3 h	4 h	0 h	18 h
Control	0.83 ± 0.02	0.90 ± 0.03	1.16 ± 0.09	1.24 ± 0.0	0.98 ± 0.03	0.57 ± 0.02	1.17 ± 0.02
Standard (a and b)	0.82 ± 0.03	0.94 ± 0.06	0.86 ± 0.02* (25.86%)	0.70 ± 0.01** (43.54%)	0.45 ± 0.09** (54.08%)	0.51 ± 0.01	0.59 ± 0.02** (49.57%)
PYZ1 (400)	0.80 ± 0.02	0.93 ± 0.04	0.99 ± 0.05 (14.66%)	0.85 ± 0.03** (31.45%)	0.57 ± 0.01** (41.83%)	0.61 ± 0.3	0.71 ± 0.01** (39.31%)
PYZ2 (400)	0.80 ± 0.02	0.87 ± 0.02	1.05 ± 0.09 (9.48%)	0.72 ± 0.01** (41.93%)	0.48 ± 0.01** (51.02%)	0.56 ± 0.2	0.62 ± 0.01** (47.41%)
PYZ3 (400)	0.81 ± 0.03	0.87 ± 0.02	0.94 ± 0.04 (18.96%)	0.77 ± 0.02** (37.90%)	0.52 ± 0.01** (46.93%)	0.58 ± 0.02	0.68 ± 0.01** (41.37%)

Values are expressed as mean ± SEM ($N = 6$).
a – Idomethacin (10 mg/kg, p.o.) was used as standard drug in carrageenan-induced paw edema method; b – aspirin (100 mg/kg, p.o.) was used as standard drug in Freund's adjuvant method.
* $P < 0.05$ compared with vehicle control (ANOVA followed by Dunnett's test).
** $P < 0.01$ compared with vehicle control (ANOVA followed by Dunnett's test).

ter et al. (1962). Rats of either sex were treated orally with pyrazolone derivatives (400 mg/kg b.w.) and standard drug indomethacin (10 mg/kg b.w.), 1 h prior to the 1% (w/v) solution injection of 0.1 ml carrageenan into plantar region of right hind paw (subcutaneously). Paw volume was measured by Plethysmometer (Model 520, IITC, Life Sciences, USA) at 0, 1, 2 and 3 h after carrageenan injection. The difference between the paw volume at 4th and 0 h measurement was calculated and taken as edema volume. Percentage inhibition in the paw was calculated by using the formula, percentage inhibition = $100 \times (1 - V_t/V_c)$, where V_t = mean increase in paw volume of test, and V_c = mean increase in paw volume of control. Percentage inhibition shown by tested compounds is recorded in Table 2.

2.5.2.2. Freund's adjuvant-induced polyarthritis model (Newbould, 1963). The arthritic syndrome was induced in rats by an injection of 0.1 ml of Freund's complete adjuvant into the subplantar region of the right hind paw. Animals were treated orally with the pyrazolone derivatives (400 mg/kg) and standard drug aspirin (100 mg/kg) once daily for 30 days. Plethysmographic determination of paw volume was performed on injected foot. Paw volume after 18 h was taken as subacute phase of inflammation and that of 30th day was observed as an index of chronic inflammation.

2.5.3. Antipyretic activity

This activity was performed on rabbits of either sex according to the reported method described by Lu et al. (2004). An aliquot of 3 ml/kg of 10% Brewer's yeast suspension was subcutaneously injected into the rabbit back. Temperature was monitored by means of thermometer inserted at least 10 cm into the rectum and recorded by calibrated thermometers. An animal was excluded from the study if the baseline temperature was not within the range of 39.2–39.8 °C. After 5 h, animals showing at least an increase of 1 °C of rectal temperature were selected for the experiment. The animals were administered with standard paracetamol (100 mg/kg, p.o.) and pyrazolone derivatives (400 mg/kg) orally. The rectal temperature was measured at 0, 1, 2 and 3 h after treatment.

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2.5.4. Acute toxicity study

The Up and Down Procedure (Dixon, 1999) was adopted to evaluate the acute toxicity of pyrazolone derivatives after oral administration in rats. Five adult, female, non-pregnant rats were randomly selected for this experiment. The animals were marked and housed individually in cages. They were fasted overnight but allowed free access to water before the administration of test compound. Dosing was initiated with 175 mg/kg and further doses were increased by a factor of 3.2. Pyrazolone derivatives were administered orally in the form of suspension with 1% CMC and observed for 48 h for signs of acute toxicity such as increased–decreased motor activity, ataxia, tremors, convulsions, sedation and lacrimation, or any instant death.

2.5.5. Subacute toxicity study (Ghosh, 1970; Dixon, 1999; Derelanko and Hollinger, 2002)

Since **PYZ2** had shown promising pharmacological activities, it was chosen for subacute toxicity study. It was administered orally to rats for 28 days at the dose of 500 and 1000 mg/kg. All animals were supplied with standard diet and water during the testing periods. At the end of the study period on 29th day the animals were anaesthetized with chloroform and blood samples immediately collected by cardiac puncture for hematological and biochemical analysis. Necropsy of all the animals was carried out and selected organs like the heart, liver and kidney were removed and preserved. The organs were physically examined, weighed and samples were collected for histopathological examinations.

2.5.5.1. Biochemical assay. The non-heparinized blood was allowed to coagulate before being centrifuged (4000 rpm for 20 min) and the serum separated. The levels of cholesterol (Chol.), glucose (Glu.) triglycerides (TG), high density lipoprotein cholesterol (HDLC), total bilirubin (TB), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were estimated using commercially available standard kits (Erba Diagnostic Kit and Span Diagnostics Ltd., India) on an automatic analyzer (Merck P. Ltd.).

2.5.5.2. Hematological assay. At the end of the experimental period, the next day after an overnight fasting, blood was collected directly from heart and used for the analysis of hematological parameters viz. Hemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count, hematocrit and blood clotting time.

2.5.5.2.1. Hemoglobin estimation. Heparinized blood was taken in the Sahli Hemoglobinometer and diluted with the 0.1 N HCl until the colour matched with the standard. The reading was taken from graduated cylinder and expressed as g/dl of blood (Wintrobe et al., 1976; Armour et al., 1965).

2.5.5.2.2. RBC count. The blood sample was diluted 1:100 with the RBC diluting fluid using Thoma pipette after vigorous mixing, a drop of resultant mixture was discharged under cover glass of Neubauer haemocytometer and corpuscles were allowed to settle for 3 min. The number of RBC in 80 small squares was counted under light microscope (Wintrobe et al., 1976; Armour et al., 1965).

2.5.5.2.3. WBC count. The blood sample was diluted 1:20 with the WBC diluting fluid using Thoma pipette after vigorous mixing a drop of resultant mixture was discharged under cover glass of Neubauer hemocytometer and corpuscles were allowed to settle for 3 min. The number of WBC in 16 small squares was counted under light microscope (Wintrobe et al., 1976; Armour et al., 1965).

