"Effect of *Euphoria longana* in Oral Cancer associated with type II Diabetes Mellitus"

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MASTER OF PHARMACY

IN

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BY

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CERTIFICATE

This is to certify that the dissertation work entitled "Effect of *Euphoria longana* extract in Oral Cancer associated in Type II Diabetes Mellitus" *submitted by Ms. LATIKA JOSHI with Regn. No. (14MPH204) in partial fulfillment for the award of Master of Pharmacy in "Pharmacology" is a bonafide research work carried out by the candidate at the Department of Pharmacology, Institute of Pharmacy, Nirma University under my guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.*

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DECLARATION

I hereby declare that the dissertation entitled "Effect of *Euphoria longana* extract in oral cancer associated in Type II Diabetes Mellitus"*, is based on the original work carried out by me under the guidance of Dr. Jigna S. Shah, Professor, Department of Pharmacology, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.*

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ABBREVIATION

HPV	Human papilloma virus
SCCs	Squamous cell carcinomas
HNCs	Head and neck cancer
IARC	International Agency for Research on cancer
PGE-2	Prostaglandin E2
NSAIDs	Non-steroidal anti-inflammatory drugs
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
TGF-β	Transforming growth factor
TNF-α	Tumour necrosis factor
PDEGF	Platelet-derived endothelial growth factor
EGF	Epidermal growth factor
FdUMP	Fluorodeoxyuridine monophosphate
DPD	Dihydropyrimidine dehydrogenase
NF-kB	Nuclear Factor kB
HUVECs	Human umbilical vein endothelial cells

ABSTRACT

Background and Objective

Oral cancer is the eighth most frequently occurring subtype of head and neck cancer worldwide. Several immunologic and metabolic changes occur in the oral mucosa due to Diabetes mellitus. There is a relationship between diabetes and oral mucosa like immunological and periodontal disease. The diabetic patients have precancerous lesions like erythroplakia and leukoplakia more than non diabetic patients. Diabetes also has the influence on microenvironment of the oral cavity and creates complications of oral mucosa. Diabetes is characterized by hyperglycemia which leads to production of reactive oxygen species, increasing the oxidative stress. This causes the modification in the lipids, proteins and DNA. Along with these mechanisms, in type II diabetes there is a condition of hyperinsulinemia due to which there is increased production of Insulin like receptor, insulin signaling and the insulin mitogenic pathway remains unregulated. These all mechanisms are responsible for initiation and progression of the cancer. The insulin levels are elevated in diabetes type II which increases concentrations of insulin-like growth factor-1(IGF-1). Elevation of these stimulates the proliferation of cells, inhibit apoptosis and increase the mitogenesis in cell lines. High level of insulin in the body also triggers the release of several pro-inflammatory cytokines, including interleukin-6 and tumor necrosis factor-alpha. These cytokines ultimately leads to inflammation and leads to malignancies of oral cavity.

Dimocarpus Longan (*Euphoria longana*) is a fruit of subtropical climate and belongs to the lychee family, Sapindaceae. The seeds of longana contain high amount of polyphenols as compared to the fruit. The major polyphenols are gallic acid, corilagin and ellagic acid. The longan is recognized for various pharmacological activities due to the huge amount of polyphenols present in the fruit that exhibit antioxidant properties. The longan seeds have been reported to have anti-MMPs activity as well as DPPH scavenging Activity. The gallic acid and ellagic acid possess activities such as antiplasmodial, antimicrobial, antioxidant and anticancer by inhibiting nuclear factor kappa-B activation. Corilagin have been reported to exert its action on TGF-β/AKT/ERK/Smad signaling pathway and has a potential anti-inflammatory activity as it inhibits the NF-kappa B resulting in reduction of pro-inflammatory cytokines and

mediators like TNF-alpha, IL-1 beta, IL-6, NO (iNOS) and COX-2. The longana fruit refresh the heart and spleen, nourish the blood, keeps the nervous system calm, cure stomachache, act as a febrifuge and antidote for poison. It also possesses pharmacological properties like, minimizing blood vessels constriction, radical scavenging activities, used in insomnia, acariasis, hernia, hemorrhages, eczema, scrofula, fatigue, anticancer (inhibits growth of colorectal carcinoma cells), and hypoglycemic.

Although Euphoria longana possess anticancer, hypoglycemic and antidiabetic activities but no reports are available on oral cancer associated with type II diabetes mellitus. Hence, based on the above mentioned facts, the objective of the present study was to study the effect of *Euphoria longana* on oral cancer associated with type II diabetes mellitus and to investigate its mechanism of action.

Materials and Methods

1. Preparation of *Euphoria longana* extract

The dried seeds of *Euphoria longana* were crushed and the powder was defatted followed by hot water extraction.

2. Pharmacological studies

4- NQO induced oral cancer in rats

The male Wistar rats were divided into six groups, with group I normal control (NC), group II Oral cancer disease control (OC) in which oral cancer was induced by applying 4- Nitroquinoloine oxide (0.5% in Propylene glycol) thrice a week on rat tongue using a paint brush of size 4 for a duration of 24 weeks. Group III was provided treatment with the *Euphoria longana* extract from day 0 (OCEL0) as a preventive therapy to oral cancer. Group IV, V and VI were treated with *Euphoria longana* extract (OCEL24), 5-fluorouracil (OCFU) and the combination of both *Euphoria longana* extract and 5-fluorouracil (OCELFU) respectively for 5 weeks after induction of 24 weeks with oral cancer.

4- NQO induced oral cancer in Diabetic rats

The male Wistar rats were divided into eight groups, with group I as normal control. Group II was diabetic control (DM) in which type II diabetes mellitus was induced using a High Fat Diet model followed by a single dose Streptozotocin injection. Group

III was diabetic oral cancer control in which oral cancer was induced after induction of diabetes, using 4- NQO oral cancer induction model for 12 weeks of duration. Group IV and V were only diabetes induced groups treated with *Euphoria longana* and standard Metformin respectively after 12 weeks of oral cancer induction for 4 weeks. Group VI, VII and VIII were diabetic oral cancer induced groups treated with *Euphoria longana* alone, standard therapy of Metformin with 5 fluorouracil and combined therapy of *Euphoria longana* with Metformin with 5 fluorouracil respectively after 12 weeks of oral cancer induction for 4 weeks. At the end of 4weeks of treatment, the glycemic parameters were measured and the animals were sacrificed for various other estimations.

Results

1. Phytochemical Tests

The phytochemical analysis confirmed the presence of flavonoid, carbohydrates, tannins, saponins and polyphenols.

2. Qualitative test

The thin layer chromatography performed for the extract revealed the presence of gallic acid, ellagic acid and corilagin in the extract.

3. Evaluation Parameters

Induction of oral cancer using 4- NQO for duration of 24 weeks developed tumor on the tongue of rats which was depicted as carcinoma in situ in histological examination. Moreover oral cancer induced in diabetic rats for 12 weeks resulted in more progression of oral cancer than in only oral cancer induced animals.

The oxidative stress was evaluated by measuring the levels of levels of MDA, GSH and SOD in the tongue tissues. The oxidative stress was significantly increased in diseased animals as compared to the normal control group of animals. The treatment showed reduction in the oxidative stress significantly as compared to the disease control groups. Moreover the combined therapy was found more effective than the treatments given alone. Also the pretreatment with *Euphoria longana* showed protective effect for oral cancer. The glycemic parameters like measurement of Glucose, glycosylated Hb and OGTT were performed and the treatment showed reduction in the glucose and HbA1c levels and improved the oral glucose tolerance in the body. Also the level of

inflammatory marker, IL-6 were measured which was significantly found elevated in the disease control groups and the treatment showed reduction in the IL-6 levels effectively. The histopathological examinations revealed the development and progression of cancer in the tongue of disease control animals where the treatment showed to improve those precancerous and cancerous conditions.

Further studies are warranted to elucidate the mechanism of action of *Euphoria longana* in oral cancer and diabetic oral cancer with special reference to TNFα, NFκB and IGF-1.

Conclusion

- Our study reveals that diabetes mellitus type II is a risk factor for development of oral cancer which might be due to the enhanced oxidative stress in diabetic condition that leads to the progression of cancer.
- The treatment with *Euphoria longana* showed protective effect in oral cancer and the combination of *Euphoria longana* with other chemotherapeutic agent might be beneficial to improve the diseased condition than using a chemotherapeutic agent alone.
- Also in the animals induced with diabetes as well as oral cancer, the combined treatment has reduced the progression of disease as compared to the treatment given alone. Hence this might prove to contribute for beneficial and therapeutic approach in the treatment of oral cancer associated with diabetes mellitus type II.

