3.0 CHAPTER -II

SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF BIS(INDOLYL)METHANES

3.1 INTRODUCTION

The chemistry of indole began in the midst of the 19th century with extensive research on the natural dye “indigo” (Sundberg, 1970; Houlihan, 1972). During the 1930s it was discovered that a number of important natural products contain indole moieties. The potent physiological properties of various traditional medicines containing indole based alkaloids have been utilized in, added stimulus to research, and many important indole syntheses were developed. During this period, the essential amino acid tryptophans (Rose, 1938) as well as the plant growth hormone indole-3-acetic acid were discovered (Kogl et al., 1933). Recently the chemistry of indoles was reviewed (Gribble, 2000).

Indoles have been widely identified as a privileged structure or pharmacophore, with representation in over 3000 natural isolates (Karthik et al., 2005) and are known to possess broad spectrum of biological and pharmaceutical activities (Fukuyama and Chen, 1994; Sundberg, 1970). Indomethacin (Asok kumar et al., 2004) and tenidap (Moore et al., 1996) are indole derivatives, which were found to possess anti-inflammatory activity along with analgesic and antipyretic properties. They inhibit production of eicosanoids like prostacyclin, thromboxanes and prostaglandins by inhibition of cyclooxygenase (cox) and thereby reduce edema.

In particular, bis(indolyl)methanes (BIMs) have received much attention in recent years (Garbe et al., 2000). Such compounds are prone to develop interesting bio-
activity and find useful applications as breast cancer preventative (Michnovicz and Bradlow, 1989) and anti-bacterial agents (Osawa and Namiki, 1983).

### 3.1.1 Bis(indolyl)methanes

#### 3.1.1.1 Biological Importance

Among various indole derivatives, diindolylmethane and 2-(indol-3-yl-methyl)-3,3’-diindolylmethane display diverse pharmacological activities and are useful in the treatment of fibromyalgia, chronic fatigue and irritable bowel syndrome (Bradfield and Bjeldanes, 1987).

A number of bis(indolyl)methanes have been reported to be isolated from terrestrial and marine natural sources, viz. parasitic bacteria, tunicates, sponges and some of these possess significant biological activities. The first report of the occurrence of diindolylalkane in nature was published when three indolic metabolites were isolated from the fungus, *Balarisia epichloe*, a parasite to pasture grasses which were known to elicit ergot-type syndrome in cattle grazing on these infected grasses (Osawa and Namiki, 1983; Fahy *et al.*, 1991; Bell, 1994; Bifulco *et al.*, 1995; Garbe *et al.*, 2000).

The bis(indolyl)methanes has been gaining increasing importance in recent years because of its potent anti-carcinogenic properties. BIM is the most active cruciferous substance for promoting beneficial estrogen metabolism in women and men (Zeligs, 1998). Hong *et al.* (2002) and Kedmi *et al.* (2003) reported the potential beneficial effects of 3, 3-diindolyl methane on the proliferation and induction of apoptosis in human prostate and breast cancer cells. The first reported naturally occurring, a fungal metabolite, was found to be toxic to fertile leghorn eggs (Porter *et al.*, 1977).
Kassouf et al. (2006) have identified 1,1-bis(3’-indolyl)-1-(p-substitutedphenyl)methanes containing p-trifluoromethyl (BIM-C-pPhCF$_3$), p-t-butyl (BIM-C-pPhtBu), and phenyl (BIM-C-pPhC$_6$H$_5$) substituents as a new class of peroxisome proliferator-activated receptor gamma (PPAR gamma) agonists that exhibit anti-tumorigenic activity.

Chen et al. (1998) reported that the BIM bound to the AhR had induced the formation of the nuclear AhR complex in MCF-7 human breast cancer cells and also inhibited E2 induced breast cancer cell proliferation and growth of 7, 12- dimethyl benz[a] anthracene (DMBA) – induced mammary tumours in female Sprague-Dawley rats at doses of 5 mg/kg every 2 days.

McDougal et al. (2000) reported the inhibition of carcinogen induced rat mammary tumour growth and other estrogen dependent responses by symmetrical dihalo substituted analogs of diindolyl methane. Dihalo BIMS (100 mg/kg / day x 3) were not estrogenic in the immature female B6C3 F1 mouse uterus; however in animals co-treated with 17 $\beta$ -estradiol (0.02 $\mu$g/mouse), some of the compounds inhibited uterine progesterone receptor binding and uterine peroxidase activity. The antitumorigenic activities of the dihalo BIMS were determined by their inhibition of carcinogen induced mammary tumour growth in female Sprague-Dawley rats. A group of dihalo BIMS exhibited anti-tumorigenic activity at doses as low as 1.0 mg/kg for 2 days and there were no significant changes in organ weights or in histopathology.

Kedmi et al. (2003) used the prostate cancer cell lines LNCaP, DU145, PC3 to examine the effects of diet derived indole derivatives, I3C and BIM on cell proliferation in vitro. The prostate cancer cell lines were treated with I3C (10-100 $\mu$M ) or BIM (10-100 $\mu$M ) for 48 h. I3C and BIM had an inhibitory effect on the growth of all cell lines.
They also demonstrated that the indole derivatives found in crucifers, indole-3-carbinol (200 μM) and 3, 3’- diindolyl methane (75 μM) induce apoptosis in three human prostate cancer cells, LNCaP, DU 145 and PC3. Induction of apoptosis by BIM is through a P53, bax, bcl-2 and fasL independent pathway.

Vanderlaag *et al.* (2006) reported the inhibition of breast cancer cell growth and induction of cell death by 1, 1-bis (3’-indolyl) methane and 5, 5’-dibromo BIM. Preliminary cell proliferation studies in MCF-7 and MDA-MB-231 cells using bromo, methyl and chloro-substituents at 4, 5, 6, 7 positions indicated that 5, 5’- dibromo BIM was the most active ring- substituted BIM. The IC₅₀ values for growth inhibition by 5, 5’- dibromo BIM were < 5 μM in both cell lines, whereas the corresponding IC₅₀’s for BIM were 10-20 μM. BIM and 5, 5’- dibromo BIM induce cell death by multiple pathways including activation of proteosome dependent degradation of cyclin D1, induction of cell necrosis, apoptosis and decreased MMP and these effects were essentially Ah receptor independent. For Most responses, 5, 5’-dibromo BIM was significantly more potent than BIM.

Hong *et al.* (2002) reported Bcl-2 family mediated apoptotic effects of 3, 3’- diindolyl methane in both estrogen receptor replete (MCF-7) and deficient (MDA-MB-231) human breast cancer cells at concentrations above 10 μM. Sudhakar *et al.* (2006) reported 1, 1’-bis (3’-indolyl) -1-(p-substituted phenyl) methanes containing p-trifluoromethyl, t-butyl and phenyl [1, 1-bis (3’-indolyl) -1- (p-phenyl) methane substituents (2.5 to 7.5 μM/l) induce peroxisome proliferator – activated receptor γ (PPAR γ) mediated transactivation in SW 480 colon cancer cells. In higher concentrations (10 μM/l), these compounds induce apoptosis which is PPAR γ
in independent. In athymic nude mice xenograft model, BIM-C-p-phenyl C₆ H₅ inhibits

tumor growth at doses of 20 and 40 mg/kg/day for 20 days. There were no significant
differences in animal weight, organ weights or histopathology in any of the treatment
groups.

3.1.1.2 Synthetic Approaches

Zhang et al. (2005) have reported the zirconium tetrachloride catalyzed synthesis

\[ 2 \text{Indole} + \text{Benzaldehyde} + 2\text{ZrCl}_4 \rightarrow \text{Bis(indolyl)methane} \]

of bis(indolyl)methanes.

Yadav et al. (2001) have reported the lithium perchlorate catalyzed synthesis of

\[ 2 \text{Indole} + \text{Benzaldehyde} + 2\text{LiClO}_4 \rightarrow \text{Bis(indolyl)methane} \]

bis(indolyl)methanes in diethyl ether.

Li et al. (2006) have reported the aminosulfonic acid catalyzed synthesis of

bis(indolyl)methanes under ultrasound irradiation.
Karthik et al. (2005) have reported the zeolite catalyzed synthesis of bis(indolyl)methanes.