2.5.5.2.4. Differential leukocyte count, platelet count and hematocrit. The heparinized blood was analyzed by digital automatic hematology analyzer/blood cell counter (Care Well Biotech Pvt. Ltd., India) in Ashok Laboratories, Jodhpur Park, Kolkata-32, India and the results were obtained.

2.5.5.2.5. Blood clotting time. The blood sample was taken with the help of a glass capillary from orbital plexus of the eye of each rat and the time was noted. Small pieces of capillary were broken from one end at every 30 s till fibrin threads of blood appeared between the broken ends of capillary (Ghai, 1990).

2.5.5.3. Histopathology. Liver, heart and kidney of animals from control and treated groups were dissected into small sections and preserved in cedar wood oil. Infiltration was done by dipping the tissues in xylene:paraffin wax in 1:1 ratio for 1 h at 60 °C and then tissues were dipped in molten paraffin for 1 h at 60 °C. The processed tissues were embedded in the molten wax for section cutting. Thin section of the paraffin blocks containing tissue was done using rotary microtome. Then the slides were stained with eosin and hematoxylin and mounted with dextrene polystyrene xylene and examined microscopically for pathological examination.

2.6. Statistical analysis

All the results were expressed as mean ± SEM. The results were analyzed by one way analysis of variance (ANOVA) followed by Dunnett's test through the computer program Graph Pad Instat 3. P value $<0.01$and <0.05 were considered statistically significant.

3. Results

Table 1 shows the analgesic activity by tail flick and acetic acid-induced writhing methods using pentazocine and indomethacin as standard drugs, respectively. All the compounds showed significant analgesic activities in both methods. **PYZ2** has shown more prominent analgesic response at 400 mg/kg b.w. since it was found to be more effective at the end of 4 h similar to the standard drug indomethacin (10 mg/kg, p.o.). Anti-inflammatory activity was screened by carrageenan-induced acute inflammation model and adjuvant-induced arthritis model and results are presented in Table 2. The results of carrageenan induced model revealed that **PYZ2** was more active at 4 h (51.02% inhibition) of paw edema which is comparable to the standard indomethacin (54.08% inhibition). The results of adjuvant-induced arthritis model showed that **PYZ2** (47.41% inhibition), activity was comparable to standard drug aspirin (49.57% inhibition) after 30 days administration. The perusal of results of antipyretic study revealed that significantly all the compounds reversed hyperthermia similar to standard drug paracetamol (100 mg/kg, p.o.) as shown in Table 3. Amongst them, **PYZ2** was found to be more active (37.8 °C) which is similar to standard paracetamol (37.46 °C). Acute toxicity and gross behavior studies revealed that the test compounds in the present investigation

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Table 3 Effect of pyrazolone derivatives on yeast induced pyrexia in rabbits.

Treatment (mg/kg, p.o.)	Rectal temperature (°C)			
	Time (h)			
	0	1	2	3
Control	41.03 ± 0.16	41.08 ± 0.13	40.86 ± 0.21	40.86 ± 0.21
Standard paracetamol (100)	40.96 ± 0.26	40.76 ± 0.28	37.93 ± 0.24**	37.46 ± 0.15**
PYZ1 (400)	40.98 ± 0.12	40.46 ± 0.12	39.76 ± 0.21*	38.76 ± 0.16**
PYZ2 (400)	40.6 ± 0.38	40.48 ± 0.07	38.65 ± 0.37**	37.8 ± 0.16**
PYZ3 (400)	40.85 ± 0.28	40.51 ± 0.21	39.71 ± 0.29*	38.13 ± 0.24**

Values are expressed as mean ± SEM ($N = 6$).

* $P < 0.05$ compared with vehicle control (ANOVA followed by Dunnett's test).

** $P < 0.01$ compared with vehicle control (ANOVA followed by Dunnett's test).

Table 4 Hematological parameters of **PYZ2** treated rats.

	Male			Female		
	Control	500 mg/kg	1000 mg/kg	Control	500 mg/kg	1000 mg/kg
RBC[a]	8.6 ± 0.23	7.9 ± 0.57*	6.5 ± 0.56*	8.35 ± 0.34	7.27 ± 0.64*	6.21 ± 0.78*
Hb[b]	15.32 ± 0.98	14.83 ± 0.76*	10.34 ± 0.72*	13.9 ± 0.57	12.83 ± 0.78*	9.43 ± 0.38*
Hr[c]	46.56 ± 2.08	46.65 ± 1.34*	46.23 ± 2.08*	44.34 ± 2.56	43.23 ± 1.9*	43.43 ± 1.3*
WBC[d]	6.73 ± 2.01	5.28 ± 1.22*	4.65 ± 2.34*	5.15 ± 1.58	4.78 ± 2.48*	4.12 ± 2.08*
Lymp.[e]	70.41 ± 6.21	69.9 ± 9.16*	68.62 ± 9.78*	84.47 ± 1.65	81.67 ± 4.6*	80.54 ± 3.2*
Mono.[f]	3.83 ± 0.12	3.2 ± 0.22*	3.6 ± 0.7*	2.9 ± 0.87	2.7 ± 0.55*	2.04 ± 0.28*
Eosi.[g]	0.7 ± 0.03	0.57 ± 0.07*	0.46 ± 0.02*	0.6 ± 0.02	0.5 ± 0.03*	0.29 ± 0.04*
PLT.[h]	912.1 ± 98	925.2 ± 102*	968.9 ± 79*	911.6 ± 78.9	947.6 ± 47.9*	928.3 ± 50.7*
CIT.[i]	2.01 ± 0.5	2.35 ± 0.76*	2.15 ± 0.65*	2.77 ± 0.19	2.49 ± 0.45*	2.65 ± 0.97*

Data are expressed as mean ± SEM ($N = 5$).

[a] Red blood cell ($\times 10^6$ mm^{-3}).

[b] Hemoglobin concentration (g/dl).

[c] Hematocrit (%).

[d] White blood cell ($\times 10^3$ mm^{-3}).

[e] Lymphocyte (%).

[f] Monocyte (%).

[g] Eosinophilic leukocyte (%).

[h] Platlets ($\times 10^3$ mm^{-3}).

[i] Clotting time (min).

