5. DISCUSSION

Inflammatory bowel disease (IBD) results in a substantial burden to individuals and society, not only because of direct and indirect medical costs, but also by causing disability. The reduction in working capacity, especially in a young and active segment of the population, is the major economic and social burden of disease that outweighs the cost of drug therapy\textsuperscript{[122]}. Despite recent advances in the treatment of IBD, much remains to be done in order to improve outcomes. Higher rates of relapse, resistance or intolerance to some of drugs, unacceptable toxicity and high cost of drugs used for treatment are major hurdles in the management of symptoms\textsuperscript{[4; 5]}. Therefore, there is a need for alternative agents that may be equally or more effective as well as being cheaper.

In recent years, there has been renewed interest in plant medicine for the treatment against different diseases, as herbal drugs are generally out of toxic effect reported from research work conducted on experimental animal models\textsuperscript{[9]}. Herbal medicines are recognized by WHO as an essential building block for primary health care, especially in developing countries like India, but the herbal and other indigenous sources have not adequately been explored for the presence of safe and effective herbal constituent for the treatment of IBD. In present study attempt has been made to cope up with the need of alternative herbal medicine for treatment of IBD, which is devoid of side effects, do not show resistance or intolerance and cheaper as compared to current medication available.

\textit{Oroxylum indicum} (Syonakh) is a traditional herbal medicine in India, China and Japan, reported in Indian ancient text “Ayurveda” to possess anti-diarrheal and antibacterial activity\textsuperscript{[10]}. It has been used as an analgesic and anti-inflammatory agent by traditional medical practitioners\textsuperscript{[11]}. This forms a good basis for its use as a curative drug against IBD. \textit{Aconitum heterophyllum} commonly known as “Atis” or “Patis” belongs to family Ranunculaceae, reported to possess significant antipyretic and analgesic properties with a high therapeutic index\textsuperscript{[12]}. The plant was used for treatment of diseases of digestive system, rheumatism and fever\textsuperscript{[12]}. It also exhibits anti-fungal and anti-bacterial activities\textsuperscript{[12]} which indicate its usefulness as a promising drug in treatment of IBD. \textit{Aegle marmelos} (L.) Correa commonly known as Bael/Bilva belonging to the family Rutaceae, frequently used for the treatment of
intermittent fever and diarrhoea, especially in chronic diarrhoeas, was reported to be effective in experimental model of IBD$^{109}$. 

Worldwide consumption of herbal medicines has markedly increased, which raised concerns about its quality, purity and safety issues. From the cultivation of medicinal herbs to the final herbal product, there are many factors which influence the quality of herbal medicines, like contamination, adulteration, misidentification etc$^{102}$. Hence it is of utmost importance to standardize the plant material since it may vary in its phytochemical content according to diverse places of collection, with different times in a year for collection, with collection at the same time and places but in different years and with different environmental factors surrounding the cultivation of a particular medicinal plant. These phytochemical alterations may vary its therapeutic effect as well and hence standardization of plant material used in this study was done with various methods like ash value, extractive value, loss on drying and phytochemical screening for various constituents, and compared with standards reported in Ayurvedic Pharmacopoeia of India. Moreover plants were authentified by experts in Botany. The parameters were found to be comparable and collected plants were considered authentic, non adulterated and devoid of contamination.

Recently, various physicians and researchers raised concerns about safety of various herbal medicines owing to lack of adequate regulations, the pharmacological complexity of herbal products, and the paucity of information on the pharmacology and toxicity of these compounds$^{123; 124}$. Thus a crucial evaluation of their safety is relevant and important. In current study, aqueous extract of root bark of $O$. indicum and roots of $A$. heterophyllum were evaluated for their toxicity and found to be safe in acute and chronic toxicity protocols. This study provides scientific evidence for safety of these plant extracts and showed no mortality or morbidity in rats. These extracts were found to be devoid of any adverse effects when evaluated by morphological, haematological and biochemical parameters. Functional Observational Battery (FOB) showed no clinical signs of neurological toxicity.