Ji et al. (2004b) have reported the iodine catalyzed synthesis of bis(indolyl)methane by grinding a mixture of indole and aldehyde in a mortar with a pestle at room temperature for several minutes.

Ji et al. (2003) have reported the synthesis of bis(indolyl)methanes catalyzed by ferric chloride hexahydrate by using ionic liquid as reaction medium.
Ji et al. (2004a) have reported the ionic liquid mediated synthesis of bis(indolyl)methanes catalyzed by indium triflate.

Recently, Nair et al. (2005) have reported the gold (III) chloride promoted synthesis of triaryl- and triheteroaryl- methanes by reaction of aldehydes and activated arenes.
Protic acids, Lewis acids, clays, Iodine, and ionic liquids are known to promote these reactions. However, many Lewis acids are deactivated or some times decomposed by nitrogen containing reactants. Even when the desired reactions proceed, more than stoichiometric amounts of Lewis acids are required because the acids are trapped by nitrogen. Many of the procedures involve expensive or less easily available reagents, long reaction time, and low yield of the products, strongly acidic conditions and cumbersome experimental and product isolation procedures. Therefore, there is a need for a greener catalytically efficient method for the synthesis of BIMs which would work under mild and economically cheaper conditions.

Almost two decades ago, steroids namely prednisolone, dexamethasone, betamethasone, etc. were considered to be the choicest anti-inflammatory drugs. Owing to the several adverse effects caused by either short-term or long-term steroid therapy, these have been more or less replaced by much safer and better-tolerated non-steroidal anti-inflammatory drugs (NSAID). The seriousness and enormous after effects of steroid therapy necessitated an accelerated research towards the development of NSAIDs since the past two decades. NSAIDs have been highly useful for treating acute, self-limited inflammatory conditions (Ashutoshkar, 2003; Geetakant et al., 2005). The development
of NSAIDs has helped in understanding the tissue mechanism of inflammation.

It is generally agreed that the NSAIDs and analgesic drugs available in the market (aspirin, phenylbutazone, oxyphenbutazone, indomethacin, tenidap, ibuprofen and ketoprofen) are highly acidic in nature and suffer from a common drawback of gastrointestinal toxicity (Geetakant et al., 2005). The search for new therapeutic agents with high margin of safety and freedom from normally associated Gastro Intestinal toxic effects such as ulceration, hemorrhage and perforation has been a priority of pharmacologists and pharmaceutical industries (Mattisan et al., 1998).

In the light of these observations, a novel approach is described in this study using commercially available and low cost catalyst to synthesize BIMs with the hope to develop safer and potent analgesic and anti-inflammatory agents without ulcerogenic activity.
3.2 OBJECTIVES

Indole derivatives are widely distributed in nature and are known to possess broad spectrum of biological and pharmaceutical activities. In particular, bis(indolyl)methanes have received much attention in recent years. Such compounds are prone to develop interesting bio-activity and constitute an active area of investigation in pharmaceutical and organic synthesis. The aim of the present investigation is

- To develop a simple, efficient and eco-friendly procedure for the synthesis of bis(indolyl)methanes
- To characterize the synthesized bis(indolyl)methanes and
- To study the possible analgesic, anti-inflammatory, ulcerogenic and anti-oxidant activities of the synthesized bis(indolyl)methanes
3.3 EXPERIMENTAL

3.3.1 Materials and Methods

Melting points were measured in capillary tubes and are uncorrected. Analytical TLC was performed on pre-coated sheets of silica gel G of 0.25 mm thickness containing PF 254 indicator (Merck, Darmstadt) to monitor the progress and completion of the reaction as well as to check the purity. Column chromatography was performed with silica gel (60-120 mesh, S.d Fine, Boisar).

Infra-red spectra were recorded as solids in KBr pellets on a Perkin-Elmer FTIR spectrometer. Nuclear Magnetic Resonance spectra were recorded on a Bruker spectrometer or JEOL spectrometer. Proton Magnetic Resonance spectra were recorded at 300 MHz or 500 MHz in CDCl₃ or CDCl₃ plus DMSO-d₆ combination with tetramethylsilane as internal standard. Carbon-13 NMR spectra were recorded at 75 MHz or 125 MHz spectrometer in CDCl₃ or CDCl₃ plus DMSO-d₆ combination using the same internal standard. The chemical shifts are given in δ ppm scale. Mass spectra were recorded on a Varian VG 70-70H mass spectrometer or on a Perkin Elmer Turbo mass spectrometer.

3.3.2 Typical procedure for the synthesis of bis-indolylmethanes

To a mixture of indoles (2.5 mmol), substituted benzaldehydes (1.25 mmol) in methanol:water (1:1), sodium bisulphite (20 mol %) was added and stirred at room temperature for appropriate time. When the reaction was complete, it was extracted with CH₂Cl₂ (3 x 10 ml). The combined organic layers were dried using anhydrous Na₂SO₄, filtered and the solvent evaporated. The crude products were purified by column
chromatography and eluted with ethyl acetate: petroleum ether mixture to afford the products.

3.3.3 Pharmacological studies

3.3.3.1 Animals

For acute toxicity and analgesic activity, healthy adult swiss albino mice of either sex weighing 25-30 g were used. For anti-inflammatory, ulcerogenic and antioxidant activities, healthy adult Wistar rats of either sex weighing between 150-200 g were used. All the animals were maintained as per the norms of the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA) and the study protocols were approved by CPCSEA and Institutional Animal Ethics Committee constituted for the purpose (IAEC/SRMC&RI/42/2005).

3.3.3.2 Materials Requirement

Sodium carboxy methylcellulose (Loba Chemie, Mumbai) ibuprofen, indomethacin, carrageenan and other chemicals used for antioxidant activity (Sigma chemicals, USA), digital pH meter (Chemslab, Chennai), Electronic balance (Sartorius BS 223 S), tissue homogenizer (POTTERS, B.Braun Biotech international), refrigerated centrifuge (Hettich Zentrifugen Micro 200R), spectrophotometer (Perkin Elmer Lambda 25 UV/Vis), digital plethysmometer LE 7500 (Spain) – an instrument used to determine the variation in volume of rodent limbs, measuring the variation in fluid level on inserting the limb into a tank. The introduction of a tissue into a container changes the level of fluid and conductivity between two platinum electrodes introduced before hand into the container. This change is expressed in ml with a resolution of 0.01 ml. The test compounds and the standards were prepared in 1% sodium carboxy methylcellulose.
3.3.4 Acute toxicity study

The study is in accordance with the OECD guidelines (1987). Groups of five mice each are administered with single oral doses of the control (1 % sodium CMC) and the test compounds $3\text{ a-h}$ at 500 mg/kg, 1000 mg/kg, and 2000 mg/kg body weight dose levels respectively. Animals were fasted overnight prior to drug administration and weights of the animals were noted. After the administration of test compounds food was withheld for a further 3-4 h. The animals were under observation for a period of 14 days. Cage side observations included, changes in skin and fur, eyes and mucous membrane (nasal), autonomic (salivation, acclimation, perspiration, piloerection, urinary incontinence, defecation), circulatory, central nervous system (ptosis, drowsiness, gait, tremors and convulsion) and behavioural pattern were studied. At the end of the test, surviving animals were weighed and necropsies of animals treated with compounds $3\text{c}$ & $3\text{d}$ were carried out and all gross pathological changes were recorded. Histopathological examination of organs showing evidence of gross pathology in animals surviving at the end of experiment was performed and the results were analyzed by veterinary pathologist.

3.3.5 Analgesic activity

3.3.5.1 Introduction

Pain is ill defined, disabling accompaniment of many medical conditions. It is often evoked by an external or internal noxious stimulus. Analgesics are the drugs which possess significant pain relieving properties by acting in the Central Nervous System (CNS) or on peripheral pain receptors without significantly affecting consciousness (Sharma and Sharma, 2007).
3.3.5.2 Principle

Painful reaction in experimental animals can be produced by applying noxious stimuli such as

i) thermal (radiant heat as a source of pain), ii) chemical (irritants such as acetic acid and bradykinin) and physical pressure (tail compression). Exposing heat to mice tail is one way of stimulating pain in cutaneous receptors, which in turn excites the thermoselective and nociceptive fibers.

