# CHAPTER TWO

## 2.1. LITERATURE REVIEW

### 2.1.1 Colorectal Cancer

Genetics, experimental, and epidemiological data suggest that colorectal cancer develops from complex interactions between inherited susceptibility and environmental factors. The current hypothesis is that adenomatous polyps are the precursors of the vast majority of colorectal cancers. Thus measures that can detect and reduce the prevalence of these adenomatous polyps can reduce the risk of colorectal cancer (MSGH, 2004).

Among colorectal cancer, some are considered to be an invasive cancers that are confined within the wall of the colon (TNM stages I and II), are curable with surgery. If untreated, they spread to regional lymph nodes (stage III), where up to 73% are curable by surgery and chemotherapy. Cancer that metastasizes to distant sites (stage IV) is usually not curable, although chemotherapy can extend survival, and in rare cases, surgery and chemotherapy together have seen patients through to a cure (Markowitz and Bertagnolli, 2009).

On the cellular and molecular level, colorectal cancer starts with a mutation to the Wnt signaling pathway. When Wnt binds to a receptor on the cell that sets in motion a chain of molecular events that ends with β-catenin moving into the nucleus and activating a gene on DNA. In colorectal cancer, genes along this chain are damaged. Usually, a gene called APC, which is a "brake" on the Wnt pathway,

is damaged. Without a working APC brake, the Wnt pathway is stuck in the "on" position (Markowitz and Bertagnolli, 2009) (Figure 2.1.).

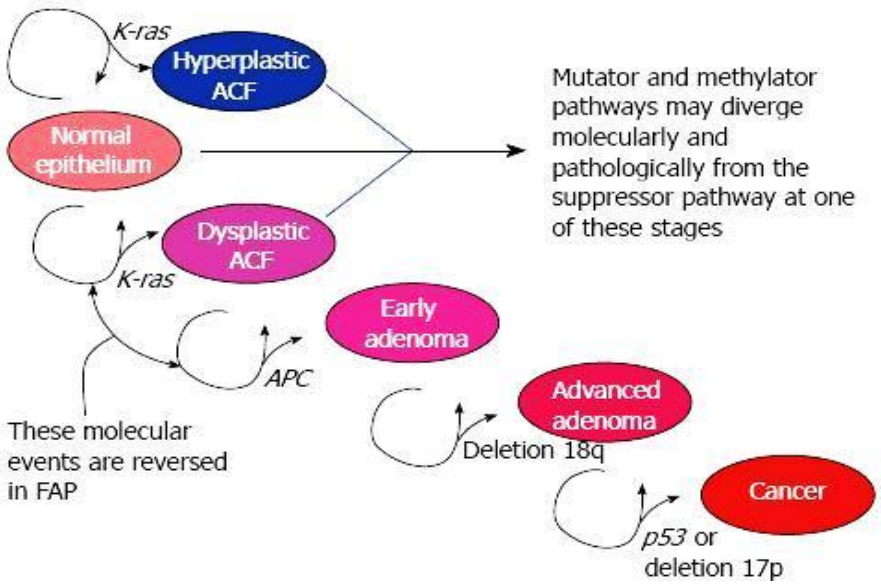


Figure 2.1. Sequential pathological stages and molecular events in colon cancer (Corpet, 2002)

More carcinogenic compounds such as Dimethyl diazene-1-oxide (Azoxymethane) and 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) have now been identified. These compounds has been demonstrated to produce colon tumors when fed to male rats for a specified period of time (Roderick, 1998).

More than 200 agents, including the above cited phytochemicals such as secondary metabolites derived from plants like *P. niruri* , and other food components like calcium or folic acid (a B vitamin), and NSAIDs like aspirin, are able to decrease carcinogenesis in pre-clinical development models: Some studies show full inhibition of carcinogen-induced tumors in the colon of rats. Other

studies show strong inhibition of spontaneous intestinal polyps in mutated mice (Min mice) (Wikipedia, 2010).

The chance of long-term survival for colon, rectal, or other cancers improves significantly with early detection. For example, it was reported that the 5-year survival rate for people whose colorectal cancer is found and treated at an early stage is > 90%, but once the cancer has spread to nearby organs the 5-year survival rate drops to 64%, and it is < 10% when the spread includes distant organs, such as the liver and lungs (Roderick, 1998).