Ulcerative colitis is characterized as inflammation of the gastrointestinal tract, limited to the rectum and colon. Four major categories of colonic involvement have been defined. Proctitis is rectum-only involvement. Proctosigmoiditis includes the rectal involvement with extension into the sigmoid region. Left-sided colitis refers to
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disease extending from rectum continuously to the splenic flexure. Pancolitis is inflammation past the splenic flexure, potentially extending to the cecum. The common features include rectal bleeding or bloody diarrhoea, abdominal pain, fever, weight loss, and malaise\textsuperscript{[125]}. These common symptoms were also observed in present study. DNBS treated rats showed decreased body weight and diarrhoea with pasty stools and presence of blood in stools. Treatment with OI\textsubscript{aq} and AH\textsubscript{aq} showed reduction in diarrhoea and improved body weight. Furthermore OI\textsubscript{aq} inhibited motility and secretory diarrhoea in castor oil induced and magnesium sulphate induced model.

Anorexia and malnutrition are the symptoms of chronic IBD due to nausea, vomiting, diarrhoea and abdominal pain. It is previously been reported that tissue injury and infection trigger the release of proinflammatory cytokines that alter normal energy regulatory mechanisms resulting in anorexia, tissue catabolism, increased metabolic rate, and loss of body weight\textsuperscript{[126]}. Similar results were obtained in DNBS induced tissue injury and increased proinflammatory cytokines in rats. In experimental colitis induced by DNBS, anorexia was reported in model control rats, while there was no significant alteration in food intake in OI\textsubscript{aq} and AH\textsubscript{aq} treated rats. This effect may be ascribed to protective and anti-inflammatory effects of these extracts.

In Ulcerative colitis (UC), inflammation is limited to the mucosa and submucosa. Neutrophils can be found infiltrating the crypts, forming crypt abscesses. Superficial erosions or ulcerations occur and can penetrate deeper into the submucosa in severe conditions. Active disease also results in indistinct crypts. The inflammatory changes extend continuously from the rectum. Mucosa is generally erythematous and friable. Superficial ulcers are often observed and pseudopolyps are characteristic of severe disease. There are areas of normal but edematous mucosa bulging around the diseased inflamed mucosa\textsuperscript{[122]}. Similar results were found in DNBS induced colitis in rats. Animals treated with DNBS alone exhibited significant increase in colonic lesion area, compared with control. OI\textsubscript{aq} and AH\textsubscript{aq} treatment decreased colonic damage induced by DNBS. Histological sections from DNBS treated rats colon showed trans-mural necrosis, along with extensive morphological disorientation, oedema and diffuse leukocyte cellular infiltrate as well as lymphocyte in submucosa. While treatment with OI\textsubscript{aq} and AH\textsubscript{aq} reduced the disorientation of mucosa, prevented
infiltration and oedema in higher dose levels (200 and 400 mg/kg doses). Weight of colon in DNBS treated rats was found to be increased, which is an indicator of oedema and increased fluid deposition in colonic tissue layers. Pretreatment with OI\textsubscript{aq} and AH\textsubscript{aq} significantly decreased colon weight, compared with model control.

Chronic inflammatory diseases are known to demonstrate acute enlargement of spleen that develops in association with various inflammatory processes and results from an increase in the defense activities of the organ. The demand for increased antigen clearance from the blood may lead to increased numbers of reticuloendothelial cells in the spleen and stimulate accelerated antibody production, with resultant lymphoid hyperplasia\textsuperscript{127}. In current study, DNBS treated rats showed increase in weight of spleen, which is an indicator of inflammation. Pretreatment with OI\textsubscript{aq} and AH\textsubscript{aq} extracts prevented this increase.

Signs of increased oxidative stress are in evidence in the intestinal mucosa of patients with ulcerative colitis and may be secondary to inflammation. This oxidative stress is apparent when there is an imbalance between the levels of natural antioxidant and reactive oxygen species (ROS)\textsuperscript{125, 128}. ROS can be generated through two pathways. The first one is via NADPH oxidase activation in infiltrated neutrophils, which induces release of large amount of ROS and oxidants derived from Mylloperoxidase (MPO)\textsuperscript{129}. Alternatively other pathway acts through Xanthine oxidase (XO) induction after periods of hypoxia\textsuperscript{130}. MPO activity was found to be increased significantly which was correlated with laboratory parameters and endoscopic grade of inflammation in IBD patients\textsuperscript{131}. MPO induced conversion of hydrogen peroxide to hypochlorus acid seems closely related to ROS-mediated tissue injury\textsuperscript{132}. While XO pathway seems to be discrete for UC. Reynolds and co-workers in one of clinical study illustrated that colonic XO activity was not elevated in UC patients\textsuperscript{133}. It was suggested that XO may not be a major source of superoxide and ROS production in UC\textsuperscript{134}. Similarly in present study MPO levels correlated well with severity of symptoms of DNBS induced colitis in rats. Model control animals showed elevated activity of MPO in colon while pretreatment with OI\textsubscript{aq} and AH\textsubscript{aq} showed significantly reduction.