INTRODUCTION

Oral cancer is the eighth most frequently occurring subtype of head and neck cancer worldwide [1] which includes the lips, buccal mucosa, the gums, the floor of the mouth below the tongue, the teeth, the front two-thirds of the tongue, and the hard palate; though retromolar trigone is usually considered as a part of oropharynx but can be included as a part of the oral cavity also. The various types of tumors that can develop in the oral cavity are divided into three categories, benign or non-cancerous, precancerous and cancerous tumors. Benign tumors include eosinophilic granuloma, fibroma, granular cell tumor, keratoacanthoma, leiomyoma, osteochondroma, lipoma, schwannoma, neurofibroma, papilloma, condyloma acuminatum, verruciform xanthoma, pyogenic granuloma, rhabdomyoma, odontogenic tumors. In precancerous conditions, the tumors' growth is harmless but later cancer can develop, like leukoplakia (gray or white patch) and erythroplakia (red patch). Cancerous tumors are those which can grow into the surrounding tissues and also spread to other body parts. The cancerous tumours are of several types including squamous cell carcinomas, verrucous carcinoma, minor salivary gland carcinomas and lymphomas. More than 90% of oral cancer is Squamous cell carcinomas [2]. The symptoms include a bleeding or non bleeding lesion, a lump, a red or white patch, or thickened or inflamed throat or mouth; a mass on neck; ear pain; or coughing up blood. The treatments for oral cancer are surgery and radiation therapy in combination or alone; for advanced disease chemotherapy or targeted therapy are also combined to radiotherapy[3].

A total number of 34,780 males and 13,550 females are estimated for the new cases to suffer from oral cancer; whereas 6,910 males and 2,660 females are estimated for death in 2016 due to oral cancer. The people suffering from oral cavity and pharynx cancer have 63% and 52% survival rates for 5- and 10-years respectively[3]. Oral cancer is highly prevalent in South and southeast parts of Asia which includes India with 90-95% oral cancers occurring as Oral Squamous cell carcinomas (OSCC) or Epidermoid carcinoma. According to a prediction of an International Agency for Research on Cancer, the incidence of cancer in India would increase from the year 2012 to 2035, as 1 million to 1.7 million respectively and the death rate would also increase from 680000 to 1-2 million during the same period. There are 20 in 100000 populations suffering from oral cancer in India which is about 30% of all cancer and about 5 people die due to oral cancer every hour per day[4].

Oral carcinogenesis involves multiple stages for its progression. There are various oncogenes and tumor suppressor genes which if over expressed, altered or mutated, lead to their enhanced function, deactivation or functions opposite to its actual. So there are multiple pathways which get altered in precancerous condition leading to cancerous condition causing changes in cell proliferation, angiogenesis[5]. Inactivation of tumor suppressive genes like p16 and p53, and overexpression of oncogenes like *PRAD1*, *H-ras* and several others lead to uncontrollable growth of cancer. *H-ras* is one of the genes responsible for cell signaling of EGFR. The major risk factors are smoke or smokeless tobacco use, excessive alcohol consumption and human papillomavirus (HPV) infection[3]. The alcohol consumed is converted into

acetaldehyde by an enzyme alcohol dehydrogenase, this acetaldehyde is cytotoxic as it produces free radicals and hydroxylated bases of DNA. The tobacco smoke creates free radicals which interacts with acive metals present in saliva and thus the anti oxidant activity of saliva is lost (29).

There are many signaling pathways that are altered in cancerous condition which causes the proliferation and differentiation in the cells and thus changes the morphology of the cancer cells. Epidermal Growth Factor Receptor (EGFR) is the most prominent oncogene which signals through the tyrosine kinase cascade and hence leads to downstream signaling which is ultimately associated with oral squamous cell carcinogenesis. The overexpression of this oncogene causes cellular proliferation, invasion and inhibition of apoptosis, angiogenesis and metastasis. Once this type I receptor tyrosine kinases or ErbB tyrosine kinase receptor get phosphorylated, it can signal via MAPK, Akt, ERK, and Jak/STAT pathways. The upregulation of EGFR and TGF-α cause dysplastic changes in the oral epithelium[5]. The activation of PI3K-AKT signal pathway is also found responsible for oral precancerous lesion which is due to the genetic mutation in PIK3CA gene[6].

Most invasive oral carcinomas are preceded by a preinvasive stage, which may last for many years. Tumor progression in epithelia has been classified as normal, hyperplastic (non-dysplastic), dysplastic carcinoma in situ and invasive carcinoma. The majority of the initial alterations of precancerous and cancerous oral lesions are not readily recognizable, on clinical or histopathological examinations. There are genetic alterations in oncogene or the tumour suppressor genes, or genomic instability, or epigenetic modifications in the genes which are responsible for altered physiology of the body cells. The cells become hypoxic and in that stress condition they release various inflammatory cytokines and pro- inflammatory mediators like TNF-1α, IL-1β, IL-6, HIF-1α, and COX-2, hence causes chornic inflammation in the tissues. These mediators cause degradation of extracellular matrix (ECM) which is the microenvironment of the cell and is regulated by Matrix Metalloproteinase (MMPs). These mediators are responsible for the overexpression of MMP due to which the ECM is degraded. Also due to chronic inflammation, neoplasia occurs that causes increased levels of VEGF, cell proliferation and angiogenesis. Nuclear factor kappa-B (NF-κB) is a transcription factor for the MMP production which gets activated by high levels of proinflammatory mediators like TNF-1^{α} by phosphorylating NF-κB p65 at S536. The overexpression of this transcription factor and NF-κB kinase are related to metastasis, invasiveness and antiapoptotic activity. Thus the dysregulation of NF-κB lead to inflammation, cancer and autoimmune diseases. The growth factors are increased due to cell proliferation and tumour formation[6]. The tumour growth factors (TGF) are over expressed where Epidermal Growth factors (EGF), VEGF and TGF-β are very specific to Oral cancer.

Diabetes is a chronic, metabolic disorder in which the blood glucose levels get elevated which causes to serious damage to the heart, blood vessels, eyes, kidneys, and nerves as the disease progresses. Diabetes is categorized into two types, Type I and Type II where Type I is due to reduced secretion of insulin, and Type II is due to the resistance towards the insulin and

impaired response to insulin by the body[7]. Type2 diabetes is the most common occurring mostly to adults characterized by increased insulin resistance in the body or the production of insulin in the body is decreased. The prevalence of Diabetes has raised dramatically over the past 3 decades globally. Type 1 diabetes which is insulin-dependent and also known as juvenile diabetes is a condition when the pancreas is not able to produce insulin by itself or very negligible amount of insulin. About 422 million people are suffering from diabetes, 1.5 million people dies each year due to diabetes are directly attributed to diabetes each year and 1 in 3 adults having an age over 18 years is overweight and 1 in 10 is obese worldwide. A target has been agreed globally to halt the rise of both diabetes and obesity by 2025. The mission of WHO Diabetes Programme is to prevent type 2 diabetes with minimum complications and maximum quality of life of all the diabetics (30).

Diabetes of both the types causes hyperglycemia, increased water intake, weight loss, excessive urine output, blurred vision, lethargy and changes in energy metabolism. Diabetes after a long duration leads to various complications such as atherosclerosis, nephropathy neuropathy and heart disease. One of the most common causes of death due to diabetes is cancer. Oral cancer accounts for approximately 4-5% of all cancers in the world. The most commonly affected are adult males, especially those who have a habit of alcoholic consumption and smokers of sixth and eighth decades of their lives. Reports suggest that the cancer patients having diabetes have worse prognosis than the non diabetics. The studies have shown that the risk of cancer is more in diabetes type II as compared to type I. The relationship between diabetes and cancer was first studied in 1855. In India, there are 25.6% and 31.3% diabetics type I and type II patients respectively having Glossitis & chronic Cheilitis. Out of these, 10.9% and 25.6% of type I and type II are suffering from Benign tumor. Further 3.2% and 11 % of type I and II patients among these are having oral manifestations like leukoplakia & erythroplakia. Gingival cancer (29%) and lip cancer (24%) are more prevalent in male diabetic patients than female which is due to smoking and drinking habits in male population.