Because the survival rates improve with early detection, considerable effort has gone into devising screening tests that might provide an "early warning system" for individuals with no symptoms as well as for those who exhibit one or more symptoms. It has been cited that if intermediate biomarkers are identified, one might be able to recognize very early stages of cancer development, long before the formation of a frank tumor. With appropriate intervention, progression of the lesion could be reversed or slowed significantly. One promising candidate for an intermediate biomarker for colon cancer, called an Aberrant Crypt Foci, or "ACF" (Wargovich et al., 2010).

On a much shorter time scale, Azoxymethane, PhIP and related heterocyclic amines generate ACF in rats when fed for a few weeks. The ACF assay also provides a quick method to screen compounds that might be effective at inhibiting the development of colon cancer, and several promising candidates for chemoprevention such as plant extracts.

As mentioned somewhere else that the development of colorectal cancer depends on many lifestyle related factors in addition to genetic factors that influence the digestive tract. Hence, identifying the point at which normal colonic epithelium becomes neoplastic, hyperplastic, dysplastic, or an early indicator of disease is of interest to many research groups. There is an overwhelming idea that aberrant crypt foci (ACF) are colon cancer precursors (Wargovich *et al.*, 2010) whose size and numbers directly correlate with the risk of developing colon cancer.

### 2.1.2. Aberrant Crypt foci (ACF)

Aberrant crypt foci were first discovered by Bird in 1987(Corpet, 2002). Treating mice with the carcinogen azoxymethane (AOM) induced the growth of colonic crypts that were larger, thicker and darker staining than normal crypts when visualized with methylene blue (Wargovich *et al.*, 2010). Wargovich*et al.* (2010) mentioned the use of ACF as biomarker in their research "Aberrant Crypt Foci: The Case for Inclusion as a Biomarker for Colon Cancer". Bird (1995) reported the role of aberrant crypt foci in understanding the pathogenesis of colon cancer, Takayama *et al.* (2005) elucidate the detection, gene abnormalities, and clinical usefulness of ACF.

Aberrant crypts were first observed in the surrounding normal colonic mucosa of patients with colon cancer in 1991 (Pretlow *et al.*, 1991). The crypt clusters found in human mucosa appear raised from the normal mucosal surface of

the colon (Pretlow *et al.*, 1991). Due to the observed rapid turnover of intestinal and colonic cells under normal conditions, it is expected that aberrant crypts would replicate at the same rate, if not faster than normal crypts. However, in humans there is conflicting evidence as to how much of an increase in replication exists, if any (Wargovich *et al.*, 2010).

This inconsistency can be due to many factors, most notable difference between the methods of sampling and analysis in various studies determining colonic epithelial cell proliferation (Jass *et al.*, 1997). Aberrant crypt replication is essentially identical to that of normal crypts with the replication process starting at the bottom of the crypt pushing cells upward and outwards to form new colonic crypts in addition to replenishing the cells in the original crypt (Fujimitsu *et al.*, 1996). This is a budding and branching process, known as crypt fission, which forms larger sized foci over time (Fujimitsu *et al.*, 1996). This process does, however, occur at an increased rate in various disease states of the bowel (Figure 2.2).