Oxidative damage represents crucial pathogenic factor in inflammatory bowel disease because intestinal inflammation is accompanied by increased production of
superoxide (O$_2^-$), nitric oxide (NO·), peroxynitrite (ONOO$^-$) and hydroxyl radicals (-OH). Among these, NO radical synthesis is governed by elevated iNOS expression in colonic mucosa of patients suffering from UC$^{[135]}$. NO has been postulated to play a dual role in the gastrointestinal tract. Continuous release of NO from a constitutive NO synthase, which is located in intestinal epithelial and lamina propria cells, neuronal terminals and endothelial cells, is involved in the physiological maintenance of motility, tone, permeability, and tissue blood flow$^{[136]}$. On the other hand, overwhelming production of NO by iNOS has been postulated to have a pathological role in IBD. This overwhelming production of NO by iNOS causes mucosal vasodilatation and modulation of intestinal water and electrolyte transport, which together increase vascular permeability$^{[137]}$. One of the underlying mechanisms of NO in inducing colonic toxicity involves interaction with superoxide to produce peroxynitrite, a potent oxidant that can damage colonic mucosa via a number of independent mechanisms, including (i) initiation of lipid peroxidation, (ii) inactivation of a variety of enzymes (most notably mitochondrial respiratory enzymes and membrane pumps)$^{[138]}$, and (iii) depletion of glutathione$^{[139]}$. Moreover, peroxynitrite can also cause DNA damage$^{[140]; 141]}$. Malondialdehyde (MDA), which is an endogenous product of enzymatic and oxygen free radical-induced lipid peroxidation, also found to be increased along with NO$^{[110]}$. Similar results were obtained in current study, DNBS treated rats showed significant rise in MDA and NO levels in colon. Animals treated with OI$_{aq}$ showed significant reduction in these levels in dose 200 and 400 mg/kg, while AH$_{aq}$ treatment was found to be ineffective. Baicalein (5,6,7-trihydroxyflavone, BAE, C$_{15}$H$_{10}$O$_5$), is a flavonoid found in root bark of O. indicum, reported to be protective against neuronal cell injuries induced by β-amyloid$^{[142]}$, oxidative stress$^{[143]}$, glutamate and glucose deprivation$^{[144]}$, and brain microglia death caused by lipopolysaccharide$^{[145]}$. Baicalein was reported to inhibit adhesion and recruitment of circulating leukocytes to tissue injury sites during inflammation$^{[146]}$ ultimately inhibiting free radical generation. Chen et al demonstrated inhibition of Lipopolysaccharide (LPS) induced NO production by baicalein$^{[147]}$. Moreover, Chrysin (flavonoid) and Biochanin-A (phytoestrogen), found in root bark of O. indicum was reported to inhibit iNOS, thereby inhibiting NO synthesis. This antioxidant effect of baicalein, chrysin and biochanin-A may involve in protective effect of OI$_{aq}$ against DNBS induced colitis.
Discussion

It is well evident from previous literature that the imbalance of ROS generation and antioxidant defense in colon is an important contributor to initiation and maintenance of inflammation in IBD\textsuperscript{[148]}. The normal mucosa is endowed with various endogenous antioxidant defense systems like catalase, superoxide dismutase, and glutathione peroxidise to remove ROS resulting from normal metabolism. From these natural antioxidants reduced colonic GSH level is crucial in inducing inflammatory changes\textsuperscript{[149]}. Previous studies showed that decrease in colonic GSH concentration following colitis induction is due to over-production of ROS that deplete GSH by inhibiting synthetic enzymes for GSH production in colonic tissue\textsuperscript{[149]}. Similar results were reported in current study showing significant decrease of GSH levels in DNBS treated rats. Levels of GSH were improved significantly with treatment of \textit{O. indica} while \textit{A. hypochondriaca} treatment was unable to show significant effect.