* All the P values are <0.05 as compared to respective controls by Dunnett's test.

were found to be non-toxic up to 5000 mg/kg b.w. Hematological changes have been observed such as depletion of hemoglobin; RBC and WBC count in dose dependent manner of **PYZ2** treated rats on either sex. In case of hematocrit, no significance difference was observed between control and treated male groups at 500 and 1000 mg/kg b.w. But this value was slightly reduced in female rats as compared to control at 500 and 1000 mg/kg b.w. (Table 4). Differential leukocyte count (DLC) determination showed eosinopenia at higher dose (1000 mg/kg b.w.). The level of lymphocyte and monocyte is slightly reduced as compared to control in dose dependent manner in **PYZ2** treated groups of either sex. The platelets count is increased in treated rats in dose dependent manner. The clotting time is altered in **PYZ2** treated groups of either sex and change is within the normal range. Biochemical parameters showed that decrease of total bilirubin and blood glucose in both 500 and 1000 mg/kg b.w. dose of PYZ2 treated groups as compared to control. The elevation of lysosomal enzymes such as ALP, AST and ALT level were observed in animals with treated **PYZ2** in dose dependent manner. No significant changes were observed in HDL, cholesterol and triglycerides level (Table 5). The histopathological studies revealed that some toxic changes have been observed in **PYZ2** treated animals at cellular level. The structural changes like cytoplasm vacuolation and degranulation of nucleus in hepatic cells were also observed. Dilated globules and atrophied glomeruli were seen in kidney. Interestingly no toxic effects were observed on myocardium in Fig. 2.

4. Discussion

The mechanism for testing analgesic was selected such that both centrally and peripherally mediated effects were investigated. The acetic acid induced abdominal constriction and tail immersion methods elucidated peripheral and central activity, respectively. This test is very useful not only for assessing analgesic drugs but also helping in the elucidation of mode of action. Acetic acid which is used as an inducer for writhing syndrome (Koster et al., 1959) causes an algesia by releasing of endogenous substances, which then excite the pain nerve ending. The abdominal constriction is related to the sensitiza-

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Table 5 Biochemical parameters of **PYZ2** treated rats.

	Male			Female		
	Control	500 mg/kg	1000 mg/kg	Control	500 mg/kg	1000 mg/kg
TB[a]	0.27 ± 0.005	0.24 ± 0.06*	0.26 ± 0.02*	0.22 ± 0.034	0.20 ± 0.07*	0.21 ± 0.02*
AST[b]	173.6 ± 29.5	178.2 ± 21.4	224.6 ± 19.6	167.7 ± 12.5	185.6 ± 19.5	191.47 ± 15.3
ALT[c]	48.2 ± 3.9	48.4 ± 3.5	65.3 ± 0.69	48.09 ± 0.92	52.2 ± 0.45	57.2 ± 0.89
HDL[d]	35.1 ± 4.28	36.2 ± 5.1	36.5 ± 4.9	36.89 ± 3.4	36.4 ± 2.4	36.8 ± 2.1
Chol.[e]	56.3 ± 3.2	55.7 ± 3.7	56.5 ± 4.6	55.8 ± 3.9	56.4 ± 5.9	54.7 ± 4.8
TG[f]	42.3 ± 14.3	42.5 ± 14.5	43.6 ± 11.8	45.3 ± 10.3	45.2 ± 9.4	44.48 ± 11.2
Glu.[g]	100.3 ± 0.87	86.7 ± 2.6*	77.07 ± 2.9*	119.2 ± 2.67	106.3 ± 1.7*	92.4 ± 2.65*
ALP[h]	39.2 ± 0.55	41.4 ± 0.76	43.6 ± 0.89	38.4 ± 0.46	39.2 ± 0.65	43.7 ± 0.83

Data are expressed as mean ± SEM ($N = 5$).
[a] Total bilirubin (mg/dl).
[b] Aspartate transaminase (U/l) S.G.O.T.
[c] Alanine transaminase (U/l) S.G.P.T.
[d] High density lipoprotein (mg/dl).
[e] Cholesterol (mg/dl).
[f] Triglycerides (mg/dl).
[g] Glucose (mg/dl).
[h] Alkaline phosphatase (U/l).
* $P < 0.05$ compared with vehicle control (ANOVA followed by Dunnett's test).

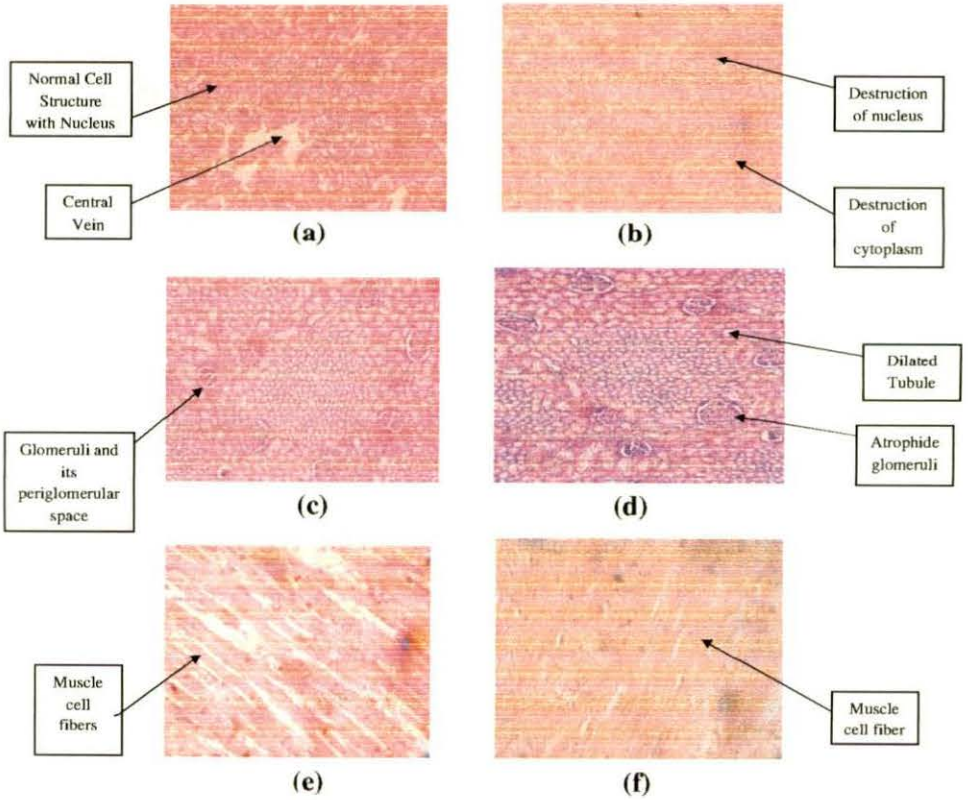

Figure 2 Histological changes of liver, kidney and heart from **PYZ2** treated rats. (a) Liver from control, (b) **PYZ2** liver from treated, (c) kidney from control, (d) **PYZ2** kidney from treated, (e) heart from control and (f) **PYZ2** heart from treated rats.

tion of nociceptive receptors to prostaglandins. Prostaglandins cause pain (Roberts and Morrow, 2001) and sensitize the skin to painful stimuli (Dray, 1995) probably because they sensitize pain receptors to mechanical and chemical stimulation (Roberts and Morrow, 2001) such as the pain eliciting effect of mediators (e.g. histamine, kinins, etc.) which are released in tissue injury and inflammation. The inhibition of the synthesis of pro-inflammatory prostaglandins is one of such thera-

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peutic targets to which some of the potent analgesic and anti-inflammatory agents of clinical relevance (e.g. NSAIDs) owe their activity (Flower and Vane, 1974). The pyrazolone derivatives administered orally (400 mg/kg); significantly inhibit acetic acid-induced writhing in rats. The result strongly suggests that the mechanism of action of pyrazolones may be linked to lipoxygenase and/or cyclooxygenase inhibition. It is possible that pyrazolone exert an analgesic effect probably by inhibiting prostaglandin synthesis by blocking cyclooxygenase enzyme.