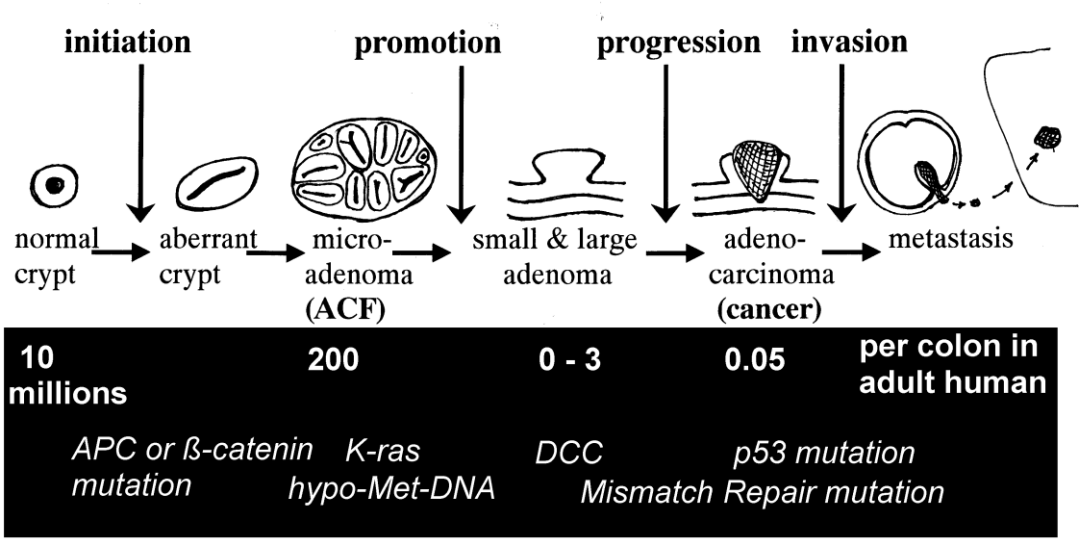


Figure 2.2. Stages of ACF formation in colon cancer (Corpet and Tache, 2002)

As mentioned, ACF were first reported in the colon epithelium of rodents treated with chemical carcinogens (Bird *et al.*, 1989). They are single to multiple crypt clusters of abnormally staining crypts after short-term staining with either methylene-blue or indigo-carmine solutions and fixation with either buffered formalin or alcohol-based fixatives (Bird, 1987).

As aforementioned, ACF are readily visible usually with the aid of a dissection microscope at a magnification of at 40x. There is a considerable wealth of literature describing the key histopathological signatures of ACF, and categorizing them in human has met with considerable controversy (Gupta *et al.*, 2007; Gupta *et al.*, 2009, Khare *et al.*, 2009, Lance and Hamilton, 2008, Pereira *et al.*, 1994). Microscopically, a distinction has been made between dysplastic ACF

and non-dysplastic ACF (often including serrated hyperplastic ACF) (Wargovich *et al.*, 2010)

Before 1990, the gold standard endpoint for chemoprevention in rodents was the incidence of macroscopic tumors and colon cancers: colon adenomas and adenocarcinomas induced by a chemical carcinogen. Though, these endpoints are clearly related to cancer, but they however, have three major drawbacks:

- a tumor requires a long time to develop (usually 5-8 months),
- each tumor must be confirmed by histology, which is long and costly, and
- each animal brings little information to the study (each rat has either no tumor or a tumor), thus large groups of rats are needed for statistical analysis (typically 30 rats or more per group).

In lieu of the above draw backs, ACF was found to be advantageous as endpoint chemoprotective screening biomarkers because (i) ACF were induced by all colon carcinogens in a dose- and species-dependant manner; (ii) their number and growth were modified by the modulators of colon carcinogenesis, and they predicted the tumor outcome in several rodent studies; (iii) they correlate with colon cancer risk, and adenoma size and number in humans; (iv) the morphological and genotypic features of ACF in human colons were similar to those in animal colons, and many alterations are similar in ACF and in tumors; (v) some ACF show dysplasia, and carcinoma were observed in rodents and humans' ACF (Corpet and Tache, 2002).

This is why ACF is now widely used as a preliminary endpoint in colon cancer chemoprevention studies, for it provides a simple and economical tool for preliminary screening of potential chemopreventive agents, and it allows a quantitative assessment of the mechanisms of colon carcinogenesis.

### 2.1.3. Azoxymethane (AOM)

Azoxymethane (methyl-methylimino-oxidoazanium) is a compound as oxide of azomethane, having molecular formula of $C_2H_6N_2O$, with chemical structure (figure 2.3.). It is carcinogenic and neurotoxic chemical, found wide application in biological research, particularly effective in inducing colon carcinomas. Besides been soluble in water, this compound is reported to be sensitive to prolonged exposure to air and elevated temperatures.