A theory proposed by various researchers depicts involvement of Tumour Necrosis Factor – alpha (TNF-\textalpha) in production of ROS. This ROS in turn activate nuclear factor-kappa B (NF-\kappaB), which then enhances further TNF-\textalpha production, propagating a vicious cycle and further damage to tissue\textsuperscript{[68; 69; 150]}. Similar evidences were found in UC depicting that the increase of pro-inflammatory mediators such as ROS, TNF-\textalpha and interleukins (IL-1\beta, IL-6, IL-8) led to inflammation cascade effects and tissue damage in the pathological progress of UC\textsuperscript{[68]}. In addition, it has been demonstrated that all these pro-inflammatory cytokines are regulated by NF-\kappaB pathway\textsuperscript{[69]}.

The nuclear transcription factor NF-\kappaB has crucial role in the pathogenesis of several human disorders, particularly those with an inflammatory component. The signalling pathways of NF-\kappaB were activated dependent on IKK. The stimulation of receptors such as TNF receptor, Toll-like receptors (TLRs) or T-cell receptor (TCR) activates a multisubunit IKK (IKK\textalpha, IKK\textbeta and IKK\textgamma), which catalyzes the phosphorylation of I\kappaB\alpha. This phosphorylation is essential for signalling the subsequent ubiquitination and proteolysis of I\kappaB\alpha, leaving NF-\kappaB free to translocate to the nucleus and promote gene transcription. Once activated, NF-\kappaB translocated to the nucleus from the cytoplasm, then activated the consensus sequence related gene, including TNF-\textalpha, IL-6, IL-2, IL-8, ICAM-1, and so forth, involved in immune and
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Increasing evidence reveals that the inhibition of NF-κB activity may lead to alleviating the severity of inflammatory diseases.

NF-κB-driven cytokine production by myeloid cells is instrumental in Colitis Associated Cancer (CAC) generation whereas NF-kB activation in intestinal epithelial cells (IECs) promotes survival of newly emerging premalignant cells.[67; 70]. In patients with UC, risk of colorectal cancer (CRC) development is much higher than in general population.[60]. Long-standing UC predisposes to development of colitis-associated cancer (CAC), the major cause of death in UC patients.[61]. Tumor-promoting effect of inflammation is widely recognized and better understood.[65]. Immune cells, which often infiltrate tumours and preneoplastic lesions, produce a variety of cytokines and chemokines that propagate localized inflammatory response and also enhance growth and survival of premalignant cells by activating transcription factors such as NF-κB.[151-153]. Similar results were found in current study. We observed significant rise in local NF-κB levels in Azoxymethane (AOM) and Dextran Sulfate Sodium (DSS) induced CAC in rat colons which were in accordance with incidence and multiplicity of tumours. Pretreatment with AM-E in dose 100 mg/kg p.o. showed significant reduction in NF-κB levels (75.36±11.45 ng/g of tissue) and incidence (30%) and multiplicity of tumours (2.8±0.82 tumours/colon) as compared with model control (NF-κB levels - 119.63±9.22 ng/g of tissue, incidence – 100% and multiplicity - 8.6±1.83 tumours/colon).

OI-F, flavonoid rich active fraction from O. indicum, in dose 100 mg/kg p.o. showed significant reduction in NF-κB levels (75.36±11.45 ng/g of tissue) and incidence (30%) and multiplicity of tumours (2.8±0.82 tumours/colon) as compared with model control (NF-κB levels - 119.63±9.22 ng/g of tissue, incidence – 100% and multiplicity - 8.6±1.83 tumours/colon). Previously chrysin, component of root bark of O. indicum, was shown to inhibit NF-κB and JNK therby decreasing cytokine production.[154]. One more constituent Biochanin-A, an isoflavone (phytoestrogen) existing in root bark of O. indicum, was reported to prevent phosphorylation and degradation of IκBα, thereby blocking NFκB activation and nuclear translocation, which in turns leads to decreased transcription of iNOS and other pro-inflammatory genes, thus preventing inflammation.[155]. All these constituents baicalein, chrysin and
biochanin-A affects synthesis of NO which justifies our observations in current study i.e. decreased NO production in rat colon due to pretreatment of OI$_{aq}$.