3.3.5.3 Method

The analgesic activity of the prepared BIMs was determined by the standard tail immersion method in mouse (Vogel and Vogel, 1998; Ghosh, 1984). Albino mice of either sex weighing 25-30 g were divided into 10 groups (comprising of control, standard and test compounds 3a-h) of five animals each. Mice were placed in individual, conical paper bags, fashioned from a square of stiff paper, folded and stapled to form a cone or pyramid. The animal tail projected from one side. At intervals, the mouse was held so that its tail was totally immersed in a bath at the temperature of 55°C. The time until the typical reaction- a violent jerk of the tail was recorded and noted as the basal reaction time. Normally, a mouse withdraws its tail within 2-4 sec. Any animal failing to respond within 2-4 sec was rejected.

The animals were administered p.o with the control (1% sodium carboxy methyl cellulose), test compounds (3a-h) and ibuprofen (standard) at a dose of 100 mg/kg as an aqueous suspension in 1% sodium carboxy methyl cellulose. Time taken for tail withdrawal response was recorded at 30 min, 1 h, 2 h and 3 h after administering the compounds. The cut-off time was fixed at 15 sec to prevent injury to the tail. Increase in
reaction time (interval for withdrawal of tail by the animal) was considered as an analgesic activity. The percentage analgesic activity was calculated by using the following formula (Daud et al., 2006).

\[
\text{Percentage analgesic activity} = \left( \frac{Ta - Tb}{Tb} \right) \times 100
\]

where \( Ta \) – reaction time for test or standard

\( Tb \) – reaction time for control

The significant difference between groups was tested by one-way ANOVA followed by Dunnett’s ‘t’ test.

3.3.6 Anti-inflammatory activity

3.3.6.1 Introduction

Inflammation is a response of the tissue to an infection, irritation or foreign substance (Turner, 1965) and is a part of the host defense mechanism. The inflammatory process involves a series of events that can be elicited by numerous stimuli (e.g. infectious agents, ischemia, antigen-antibody interaction and thermal or other physical injuries (Paul, 1996).

3.3.6.2 Principle

The inflammatory reaction is readily produced in the rats in the form of paw oedema with the help of irritants. Substances such as carrageenan, formalin, bradykinin, histamine, 5-hydroxy tryptamine, mustard or egg white when injected in the dorsum of the foot of rats, they produce acute paw oedema within a few minutes of the injection. Carrageenan - induced paw oedema is the most widely used model for studying the anti-inflammatory activity of new compounds. Carrageenan is a sulphated polysaccharide
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obtained from seaweed (Rhodophyceae) and by causing the release of histamine, 5-hydroxy tryptamine, bradykinin and protaglandins, it produces inflammation and oedema.

3.3.6.3 Method

The anti-inflammatory activity was evaluated using carrageenan induced rat hind paw oedema method (Winter et al., 1962). The animals fasted for 24 h were divided into control, standard and test groups each consisting of five rats. The first group of rats was treated with sodium carboxy methyl cellulose (1% w/v) suspension (control), second group was administered per orally with a dose of 10 mg/kg of indomethacin and the third group was treated with 100 mg/kg of suspension of the test compounds (3a-h). After 30 min, the animals were injected subcutaneously with 0.1 ml of freshly prepared suspension of carrageenan solution (1% in 0.9% saline) into the sub plantar region of the right hind paw of rats. The volume of hind paw was measured using a Digital plethysmometer LE 7500 (Spain) both in control and in animals treated with standard and test compounds at 60, 120, 180, 240 and 300 min after carrageenan challenge. The initial paw volume was measured within 30 sec of the injection. The anti-inflammatory activity was expressed as a percentage inhibition of the inflammation in treated animals in comparison with the control group (Bansal et al., 2001).

Percentage inhibition of oedema = (1-Vt/Vc) 100 where Vc and Vt are the mean relative changes in the volume of paw oedema in the control and drug treated groups respectively. The results are summarized in table and the data presented are mean ± SE. The significant difference between groups were statistically analysed by one-way ANOVA followed by Dunnett’s ‘t’ test.
3.3.7 Ulcerogenic liability

3.3.7.1 Method

The ulcerogenic liability was determined by the method of Djahanguiri (1969). The animals of group 3 treated with 100 mg/kg of suspension of the test compounds (3a-h) after studying anti-inflammatory activity were sacrificed 8 h after dosing and the stomach is removed and opened along the greater curvature and was washed with 0.9% saline and microscopically examined to assess for severity of histopathological changes if any and the results were analyzed by veterinary pathologist. The severity of ulcers will be assigned according to the arbitrary scoring system and the ulcer index was calculated (Kulkarni, 1999).

3.3.8 Antioxidant study

Antioxidant investigations of bis(indolyl)methanes were studied by both in vitro and in vivo antioxidant models of screening. The in vitro antioxidant activity was studied by 1,1-diphenyl, 2-picryl hydrazyl (DPPH) and nitric oxide induced free radical assay methods. The in vivo antioxidant activity involves measurement of lipid peroxidation, SOD, GSH, catalase and ascorbic acid.

The animals used in anti-inflammatory activity were anaesthetized with ether and sacrificed by humane method. The liver and stomach were dissected out, washed with ice-cold saline and a 10% (W/V) homogenate was prepared in ice-cold tris buffer (10 mM, pH 7.4) using a homogenizer. The homogenates were centrifuged at 5000 rpm for 20 min. The clear supernatant was used for the assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione, ascorbic acid and protein. Detection and
measurement of LPO is the evidence cited to support the involvement of free radicals (Naito, 1995).

3.3.9 Lipid peroxidation

3.3.9.1 Introduction

Lipid peroxidation (LPO) is defined as a type of oxidative degradation of polyunsaturated fatty acid (PUFA) (Tappel, 1973) set into notion by free radicals. Free radicals may be formed both enzymatically and non-enzymatically. These free radicals, because of their high reactivity, may undergo a number of reactions including i) electron transfer to molecular oxygen generating superoxide anion radical and other active oxygen species; ii) hydrogen atom abstraction possibly leading to auto-oxidation of PUFA; and iii) covalent binding to tissue macromolecule by radical addition to carbon – carbon double bonds or by radical combination (Smith et al., 1984; Halliwell and Gutteridge, 1985). The process of LPO has been related to phenomena such as inflammation, aging, cancer and toxicity of xenobiotics (Sies, 1985).

LPO is thought to be an important biological consequence of oxidative cellular damage (Plaa and Witschi, 1976), which results in structural damage to cellular membranes, with release of cell and organelle contents, loss of essential fatty acids, erosion of antioxidant production and formation of cytotoxic, aldehyde and peroxide production.

3.3.9.2 Principle

The lipids in the cell membranes are highly susceptible to peroxidase damage and are broken into number of small units to form malonyldialdehyde. This reacts with
thiobarbituric acid to form thiobarbituric acid reacting substance (TBARS) which has a pink color with absorption maximum at 532 nm.

3.3.9.3 Reagents

- Trichloro acetic acid (TCA): 10 %
- Thiobarbituric acid (TBA): 46 mM – 6.63 g in 1000 ml of 1 M sodium nitoxide

3.3.9.4 Procedure

Quantitative measurement of lipid peroxidation in the tissues was measured by the method of Ohkawa et al. (1979). To 0.1 ml of the tissue homogenate, 3 ml of ice cold 10 % TCA was added. The tubes were mixed well and 2 ml of TBA was added. The tubes were kept is boiling water bath for 20 min. After cooling, the tubes were centrifuged and the absorbance of the supernatant was read at 532 nm using a spectrophotometer. Malonyldialdehyde (MDA) content was calculated using a molar extinction co-efficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ Cm}^{-1}$ and expressed as nanomoles of TBARS / mg of protein.