The inflammatory response involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair (Vane and Bolting, 1995) which are aimed at host defense and usually activated in most disease conditions. These different reactions in the inflammatory response cascade are therapeutic targets which anti-inflammatory agents interfere with to suppress exacerbated inflammatory responses usually invoked in such disorders such as rheumatoid arthritis, infection or injury.

The non-steroidal anti-inflammatory drugs exert anti-inflammatory effect principally by inhibiting the synthesis of prostaglandin an eicosanoid mediator of the inflammatory response (Foegh and Ramwell, 2001). The most widely used primary test to screen new anti-inflammatory agent's ability to reduce local edema induced in the rat paw by injection of an irritant agent. Carrageenan-induced edema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The carrageenan test was selected because of its sensitivity in defecting orally active anti-inflammatory agents particularly in the acute phase of inflammation (Dirosa et al., 1971). The subplantar injection of carrageenan in rats leads to paw edema. The early phase (1–2 h) carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissues surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorph nuclear cells and prostaglandins produced by tissues macrophages (Brito and Antonio, 1998). The pyrazolone derivatives reduced the carrageenan-induced paw edema in rats. It may be due to inhibition of cyclooxygenase which activates prostaglandin synthesis followed by prevention of inflammatory mediator's release.

In Freund's adjuvant-induced polyarthritis model, treatment with pyrazolone derivatives showed significant inhibitory effect on injected hind paw edema and maximum inhibition was observed on the 30th day. In the present study, the increased lymphocyte count and migration of leucocytes into the inflamed area of arthritic rats were significantly prevented with the treatment of the pyrazolone derivatives and the standard drug as reflected from the significant decrease in total WBC count (Saraf et al., 1989).

Fever results due to generation of mediators such as IL-1β, IL-6, interferons and TNF-α cytokines increase the synthesis of prostaglandin which elevates the body temperature. From the results of antipyretics study, it can be suggested that pyrazolone derivatives produce the antipyretic action by inhibiting the prostaglandin synthesis by blocking cyclooxygenase isoenzymes, platelet thromboxane synthesis, and prostanoids synthesis (Graham and Scott, 2003; Bentur and Cohen, 2004).

Inflammatory process is characterized by the involvement of multiple inflammatory cells of the WBC (Kytridis and Manetas, 2006). WBC and indices relating to it such as lymphocytes usually show increase in activity in response to toxic environment (Robins, 1974). In this study, WBC was significantly altered. The lymphocytes, the main effector cells of the immune system (McKnight et al., 1999) showed marginal decrease thus suggesting that the pyrazolones only exerted minimal challenge on the immune system of the animals. Moreover, the decreased level of lymphocytes and monocytes clearly proving that pyrazolone derivatives are responsible for agranulocytosis. Our result is consistent with the result obtained from previous researchers (Bentur and Cohen, 2004; Uetrecht et al., 1995). Generally pyrazolones are associated with toxic effects such as thrombocytopenia (Flower, 1983; Yu, 1974) but in this study **PYZ2** elevated the level of platelets at 500 and 1000 mg/kg b.w. This proves that the structural modification of pyrazolones may reduce the mentioned toxic effect.

The hematocrit raises when the number of red blood cells increases or when the plasma volume is reduced, as in erythrocytosis. It falls to less than normal, indicating anemia (Hoffman et al., 2005; Mcpherson and Pincus, 2007). In both male and female rats, the hematocrit value is within the normal range (male 40–50% and female 36–44%) (Table 4). There is increasing evidence that lysosomal enzymes (ALT, AST and ALP) play an important role in the development of acute and chronic inflammation (Jannoff and Zweifach, 1964; Anderson et al., 1971).

Most of anti-inflammatory drugs exert their beneficial effect by inhibiting either release of lysosomal enzymes or by stabilizing lysosomal membrane which is one of the major events responsible for the inflammatory process. The stabilization of lysosomal membranes is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extracellular release (Chou, 1997). Some NSAIDs like indomethacin and acetylsalicylic acid are known to possess membrane stabilizing properties (Murugesh et al., 1981; Furst and Munster, 2001) which may contribute to the potency of their anti-inflammatory effect. In a toxic environment, blood level of AST and ALT are known to significantly increase (Adam, 1998; Crook, 2006). These two classical enzymes are reliable indices of liver toxicity. From the subacute toxicity it can be assumed that the increased level of alkaline phosphatase, AST and ALT level may be responsible for the tissues damages in the liver and kidney. This was confirmed by the histological study in which tissue morphology showed mild changes on liver and kidney.

The histopathological studies revealed that **PYZ2** has mild toxicity on liver and kidney with the exception on heart. This supports that pyrazolones have no toxic effect on heart on 500 and 1000 mg/kg b.w. The present experimental findings of both pharmacological and toxicological parameters suggest that pyrazolone derivatives are the promising non-steroidal anti-inflammatory agents. Hence it can be concluded that it is worthwhile to modify the structure to obtain more potent and less toxic compound.

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EVALUATION OF ANTIOXIDANT POTENTIAL OF PYRAZOLONE DERIVATIVES

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ABSTRACT

In this article the antioxidant property of pyrazolones derivatives (PYZ1 to PYZ10) are reported. It was assessed by estimation of Malonaldehyde (MDA) and 4-Hydroxyl-2-noneal (4-HNE) as lipid peroxidation markers in myocardial ischemic reperfusion injury. The inhibition of lipid peroxidation was compared with the standard ascorbic acid. Among synthesized derivatives PYZ2, PYZ3, PYZ7, PYZ8, PYZ9, and PYZ10 were found to have potent antioxidant effect against MDA marker. In case of 4-HNE, PYZ4, PYZ5, PYZ6, PYZ7, PYZ8, PYZ9 and PYZ10 were found to have effective antioxidant activity and the rest of the compounds are moderately active. Comparatively PYZ7, PYZ8, PYZ9 and PYZ10 are having effective role to control both MDA and 4-HNE generation. All the experimental data were statistically significant at $p<0.05$ level. Interestingly, beyond its NSAID property, this study explores the protective role of pyrazolone derivatives in ischemic heart injury.