Several immunologic and metabolic changes occur in the oral mucosa due to Diabetes mellitus. There is a relationship between diabetes and oral mucosa like immunological and periodontal disease. The diabetic patients have precancerous lesions like erythroplakia and leukoplakia more than non diabetic patients. Almost 90% of systemic metabolic disorders lead to oral mucosal damage. Diabetes also has the influence on microenvironment of the oral cavity and creates complications of oral mucosa like lesions in the mucosa and tongue, change in saliva composition, Xerostomia, osteolytic lesions, odontogenic abscess, granulomas, Gingivitis and periodontitis and loss of teeth. Diabetes is characterized by hyperglycemia which leads to production of reactive oxygen species, increasing the oxidative stress. This causes the modification in the lipids, proteins and DNA. Along with these mechanisms, in type II diabetes there is a condition of Hyperinsulinemia due to which there is increased production of Insulin like receptor, also insulin signaling and insulin mitogenic pathway remains unregulated. These all mechanisms are responsible for intiation and progression of the cancer.[7] The hypothesized biological mechanisms is related to the effect of insulin and insulin-like growth factors (IGFs)

axis, which would trigger intracellular signaling cascades with mitogenic and antiapoptotic effects. The insulin levels are elevated in diabetes type II which increases concentrations of insulin-like growth factor-1(IGF-1). Elevation of these both stimulates the growth of cell by proliferation of the cells, apoptosis inhibition and increasing the mitogenesis in cell lines. High level of insulin in the body also triggers the release of several pro-inflammatory cytokines, including interleukin-6 and tumor necrosis factor-alpha. These cytokines ultimately leads to inflammation and leads to malignancies of oral cavity.[1]

Currently there are several drugs available for the treatment of oral cancer and Diabetes mellitus. Treatment for oral cancer is surgery, radiotherapy, chemotherapy, targeted therapy and palliative therapies. The chemo drugs most often used for oral cancer are Cisplatin, Carboplatin, 5fluorouracil (5FU), Paclitaxel (Taxol®) and Docetaxel (Taxotere®). Few drugs are there which are also used for oral cancer but less often, they are Methotrexate, Ifosfamide (Ifex®) and Bleomycin. A combined therapy of a chemo drug with some other drug can be used to treat oral cancer. Most frequently used combination is cisplatin and 5FU. Surgery includes tumor resection, mohs micrographic surgery (cancers of the lip), glossectomy (removal of the tongue), mandibulectomy (removal of the jaw bone), maxillectomy, robotic surgery, reconstructive surgery and neck dissection. Radiation therapy uses X rays or particles of high energy to slow down the growth of cancer cells or destroy them. Radiation therapy includes external beam radiation therapy and brachytherapy. Targeted drug therapy is a special type of chemotherapy which has an advantage of small differences between normal cells and cancer cells. Targeted therapy can be used alone or in combination with other treatments. There are differen types of targeted therapies which include Signal transduction inhibitors like EGFR inhibitors (cetuximab and erlotinib), *HER2 inhibitors* (trastuzumab and pertuzumab), *BCRABL inhibitors* (imatinib and dasatinib), *ALK inhibitors* (crizotinib and ceritinib, *BRAF inhibitors* (vemurafenib and dabrafenib); angiogenesis inhibitors like bevacizumab and ramucirumab); Apoptosis inducing drugs like *proteasome inhibitors*, such as bortezomib and carfilzomib; Immunotherapy drugs such as pembrolizumab and ipilimumab; monoclonal antibodies attached to toxins like brentuximab vedotin and adotrastuzumab emtansine.[2]

Treatment for diabetes includes the drugs like biguanides sulphonylureas, thiazolidinones (TZDs) and Dipeptidyl peptidase-4 (DPP-4) inhibitors. Biguanides act by inhibiting the glucose production in liver, as it enhances the disposal of glucose. The widely used drug is metformin which reduces the glucogenesis, increase the insulin sensivity and uptake of glucose by peripheral tissues. It also has shown anticancer effects by inhibiting the phosphorylation of IGD, Akt and extracellular signaling pathway. Sulfonylureas (insulin secretatogogues), thiazolidinones (Peroxisome proliferator-activator receptors) and Dipeptidyl peptidases inhibitors (gliptins) (31).

Dimocarpus Longan (*Euphoria longana*) is a fruit of subtropical climate and belongs to the lychee family, Sapindaceae. The seeds of longana contain high amount of polyphenols as compared to the fruit. There are 11 known phenolic components present in the seed, they are gallic acid, corilagin, ellagic acid methyl gallate, 4-O-a-L-arabinofuranosirde, an A-type

proanthocyanidin trimer, isomallotinic acid, carboxylate, methyl brevifolin, geraniin, chebulagic acid and (-)-epicatechin. Out of these the major ones are gallic acid, corilagin and ellagic acid[8].

The longan is recognized for various pharmacological activities due to the huge amount of polyphenols present in the fruit that exhibit antioxidant properties. The longan seeds have been reported to have anti-MMPs activity as well as DPPH scavenging activity[9]. The gallic acid and ellagic acid possess activities such as antiplasmodial, antimicrobial, antioxidant and anticancer by inhibiting nuclear factor kappa-B activation. Corilagin have been reported to exert its action on TGF-β/AKT/ERK/Smad signaling pathway and provide the therapeutic benefit by reducing the growth of ovarian cancer cells. Some in-vitro cell line studies have suggested that corilagin arrest the growth of cell at G2/M phase[10]. Also it has a potential anti-inflammatory activity as it inhibits the NF-kappa B nuclear translocation at genetic and protein levels resulting in reduction of pro-inflammatory cytokines and mediators like TNF-alpha, IL-1 beta, IL-6, NO (iNOS) and COX-2. The longana fruit refresh the heart and spleen, nourish the blood, keeps the nervous system calm, cure stomachache, act as a febrifuge and antidote for poison. It also possesses pharmacological properties like, minimizing blood vessels constriction, radical scavenging activities, used in insomnia, acariasis, hernia, hemorrhages, eczema, scrofula, fatigue, anticancer (inhibits growth of colorectal carcinoma cells), and hypoglycemic[11].

Although *Euphoria longana* possess anticancer, hypoglycemic and antidiabetic activities but no reports are available on oral cancer associated with type II diabetes mellitus. Hence, based on the above mentioned facts, the objective of the present study is to study the effect of Euphoria longana on oral cancer associated with type II diabetes mellitus and to investigate the mechanism of action.

3. REVIEW OF LITERATURE

3.1 Introduction to oral cancer

Oral cancer is the eighth most frequently occurring subtype of head and neck cancer worldwide [1] which includes the lips, buccal mucosa, the gums, the floor of the mouth below the tongue, the teeth, the front two-thirds of the tongue, and the hard palate; though retromolar trigone is usually considered as a part of oropharynx but can be included as a part of the oral cavity also. Leukoplakia and erythroplakia are terms used to describe certain types of abnormal tissue that can be seen in the mouth or throat. Leukoplakia is a white or gray patch and Erythroplakia is a flat or slightly raised, red area that often bleeds easily if it is scraped. Erythroleukoplakia is a patch with both red and white areas. Dysplasia is graded as mild, moderate, or severe, based on how abnormal the tissue looks under the microscope. Knowing the degree of dysplasia helps predict how likely it is to progress to cancer or to go away on its own or after treatment. More than 9 of 10 cancers of the oral cavity and oropharynx are squamous cell carcinomas, also called *squamous cell cancers*. These cancers begin in early forms of squamous cells, which are flat, scale-like cells that normally form the lining of the mouth and throat. The earliest form of squamous cell cancer is called *carcinoma in situ,* meaning that the cancer cells are present only in the outer layer of cells called the *epithelium*. This is different from invasive squamous cell carcinoma, where the cancer cells have grown into deeper layers of the oral cavity or oropharynx.

3.2 Risk factors for Oral Cancer

A risk factor is anything that changes a person's chance of getting a disease such as cancer. Different cancers have different risk factors.

a) Use of Tobacco

The risk of tobacco for oral cancer depends on duration for which the tobacco is in contact with oral cavity either by chewing or by smoking. "Smokers are many times more likely than non-smokers to develop these cancers. Tobacco smoke from cigarettes, cigars, or pipes can cause cancers anywhere in the mouth or throat, as well as causing cancers of the larynx (voice box), lungs, esophagus, kidneys, bladder, and several other organs. Pipe smoking is a particularly significant risk for cancers in the area of the lips that touch the pipe stem. Oral tobacco products (snuff or chewing tobacco) are linked with cancers of the cheek, gums, and inner surface of the lips." Using oral tobacco products for a long time poses an especially high risk. The tobacco smoke creates free radicals which interacts with active metals present in saliva and thus the anti oxidant activity of saliva is lost (29).