3.3.10 Total reduced Glutathione

3.3.10.1 Introduction

Glutathione, a tripeptide containing γ-glutamic acid, cysteine and glycine is widely distributed in biological systems. In addition to the role of GSH as a substrate for the H$_2$O$_2$ removing enzyme glutathione peroxidase and for dehydroascorbate reductase, it acts as a scavenger of hydroxyl radicals and singlet oxygen (Halliwell and Gutterridge, 1989). The oxidation-reduction state of the reduced glutathione-oxidized glutathione is of major importance in cellular metabolism since it is the largest mobile thiol redox system of the cell (Arios and Jacoby, 1976; Sies and Wendel, 1978). When exposed to a large flux of H$_2$O$_2$ and / or hydroxyl radicals, the GSH/GSSG ratio cannot be maintained at its
normal high value and GSSG accumulates. GSSG is known to inactivate many enzymes by forming mixed disulphides with them. Reduced glutathione was measured according to the method of Moron et al. (1979).

\[
2 \text{G-SH} + \text{H}_2\text{O}_2 \xrightarrow{\text{glutathione}} \text{G-S-S-G} + 2\text{H}_2\text{O}
\]

(Reduced form) peroxidase (oxidised form)

3.3.10.2 Principle

This method was based on the reaction of reduced glutathione with dithio-bis-nitrobenzoic acid (DTNB) to give a compound which has a yellow color with absorption maximum at 412 nm.

3.3.10.3 Reagents

- Phosphate buffer: 0.2 M, pH 8.0
- DTNB: 0.6 mM in 0.2 M phosphate buffer, pH 8.0
- TCA: 10%
- Glutathione standard: 20 mg in 100 ml distilled water

3.3.10.4 Procedure

Equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.5 ml of supernatant, 2 ml of phosphate buffer and 0.5 ml of DTNB reagent was added. The yellow color developed was read at 412 nm against a blank containing 10% TCA instead of sample. A series of standards were treated in a similar manner.

The amount of glutathione in the tissues was expressed as \(\mu g/\) mg of Protein.
3.3.11 Superoxide Dismutase

3.3.11.1 Introduction

SOD catalytically scavenges the superoxide radical and this provides a first line of defense against free radical damage. The reaction catalyzed by this enzyme is a disproportion of dismutation of superoxide radicals (Barber and Bernheim, 1967).

\[
O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \text{ (ground state)}
\]

\( O_2^- \) generated is capable of oxidizing oxyhemoglobin to methemoglobin. Hence it has been proposed that a major function of SOD in the red cell is to prevent the formation of methemoglobin (Lynch et al., 1977).

3.3.11.2 Principle

The degree of inhibition of auto-oxidation of pyrogallol, at an alkaline pH, by SOD was used as a measure of the enzyme activity. The change in absorbance was measured at 470 nm.

3.3.11.3 Reagents

- Tris-HCl: 0.1 M, pH 8.2 containing 2 mM of diethylene triamine penta acetic acid (DETAPA)
- Tris-HCl buffer: 0.05 M, pH 7.4
- Pyrogallol stock solution: 25.2 mg of pyrogallol was dissolved in 1 ml of 0.05 M Tris-HCl buffer, pH 7.4 in an aluminium foil wrapped and stoppered test tube.
- Pyrogallol working solution: At the time of assay, 0.5 ml was diluted to 50 ml with 0.05 M Tris-HCl buffer, pH 7.4 to give a 2 mM solution and shielded from exposure to light.
- Absolute ethanol
• Chloroform

3.3.11.4 Procedure

SOD activity was analysed by the method of Marklund and Marklund (1974). To 0.5 ml of tissue homogenate, 0.25 ml of absolute ethanol and 0.15 ml of chloroform were added. After 15 min of shaking in a mechanical shaker, the suspension was centrifuged and the supernatant obtained constituted the enzyme extract.

The reaction mixture for auto oxidation consisted of 2 ml of the buffer containing DETAPA, 0.5 ml of 2 mM pyrogallol and 2 ml of water. Initially, the rate of auto-oxidation of pyrogallol was noted at 420 nm for 3 min at an interval of every minute.

The assay mixture for the enzyme contained 2 ml of 0.05 M Tris – HCl buffer, 0.5 ml of pyrogallol, 0.5 ml of supernatant and 1.5 ml of water. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted.

The concentration of SOD was expressed as U / mg of protein. One unit corresponds to the amount of enzyme that inhibited the auto-oxidation reaction by 50%.

3.3.12 Catalase

3.3.12.1 Introduction

Catalase constitutes a mutually supportive team of defense against ROS. Catalase is a haemoprotein, localized in the peroxisomes or microperoxisomes. It catalyses the decomposition of hydrogen peroxide to water and oxygen and thus protecting cells from oxidative damage of hydrogen peroxide and OH.

3.3.12.2 Reagents

• Phosphate buffer: 50 mM
• Hydrogen peroxide: 30 mM in phosphate buffer
3.3.12.3 Procedure

Catalase activity was measured by the method of Colowick et al. (1984). To 1.2 ml of 50 mM phosphate buffer, 0.1 ml of tissue homogenate was added. The enzyme reaction was started by the addition of 1 ml of freshly prepared 30 mM hydrogen peroxide solution. The rate of decomposition of hydrogen peroxide was measured at 240 nm for 3 min at 30 sec interval. Activity of catalase was expressed as U/ mg of protein.

3.3.13 Protein

3.3.13.1 Introduction

Protein is used to estimate diseased condition and increased levels of protein were found in dehydration, multiple myeloma, cancer, chronic liver disease and decreases in malnutrition.

3.3.13.2 Principle

The aromatic amino acids present in proteins react with Folin-Ciocalteau reagent which contains phosphomolybdic acid and tungstate to produce a blue colored complex which absorbs maximally at 620 nm.

3.3.13.3 Reagents

- Lowry’s reagent: - Solution A- 2 % sodium carbonates in 0.1 N Sodium hydroxide.
  - Solution B- 0.5 % Copper sulphate in 1 % sodium potassium tartrate.
  - 50 ml of A was mixed with 1 ml of B just before use.
- Folin-Ciocalteau reagent:- Dilute the reagent with water (1:2) just before the experiment.
• Standard protein: Standard bovine serum albumin (BSA) containing 20 mg in 100 ml was prepared.

3.3.13.4 Procedure

The protein level was determined by the method of Lowry et al. (1951). To 0.1 ml of the sample with 0.9 ml water, 5 ml Lowry’s regent was added, shaken well and allowed to stand for 10 min. Then 0.5 ml of Folin Ciocalteau reagent was added, shaken well and kept at room temperature for 20 min. A series of standard solutions containing BSA and blank were treated in a similar manner. The color developed was measured at 620 nm. The protein content in the tissue homogenate was expressed as mg/ gm of tissue.

3.3.14 Ascorbic acid

3.3.14.1 Introduction

Ascorbic acid (vitamin C) is well known for its direct free radical quenching ability. It has been implicated in regulating activites in the body by virtue of its ability to promote collagen synthesis, its role and ability to protect cells from free radicals.

3.3.14.2 Principle

Ascorbic acid is oxidized by copper to form dihydroascorbic acid and diketogluonic acid. These products react with DNPH to form a complex which in presence of strong sulphuric acid undergoes rearrangement to form a product with absorbance maxima at 520 nm. The reaction is carried out in the presence of thio-urea to provide a reducing medium to prevent interference from non-ascorbic acid chromogen.

3.3.14.3 Reagents

• Trichloro acetic acid (TCA): 5 %
• DNPH-Thiourea-Copper sulphate reagent (DTC): 3 g of dinitrophenylhydrazine, 0.4 g thiourea, 0.05 g of copper sulphate were dissolved in 9.0 N sulphuric acid and made up to 100 ml with the same.

• Sulphuric acid: 65 % ice cold

• Standard ascorbic acid: 10 mg was dissolved in 100 ml of 5 % TCA

3.3.14.4 Procedure

Ascorbic acid was estimated by the method of Omaye et al. (1979). To 0.5 ml of homogenate, 5 % ice cold TCA was added for precipitation. A protein free filtrate is obtained by centrifuging for 20 min at 3500 rpm. One ml of the supernatant was mixed with 0.2 ml of DTC and incubated for 3 h at 37°C. Then 1.5 ml of 65% ice- cold sulphuric acid was added, mixed well and allowed to stand at room temperature for 30 min and read at 520 nm. A series of standards were treated in a similar manner. The concentration of ascorbic acid was expressed as μg/ mg of protein.

3.3.14.5 Statistical Analysis

The data were expressed as mean ± SEM and analyzed statistically using one way ANOVA followed by Dunnett’s ‘t’ test. P values <0.01 and <0.001 were considered as significant.