Key words: Lipid peroxidation, antioxidants, Malonaldehyde, 4-Hydroxyl-2-nonenal, pyrazolone

INTRODUCTION

Pyrazolone derivatives have been used in patients with acute brain infarction since April 2001 in Japan [1]. These derivatives have been shown to be effective against brain edema after ischemia and reperfusion injury in animal models [2] and in stroke patients [3]. Moreover, it has been shown that these molecules have preventive effects on myocardial injury following ischemia and reperfusion in the rat heart [4] and in patients with acute myocardial infarction [5]. Several lines of evidence have demonstrated that oxidative stress plays an important role in the pathogenesis and development of cardiovascular diseases, including hypertension, hypercholesterolemia, diabetes mellitus, atherosclerosis, and myocardial infarction, angina pectoris, and heart failure [6]. Lipid peroxidation is found to cause formation of

atherosclerotic plaques, neurological disorders, cancer, diabetes mellitus, myocardial infarction [7] and ageing. Lipid peroxidation is associated with ischaemia-reperfusion injury and hyperoxic lung injury. The peroxides derived from lipid peroxidation such as MDA (TBARS) and 4-HNE have been strongly associated with myocardial ischemic reperfusion injury [8, 9]. It is expected that pyrazolone derivatives have beneficial effects on coronary artery and myocardial cells after ischemic and post ischemic myocardial injury in patients with ischemic heart diseases, including acute myocardial infarction and angina pectoris. Some animal studies using acute myocardial ischemia-reperfusion models have suggested the protective effects of pyrazolone derivatives on myocardial damage. By taking this research finding as evidence, the present study is focused to explore the antioxidant potential of pyrazolone derivatives.

MATERIALS AND METHODS

Pyrazolone derivatives were synthesized in our laboratory by reported procedure [10], and used as test drugs in the experiments at 100 mg/kg body weight. The physical data of the synthesized compounds have been given in Table.1.Thiobarbituric acid (TBA) was obtained from Loba Chemie, India. 2, 4-dinitrophenyl hydrazine (DNPH) and 1, 1, 3, 3-tetramethoxy propane (TMP) were obtained from Sigma Chemicals, USA. Ferrous sulphate, trichloroacetic acid (TCA), hydrogen peroxide, ascorbic acid, potassium dihydrogen phosphate, potassium hydroxide, hexane, methanol and HCl were of analytical grade and obtained from Ranbaxy Fine Chemicals. 4-HNE (4- Hydroxy- 2- Noneal) was obtained from Ranbaxy Ltd. as a gift sample. All other chemicals and reagents used were of analytical grade.

Experimental animals

Toxicological studies of the pyrazolone derivatives (as suspension in 0.5% w/v carboxy methylcellulose) were carried out by standard method in oral dose of 100 to 1500 mg/kg body weight in albino mice. The LD_{50} of the test compounds was found at 1000 mg/kg.b.w. The one tenth of the LD_{50} (100mg/kg) was considered to be the dose of test compounds. Male albino rats, weighing between 150-200gm were included in the study. Rats were housed in the departmental animal house at an ambient temperature of 25°C, under a 12 h dark -12 h light, cycle, for the whole period of the study. Experiments were carried out according to the guidelines of the animal ethics committee of the institute. Animals were fasted overnight and were divided in to twelve group's i.e. control, standard and different test groups each consisting of three animals. Rats in the control group received the vehicle solution without

drugs, rats in the standard group received the standard ascorbic acid (100mg/kg p.o) and the pyrazolone derivatives were administered orally to the test group of rats.

***In vitro* myocardial ischemic-reperfusion injury [11]**

After 48 h the rats were anaesthetized with ether, the chest opened and the heart along with one cm of ascending aorta attached was quickly removed and dipped in ice-cold saline. The hearts were then mounted on Langendorff's apparatus and perfused with Krebs's Hensleit (K-H) buffer at a constant pressure of 60-70mm Hg at 37°C, and aerated with a mixture of O_2 (95%) and CO_2 (5%). Following an initial period of 5 min of stabilization, the flow was stopped for 9 min (ischemia) followed by perfusion with K-H buffer for 12 minutes (reperfusion). Then the heart was removed from the apparatus and subjected for the biochemical estimation.

***In vitro* antioxidant activity of pyrazolone derivatives**

Estimation of TBARS [12]

TBARS activity in the myocardium was determined by a modified version of the method described by Okhawa et.al. 1979. Hearts were homogenized in 10% trichloroacetic acid at 4°C. 0.2 ml homogenate was pippeted in to a test tube followed by the addition of 0.2 ml of 8.1% sodiumdodecyl sulphate (SDS), 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA. All tubes were boiled for 60min at 90°C and then cooled on ice. 1.0 ml double distilled water and 5ml of n-Butanol: pyridine (15:1v/v) mixture was added to the tubes and centrifuged at 4000 rpm for 10 minute. The absorbance of developed colour in organic layer was measured at 532nm. TBARS activity was determined, from the standard curve of TBA adduct formation when various concentration of commercially available 1, 1, 3, 3-tetramethoxypropane was subjected to the above procedure (Fig. 1 and 2). The concentration of MDA was expressed in nM.

Estimation of 4-HNE [13]

The heart homogenate was prepared as the procedure mentioned above. 2ml of filtrate was taken in a 13x100mm glass tube with cap. 1ml of DNPH was added to all the tubes containing heart homogenate, mixed thoroughly and set aside for 1h to react with 4-HNE. Then the formed adduct of 4-HNE and DNPH was extracted by hexane, which was evaporated under argon at 40°C. After cooling, 2 ml of methanol was added to all the samples and the absorbance was measured at 350 nm in the spectrophotometer. The quantity of 4-HNE was calculated by linear regression analysis. The conc. of 4-HNE present in myocardial tissues was expressed in nM (Fig. 3 and 4).

Table 1: Physical data of the synthesized compounds

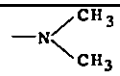
Compound code	-R	Molecular formula	Mol. weight
PYZ1		$C_{13}H_{15}N_3O$	239
PYZ2	Phenyl ethenyl	$C_{13}H_{12}N_2O$	212
PYZ3	$-OCH_3$(2,3)	$C_{13}H_{14}N_2O_3$	246
PYZ4	$-NO_2$	$C_{11}H_9N_3O_3$	231
PYZ5	Cl	$C_{11}H_9N_2OCl$	221
PYZ6	H	$C_{11}H_{10}N_2O$	186
PYZ7	$-CH_3$	$C_6H_8N_2O$	128
PYZ8	4-OCH_3	$C_{12}H_{12}N_2O_2$	216
PYZ9	4-OCH_3	$C_{12}H_{12}N_2O_2$	216
PYZ10	OH	$C_{11}H_{10}N_2O_2$	202

Table 2: *In vitro* antioxidant activity of pyrazolone derivatives by MDA and 4-HNE model

Compounds code	Dose (100mg/kg)	MDA (nM) ($\bar{X}$ ± S.E)	4-HNE(nM) ($\bar{X}$ ± S.E)
Control	-	18.87±0.18	97.59±0.11
PYZ1	100	16.27±0.21	95.21±0.18*
PYZ2	100	15.27±0.19	94.21±0.21
PYZ3	100	15.38±0.18	90.56±0.19
PYZ4	100	16.28±0.20	79.18±0.10
PYZ5	100	17.28±0.22	80.28±0.21*
PYZ6	100	17.01±0.21	80.18±0.11
PYZ7	100	15.01±0.19	79.23±0.20
PYZ8	100	14.29±0.22*	77.19±0.14
PYZ9	100	14.78±0.18*	76.28±0.16*
PYZ10	100	14.23±0.21*	76.23±0.23