(b) Drinking alcohol

Drinking alcohol increases the risk of developing oral cavity and oropharyngeal cancers. About 7 out of 10 patients with oral cancer are heavy drinkers. [2] The alcohol consumed is converted into acetaldehyde by an enzyme alcohol dehydrogenase, this acetaldehyde is cytotoxic as it produces free radicals and hydroxylated bases of DNA. The risk of these cancers is even higher

in people who both smoke and drink alcohol, with the highest risk in heavy smokers and drinkers.

(c) Betel quid and gutka

In Southeast Asia, South Asia, and certain other areas of the world, many people chew betel quid, which is made up of areca nut and lime wrapped in a betel leaf. Many people in these areas also chew gutka, a mixture of betel quid and tobacco. People who chew betel quid or gutka have an increased risk of cancer of the mouth.

(d) Human papilloma virus (HPV) infection

Human papilloma virus (HPV) is a group of more than 150 types of viruses. They are called *papilloma viruses* because some of them cause a type of growth called a papilloma. Papillomas are not cancers, and are more commonly called *warts*. Infection with certain types of HPV can also cause some forms of cancer, including cancers of the penis, cervix, vulva, vagina, anus, and throat. Other types of HPV cause warts in different parts of the body. HPV can be passed from one person to another during skin-to-skin contact. One way HPV is spread is through sex, including vaginal and anal intercourse and even oral sex. HPV types are given numbers. The type linked to throat cancer (including cancer of the oropharynx) is HPV16. Most people with HPV infections of the mouth and throat have no symptoms, and only a very small percentage develop oropharyngeal cancer. Oral HPV infection is more common in men than in women.

(e) Gender

Oral and oropharyngeal cancers are about twice as common in men as in women. This might be because men have been more likely to use tobacco and alcohol in the past. This is changing, but the recent rise in HPV-linked cancers has been mainly among younger men, so it is still likely to occur more often in men in the near future.

(f) Age

Cancers of the oral cavity and oropharynx usually take many years to develop, so they are not common in young people. Most patients with these cancers are older than 55 when the cancers are first found. But this may be changing as HPV-linked cancers become more common. People with cancers linked to HPV infection tend to be younger.

(g) Ultraviolet (UV) light

Sunlight is the main source of UV light for most people. Cancers of the lip are more common in people who have outdoor jobs where they are exposed to sunlight for long periods of time.

(h) Poor nutrition

Several studies have found that a diet low in fruits and vegetables is linked with an increased risk of cancers of the oral cavity and oropharynx.

(i) Weakened immune system

Oral cavity and oropharyngeal cancers are more common in people who have a weak immune system. A weak immune system can be caused by certain diseases present at birth, the acquired immunodeficiency syndrome (AIDS), and certain medicines (such as those given after organ transplants).

(j) Graft-versus-host disease

Graft-versus-host disease (GVHD) is a condition that sometimes occurs after a stem cell transplant. During this medical procedure, blood stem cells from a donor are used to replace bone marrow that has been destroyed by disease, chemotherapy, or radiation. GVHD occurs when the donor stem cells recognize the patient's cells as foreign and launch an attack against them. GVHD can affect many tissues of the body, including those in the mouth. This increases the risk of oral cancer, which can occur as early as 2 years after GVHD.

(k) Genetic syndromes

People with certain syndromes caused by inherited defects (mutations) in certain genes have a very high risk of mouth and throat cancer. Fanconi anemia is a condition that can be caused by inherited defects in several genes that contribute to repair of DNA. People with this syndrome often have blood problems at an early age, which may lead to leukemia or aplastic anemia. They also have a very high risk of cancer of the mouth and throat. Dyskeratosis congenita is a genetic syndrome that can cause aplastic anemia, skin rashes, and abnormal fingernails and toenails. People with this syndrome also have a very high risk of developing cancer of the mouth and throat at an early age.

3.3 Controversial Risk factors

(a) Mouthwash

Some studies have suggested that mouthwash with high alcohol content might be linked to a higher risk of oral and oropharyngeal cancers. But recent research has questioned these results. Studying this possible link is complicated by the fact that smokers and frequent drinkers (who already have an increased risk of these cancers) are more likely to use mouthwash than people who neither smoke nor drink.

(b) Irritation from dentures

It has been suggested that long-term irritation of the lining of the mouth caused by poorly fitting dentures is a risk factor for oral cancer. But many studies have found no increased risk in denture wearers overall. Poorly fitting dentures can tend to trap agents that have been proven to cause oral cancer, such as alcohol and tobacco particles, so denture wearers should have them checked by a dentist regularly to ensure a good fit. All denture wearers should remove their dentures at night and clean and rinse them thoroughly every day.

3.4 Cause of Oral cancer

Tobacco or heavy alcohol use may cause these cancers by damaging the DNA of cells that line the inside of the mouth and throat. DNA is the chemical in each cell that makes up our *genes*. However, DNA affects more than how we look. Some genes called protooncogenes can help control when cells grow and divide. DNA changes can change these into genes that promote cell division that are called *oncogenes*. Some genes that slow down cell division or make cells die at the right time and are called *tumor suppressor genes*. DNA changes can turn off tumor suppressor genes, and lead to cells growing out of control. Cancers can be caused by DNA changes that create oncogenes or turn off tumor suppressor genes. When tobacco and alcohol damage the cells lining the mouth and throat, the cells in this layer must grow more rapidly to repair this damage. The more often cells need to divide, the more chances there are for them to make mistakes when copying their DNA, which may increase their chances of becoming cancerous. Many of the chemicals found in tobacco can damage DNA directly.

3.5 Pathology of Oral Cancer

Oral carcinogenesis involves multiple stages for its progression. There are various oncogenes and tumor suppressor genes which if over expressed, altered or mutated, lead to their enhanced function, deactivation or functions opposite to its actual. So there are multiple pathways which get altered in precancerous condition leading to cancerous condition causing changes in cell proliferation, angiogenesis[5]. Inactivation of tumor suppressive genes like p16 and p53, and overexpression of oncogenes like *PRAD1*, *H-ras* and several others lead to uncontrollable growth of cancer. *H-ras* is one of the genes responsible for cell signaling of EGFR (29). The major risk factors are smoke or smokeless tobacco use, excessive alcohol consumption and human papillomavirus (HPV) infection[3]. The alcohol consumed is converted into acetaldehyde by an enzyme alcohol dehydrogenase, this acetaldehyde is cytotoxic as it produces free radicals and hydroxylated bases of DNA. The tobacco smoke creates free radicals which interacts with acive metals present in saliva and thus the anti oxidant activity of saliva is lost (29).

There are many signaling pathways that are altered in cancerous condition which causes the proliferation and differentiation in the cells and thus changes the morphology of the cancer cells. Epidermal Growth Factor Receptor (EGFR) is the most prominent oncogene which signals through the tyrosine kinase cascade and hence leads to downstream signaling which is ultimately associated with oral squamous cell carcinogenesis. The overexpression of this oncogene causes cellular proliferation, invasion and inhibition of apoptosis, angiogenesis and metastasis. Once this type I receptor tyrosine kinases or ErbB tyrosine kinase receptor get phosphorylated, it can signal via MAPK, Akt, ERK, and Jak/STAT pathways. The upregulation of EGFR and TGF-α cause dysplastic changes in the oral epithelium[5]. The activation of PI3K-AKT signal pathway is also found responsible for oral precancerous lesion which is due to the genetic mutation in PIK3CA gene[6].

Most invasive oral carcinomas are preceded by a preinvasive stage, which may last for many years. Tumor progression in epithelia has been classified as normal, hyperplastic (non-dysplastic), dysplastic carcinoma in situ and invasive carcinoma. The majority of the initial alterations of precancerous and cancerous oral lesions are not readily recognizable, on clinical or histopathological examinations. There are genetic alterations in oncogene or the tumour suppressor genes, or genomic instability, or epigenetic modifications in the genes which are responsible for altered physiology of the body cells. The cells become hypoxic and in that stress condition they release various inflammatory cytokines and pro- inflammatory mediators like TNF-1α, IL-1β, IL-6, HIF-1α, and COX-2, hence causes chornic inflammation in the tissues. These mediators cause degradation of extracellular matrix (ECM) which is the microenvironment of the cell and is regulated by Matrix Metalloproteinase (MMPs). These mediators are responsible for the overexpression of MMP due to which the ECM is degraded. Also due to chronic inflammation, neoplasia occurs that causes increased levels of VEGF, cell proliferation and angiogenesis. Nuclear factor kappa-B (NF-κB) is a transcription factor for the MMP production which gets activated by high levels of proinflammatory mediators like TNF-1^{α} by phosphorylating NF-κB p65 at S536. The overexpression of this transcription factor and NF-κB kinase are related to metastasis, invasiveness and antiapoptotic activity. Thus the dysregulation of NF-κB lead to inflammation, cancer and autoimmune diseases. The growth factors are increased due to cell proliferation and tumour formation[6]. The tumour growth factors (TGF) are over expressed where Epidermal Growth factors (EGF), VEGF and TGF-β are very specific to Oral cancer.