3.3.15 DPPH radical scavenging activity

3.3.15.1 Introduction

DPPH is a stable free radical which can be used to determine the antioxidant activities of various compounds. This activity was measured by the spectrophotometric method and it shows deep violet color, characterized by an absorption band in ethanol solution at 570 nm.
3.3.15.2 Principle

When a DPPH solution is mixed with that of a substance that can donate a hydrogen atom, the free radical DPPH is reduced to corresponding hydrazine.

\[
\text{DPPH} \quad \downarrow \quad \text{H}^+ \\
\]

Diphenyl picryl hydrazine

3.3.15.3 Reagents

- DPPH (200 μM) in ethanol

3.3.15.4 Method

DPPH scavenging activity was measured by the spectrophotometric method (SreeJayan and Rao, 1996). To an ethanolic solution of DPPH (200 μM), 0.05 ml of test compounds (3a-h) dissolved in ethanol were added at different concentrations (2-1000
An equal amount of ethanol was added to the control. After 20 min., the decrease in absorbance of test compounds (due to quenching of DPPH free radical) was read at 517 nm. Degree of DPPH radical scavenging activity was expressed as

\[
\text{Percentage inhibition} = \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100
\]

where \(A_{\text{control}} = \) Absorbance of DPPH alone and \(A_{\text{test}} = \) Absorbance of DPPH with different concentrations of test compounds.

### 3.3.16 Scavenging of nitric oxide radical

#### 3.3.16.1 Introduction

Nitric oxide (NO) has a very high affinity to even low concentrations of super oxide anion (O\(_2^\cdot\)). In situations of oxidative stress, where super oxide free radicals are in plenty, NO reacts very rapidly to form an unstable peroxynitrite moiety (ONOO\(^\cdot\)). The latter instantly gets decomposed to hydroxyl (OH\(^\cdot\)) and nitrite(NO\(_2^\cdot\)) free radicals which are responsible for the cytotoxic actions of NO.

\[
\text{NO} + \text{O}_2^\cdot \rightarrow \text{ONOO}^\cdot \rightarrow \text{NO}_2^\cdot + \text{OH}^\cdot
\]

The peroxynitrite (ONOO\(^\cdot\)), hydroxy (OH\(^\cdot\)) and nitrite (NO\(_2^\cdot\)) free radicals possess a high affinity for the sulfhydryl groups (SH groups) and thus inactivate a number of metabolically important –SH bearing enzymes. Thus, scavengers of O\(_2^\cdot\) anion such as SOD may protect degradation of NO, enhancing its potency and prolonging its duration of action.

#### 3.3.16.2 Principle

Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction (SreeJayan and Rao, 1997).
3.3.16.3 Reagents

- Sodium nitrobrusside (5 mM) in phosphate buffer solution
- 0.1% naphthyl ethylene diamine dihydrochloride in water
- 1% sulphanilamide in phosphoric acid

3.3.16.4 Method

Sodium nitroprusside (5 mM) in phosphate buffer solution was incubated with different concentrations (2-1000 μg) of test compounds dissolved in phosphate buffer at 25°C for 5 h. Control experiments without the test compounds but with equivalent amounts of buffer were conducted in an identical manner. After 5 h, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent. Griess reagent was prepared by mixing equal volumes of 0.1% naphthyl ethylene diamine dihydrochloride in water and 1% sulphanilamide in phosphoric acid. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene diamine was read at 546 nm. The experiment was repeated in triplicate.
3.4 SPECTRAL DATA

3,3’-Bis-indolylmethane (3a)

Yield: 90%; mp: 89-90°C; IR (KBr): 3415, 1638, 1459, 741 cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\)): \(\delta 7.82-7.10\) (m, 10H), 6.42 (s, 2H), 5.65 (s, 2H); \(^13\)C NMR (CDCl\(_3\)): \(\delta 140.2, 136.2, 131.2, 130.5, 128.2, 126.4, 123.9, 1212.1, 117.3, 111.8, 39.2\); MS m/z: 246 (M+); Anal. Calcd for C\(_{17}H_{14}N_2\): C, 82.90; H, 5.73; N, 11.37. Found: C, 82.85; H, 5.69; N, 11.32.

3,3’-Bis-indolyl-(4-methylphenyl)methane (3b)

Yield: 92%; mp: 94-96°C; IR (KBr): 3402, 3040, 1697, 1450, 1214, 1089, 742 cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\)): \(\delta 7.93\) (br s, 2H), 7.33-6.80 (m, 12H), 6.77 (s, 2H), 5.75 (s, 1H), 2.21 (s, 3H); \(^13\)C NMR (CDCl\(_3\)): \(\delta 142.3, 137.0, 135.0, 129.7, 129.0, 128.6, 127.0, 123.8, 121.2, 119.5, 118.5, 111.8, 39.0, 21.0\); MS m/z: 336 (M+); Anal. Calcd for C\(_{24}H_{20}N_2\): C, 85.68; H, 5.99; N, 8.33. Found: C, 85.58; H, 5.96; N, 8.30.

3,3’-Bis-indolyl-(2-nitrophenyl)methane (3c)

Yield: 94%; mp: 217-219°C; IR (KBr): 3422, 3052, 1592, 1507, 1456, 1340 cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\)): \(\delta 8.15\) (d, 2H, \(J = 8.8\) Hz), 8.04 (br s, 2H), 7.52 (d, 2H, \(J = 8.8\) Hz), 7.40 (d, 2H, \(J = 8.0\) Hz), 7.35 (d, 3H, \(J = 8.0\) Hz), 7.00-7.05 (m, 3H), 6.70 (s, 2H), 6.00 (s, 1H); \(^13\)C NMR (CDCl\(_3\)): \(\delta 141.8, 137.6, 135.2, 129.2, 129.4, 128.0, 127.6, 122.8, 120.2, 119.2, 118.5, 112.8, 39.5\); MS m/z: 367 (M+); Anal. Calcd for C\(_{23}H_{17}N_3O_2\): C, 75.19; H, 4.66; N, 11.44. Found: C, 75.11; H, 4.69; N, 11.39.

3,3’-Bis-indolyl-(3-methoxy-4-hydroxyphenyl) methane (3d)

Yield: 86%; mp: 99-101°C; IR (KBr): 3500, 3412, 1634, 1383, 741 cm\(^{-1}\); \(^1\)H NMR
(CDCl₃): δ 9.81 (br s, 2H), 7.91 (s, 1H), 7.39-6.80 (m, 11H), 6.63 (s, 2H), 5.80 (s, 1H), 3.74 (s, 3H); ¹³C NMR (CDCl₃): δ 143.8, 136.7, 136.1, 127.0, 123.5, 121.9, 121.3, 119.9, 119.2, 114.0, 111.4, 55.8, 39.8; MS m/z: 368 (M+); Anal. Calcd for C₂₄H₂₀N₂O₂: C, 78.24; H, 5.47; N, 7.60. Found: C, 78.19; H, 5.41; N, 7.70.

3,3’-Bis-indolyl-(4-chlorophenyl)methane (3e)

Yield: 94%; mp: 89-90°C; IR (KBr): 3415, 1638, 1459, 741 cm⁻¹; ¹H NMR (CDCl₃): δ 7.77-7.03 (m, 14H), 6.53 (s, 2H), 5.87 (s, 1H); ¹³C NMR (CDCl₃): δ 142.5, 136.5, 131.6, 130.9, 129.9, 128.2, 126.7, 123.5, 121.9, 118.9, 115.1, 111.1, 39.5; MS m/z: 356 (M+), 358 (M+2); Anal. Calcd for C₂₃H₁₇ClN₂: C, 77.41; H, 4.80; N, 7.85. Found: C, 77.36; H, 4.82; N, 7.81.

3,3’-Bis-indolyl-(4-methoxyphenyl)methane (3f)

Yield: 88%; mp: 187-188°C; IR (KBr): 3410, 1609, 1508, 1455 cm⁻¹; ¹H NMR (CDCl₃): δ 7.94 (br s, 2H), 7.35-7.40 (m, 4H), 7.19 (s, 2H), 7.17 (t, 2H, J = 7.3 Hz), 7.00 (t, 2H, J = 7.3 Hz), 6.82 (d, 2H, J = 8.3 Hz), 6.66 (s, 2H), 5.84 (s, 1H), 3.78 (s, 3H), 7.77-7.03 (m, 14H), 6.53 (s, 2H), 5.87 (s, 1H); ¹³C NMR (CDCl₃): δ 140.5, 136.5, 130.6, 130.1, 129.7, 128.5, 126.0, 123.7, 121.4, 117.6, 115.9, 112.1, 55.8, 40.1; MS m/z: 352 (M+); Anal. Calcd for C₂₄H₂₀N₂O: C, 81.79; H, 5.72; N, 7.95. Found: C, 81.72; H, 5.82; N, 7.98.