$\bar{X}$ = Mean concentration S.E. = Standard Error

* The values are significant at $P < 0.05$

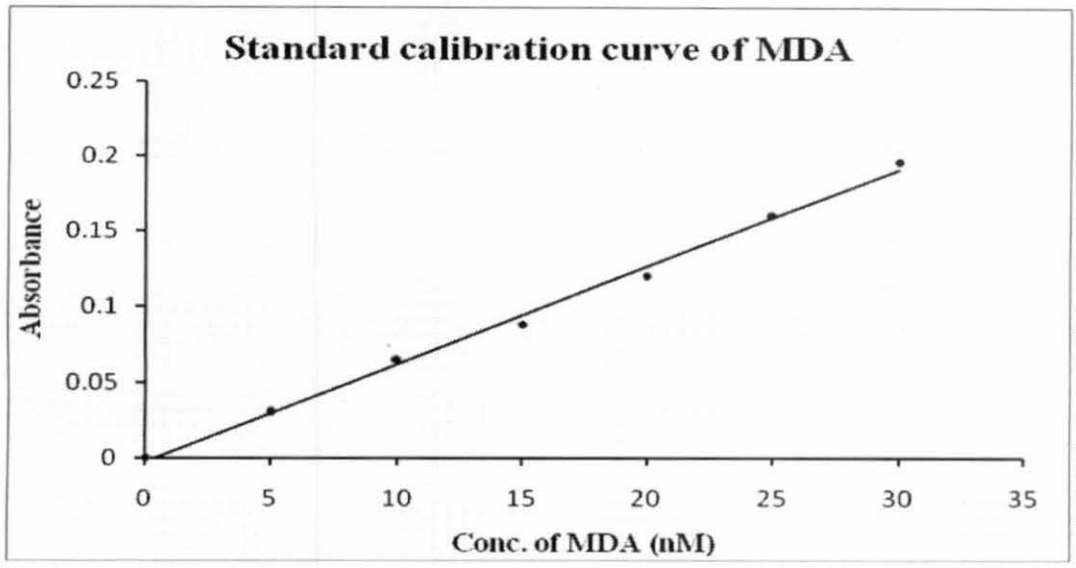


Fig. 1: Standard calibration curve of MDA estimation

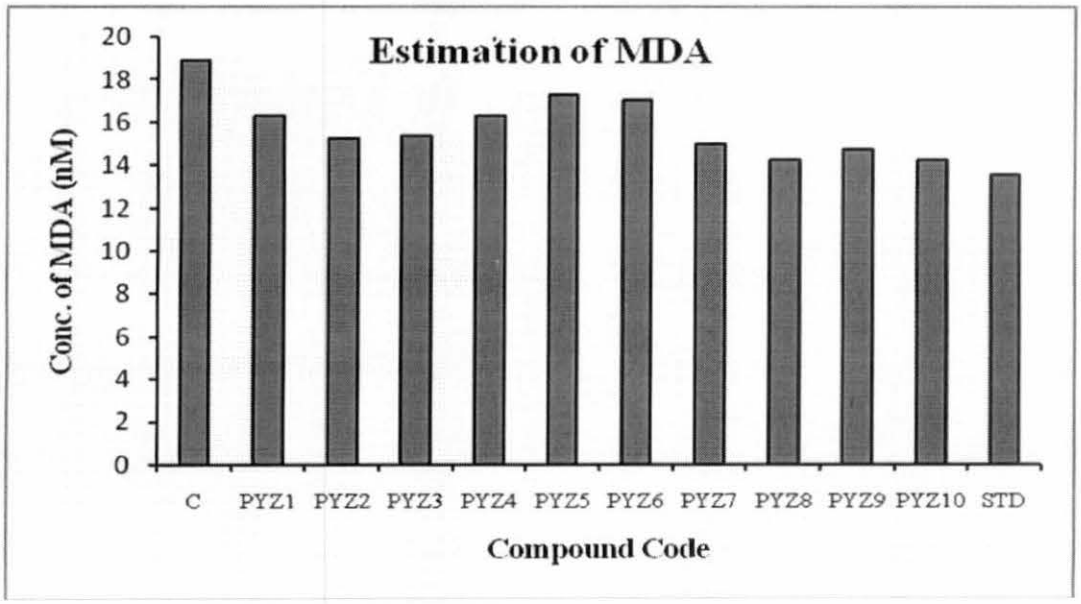


Fig. 2: Antioxidant activity of Pyrazolone derivatives on MDA suppression model C-Control, Std-Ascorbic acid