3.6 Signs and symptoms of oral cavity cancer

· The most common symptom is a sore in the mouth that does not heal and pain in the mouth.
· A lump or thickening in the cheek.
· A white or red patch on the gums, tongue, tonsil, or lining of mouth.
· A sore throat or a feeling that something is caught in the throat.
· Trouble chewing or swallowing
· Trouble moving the jaw or tongue
· Numbness of the tongue or other area of the mouth
· Swelling of the jaw that causes dentures to fit poorly or become uncomfortable
· Loosening of the teeth or pain around the teeth or jaw
· Voice changes
· A lump or mass in the neck
· Weight loss
· Constant bad breath

3.7 Stages of oral cancer

Staging of cancer is useful for treatment, planning, prognosis and comparison of the outcomes of the treatment. On diagnosis, all oral cancers are clinically staged using the

TNM (tumor, node, and metastasis) classification system. The T refers to the size of primary neoplasm, N refers to the extent of lymph node involvement and M refers to the presence of metastasis. The identification of oral cancer at stages I and II in comparison to cancer at stages III and IV is an important determination for planning of the treatment. There are 56 recent technology such as computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography which are useful in further determination of tumor size, nodal involvement and dissemination. The major limitation of the TNM classification system is that it is unable to account for the unique biological behavior of a given oral cancer.

3.8 Treatment for Oral cancer

Treatment for oral cancer is surgery, radiotherapy, chemotherapy, targeted therapy and palliative therapies. The chemo drugs most often used for oral cancer are Cisplatin, Carboplatin, 5fluorouracil (5FU), Paclitaxel (Taxol®) and Docetaxel (Taxotere®). Few drugs are there which are also used for oral cancer but less often, they are Methotrexate, Ifosfamide (Ifex®) and Bleomycin. A combined therapy of a chemo drug with some other drug can be used to treat oral cancer. Most frequently used combination is cisplatin and 5FU. Surgery includes tumor resection, mohs micrographic surgery (cancers of the lip), glossectomy (removal of the tongue), mandibulectomy (removal of the jaw bone), maxillectomy, robotic surgery, reconstructive surgery and neck dissection. Radiation therapy uses X rays or particles of high energy to slow down the growth of cancer cells or destroy them. Radiation therapy includes external beam radiation therapy and brachytherapy. Targeted drug therapy is a special type of chemotherapy which has an advantage of small differences between normal cells and cancer cells. Targeted therapy can be used alone or in combination with other treatments. There are differen types of targeted therapies which include Signal transduction inhibitors like EGFR inhibitors (cetuximab and erlotinib), *HER2 inhibitors* (trastuzumab and pertuzumab), *BCRABL inhibitors* (imatinib and dasatinib), *ALK inhibitors* (crizotinib and ceritinib, *BRAF inhibitors* (vemurafenib and dabrafenib); angiogenesis inhibitors like bevacizumab and ramucirumab); Apoptosis inducing drugs like *proteasome inhibitors*, such as bortezomib and carfilzomib; Immunotherapy drugs such as pembrolizumab and ipilimumab; monoclonal antibodies attached to toxins like brentuximab vedotin and adotrastuzumab emtansine.[2]

3.9 Prevention for Oral Cancer

(a)Limit smoking and drinking

Tobacco and alcohol are among the most important risk factors for these cancers. Not starting to smoke is the best way to limit the risk of getting these cancers. Quitting tobacco also greatly lowers your risk of developing these cancers, even after many years of use. The same is true of heavy drinking.

(b) Avoid HPV infection

The risk of infection of the mouth and throat with the human papilloma virus (HPV) is increased in those who have oral sex and multiple sex partners. These infections are also more common in smokers, which may be because the smoke damages their immune system or the cells that line the oral cavity. These infections are common and rarely cause symptoms. Although HPV infection is linked to oropharyngeal cancer, most people with HPV infections of the mouth and throat do not go on to develop this cancer. In addition, many oral and oropharyngeal cancers are not related to HPV infection. In recent years, vaccines that reduce the risk of infection with certain types of HPV have become available. These vaccines were originally meant to lower the risk of cervical cancer, but they have been shown to lower the risk of other cancers linked to HPV as well, such as cancers of the anus, vulva, and vagina. HPV vaccination may also lower the risk of mouth and throat cancers, but this has not yet been proven. Since these vaccines are only effective if given before someone is infected with HPV, they are given when a person is young, before they are likely to become sexually active.

(c) **Limit exposure to ultraviolet (UV) light**

Ultraviolet radiation is an important and avoidable risk factor for cancer of the lips, as well as for skin cancer. If possible, limit the time you spend outdoors during the middle of the day, when the sun's UV rays are strongest. If you are out in the sun, wear a wide brimmed hat and use sunscreen and lip balm with a sun protection factor (SPF) of at least 15.

(d) Eat a healthy diet

A poor diet has been linked to oral cavity and oropharyngeal cancer; although it's not exactly clear what substances in healthy foods might be responsible for reducing the risk of these cancers. In general, eating a healthy diet is much better than adding vitamin supplements to an otherwise unhealthy diet. The American Cancer Society recommends eating a healthy diet that emphasizes plant foods. This includes eating at least 2½ cups of vegetables and fruits every day. Choosing whole-grain breads, pastas, and cereals instead of refined grains, and eating fish, poultry, or beans instead of processed meat and red meat may also help lower your risk of cancer.

(e) Wear properly fitted dentures

Avoiding sources of oral irritation (such as dentures that don't fit properly) may also lower your risk for oral cancer.

(f) Treat pre-cancerous growths

Areas of leukoplakia or erythroplakia in the mouth sometimes progress to cancer. Doctors often remove these areas, especially if a biopsy shows they contain areas of dysplasia (abnormal growth) when looked at under a microscope. But removing areas of leukoplakia or erythroplakia does not always prevent someone from getting oral cavity cancer. Studies have found that even when these areas are completely removed, people with certain types of erythroplakia and leukoplakia still have a higher chance of developing a cancer in some other area of their mouth. This may be because the whole lining of the mouth has probably been

exposed to the same cancer-causing agents that led to these pre-cancers (like tobacco). This means that the entire area may already have early changes that can lead to cancer. This concept is called *field cancerization*. It is important for patients who have had these areas removed to continue having checkups to look for cancer, and for new areas of leukoplakia or erythroplakia.

(g) Chemoprevention

Chemoprevention is particularly needed for people who have a higher risk of these cancers, such as those with leukoplakia or erythroplakia. Several kinds of drugs have been studied for oropharyngeal cancer chemoprevention, but most of the research has focused on drugs related to vitamin A (retinoids). The retinoids can cause some areas of leukoplakia to shrink or even go away temporarily. But these studies have not found a long-term benefit in preventing cancer or helping patients live longer. At the same time, most of these drugs have bothersome and even serious side effects.

3.9 Diabetes- A Risk factor for Oral Cancer

Diabetes is a chronic, metabolic disorder in which the blood glucose levels get elevated which causes to serious damage to the heart, blood vessels, eyes, kidneys, and nerves as the disease progresses. Diabetes is categorized into two types, Type I and Type II where Type I is due to reduced secretion of insulin, and Type II is due to the resistance towards the insulin and impaired response to insulin by the body[7]. Type2 diabetes is the most common occurring mostly to adults characterized by increased insulin resistance in the body or the production of insulin in the body is decreased. The prevalence of Diabetes has raised dramatically over the past 3 decades globally. Type 1 diabetes which is insulin-dependent and also known as juvenile diabetes is a condition when the pancreas is not able to produce insulin by itself or very negligible amount of insulin. About 422 Million people are suffering and from diabetes, 1.5 million people dies each year due to diabetes are directly attributed to diabetes each year and 1 in 3 adults having an age over 18 years is overweight and 1 in 10 is obese worldwide. A target has been agreed globally to halt the rise of both diabetes and obesity by 2025. The mission of WHO Diabetes Programme is to prevent type 2 diabetes with minimum complications and maximum quality of life of all the diabetics (30).