3,3’-Bis-indolyl-(phenyl)methane (3g)

Yield: 91%; mp: 125-126°C; IR (KBr): 3416, 1634, 1378, 737 cm⁻¹; ¹H NMR (CDCl₃): δ 7.58 (br s, 2H), 7.45-7.18 (m, 13H), 6.51 (s, 2H), 5.91 (s, 1H); ¹³C NMR (CDCl₃): δ 137.1, 134.3, 133.7, 130.2, 130.1, 130.0, 127.1, 125.1, 123.0, 119.4, 118.9, 114.3, 39.2; MS m/z: 322 (M+); Anal. Calcd for C₂₃H₁₈N₂: C, 85.68; H, 5.63;
N, 8.69. Found: C, 85.62; H, 5.68; N, 8.63.

3,3’-Bis-indolyl-(3-nitrophenyl)methane (3h)

Yield: 96%; mp: 261-262°C; IR (KBr): 3468, 3060, 1649, 1569, 1240, 769 cm⁻¹; ¹H NMR (CDCl₃): δ 8.18 (br s, 2H), 8.06 (d, 1H, J = 7.2 Hz), 7.94 (s, 2H), 7.67 (d, 1H, J = 7.5 Hz), 7.41 (m, 4H), 7.17 (t, 2H, J = 7.2 Hz), 7.03 (d, 2H, J = 7.2 Hz), 6.59 (s, 2H), 5.96 (s, 1H); ¹³C NMR (CDCl₃): δ 136.6, 134.8, 129.1, 126.5, 123.7, 123.5, 122.2, 121.4, 119.5, 118.1, 111.3, 39.9; MS m/z: 367 (M⁺); Anal. Calcd for C₂₃H₁₇N₃O₂: C, 75.19; H, 4.66; N, 11.44. Found: C, 75.41; H, 4.69; N, 11.54.
3.5 RESULTS AND DISCUSSION

The reaction of indole with aromatic aldehydes catalyzed by sodium bisulphite to bis(indolylmethane) derivatives were studied (Scheme 2). The results obtained with various substituted aldehydes are given in Table 2.1

![Scheme 2]

Table 2.1 Synthesis of bis(indolylmethanes)

<table>
<thead>
<tr>
<th>Product</th>
<th>R</th>
<th>Yield (%)</th>
</tr>
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<tbody>
<tr>
<td>3a</td>
<td>HCHO</td>
<td>90</td>
</tr>
<tr>
<td>3b</td>
<td>p-methyl benzaldehyde</td>
<td>92</td>
</tr>
<tr>
<td>3c</td>
<td>o-nitro benzaldehyde</td>
<td>94</td>
</tr>
<tr>
<td>3d</td>
<td>m-methoxy 4-hydroxybenzaldehyde</td>
<td>86</td>
</tr>
<tr>
<td>3e</td>
<td>p-chloro benzaldehyde</td>
<td>94</td>
</tr>
<tr>
<td>3f</td>
<td>p-methoxy benzaldehyde</td>
<td>88</td>
</tr>
<tr>
<td>3g</td>
<td>Benzaldehyde</td>
<td>91</td>
</tr>
<tr>
<td>3h</td>
<td>m-nitro benzaldehyde</td>
<td>96</td>
</tr>
</tbody>
</table>
The IR spectrum of $3g$ showed the NH stretching frequency at 3416 cm$^{-1}$. The proton NMR spectrum (spectrum 2.1) exhibits indolic NH protons at $\delta$ 7.58. The aromatic protons appeared as follows. $\delta$ 7.45-7.18 (m, 13H), 6.51 (s, 2H). The methyne proton was observed at $\delta$ 5.91. $^{13}$C NMR spectrum (spectrum 2.2) exhibited the aromatic signals in the range $\delta$ 137.1-114.3 and methyne signal at $\delta$ 40.0. The mass spectrum showed the molecular ion peak m/z at 322 (spectrum 2.3).

3.5.1 Acute toxicity

The test compounds were made as suspension in 1% in sodium carboxy methyl cellulose. The test compounds 3a-h were tested in mice (500 mg/kg, 1000 mg/kg, and 2000 mg/kg, p.o.) after 14 days of administration for their safety as per OECD guidelines (1987). The compounds did not produce any significant changes in the body weight (Table 2.2), food, water intake and other behavioural patterns. There was also no change in the haematological parameters (Table 2.4) and organ weights of treated animals (Table 2.3) compared to control. No mortality was observed in the control and compound treated groups. Histopathological examination of organs viz. heart, liver, brain, lung, kidney, spleen, stomach, testis and ovary in animals treated with compounds 3c & 3d were studied.

Results of the histopathological studies revealed that compound 3c (500 mg/kg, 1000 mg/kg, 2000 mg/kg), compound 3d (500 mg/kg) and control did not show any pathological changes (Fig. 2.1a, 2.2a). Compound 3d at a dose of 1000 mg/kg, p.o. produced mild swelling and narrowing of sinusoid in liver (Fig. 2.1b). Compound 3d at 2000 mg/kg dose level induced diffuse severe degeneration and necrosis of the tubular epithelial cells in kidney (Fig. 2.2b). Compound 3d at 1000 mg/kg dose level induced mild
degeneration and necrosis of tubular epithelial cells with accumulation of eosinophilic casts in the tubular lumen (Fig. 2.2c). Compound 3d at 2000 mg/kg dose level induced congestion of blood vessels, mild degeneration in tubular epithelial cells, condensation and vacuolation of cytoplasm with margination of nucleus (Fig. 2.1c) and Focal coagulation necrosis of hepatocytes in proximity to central vein with infiltration of lymphocytes and mononuclear cells around necrotic area in liver (Fig. 2.1d). The other vital organs examined did not show any changes.

3.5.2 Analgesic activity

The analgesic activity of the prepared BIMs (3a-h) was determined by the tail immersion method (Ghosh, 1984; Vogel and Vogel, 1998). In this investigation, it has been observed that all the tested BIMs show highly significant analgesic activity without ulcerogenic liability.

Among these, compounds 3b, 3g and 3h (284.4 % -306 % analgesic activity) showed enhanced activity and comparable to those of ibuprofen, which is used as a reference standard (309 % analgesic activity). Compounds 3c and 3d (310.5 % & 414 % activity) were found to be more potent than the standard drug ibuprofen while compounds 3a, 3e and 3f gave moderate results (Table 2.5 and Fig. 2.3).

3.5.3 Anti-inflammatory activity

The anti-inflammatory activity of the prepared bis(indolyl)methanes was determined by the carrageenan induced paw oedema standard method in rats (Winter et al., 1962). The inflammatory response induced by carrageenan is characterized by a marked oedema formation resulting from the release of several mediators such as histamine, serotonin and bradykinin which is subsequently sustained by the release of
prostaglandin and nitric oxide produced by inducible isoforms of cyclooxygenase (cox-2) and nitric oxide synthase (iNOS) respectively (DiRosa et al., 1971; Seibert et al., 1994; Selvamani et al., 1996; Nantel et al., 1999).

In this investigation, it has been observed that all the tested BIMs (3a-h) show highly significant anti-inflammatory activity without ulcerogenic liability. From the obtained data, compounds having 2-nitrophenyl (3c), 3-methoxy 4-hydroxyphenyl (3d) and 3-nitrophenyl substitution (3h) revealed enhanced anti-inflammatory activity. Compounds 3a, 3b, 3e, 3f and 3g showed moderate activity (Table 2.6 and Fig. 2.4). The observed anti-inflammatory activity may be due to an inhibitory effect exerted predominantly on the mediators of inflammation.

3.5.4 Ulcerogenic activity

The ulcerogenic liability of the prepared compounds (3a-h) was determined in Wistar rats following the previously reported standard method (Djahanguiri, 1969). Histopathological examination of stomach did not reveal presence of any ulcer (Fig. 2.5a-d). Thus besides having the analgesic and anti-inflammatory activity, the compounds were devoid of ulcerogenicity.