Figure 2.3. Chemical structure of Azoxymethane (methyl-methylimino-oxidoazanium)

The use of azoxymethane to induce foci of aberrant crypts in rats has been reported in many literatures (Adler, 2005, Bagalkotkar et al., 2006, Rajeshkumar et

al., 2002, Shimpo et al., 2001, Velmurugan et al., 2008, Verghese et al., 2002, Wargovich et al., 2010). Tanaka *et al.* (2001) studied the Chemoprevention effect of zerunbone isolated from *Zingiber zerumbet* by using azoxymethane to induce aberrant crypt foci in male F344 rats. The researchers induced ACF in these rats by subcutaneous injections of AOM (15 mg/kg body weight) for three weeks. They assessed the effects of zerumbone on cell proliferation activity by counting silver-stained nucleolar organizer regions protein (AgNORs) in colonic cryptal cell nuclei.

Anjana *et al*.(1997) reported the induction of aberrant crypt foci using azoxymethane while studying the effect of phytic acid and green tea in interactive suppression of the ACF. Magnuson *et al*. (2009) published an extensive literature on the increased susceptibility of adult rats to AOM-induced aberrant crypt foci. Verghese *et al*. (2002) studied the suppression of AOM-induced ACF using dietary insulin.

In general, azoxymethane is commonly used as a model for colon cancer induction. It can specifically induce colon cancer similar to the pathogenesis of human sporadic colon cancer. Thus, it has been extensively used in the study of the molecular biology, prevention and treatment of colon cancer. After administration, AOM is metabolised into methylazoxymethanol by CYP2E1, which causes DNA mutations. Mutation of K-ras activates this pathway and its downstream PI3K/Akt pathway and Mitogen-activated protein kinase (MAPK) pathway (Chen and Huang, 2009). Mutation of β-catenin also prevents it from being degraded by

GSK-3 and accumulation of β-catenin leads to cell proliferation. TGFβ, a pro-apoptotic protein, is inhibited. These changes form the basis of AOM carcinogenesis.

#### 2.1.3.1. Azoxymethane (AOM) metabolism

Azoxymethane does not interact with DNA directly. It has to be activated *in vivo* to develop carcinogenesis. Azoxymethane is metabolised by cytochrome P450, specifically isoform CYP2E1. The first step is the hydroxylation of the methyl group of AOM to form methylazoxymethanol (MAM). Methylazoxymethanol then breaks down into formaldehyde and a highly reactive alkylating species, probably the methyldiazonium. This chemical actually causes alkylation of DNA guanine to O6-MEG and to O4-methylthymine (Chen and Huang, 2009). These mutations can initiate tumorigenesis through several key genes in intracellular signal pathways. The inhibition of CYP2E1 (for example by disulfiram, an agent used for avoidance therapy in alcohol abuse) has been shown to prevent chemical carcinogenesis (Chen and Huang, 2009). In CYP2E1 knockout mice, O6-MEG formation and colon polyp numbers decrease in response to AOM treatment (Chen and Huang, 2009).

#### 2.1.3.2. Mechanisms for AOM Causing Colon Cancer

Several activation pathways have been revealed to explain the mechanism of AOM-induced colon cancer (Figure 2.4). These include K-ras, β-catenin and TGFβ. However, there is no unified explanation for the mechanism of this model. K-ras is a small G-protein that regulates both MAPK and PI3K/Akt intracellular

signal pathways, which, in turn, regulate cell growth, proliferation and glucose metabolism. In K-ras pathway, K-ras plays an important role in the carcinogenesis of colon cancer. Azoxymethane has been shown to cause a K-ras gene transversion mutation from G:C to A:T at codon 12 deriving from O6-methyl-deoxyguanine adducts. It changes glycine to aspartic acid. This mutation causes the activation of the K-ras protein.