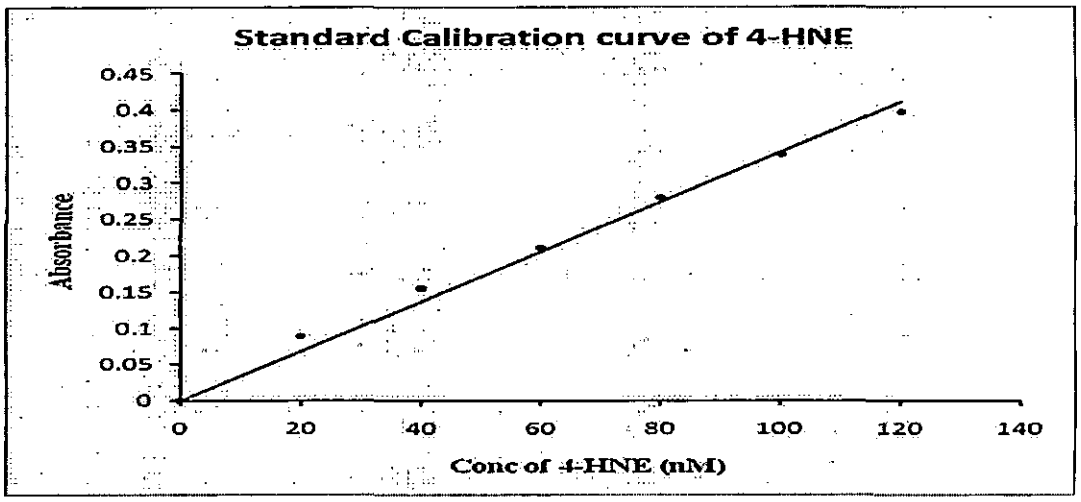


Fig. 3: Standard calibration curve of 4-HNE estimation

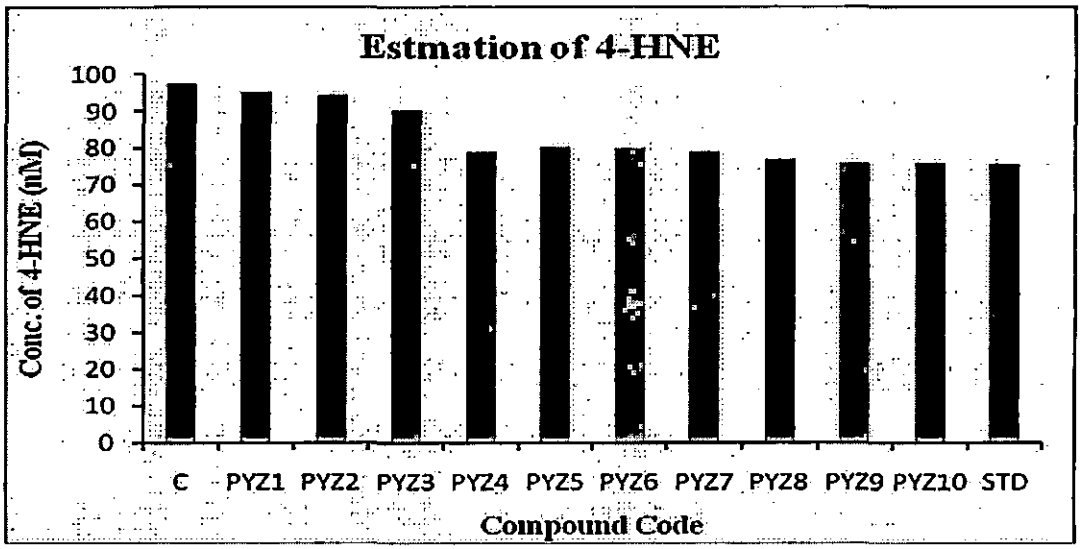


Fig.4: Antioxidant activity of Pyrazolone derivatives on 4-HNE suppression model C-Control, Std-Ascorbic acid

RESULTS AND DISCUSSION

The experimental study was based on the estimation of MDA and 4-HNE and their suppression by the pyrazolone derivatives are presented in Table-2. From the experimental results, it has been proved that the pyrazolone derivatives have significant antioxidant activity. The quantification of MDA and 4-HNE can be directly correlated with the lipid peroxidation inhibition capacity of the pyrazolone derivatives. The toxic radicals' quantification is also an indicator to monitor the overall

progress of lipid peroxidation which is associated with myocardial ischemic reperfusion injury. The antioxidant activity of pyrazolone derivative was compared with standard antioxidant (ascorbic acid). The results were analyzed statistically and found significant at $P<0.05$ level.

ACKNOWLEDGEMENT

The authors are thankful to the Director Dr. H. P. Chhetri, Himalayan Pharmacy Institute, Majhitar, East Sikkim who provided the facilities to carry out the research work. The authors are also grateful to Dr. L. Sutharson H.O.D. of Pharmacology, HPI, Sikkim for his valuable suggestions.

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ANTIPYRETIC ACTIVITY OF SOME PYRAZOLONE DERIVATIVES

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Pyrazolone ring systems represent an important class of compounds not only for their theoretical interest but also for their anti-inflammatory, analgesic, antipyretic, hypoglycemic agent, fungicide, antimicrobial and some of them have been tested as potential cardiovascular drugs including hypertension, hypercholesterolemia, atherosclerosis, myocardial infarction, angina pectoris, and heart failure. Moreover, it has been proved that these molecules have preventive effects on myocardial injury following ischemia and reperfusion in the rat heart and in patients with acute myocardial infarction. Hence much attention is being paid for the synthesis of pyrazolone derivatives. In continuation of our work, novel pyrazolone derivatives were synthesized and screened for their antipyretic activity. Here 3-Methyl-4-substituted benzylidene-pyrazol-5-ones **(PYZ1-10)** were synthesized by the condensation of 3-methyl-pyrazol-5-one with substituted aliphatic and aromatic aldehydes. Their structures have been elucidated by UV, IR, ^{1}H NMR and Mass spectral data. The antipyretic activity of the synthesized compounds was evaluated using Brewer's yeast induced pyrexia in rabbit model. In this technique the animals were administered with standard paracetamol (100 mg/kg) and pyrazolone derivatives (400 mg/kg) orally. The rectal temperature was measured at 0, 1, 2 and 3 hour after treatment. The experimental data were statistically significant at $p<0.05$ and $p<0.01$ level.

Addendum/Corrigendum

1. **The PhD thesis is not certified and signed by guide at the time of the submission.**

   Ans: As per the North Bengal University guidelines for submitting thesis, the certificate from supervisor was not attached with thesis but submitted to Registrar's office as a part of record. (Guideline for submitting PhD thesis issued by NBU Registrar: item no.6.

   The scholars are requested not to attach copy of the certificate issued by the supervisor and co supervisor if any, in the thesis as the same shall be retained in the office of the Registrar as part of record.)

2. **In Introduction Section, there are a number of references which are cited in text but their details are not mentioned in section 1.7 (page 31) eg. Yello et al. 2007, Jennings 1960, Ishii et al 2005, Holcomb 1963, Pavithron et al 2007 are some of the example.**

   Ans: References which are cited in text but their details are not mentioned in section 1.7 (page 31) are given as follows.

   There is some typographical error in yello et al 2007, the correct reference is

   a) Yellon DM, Hausenloy DJ, Myocardial reperfusion injury, *New Engl J Med. 2007;* 357:1121-1135.

   b) Jennings RB, Summers HM, Smyth GA, Flack HA, Linn H. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog, Arch Pathos. 1960; 70:68-78.

   c) Ishii H, Icsimiya S, Kanashiro M, et al. Impact of a single intravenous administration of nicorandil before reperfusion in patients with ST-segment-elevation myocardial infarction, *Circulation.* 2005; 112:1284-1288.

d) P. Pavithran, H Nandeesha, Madan Mohan, Za Charich Bobby, V Sathiapriya, Padnabha Shenoy, Shirdas Sunil and P. Shyma. Dyslipedemia antedates occurrence of clinical hypertension in non- diabetic and non obese male subjects. *Ind. J. Physiology Pharmacol.* 2007; 51: 96-98.

e) Holcomb, G.N. *J.Pharm.Sciences,* 1963; 55: 125.

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g) Zang XF, Tan BK. Anti hyperglycemic and anti oxidant properties of *andrographis paniculata* in normal and diabetic rats.*Clin.Exp.Pharmacol.Physiol.*2000; 27: 358-363.

3. **In page 4, reference of Kloner et al states for 1993 but in reference cited in page 33 reveals it for 1983.**

   Ans: It is a typographical error and should be read as 1983

4. **In the review of Literature, there are some typographical errors.**

   Ans: In page 60 and 61 edaravone is the correct name

5. **The reason for carrying out studies of synthesized derivatives with respect to enzymes and cardiovascular parameters at 10mg/kg (Refer table 5.2 to 5.4) is not clear. Suddenly why antioxidant, anti inflammatory, antipyretic and analgesic studies was carried at 100mg/kg (Table 5.5 to 5.8)? Scientific paper published in Saudi Pharma Journal reveals that three compounds and all activities were studied at 400mg/kg. This anomaly of dose is not clear.**

Ans: A dose of 10 mg/kg b.w. was chosen for cardiovascular studies based on the report of Tsujimoto *et al,* 2005. Though 10 mg/kg b.w. dose is effective in CVS but this dose does not show any significant effect in analgesic, antipyretic activity. Hence 100 mg/kg b.w. was chosen for these activities.