3.5.5 Antioxidant activity

3.5.5.1 In vitro antioxidant activity

Several concentrations ranging from 2-1000 µg of BIMs (3a-h) were tested for their in vitro antioxidant activity. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner. The maximum % inhibition in DPPH and NO model were found to be 84.64 and 53.15 for the compounds 3d and 3c respectively at 1 mg concentration (Table 2.7 and 2.8, Fig. 2.6 and 2.7).
DPPH is a relatively stable free radical and the assay determines the ability of BIMs to reduce DPPH radicals to the corresponding hydrazine by converting the unpaired electrons to paired ones. Antioxidants can act by converting the unpaired electrons to paired ones. The dose dependent inhibition of DPPH radicals indicates that BIMs causes reduction of DPPH radicals in a stoichometric manner (Blois, 1958).

In the NO model, the nitrite produced by incubation of solutions of sodium nitroprusside in standard phosphate buffer was reduced by the BIMs. This may be due to the antioxidant activity of the BIMs which compete with oxygen to react with nitric oxide (Narcocci et al., 1994), thereby inhibiting the generation of nitrite.

During the inflammatory phase, from the macrophages, the reactive free radical nitric oxide (NO) is synthesized by inducible NO synthase. Accumulating evidence indicates that excessive production of NO plays a pathogenic role in both acute and chronic inflammations (Clancy et al., 1998). Manipulation of NO free radical can be a potential and promising therapeutic area in treating inflammation (Mittal et al., 2003).

Since the entire test compounds exhibited \textit{in vitro} NO free radical scavenging activity, the same may be responsible for the observed anti-inflammatory effect of BIMs.

\textbf{3.5.5.2 \textit{In vivo} antioxidant activity}

Several reports pointed out the involvement of free radicals in the process of inflammation (Sudheer kumar, 2004). Normally endogenous intra cellular antioxidant protects the tissue from injury by free radicals. Therefore development of antioxidant drug could be beneficial as an anti-inflammatory agent (Shenoy and Shirwaikar, 2002).

When the production of oxygen species is increased or when the production of antioxidants is diminished, the state is oxidative stress and results in serious cell damage.
Oxidative stress has been implicated in the pathophysiology of many diseases and conditions including diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing (Marx, 1987). Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the LPO and thus prevent disease (Youdim and Joseph, 2001).

In this study, rat treated with carrageenan developed an oxidative stress which was observed from a significant (P<0.001) increase in tissue MDA and protein levels and significant (P<0.001) decrease in tissue SOD, GSH, CAT and ascorbic acid levels when compared to normal rats. Following BIM treatment, there was highly significant (P<0.001) reduction in tissue MDA and protein levels and highly significant increase in SOD, GSH, CAT and ascorbic acid when compared to carrageenan treated rats which suggests the antioxidant activity of test compounds (Table 2.9 and Fig. 2.8 – 2.12)
3.6 SUMMARY

Synthesis, acute toxicity, analgesic, anti-inflammatory, ulcerogenic and antioxidant activities of bis(indolyl)methanes in mice and rats were studied. A series of bis(indolyl)methanes have been synthesized by stirring a mixture of indole and aldehydes in methanol:water (1:1) containing catalytic amount of sodium bisulphite at room temperature. In acute toxicity study, no mortality was observed in the compound treated groups (3a-h) at 500, 1000 and 2000 mg/kg dose levels. Histopathological examination of vital organs of mice treated with compound 3c did not show any pathological changes and was found to be safe even at 2000 mg/kg dose level. However, further detailed toxicological investigations are required particularly to elucidate their chronic toxicity. The results obtained clearly indicate that the compounds discussed here showed highly significant analgesic (227.8 - 414.0 % analgesic activity) and anti-inflammatory activities (40.3 - 52.9 % inhibition of oedema) without an ulcerogenic liability. Compounds 3c and 3d appear to be the most active derivatives in this series. These observations may promote the synthesis of more active bis(indolyl)methanes in future. The mechanism involved in these activities has to be explored.
3.7 REFERENCES


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## Table 2.2 Body weight gain (g)

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<th>Compound name</th>
<th>Concentration in mg/kg</th>
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<tr>
<td></td>
<td>500</td>
</tr>
<tr>
<td>3c</td>
<td>2.33±0.02</td>
</tr>
<tr>
<td>3d</td>
<td>2.32±0.014</td>
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<td>Control</td>
<td></td>
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Values are Mean±SE (n=5). P>0.5 in treated groups compared with control (student’s t – test)

## Table 2.3 Effect of compounds on organ weight (g/100 g body weight)

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<thead>
<tr>
<th>Organ name</th>
<th>Compound 3c concentration</th>
<th>Compound 3d concentration</th>
<th>Control</th>
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<tbody>
<tr>
<td></td>
<td>500 mg/kg</td>
<td>1000 mg/kg</td>
<td>2000 mg/kg</td>
</tr>
<tr>
<td>Heart</td>
<td>0.582±0.0009</td>
<td>0.585±0.0005</td>
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<td>Kidney</td>
<td>1.314±0.0013</td>
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<td>Liver</td>
<td>4.81±0.004</td>
<td>4.821±0.002</td>
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<td>Lung</td>
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<td>0.6698±0.001</td>
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Values are Mean±SE (n=5). P>0.5 in treated groups compared with control (student’s t – test)
Table 2.4 Effect of BIMs on haematological parameters

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<tr>
<th>Parameters</th>
<th>Compound 3c 500 mg/kg</th>
<th>Compound 3c 1000 mg/kg</th>
<th>Compound 3c 2000 mg/kg</th>
<th>Compound 500 mg/kg</th>
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<th>Control</th>
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<td>R.B.C.</td>
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<td>Hb (g %)</td>
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<td>15.4</td>
<td>15.6</td>
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<td>W.B.C. (*10^3/mm3)</td>
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<td>Neutrophil (%)</td>
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<td>44</td>
<td>37</td>
<td>41</td>
<td>45</td>
<td>41</td>
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</table>

(n=5, values are Mean±SE)
Table 2.7 Effect of BIMs on DPPH *in vitro* free radical model

<table>
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<th>Concentration (μg)</th>
<th>3a</th>
<th>3b</th>
<th>3c</th>
<th>3d</th>
<th>3e</th>
<th>3f</th>
<th>3g</th>
<th>3h</th>
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<td>40.26</td>
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% inhibition of ascorbic acid 20 μg (reference standard) was found to be 94.19%. Values are mean of 3 replicates.
Table 2.8 Effect of BIMs on nitric oxide free radical method

<table>
<thead>
<tr>
<th>Concentration (μg)</th>
<th>3a</th>
<th>3b</th>
<th>3c</th>
<th>3d</th>
<th>3e</th>
<th>3f</th>
<th>3g</th>
<th>3h</th>
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<tr>
<td>1000</td>
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<td>32.49</td>
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<td>51.76</td>
<td>33.75</td>
<td>39.55</td>
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<td>37.78</td>
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<td>18.14</td>
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<td>39.80</td>
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</table>