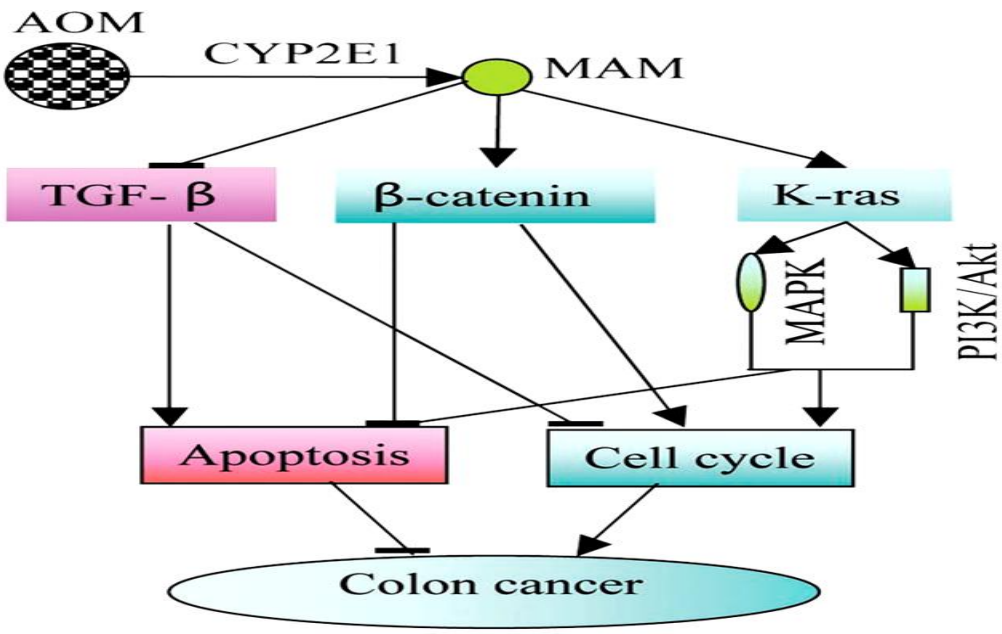


Figure 2.4 Azoxymethane (AOM) mechanism of colon cancer (Chen and Huang, 2009)

In fact, both pathways play important roles in the carcinogenesis of many types of cancers including colon cancer. This is similar to human colon cancer. A study showed that pEGFR, pAkt and pMAPK are increased in colon tumours compared to normal colon tissue (Messersmith *et al.*, 2005). The PI3K/Akt

pathway is important in colon cancer and 20% of patients have PIK3CA mutations(Messersmith *et al.,* 2005). The activation of PI3K/Akt can increase cell survival pathways via phosphorylation of downstream targets, including NFκB, and Bcl-xl. PI3K/ Akt also blocks p53 and the forkhead/Fas-ligand to decrease apoptosis (Messersmith *et al.*, 2005).

In the cell cycle pathway, PI3K/Akt deactivates glycogen synthase kinase 3 (GSK3) and promotes cyclin D1 and mycto increase cell proliferation. In the cell growth pathway,PI3K/Akt activates the mammalian target of rapamycin (mTOR),a conserved Ser/Thr kinase to increase cell size. Whether mToractivity is increased in the AOM model has not yet been elucidated (Messersmith *et al.*, 2005).Downstream of PI3K/Akt, COX2 has also been shown to beinvolved in the carcinogenesis of AOM (Messersmith *et al.*, 2005). Activated Ras stimulates the serine/threonine-selective protein kinase: Raf kinase, which is an oncogene. The protein encoded has regulatory and kinase domains. Ras binds to CR1 in there gulatoryregion and phosphorylates CR2, which is rich in serine/threonine. This leads to activation of CR3 in the kinase region. It then, in turn, activates MAPK and ERK kinase (MEK), which activates mitogen-activated protein kinase (MAPK) and ERK. MAPK and ERK promote carcinogenesis via target proteins like c-myc, CREB, RSK, Mcl1, p16, Rb and cyclins. Inhibition of these pathways has been demonstrated to cause cancer cell death (Messersmith *et al.*, 2005). Over expression of cell cycle promoters, cyclin D1, may contribute to the AOM model as well. Cdk4 has been detected in the early stages in the AOM cancer induced mouse colon.

**β-catenin pathway**, β-cateninplays an important role in cell adhesion and also is an oncogenic protein. It associates with cadherin or a-catenin to link the actin cytoskeleton. It is also a co-transcriptional activator of genes in the Wnt signal pathway.

In the free form, it associates with the scaffolding proteins, axin and Apc, and is phosphorylated by GSK-3β resulting in degradation by the proteasome(Messersmith *et al.*, 2005). The N-terminus of β-catenin is also mutated in some cases, so that β-catenin cannot form the complex and be degraded. Thus, free β-catenin is increased and binds with the T-cell factor/lymphoid enhancer factor TCF/LEF to form a complex, which activates gene transcription and cell proliferation. It targets *c-myc*and *cyclinD1* genes, which are well known carcinogens (Messersmith *et al.*, 2005).