Diabetes of both the types causes hyperglycemia, increased water intake, weight loss, excessive urine output, blurred vision, lethargy and changes in energy metabolism. Diabetes after a long duration leads to various complications such as atherosclerosis, nephropathy neuropathy and heart disease. One of the most common causes of death due to diabetes is cancer. Oral cancer accounts for approximately 4-5% of all cancers in the world. The most commonly affected are adult males, especially those who have a habit of alcoholic consumption and smokers of sixth and eighth decades of their lives. Reports suggest that the cancer patients having diabetes have worse prognosis than the non diabetics. The studies have

shown that the risk of cancer is more in diabetes type II as compared to type I. The relationship between diabetes and cancer was first studied in 1855. In India, there are 25.6% and 31.3% diabetics type I and type II patients respectively having Glossitis & chronic Cheilitis. Out of these, 10.9% and 25.6% of type I and type II are suffering from benign tumor. Further 3.2% and 11 % of type I and II patients among these are having oral manifestations like leukoplakia & erythroplakia. Gingival cancer (29%) and lip cancer (24%) are more prevalent in male diabetic patients than female which is due to smoking and drinking habits in male population.

Several immunologic and metabolic changes occur in the oral mucosa due to Diabetes mellitus. There is a relationship between diabetes and oral mucosa like immunological and periodontal disease. The diabetic patients have precancerous lesions like erythroplakia and leukoplakia more than non diabetic patients. Almost 90% of systemic metabolic disorders lead to oral mucosal damage. Diabetes also has the influence on microenvironment of the oral cavity and creates complications of oral mucosa like lesions in the mucosa and tongue, change in saliva composition, Xerostomia, osteolytic lesions, odontogenic abscess, granulomas, Gingivitis and periodontitis and loss of teeth. Diabetes is characterized by hyperglycemia which leads to production of reactive oxygen species, increasing the oxidative stress. This causes the modification in the lipids, proteins and DNA. Along with these mechanisms, in type II diabetes there is a condition of Hyperinsulinemia due to which there is increased production of Insulin like receptor, also insulin signaling and insulin mitogenic pathway remains unregulated. These all mechanisms are responsible for intiation and progression of the cancer.[7] The hypothesized biological mechanisms is related to the effect of insulin and insulin-like growth factors (IGFs) axis, which would trigger intracellular signaling cascades with mitogenic and antiapoptotic effects. The insulin levels are elevated in diabetes type II which increases concentrations of insulin-like growth factor-1(IGF-1). Elevation of these both stimulates the growth of cell by proliferation of the cells, apoptosis inhibition and increasing the mitogenesis in cell lines. High level of insulin in the body also triggers the release of several pro-inflammatory cytokines, including interleukin-6 and tumor necrosis factor-alpha. These cytokines ultimately leads to inflammation and leads to malignancies of oral cavity.[1]

Euphoria longana

Euphoria longana (Lour.) is the glamorous lychee, belonging to Sapindaceae family. It is also known as Dragon's Eye or eyeball, and in Cuba it is known as Mamoncillo chino and has been referred to as the "little brother of the lychee" or “li-chihnu". Botanically, it is placed in a separate genus, and is currently designated as *Dimocarpus longana* Lour. The fruit of *Euphoria longana* (Lour.) is one of the most chosen tropical fruits in China, in the provinces of Kwangtung, Kwangsi, Schezwan and Fukien, between elevations of 500 and 1,500 ft (150-450 m). In Indian literature, the native of the longana is not only China but also south-western India (Assam, Garo hills, Bengal, elsewhere) and Asia[12]. *Euphoria longana (Lour.)* grow in cool weather over the 3 winter months. Poor blooming of longana is observed after a warm winter. *Euphoria longana (Lour.)* thrives best on a sandy loam and nearly as well on moderately acid, somewhat organic sand.

After drying in the shade for 4 days, seed should be planted without delay because the seeds lose viability hastily, not more than 3/4 in (2 cm) deep otherwise they may send up more than one sprout. Germination takes place within a week or 10 days. The trees are fertilized after harvesting of fruit, during the blooming season, at which time the proportion of nitrogen is reduced. Fresh, rich soil is added around the base of longana year to year. The longana seed contains thirteen polyphenols including gallic acid, corilagin, ellagic acid, chebulagic acid, 4-O-α-L arabinofuranoside, isomallotinic acid and geraniin etc.[13] [3].

Traditionally seeds of *Euphoria longana (Lour.)* are used in the treatment of acariasis, hernia, wound haemorrhages, eczema and scrofula[14] [4]. Pharmacologically, it possess memory enhancing[15] [5], immunomodulatory[16] [6], anti-oxidant, anti inflammatory[17] [7], antiobesity, hypolipidemic [18][8], neuroprotective activity in focal cerebral ischemia[19] [9], antifungal activity[20] [10] and anticancer activity in lung, cervix, hepatocellular, breast, colon cancer.

Botany:

The tree of *Euphoria longana (Lour.)* is 30 or 40 ft (9-12 m) in height and 45 ft (14 m) in width, with rough-barked trunk to 21/2 ft (76.2 cm) thick and long, spreading, slightly drooping, heavily foliaged branches. The evergreen, alternate, paripinnate leaves have 4 to 10 opposite leaflets, elliptic, ovate-oblong or lanceolate, blunt-tipped; 4 to 8 in (10-20 cm) long and 13/8 to 2 in (3.5-5 cm) wide; leathery, wavy, glossy-green on the upper surface, minutely hairy and grayish-green beneath. New growth is wine-colored and showy. The flowers of *Euphoria longana (Lour.)* are pale-yellow, with 5 to 6 petals, hairy-stalked, larger than those of the lychee. The fruit of *Euphoria longana* (Lour.) in drooping clusters are globose, 1/2 to 1 in (1.25-2.5 cm) in diameter, with thin, brittle, yellow-brown to light reddish-brown rind, more or less rough (pebbled), and the protuberances are much less prominent than the lychee [11][4].

Figure 1: *Euphoria longana* (Lour.) leaves, flowers, fruits and seeds

Traditional and ethno medicinal uses

The seeds of *Euphoria longana (Lour.)* have saponin content, are used like soapberries (Sapindus saponaria L.) for shampooing the hair. The seeds of longana have long been used as a folk medicine in China for treatment of acariasis, hernia, wound haemorrhages, eczema and scrofula [4]. *Euphoria longana (Lour.)* fruit is also known to invigorate the heart, spleen, nourish the blood and have a calming effect on the nervous system. A spoonful of *Euphoria longana (Lour.)* tonic made of equal quantities of its flesh and sugar simmered in water till it is reduced to a syrup consistency is recommended twice a day. A dried flesh decoction is taken as a tonic in the treatment for insomnia and neurasthenic neurosis. In Vietnam, the seed is pressed against snakebite in the belief that it will absorb the venom. The seeds are administered to counteract heavy sweating and pulverized kernel. It contains saponin, tannin and fat, which serves as a styptic (substance that draws together or constricts body tissues and is effective in stopping the flow of blood or other secretions). The fruit is a rich source of antioxidants and has antityrosinase, antiglycated and immunity modulator activity. It also helps in prevention of cancers of lung, colon, liver, cervical and breast which have been observed in vitro. [11] The *Euphoria longana (Lour.)* fruit adds luster, shine and suppleness to the skin. *Euphoria longana* is also used as a memory enhancer [15][5], immunomodulator [6], anti-oxidant [7], antiobesity and hypolipidemic [8] and antifungal [10]. Further it is also reported to have protective effect against cerebral ischemia [9]. *Euphoria Longana* (Lour.) is used as remedy for stomachache, insomnia, amnesia and dropsy [13].

Phytochemistry

The leaves, fruit and seed of *Euphoria Longana (Lour.)* contain a huge amount of polyphenols, polysaccharides, tannins, proteins and some other secondary metabolites. Due to the presence of these metabolites in high amount, it is difficult to extract the genomic DNA as there is an irreversible binding of phenolic compounds and polysaccharides with the nucleic acids. Hence to eliminate the influence of phenolic compounds and polysaccharides, a valid combination (β-mercaptoethanol, PVP40 and PVPP were used at different stages) was adopted and was found successful not only for *Euphoria longana* but also for the extraction of DNA from mango, lichi and many more [12]. The hexane extract of longana seed contains predominantly long-chain fatty acids with major contributions from palmitic and oleic acids. The polyphenolic fraction is dominated by corilagin, gallic acid, ellagic acid, geraniin and 4-O-α-l-arabinokfuranoside [3]. Flavonoids such as (-)-catechin, epicatechin, rutin and phenolic acids including chlorogenic acid, vanillic acid, p-coumaric acid, ferulic acid, sinapic acid and syringic acid are also found in it. It also contains tannins like proanthocyanindins, ascorbic acid and isomallotinic acid. The major volatile compounds are trans-caryophyllene, linalool oxide and α-humulene [14]. The seed oil of *Euphoria longana*, Sapindaceae, contains 17.4% of 9,10-methyleneoctadecanoic (dihydrosterculic) acid. The presence of smaller amounts, less than 1%, of cyclopropanoid fatty acids of different chain lengths is indicated by GLC and TLC analyses of the methyl esters. The other major fatty acids in this oil are: 16:0 (19%), 18:0 (7%), 18:1 (36%), 18:2 (6%), 18:3 (5%) and 20:0 (4%). The oil contains considerably larger amounts of cyclopropanoid fatty acids than in other seed oils previously reported [15]. The seeds of longana extracted in hot water and ethanol also results in high phenolic content. Comparatively, the hot water crude extract

contains high amount of polyphenols (41.2 ± 0.2mg GAE/g) than ethanolic crude extract (1.7 ± 0.1 mg GAE/g). These extracts can be combined with a concentrate of strawberry fruit to develop a beverage having several beneficial health effects [16].