Values are mean of 3 replicates. % inhibition of reference standard (ascorbic acid - 20μg) is 57.3%
<table>
<thead>
<tr>
<th>Compounds</th>
<th>LPO (nm/ mg protein)</th>
<th>Reduced glutathione (μg/ mg protein)</th>
<th>Catalase (U/ mg protein)</th>
<th>SOD (U/ mg protein)</th>
<th>Ascorbic acid (μg/ mg protein)</th>
<th>Protein (mg/ g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Stomach</td>
<td>Liver</td>
<td>Stomach</td>
<td>Liver</td>
<td>Stomach</td>
</tr>
<tr>
<td>Normal</td>
<td>3.20±0.043</td>
<td>4.22±0.06</td>
<td>5.64±0.048</td>
<td>7.3±0.071</td>
<td>5.32±0.09</td>
<td>7.49±0.12</td>
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<tr>
<td>Control</td>
<td>6.43±0.043</td>
<td>7.23±0.03</td>
<td>2.41±0.041</td>
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<td>1.46±0.051</td>
<td>2.7±0.130</td>
</tr>
<tr>
<td>3a</td>
<td>4.6±0.13</td>
<td>5.44±0.051</td>
<td>4.34±0.087</td>
<td>4.26±0.051</td>
<td>2.42±0.037</td>
<td>3.95±0.06</td>
</tr>
<tr>
<td>3b</td>
<td>3.52±0.11</td>
<td>5.36±0.09</td>
<td>3.94±0.051</td>
<td>4.36±0.051</td>
<td>2.56±0.045</td>
<td>3.75±0.064</td>
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<tr>
<td>3c</td>
<td>4.0±0.011</td>
<td>5.06±0.057</td>
<td>4.72±0.058</td>
<td>4.8±0.051</td>
<td>2.98±0.058</td>
<td>4.48±0.081</td>
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<tr>
<td>3d</td>
<td>3.21±0.015</td>
<td>4.75±0.043</td>
<td>5.05±0.084</td>
<td>5.2±0.045</td>
<td>3.0±0.073</td>
<td>4.63±0.072</td>
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<tr>
<td>3e</td>
<td>4.92±0.19</td>
<td>5.24±0.14</td>
<td>4.24±0.18</td>
<td>4.34±0.04</td>
<td>2.52±0.037</td>
<td>3.83±0.045</td>
</tr>
<tr>
<td>3f</td>
<td>4.56±0.121</td>
<td>5.3±0.071</td>
<td>4.32±0.066</td>
<td>4.24±0.051</td>
<td>2.56±0.114</td>
<td>4.46±0.047</td>
</tr>
<tr>
<td>3G</td>
<td>3.62±0.102</td>
<td>5.16±0.103</td>
<td>4.3±0.071</td>
<td>4.48±0.037</td>
<td>2.61±0.071</td>
<td>4.32±0.078</td>
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<tr>
<td>3H</td>
<td>3.58±0.13</td>
<td>5.04±0.092</td>
<td>4.34±0.093</td>
<td>4.36±0.051</td>
<td>2.46±0.051</td>
<td>4.39±0.058</td>
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<tr>
<td>Reference standard (indomethacin)</td>
<td>4.18±0.04</td>
<td>4.33±0.03</td>
<td>4.42±0.037</td>
<td>5.58±0.058</td>
<td>3.0±0.071</td>
<td>5.34±0.103</td>
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</tbody>
</table>

Values are mean ± SEM (n=5). One way ANOVA followed by Dunnet’s ‘t’ test was used. Control group compared with normal group (p<0.01). Test groups were compared with control group (p<0.001).
Spectrum 2.1 $^1$H NMR of BIM (3g)
Chapter II

Spectrum 2.2 $^{13}$C NMR of BIM (3g)
Spectrum 2.3 Mass spectrum of BIM (3g)

Scan: 27
Base: m/z 322; 22.2% FS TIC: 95384
R.T.: 0:36

#Ions: 52
Fig. 2.1a-d

a) H&E stained histopathological section of mice liver (160x)

Control mice liver section

b) H&E stained histopathological section for 3d-1000 mg/kg section of mice liver (160x)

Swelling of hepatocytes around portal Triad with granularity of cytoplasm, prominent Kupfer cells

c) H&E stained histopathological section of mice liver (320x) for 3d – 2000 mg/kg

Congestion of blood vessels, mild degeneration in tubular epithelial cells, condensation and vacuolation of cytoplasm with margination of nucleus

d) H&E stained histopathological section of mice liver (320x) for 3d – 2000 mg/kg

Focal coagulation necrosis of hepatocytes in proximity to central vein with infiltration of lymphocytes and mononuclear cells around necrotic area
Fig. 2.2a-c
a) Histopathological H&E stained section of mice kidney (160x)

Control mice kidney section

b) Histopathological H&E stained section of mice kidney (320x) for 3d – 2000 mg/kg

Diffuse severe degeneration and necrosis of the tubular epithelial cells

c) Histopathological H&E stained section of mice kidney (320x) for 3d – 1000 mg/kg

Mild degeneration and necrosis of tubular epithelial cells with accumulation of eosinophilic casts in the tubular lumen
### Table 2.5 Analgesic Activity of BIMs

<table>
<thead>
<tr>
<th>Compound No</th>
<th>Mean Tail withdrawal latencies (sec) ± SE</th>
<th>% Analgesic activity</th>
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<td>0 min</td>
<td>30 min</td>
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<tr>
<td>Control</td>
<td>2.4±0.055*</td>
<td>2.38±0.071*</td>
</tr>
<tr>
<td>3a</td>
<td>3.16±0.07*</td>
<td>5.22±0.06*</td>
</tr>
<tr>
<td>3b</td>
<td>2.34±0.0341*</td>
<td>5.52±0.09*</td>
</tr>
<tr>
<td>3c</td>
<td>2.28±0.06*</td>
<td>5.36±0.04*</td>
</tr>
<tr>
<td>3d</td>
<td>2.0±0.10*</td>
<td>3.8±0.114*</td>
</tr>
<tr>
<td>3e</td>
<td>3.08±0.04*</td>
<td>5.58±0.04*</td>
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<tr>
<td>3f</td>
<td>3.2±0.071*</td>
<td>5.22±0.06*</td>
</tr>
<tr>
<td>3g</td>
<td>2.14±0.065*</td>
<td>5.16±0.068*</td>
</tr>
<tr>
<td>3h</td>
<td>2.3±0.071*</td>
<td>6.16±0.087*</td>
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<td>Ibuprofen (reference standard)</td>
<td>2.44±0.07*</td>
<td>4.56±0.04*</td>
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Statistical analysis was carried out by using one-way Anova (F-test) followed by Dunnett’s ‘t’ test. *Significantly different from the control value at p<0.001.
Fig. 2.3 Analgesic Activity of BIMs
Table 2.6 Anti-inflammatory activity of the bis(indolyl)methanes

<table>
<thead>
<tr>
<th>Compound No</th>
<th>Mean values (± SE) of oedema Volume at different intervals</th>
<th>Percentage of anti-inflammatory activity at different intervals</th>
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<tbody>
<tr>
<td></td>
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<td>120 min</td>
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<tr>
<td>Control</td>
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<td>3b</td>
<td>1.296± 0.04*</td>
<td>1.192± 0.013*</td>
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<tr>
<td>3c</td>
<td>1.202± 0.03*</td>
<td>1.184± 0.023*</td>
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<tr>
<td>3d</td>
<td>1.196± 0.008*</td>
<td>1.158± 0.019*</td>
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<tr>
<td>3e</td>
<td>1.328± 0.03*</td>
<td>1.344± 0.026*</td>
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<tr>
<td>3f</td>
<td>1.30± 0.01*</td>
<td>1.228± 0.032*</td>
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<tr>
<td>3g</td>
<td>1.43± 0.013*</td>
<td>1.206± 0.085*</td>
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<tr>
<td>3h</td>
<td>1.348± 0.024*</td>
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<td>Indomethacin (reference standard)</td>
<td>1.14± 0.017*</td>
<td>1.005± 0.023*</td>
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</table>

Statistical analysis was carried out by using one-way Anova (F-test) followed by Dunnett’s t test. *Significantly different from the control value at p<0.001
Fig. 2.4  Anti-inflammatory activity of the bis(indolyl)methanes
Fig. 2.5a-d Histopathological H&E stained section of rat stomach (160 x)
Fig. 2.6 Effect of BIMs on DPPH *in vitro* free model

![Effect of BIMs on DPPH invtro free model](image)

Fig. 2.7 Effect of BIMs on nitric oxide free radical method

![Effect of BIMs on nitric oxide free radical method](image)
Fig. 2.8 Antioxidant activity of BIMs

![Graph showing antioxidant activity of BIMs in liver and stomach. The x-axis represents compounds, and the y-axis represents LPO (nm/mg protein). The graph compares normal, control, and different compounds (3a to 3h) in both liver and stomach.]

Fig. 2.9 Antioxidant activity of BIMs

![Graph showing antioxidant activity of BIMs in liver and stomach. The x-axis represents compounds, and the y-axis represents reduced glutathione (microgram/mg protein). The graph compares normal, control, and different compounds (3a to 3h) in both liver and stomach.]

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Fig. 2.10 Antioxidant activity of BIMs

Fig. 2.11 Antioxidant activity of BIMs
Fig. 2.12 Antioxidant activity of BIMs