Azoxymethane causes β-catenin mutations at codons 33 and 41, which are the serine and threonine residues that are targets for GSK-3β phosphorylation. This leads to the accumulation of β-catenin for the carcinogenesis. It has been shown that AOM treatment increases both β-catenin and cyclin D (Messersmith *et al.*, 2005).

**TGFβ pathway**. Transforming growth factor-β (TGFβ) including isoforms 1, 2 and 3 can inhibit cell growth, proliferation and the cell cycle progression and thus has an anti-tumour effect. Defects in TGFβ signalling have been found in 20–30% of colon cancer patients. The activity of the TGFβ pathway is decreased after AOM treatment, which mediates AOM-induced colon cancer. It has been

demonstrated that the active form of TGFβ is decreased in AOM treated mice (Messersmith *et al.*, 2005). Messersmith et al. (2005) cited that TGF-β induces apoptosis through several signal pathways. First, TGFβ forms dimers and binds to its type 2 receptor. This complex then associates and phosphorylates its type 1 receptor. The type 1 receptor in turn phosphorylates receptor-regulated SMAD (R-SMAD) to cause apoptosis. Second, the activated type 2 receptor binds to death associated protein 6 to induce apoptosis. Third, TGFβ has also been shown to inhibit the phosphorylation of the p85 subunit of PI3K/Akt induced by GM-CSF in several myeloid leukemia cell lines, including MV4-11, TF-1 and Tf-1a.

### 2.1.4 *Phyllanthus niruri* (gale of the wind)

*P. niruri* is an annual herbaceous plant that inhabits the tropical and subtropical regions of both hemispheres. Commonly found in coastal areas. It is a relative of the spurges, belonging to the leaf flower genus (*Phyllanthus)* of Family Euphorbiaceae. In terms of height, this plant grows 50 to 70 centimeters tall and bears ascending herbaceous branches, with smooth and light green bark (Figure 2.5). It bears numerous pale green flowers which are often flushed with red. The fruits are tiny, smooth capsules containing seeds (Wikipedia, 2010).

Figure 2.5 *Phyllanthus niruri* L. (Wikipedia, 2010)

Systematically according to the United State Department of Agriculture (USDA), Natural Resources Conservation Service; this plant is classified as follows:

Kingdom Plant – Plants

Subkingdom Tracheobionta – Vascular plants

Superdivision Spermatophyta – Seed plants

Division Magnoliophyta – Flowering plants

Class Magnoliopsida – Dicotyledons

Subclass Rosidae

Order Euphorbiales

Family Euphorbiaceae – Spurge family

Genus *Phyllanthus* L. – leafflower

Species *Phyllanthus niruri* L. – gale of the wind

This plant is popular in folk medicine, whole plant, fresh leaves and fruits are used in the treatment of various diseases, particularly hepatitis and other viral infection (Wang, 2000). The plant has long been used in Brazil and Peru as an herbal remedy for Kidney stones. Research among sufferers of Kidney stones has shown that, while intake of *P. niruri* didn't lead to a significant difference in either stone voiding or pain levels, it may reduce urinary calcium, a contributing factor to stone growth (Nishiura *et al.*, 2004). In addition, one study conducted on rats showed that an aqueous solution of *P. niruri* may inhibit kidney stone growth and formation in animals that already have stones (Freitas *et al.*, 2002).

The plant is of medicinal importance for numerous ailments like dysentery, influenza, vaginitis, tumors, diabetes, diuretics, jaundice, kidney stone, dyspepsia, anti-hepatotoxic, anti-hepatitis-B, anti-hyperglycemic and also as antiviral and antibacterial (Chopra *et al.*, 1986). Extracts of this herb have shown promise in treating a wide range of human diseases. Some of the medicinal properties suggested by numerous preclinical trials are anti-hepatotoxic, anti-lithic, anti-hypertensive, anti-HIV and anti-hepatitis B (Bagalkotkar *et al.*, 2006b).