Previous studies suggested that cytokines or growth factors produced upon NF-κB activation in intestinal myeloid cells stimulate proliferation of premalignant IECs generated during early stages of CAC tumorigenesis. Inactivation of NF-κB in myeloid cells through ablation of IKKβ, protein kinase required for its activation, inhibits production of inflammatory mediators, including cytokines such as IL-6 and TNF-α, and prevents IEC proliferation during CAC induction. As a result, tumor load is reduced due to a decrease in tumor frequency and size$^{[67]}$. One of the NF-κB-dependent tumor growth factors released by myeloid cells could be IL-6, a multifunctional cytokine important for immune responses, cell survival, apoptosis, and proliferation$^{[156;157]}$. IL-6 binds to soluble or membrane-bound IL-6 receptor (IL-6Ra) polypeptides that signal by interacting with membrane-associated gp130 subunit, whose engagement triggers activation of Janus kinases (JAKs), and downstream effectors Stat3, Shp2-Ras, and phosphatidylinositol 3-kinase (PI3K)-Akt$^{[156]}$. IL-6 is also critical for T cell survival and differentiation and therefore has a central pathogenic role in T cell-dependent autoimmune disorders, including IBD$^{[72;73]}$. By regulating differentiation and survival of pathogenic T helper (Th) cells, IL-6 can perpetuate chronic inflammation and ensure continuous production of cytokines and growth factors required for malignant cell survival and growth. IL-6 also has an important role in tissue homeostasis and regeneration$^{[72]}$, suggesting that it may have direct prosurvival and protumorigenic effects. Several studies have demonstrated a correlation between circulating or local IL-6 levels and clinical activity of IBD$^{[158]}$. IL-6 protein and mRNA are also often upregulated in serum and tumor samples of humans and mice suffering from breast, prostate, lung, liver and colon cancer$^{[159]}$. IL-6 enhances proliferation of human colon carcinoma cells in vitro, and interference with IL-6 signalling during late stages of CAC development slows down tumour growth$^{[160;161]}$. We observed significant rise in local IL-6 levels in CAC induced rat colons in current study which were in accordance with incidence and multiplicity of tumours. Pretreatment with AM-E in dose 100 mg/kg p.o. showed significant reduction in IL-6 levels (162.64±11.37 pg/g of tissue) and incidence (30%) and multiplicity of tumours (2.8±0.82 tumours/colon) as compared with model control
Pathogenesis of IBD involves participation of Histamine and their receptors and was reported in previous literature. Previous findings suggest that Histamine H1 receptor (H1R) induces activation of phospholipase C via pertussis toxin (PTX) resistant Gq/11-proteins[162]. Stimulation of phospholipase C generates inositol-1,4,5-trisphosphate and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate derived from plasma membranes. Inositol-1,4,5-trisphosphate releases Ca\(^{2+}\) from intracellular calcium stores, and subsequently activates store-operated calcium channels, whereas DAG activates specific isoforms of Phosphokinase C (PKC)[162; 163].

It has been reported that PKC might be involved in activation of ERK and NF-κB pathways in response to several stimuli[164-172]. Not only oxidative damage and TNF-α pathway leads to activation of NF-κB but histamine stimulated PKC mediated activation of NF-κB can also be a possible mechanism of cytokine stimulation. ERK is a well-characterized mitogen-activated protein kinase (MAPK) and is primarily associated with the regulation of proliferation, anti-apoptosis, differentiation and gene expression (e.g. cytokine gene expression)[166; 173], while NF-κB activation by PKC stimulates production of IL-6 levels thereby promoting inflammation. In current study, phytosterol rich AM-E fraction, obtained from A. marmelos, was found to inhibit response of histamine on H1 receptors dose dependently. This might be the reason behind inhibition of NF-κB and IL-6 levels by AM-E in AOM and DSS induced CAC model.