PHARMACOLOGICAL INVESTIGATIONS

Effect on Central Nervous System

Euphoria longana (Lour.) improves memory performance in the passive avoidance task. Treatment with longana fruit extract increase the expression of BDNF, pCREB, pERK, DCX and BrdU in the hippocampus. BDNF is coupled for activation of ERK and CREB. The BDNF binds to its receptor and activates downstream signaling mediators such as, phosphatidyl-inositol 3 kinase, phospholipase C gamma and ERK1/2). It also increased the synthesis of its own expression and a wide variety of translational-machinery proteins which is required for memory consolidation. The number of DCX- immunopositive cells were significantly increased in the *Euphoria longana (Lour.)* treated-treated group [5]. Adenosine isolated from the pulp or flesh of *Euphoria longana* produces significant anxiolytic activity at a dose of 30mg/kg sc. High doses of Adenosine may cause a sedative action. It has been reported that the anxiolytic like activity is due to the activation of central Adenosine A1 receptor selectively whereas activation of A2 receptor causes the depression of the locomotor activity [17].

Euphoria longana Steud contains polysaccharide which possesses a broad spectrum of biological, pharmacological and therapeutic activities, such as: antioxidant, immune regulation, anti-inflammatory and promoting intelligence. The ischemic cerebrovascular disease (ICVD) occurs due to shortage of blood supply leads to ischemia and hypoxia in the brain, which causes softening of the brain. Oxidative stress play an important role in the regulation of processes involved in ischemia/ reperfusion injury by modulating various signal pathways such as influencing the synthesis of antioxidant enzymes, inflammatory, apoptosis and cell proliferation pathways. The surgery was performed by middle cerebral artery occlusion (MCAO) in Sprague-Dawley rats. There was no significant difference on neurological score produced by *Euphoria longana* polysaccharides. But *Euphoria longana* Steud significantly reduced the MDA content and increased SOD, GSH, GSH-Px, MPO activity, concentrations of TNF and IL-1 in the brain tissues. It also significantly decreased the protein expression of Bax and increased the protein expression of Bcl-2 (p50.01 or p50.05), and is capable of alleviating I/R injury by a mechanism that may involve decrease in oxidative stress [9].

The neuroprotective effect of longana flower water extract was investigated in vitro study in which lipid peroxidation of brain homogenates was observed in concentration-dependent manner. The extract was found with more antioxidative activity than that of glutathione or Trolox. The ex vivo study found that the basal lipid peroxidation (0 °C) and lipid peroxidation are reduced in the brain homogenates treated with the extract (500 mg/day). The extract treated rat brains were more resistant to oxidative stress. Parkinsonian animal model was employed to demonstrate that the oral administration of extract (125–500 mg/kg/day) in dose-dependent manner attenuated 1-methyl-4-phenylpyridinium (MPP^+)-induced

neurotoxicity in the nigrostriatal dopaminergic system of rat brain [18]. Thus the longana extract is effective as antioxidative, anti-inflammatory, anti-apoptotic, and protects from neurodegenerative diseases including Parkinsonism as well.

Immunomodulatory effect of *Euphoria longana*

The immunomodulatory function of longana polysaccharide-protein complex (LP3) was tested in immunosuppressed mice models. The LP3 increases antibody production against chicken red blood cell (CRBC), concanavalin A (ConA)-induced splenocyte proliferation, macrophage phagocytosis, NK cell cytotoxicity against YAC-1 lymphoma cell, and interferon-gamma (INF-γ) and interleukin-2 (IL-2) secretion in serum at a dose of 100 mg/kg/d. The immunomodulatory effects of longana except of splenocytes and macrophages ($P > 0.05$), were also observed in mice administered with 50 or 200 mg/kg/d LP3 ($P < 0.05$) [19][16][27]. The extract with medium dose (200 mg/kg) and low-dose (100 mg/kg) had potent immuno-modulatory effects in S180 tumour mice model and exhibited significant effect on delayed-type hypersensitivity (DTH) response and macrophage phagocytosis. The longan significantly stimulates macrophage phagocytosis against the neutral red and NK cell cytotoxicity against YAC-1 lymphoma cell [20]. The longana pulp has strong immunomodulatory activity due to the presence of LP3 and hence confirms its good potential as an immunotherapeutic adjuvant [19].

Anti-oxidant and anti-inflammatory properties of *Euphoria longana* (Lour.)

The free radicals are able to induce biological damage and pathological events, such as inflammation, aging, and carcinogenesis. The longana contains gallic acid and ellagic acid which shows significant inhibition of NO production, histamine release and pro-inflammatory cytokine production in mast cells. The WLP has been found to potentially inhibit the activity of NO in a cellular model of inflammation, which was estimated by using the LPS-activated macrophages. These macrophages produce NO radicals that were measured in the form of nitrites with the help of Griess reaction in the culture medium. In this study, the NO production was reduced by WLP with an IC50 value 179.8 μg/mL. This suggests WLP, as a potential inhibitor of NO related inflammatory pathways. Additionally, WLP was found nontoxic to the cells and was confirmed after performing a cell viability test using MTT assay. WLP also exhibited protective effect in a dose dependent manner on liposome damage (12.6–37.0%), induced by the Fe^{3+}/H_2O_2 reaction in the range of 50–200 μg/mL. This implies that WLP could protect lipid molecules against oxidative damage due to its antioxidant properties [21] .

Moreover, the longana flower extract was also found to exhibit suppressive effect on production of NO and prostaglandin E2 (PGE2) on lipopolysaccharide-stimulated RAW 264.7 cell model. However the inhibitory effects were due to suppression of nitric oxide synthase protein expression probably by proanthocyanidin present in it. The NO production was inhibited in a concentration dependent manner significantly [22]. The longana pulp or fruit pericarp extraction with the aid of ultra pressure results in high polyphenolic content including

gallic acid, ellagic acid and corilagin; corilagin being present in the highest amount has been identified and quantified using HPLC. This ultra pressure aided extract has high antioxidant and antityrosinase activities [23]. Adenosine isolated from the pulp or flesh of Euphoria longana produces an analgesic activity at a dose of 100-200mg/kg po in mice by inhibiting the writhing induced with acetic acid. However, the extract inhibited the writhing at a dose of 3g/kg [17].

Antiobesity and Hypolipidemic Effects of Euphoria longana (Lour.)

The obesity has been regarded as a chronic disease by WHO and FDA since 1996. It is considered as one cause of heart disease, hypertension, diabetes, fatty liver, and is even related to certain cancers. Imbalance in fat or positive energy intake is one of the most important environmental factors resulting in obesity, and it could not only cause the accumulation of excessive body fat but also increase serum lipids. Plenty of polyphenols, i.e. phenolic acids and flavonoids, were found in longana flower water extract (LFWE) through spectrophotometric and HPLC analyses. Antiobesity and hypolipidemic effect of LFWE measured by body weight, size of epididymal fat, serum triglyceride level, atherogenic index and hepatic lipids. These parameters were decreased in HCD rats which might be because of down regulation of pancreatic lipase activity, sterol regulatory element binding protein-1c (SREBP-1c) and fatty acid synthase (FAS) gene expressions, as well as dysregulation of LDL receptor (LDLR) and peroxisome proliferator-activated-receptor-alpha (PPAR-R) gene expressions, and also increased faecal triglyceride excretions [8].