*P. niruri* extract was shown to inhibit DNA polymerase of hepatitis B virus and related hepatitis viruses and the extract of this plant contains several bioactive molecules such as lignans, phyllanthin, hypophyllanthin, flavonoids, glycosides and tannins (Rajeshkumar *et al.*, 2002). *P. niruri* was used for treating liver ailment

and possess, antitumor antiviral, anticarcinogenic (Rajeshkumar *et al.*, 2002), antioxidant (Harish and Shivanandappa, 2006). Though some literatures highlighted the gastro-protective activities of some Phyllanthus species like Shokunbi and Odetola (2008) who reported the gastro-protective and antioxidant activities of *P. amurus*, and Abdulla *et al.* (2010) reported the gastro-protective activity of *P. niruri* for the first time.

Oxidative stress plays an important role in the pathogenesis of various diseases including gastric ulcer, with antioxidants being reported to play a significant role in the protection of gastric mucosa against various necrotic agents (Trivedi and Rawal, 2001). Studies on *Phyllanthus amarus* have showed the in vivo gastro-protective and antioxidant activities of acetone and aqueous gastric ulcer (Shokunbi and Odetola, 2008).

### 2.1.5. Antioxidants and lipid peroxidation assay

Lipid peroxidation assay refers to oxidative degradation of lipids (Fig. 2.6). It is the process whereby free radicals electrons from the lipids in the cell membranes, resulting in cell damage (Leonard, 2000). Peroxidation of lipids containing polyunsaturated fatty acids, in particular, impairs the structure of biological membranes and this has a significant role in pathogenesis of many diseases (Kuzu, 2007).

Antioxidants neutralize these free radicals and pair with the free radical changing them from harmful to non-harmful or actually repair the cell damage.

Phenolic antioxidant and their derivative have been known for a long period of time. They have widespread applications in pharmaceutical, textiles, plastics, polymers, oil, pesticides, dye stuffs, explosives, fluorescent brightener and many other industries (Cotelle, 2001).

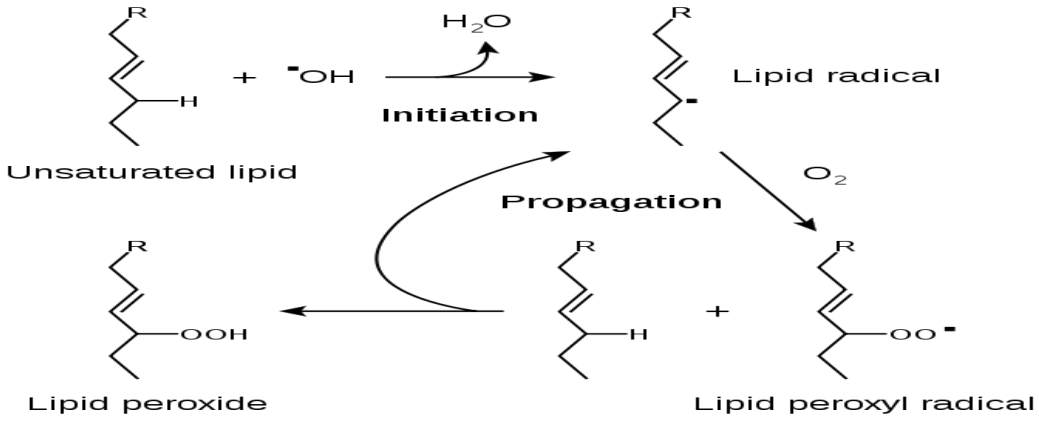


Figure 2.6 Lipid peroxidation mechanism (Wikipedia, 2010).

Malondialdehyde (MDA) was reported to be one of the major secondary oxidation products of peroxidized polyunsaturated fatty acids. It has been reported to have mutagenic and cytotoxic effects (Mateos, 2005). MDA has been found elevated in various diseases related to free radical damage. Hence, it has been spectrophometrically and used as a biomarker for the assessment of lipid peroxidation in biological and medical sciences (Mateos, 2005). It is simple, sensitive and inexpensive method to reflect the effect of oxidative stress on the levels of lipid peroxidation in cells; it has been adapted in biological samples, such as rat and human plasma, urine, other tissues organs like, liver and lung. Its feasibility as a biomarker for oxidative stress *in vivo* in a rat model has been reported (Mateos, 2005).

Therefore based on the above mentioned literatures, this study was undertaken in rats to evaluate for any chemoprotective properties of non-aqueous extracts of *P. niruri* against foci of aberrant crypts.