CD4\(^+\) T cells are thought to be major players in the processes leading to inflammatory bowel disease (IBD). Increased mucosal infiltration and activation of CD4\(^+\) T lymphocytes are the major cause for CD and UC[174]. Ca\(^{2+}\) influx across the plasma membrane following stimulation of Jurkat T-cells or peripheral blood T-lymphocytes is a necessary signal for T-cell activation[175]. Following cross-linking of T cell receptor, a number of signaling cascades are activated, one of which results in Inositol - 1,4,5 - triphosphate (Ins-1,4,5-P3) production. Ins-1,4,5-P3 initiates Ca\(^{2+}\) signalling in T-cells by two coupled processes: Ca\(^{2+}\) release from the endoplasmic reticulum through Ins-1,4,5-P3 receptor channel and subsequent activation of plasma membrane Ca\(^{2+}\) channels. Activation of Ca\(^{2+}\) channels is dependent on the Ca\(^{2+}\) filling
state of endoplasmic reticulum. A low Ca\(^{2+}\) concentration in endoplasmic reticulum provides signal to activate plasma membrane Ca\(^{2+}\) channels, whereas a high Ca\(^{2+}\) concentration in endoplasmic reticulum terminates the response\[^{176}\]. These channels are therefore referred to as store-operated Ca\(^{2+}\) channels. In T-lymphocytes, the store-operated Ca\(^{2+}\) channels are highly Ca\(^{2+}\) selective\[^{177}\] and are also termed CRAC channels (Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels) \[^{175; 178; 179}\]. These channels provide Ca\(^{2+}\) influx necessary for T-cell activation\[^{180}\], and their absence correlates with greatly reduced T-cell functionality resulting in severe combined immunodeficiencies in humans\[^{181-183}\]. These findings as well as numerous other observations\[^{175}\] stress the conclusion that CRAC channel activity determines T-cell reactivity and ultimately inflammatory responses.

Dysregulated Ca\(^{2+}\) responses have been associated with several autoimmune and inflammatory diseases, including systemic lupus erythematosus, rheumatoid arthritis, psoriasis, and IBD\[^{184}\]. Consequently, CRAC channels have been proposed as potential target in the therapeutic management of autoimmune and inflammatory disorders\[^{42}\]. In present study OI-F, fraction obtained from *O. indicum* was found to inhibit CRAC channel. Chrysin, a major component of OI-F, was reported previously for decreasing intracellular calcium levels in mast cell, inhibiting mast cell degranulation\[^{185}\]. This inhibits nuclear factor-κB and caspase-1 dependent release of pro-inflammatory cytokines like tumor necrosis factor-α, IL-1β, IL-4, and IL-6 in mast cells\[^{185}\]. Similar results were found in current study, OI-F inhibited CRAC channels *in vitro* and decreased NF-κB and IL-6 levels in AOM-DSS induced CAC in rats.

In conclusion, *O. indicum* and *A. marmelos* were found to be effective against experimentally induced colitis and may proved to be promising targets for the treatment of IBD, while *A. heterophyllum* was ineffective. OI-F, a flavonoid rich fraction obtained from *O. indicum* was shown to inhibit NF-κB and IL-6 levels mediated by inhibition of CRAC channels, thereby inhibiting symptoms of colitis and progression of colitis associated colon cancer in rats. AM-E was found to inhibit NF-κB and IL-6 levels mediated by alteration in activity of histamine on H1 receptors, thereby inhibiting symptoms of colitis and progression of colitis associated colon cancer in rats.
Discussion

Alternative medicines, especially herbal constituents are always the topic of discussion in scientific community because of concerns about its quality, purity, safety and efficacy\cite{123,124,186}. These drugs are rejected by modern medical practitioners because of contamination, adulteration, misidentification and paucity of information on the pharmacology and toxicity of these compounds\cite{124}. Preparations depicted in Ayurveda are tedious to prepare and inappropriate in current scenario. To cope up with this problem, plants proposed in Ayurveda were processed for systematic extraction procedures to isolate specific active constituents (which will be potent and efficacious) and incorporate it in to established formulations to dispense it. The first step for this is to isolate specific fractions, evaluate its pharmacological potential and discover its exact mechanism of action. Current study followed this trend and provided sufficient evidence for safety, efficacy and potency of active fractions OI-F and AM-E obtained from \textit{O. indicum} and \textit{A. marmelos} respectively. The data obtained can be useful in the future for dose range finding, preparation of pharmaceutical formulations and clinical studies.