Ref 1: Tsujimoto I, Hikoso S, Yamaguchi O, *et al.* The antioxidant edaravone attenuates pressure overload-induced left ventricular hypertrophy. Hypertension. 2005; 45, 921-6.

Indeed, the analgesic, anti inflammatory and antipyretic activity was not directly related to cardiovascular studies. But some of the research reports like Entman *et al* 1991; Chien GL *et al 1994* have shown the evidence that myocardial injury is associated with inflammatory response and hypothermia. Based on these references the synthesized compounds were screened for analgesic, anti-inflammatory and antipyretic activity.

Ref 2: Entman, ML, Michael LH, Rossen RD, Dreyer WJ, Anderson DC, Taylor A.A, Smith, CW. Inflammation in the course of early myocardial ischemia. *FASEB J.* 1991; 5: 2529–2537.

Ref 3: Chien GL, Wolf RA, Davis RF, Van Winkle DM. "Normothermic range" temperature affects myocardial infarct size.*Cardiovasc Res*1994; 28:1014 -1017.

The peroxides derived from lipid peroxidation such as MDA (TBARS) and 4-HNE have been strongly associated with myocardial ischemic reperfusion injury (Blasig *et al.*, 1995; Ski et al., 2008). Based on this fact, the compounds have been screened for antioxidant activity.

In chronic toxicity study high dose 400 mg/kg was selected since these compounds were safe up to 5000 mg/kg during acute toxicity study. So the dose 400mg/kg was selected for

chronic toxicity study. Moreover, as far as the time is concerned, the chronic toxicity study was limited to three compounds only and the complete study would take several months.

The instrument name to be cited in page 104 where the heart was stored in liquid nitrogen.

Ans: As per the protocol the heart has to be stored in liquid nitrogen but alternatively dry ice was used in my present experiment.

. The % inhibition of rat paw edema (Table 5.6, page 174) needs to be checked as there are large numbers of calculation error in % inhibition of edema in rats.

Ans: The calculation error in % inhibition of edema in rats was detected and submitted herewith after correction. (Table 5.6, page no 174)

. Why was Freund's adjuvant induced polyarthritic model not shown in the thesis though published paper reveals some data?

Ans: Freund's adjuvant induced polyarthritic model was not shown in the thesis because it was done for only three compounds where as Carrageenan induced rat paw edema was done for 10 compounds. Hence it was not included in the thesis.

. The induction of fever by subcutaneous administration of 10% yeast in rabbits is questionable. The rectal temperature should have been measured 4 hrs also after treatment. The reference cited Lu et al (2004) (page 109) is not there in the list.

Ans: As per the procedure given in reference Lu et al 2004, the antipyretic study was done even though the LPS is the suitable pyrogen to induce fever in rabbit.

Lu, W.L., Zhang, Q., Zheng, L., Wang, H., Li, R.Y., Zhang, L.F., 2004. Antipyretic, analgesic and anti inflammatory activities of ketoprofen beta-cyclodextrin inclusion complexes in animals. *Biol. Pharm. Bull.* 27 (10), 1516–1520.

10. In discussion portion (page 187), it is mentioned that all data (paragraph 1 of page 187) are significant at $p<0.05$. But result section (table-5.5, page 171) does not match with the information stated.

Ans: The experimental data were significant at $p<0.05$ for compounds PYZ 8, PYZ 9, PYZ 10 in MDA estimation. But for 4-HNE estimation, the results are significant at $p<0.05$ for compounds PYZ 1, PYZ 5, PYZ 9.

11. The structure activity relationship should be more lucid and a conclusion needs to be made regarding which of ten compounds has maximum activity with least toxicity.

Ans: In addition to SAR mentioned in page 182-183, the following may further be added.

a) The electron donor increases activity as compared to electron withdrawing substituent. This reveals that electron donor (OCH_3) may contribute to increase lipophilicity, which may play very vital role to enhance the biological response.
b) Even though alkoxy (OCH_3), hydroxyl groups are electron donating group, amongst them –OH group substituted pyrazolone is more active than the alkoxy group substituent.
c) The –OH group can facilitate the molecule to undergo keto enol tautomerism without any hindrance.
d) Amongst PYZ 8, 9 & 10 the compound PYZ 10 is more active. It may be due to the presence of hydroxyl group.
e) The increased activity in pyrazolone (PYZ 10) may be due to the capacity to impart keto- enol tautomerism in the molecules.

Based on these SAR, it can be concluded that PYZ 10 is more active amongst the synthesized congeneric series.

: 5.6. Anti inflammatory activity of pyrazolone compounds on carrageenan-induced :dema in rat

›ds /p o	Paw volume in ml, Mean ± SEM (% inhibition of paw edema)			
	1h	2 h	3 h	4 h
rol	0.97 ± 0.016	0.93 ± 0.017	0.91 ± 0.013	0.89 ± 0.013
rin	0.61±0.008(37.11)**	0.53±0.011(43.01)**	0.41±0.008(54.94)**	0.3±0.006(66.29) **
Z1	0.84±0.007(13.40) *	0.65± 0.007 (30.10) *	0.55±0.004 (39.56)*	0.42±0.003(52.80) *
Z2	0.83±0.005(14.43) *	0.67±0.008(27.95)*	0.63±0.005 (30.76)*	0.43±0.003 (51.68) *
Z3	0.88±0.009 (9.27) *	0.68±0.009(26.88)*	0.50 ±0.023(45.05)*	0.411±0.004(53.82)*
Z4	0.88±0.009 (9.27) *	0.68±0.008(26.88)*	0.51 ±0.007(43.95)*	0.40±0.004 (55.05)*
Z5	0.65±0.009(32.98)**	0.55±0.005(40.86)**	0.4 ±0.006(56.04)**	0.30±0.003(66.29)**
Z6	0.69±0.007(28.86)**	0.56±0.006(39.78)**	0.46±0.007(49.45)**	0.31±0.004(65.16)**
Z7	0.65±0.006(32.98)**	0.56±0.008(39.78)**	0.46±0.003(49.45)**	0.38 ± 0.003(57.30)**
Z8	0.73 ± 0.010 (24.74) *	0.61±0.011 (34.40) *	0.51 ± 0.004(43.95)*	0.46±0.005 (48.31) *
Z9	0.75±0.006 (22.68) *	0.66±0.010 (29.03) *	0.53 ± 0.005(41.75)*	0.5 ± 0.006 (43.82)*
Z10	0.63±0.010(35.05)**	0.56±0.015(39.78)**	0.43±0.006(52.74)**	0.36±0.004(59.55)**

:0.01vs Control, * p<0.05vs Control (n=6)

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