In vitro antifungal activities of longan seed extract

The antifungal activities of longan fruit extract and its active compound was measured by observingits activity against the opportunistic yeasts (Candida species and Cryptococcus neoformans). Ellagic acid showed the most potent antifungal activity followed by corilagin and gallic acid, respectively. Ellagic acid inhibited Candida parapsilosis and Candida neoformans more effectively than Candida krusei and also some Candida albicans clinical strains. Only corilagin and gallic acid possess weak to moderate antibacterial effects against Staphylococcus aureus and Streptococcus mutants respectively. The effervescent granules of longana (5% extract) significantly reduced adhesion of Candida albicans to acrylic strips and the mouthwash containing 0.5% extract also shows good antifungal activity as compared to a commercial product[10]. The longan seed extract and its polyphenolic compounds can be used as an antifungal agent in oral care products for the treatment of opportunistic yeast infection.

Antitumor or anticancer activity

Longana flower and seed possess an anticancer activity on lung (in vivo), hepatocellular, cervix, breast carcinoma and colorectal cell line in vitro**.** In the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay, water-soluble longan seed polysaccharide (WLSP) showed an antiproliferative activity by inhibiting the proliferation of A549 human lung cancer cells. This inhibition is consistent with the amount of lactate dehydrogenase (LDH) released

from A549 cells both in vitro as well as in vivo. The extract ability to cause cell cycle arrest in G1 phase, activation of caspase3 and 9 and cleavage of PARP, indicates an earlier mitochondrial related apoptosis which might be triggered by A549 cells. Hence apoptosis was assessed by TUNEL analysis of hepatocellular carcinoma cell line Hep-G2, cervix carcinoma cell line C33A and breast carcinoma cell line MDA-MB-231. The S phase was increased in LSE-treated C33A and MDAMB-231 cells, while the G1 phase was increased in A549 cells. The LSE systemically suppressed cyclin D1 and cyclin A expression and concomitantly enhanced the expression of CIP/p21 and KIP/p27. The proliferative and colony forming activities of cancer cell lines were suppressed up to 40 to 60% gradually by the increasing concentrations of LSE 10-100 µg/mL [24].

LFE (25–400 µg/ml) inhibit proliferation in a dose- and time-dependent manner on two colorectal cancer cell lines, SW-480 and Colo 320DM by blocking the S phase which is due to the accumulation of cyclin E and a decrease in cyclin A. Additionally, in Colo 320DM cells, it increase rhodamine 123-negative cells, DNA fragmentation and caspase 3 levels and thus leads to apoptosis which is due to the suppression of Bcl-2 protein levels [25]. The polysaccharides extracted from longan pulp using an ultrasonic extraction procedure (UELP) possess radicals scavenging and antitumor activities which were investigated in S180 tumor mice models. UELP completely scavenges the hydroxyl radicals and a,a-diphenyl-1-picrylhydrazyl (DPPH) radicals, and exhibit significant effect on delayed-type hypersensitivity (DTH) response and ConA-stimulated splenocyte proliferation [20].

Angiogenesis is a critical event in cancer metastasis, via delivery of needed oxygen and nutrients to tumour cells. Anti-angiogenesis is one of the strategy for controlling cancer progression. The dried longan seeds were found to have an antiangiogenic effect in colon adenocarcinoma cells (SW480 cells) and human umbilical vein endothelial cells (HUVECs). Seeds are found rich with gallic acid, ellagic acid, and combination of both in first, second and third fractions respectively, as observed by Sephadex LH-20 column chromatography. The longan seed fraction was effective on vascular endothelial cell growth factor (VEGF) secretion, expression and colony formation of SW480 cells only at a concentration of100 µg/mL. The antigelatinase activity of each fraction was found to exhibit a direct effect on MMP-2 and MMP-9 by causing Zn^{2+} chelation. The fraction modulated indirectly the suppression of zymogen activators and thereby MMP 2 and MMP 9 activity [26]. The dried longana seed fraction was potential angiogenic inhibitor not only by interrupting VEGF secretion and expression in SW480 cells but also abrogation of cell proliferation activity of gelatinase and tube formation of HUVECs. The oxidative stress and free radicals can convert pro-MMP into active MMP by binding to the thiol group of a cysteine in the prodomain and interrupting the interaction of Cys-Zn^{2+} on MMP. The polyphenols rich extract of seed have antioxidant activity which can be correlated to anti-MMP activity by decreasing the oxidative stress and free radicals [27].

Anti gout activity of Euphoria longana seed

The enzyme Xanthine oxidase (XOD) is responsible for the conversion of hypoxanthine to xanthine and further into uric acid by oxidation. Allopurinol inhibits this enzyme and thereby the uric acid production is blocked and has been the essential drug to be used in hyperuricemic condition before the approval of new medicines, and also has various adverse effects [28]. The urate excreted from the kidney is about 60-70% and rest is excreted through the gut [29]. The major constituents like gallic acid, ellagic acid and corilagin present in longana inhibited XOD enzyme in vitro in a dose dependent manner with IC50 of 277.78 g/ml but were not as effective as Allopurinol. The longana seed extract is non toxic to the liver cells but has a little effect on cell viability at a concentration of 200_g/ml. It promotes cell growth in Clone 9 liver cells at low concentrations of 50 and 100_g/ml where allopurinol increased the cellular XOD acitivity significantly at 24 and 48 h, but LSE did not. GLUT1 and GLUT9 both were induced dose-dependently by LSE in HepG2 cell culture, but LSE or allopurinol alone induce GLUT1 but not GLUT9 in the liver. LSE and allopurinol decreased GLUT9 protein level from the liver in hyperuricemia induced rat model. Treatment with LSE significantly reduced both serum uric acid level and xanthine oxidase activity ($p < 0:05$). LSE could be an alternative for allopurinol inpaients with certain genetic backgrounds (e.g. HLA-B*5801 allele) or at least in combination therapy to minimize the side effects of other uricosuric agents in particular for long-term treatment [28].

Effect of Euphoria longana plant Rich in Polyphenols on Metabolic syndrome

Longana flower contains high amount of polyphenols (total polyphenol, 548.2 ± 12.7 mg/g; total flavonoids, 139.3 ± 0.2 mg/g; and proanthocyanidins, 112.5 ± 5.2 mg/g) and thus possesses antioxidant activity which is credited to proanthocyanidin A2 and (-)-epicatechin. The water extract of longana flower (LFWE) hold an± influencial effect on metabolic syndrome as it improves insulin resistance by enhancing the expression of proteins involved in insulin signaling pathway, comprising of insulin receptor substrate-1 (IRS-1) and glucose transporter in a High Fructose fed rat model. LFWE possess DPPH scavenging activity of (IC50 3.75 µg/mL) which is fairly higher than that of seed extract (IC50 11.6 µg/mL). LFWE administration improves the raised blood pressure, insulin resistance in fructose-induced model for metabolic disorder and regulates the uptake of glucose in adipocyte via GLUT4 whose expression is significantly increased by LFWE. Treatment with LFWE in the animals with metabolic disorder improves oxidative stress, levels of plasma TBARS and decreases liver GRd enzyme activity [30]. The major components present in the flower, fruit, peel and seed are gallic acid, ellagic acid and corilagin [31]. Gallic acid (20 mg/kg) reduces weight gain, fasting blood glucose and plasma insulin levels in diabetic rats and significantly enhances the peroxisome proliferator-activated receptor γ (PPARγ) expression in the adipose tissue and slightly in the liver and skeletal muscle. It improves insulin-dependent glucose transport in adipose tissue by acting on glucose transporter protein 4 (GLUT4) PI3K/ p-Akt dependent pathway [32]. The polysaccharides are present in the longana pulp which exhibit anti glycated activities which might be due to the antioxidant activity of polysaccharides and the structure they have. This antiglycated activity was evaluated against Aminoguanidine which is a clinically used glycation

inhibitor [33]. This indicated that longan plant would provide strong evidence for the beneficial effects on metabolic disorders.

Toxicity studies

The administeration of longana extract (500, 2000 and 5000 mg/kg) for 14 days produces no abnormal clinical signs, mortality or any other morphological alterations in the animals. The oral administration of extract was found to have LD50 higher than 5000 mg/kg in both male and female mice. The NOAEL (no observed-adverse-effect-level) of LE was at a dose of 5000 mg/kg. After 13-week of administration none of the effect was seen on body weights of rats at doses 50, 250, and 500 mg/kg whereas food consumption in rats treated with medium dose (250 mg/kg) was significantly decreased at week 3 and 9 respectively. Also there were no significant effects seen on haematological parameters and relative organ weights of rats. The toxicological studies reveals that oral administration of the extract for acute and repeated doses (4 and13 weeks) causes no toxic effects in the rats [34].

4. MATERIALS AND METHODS

4.1 Phytochemical Studies

4.1.1 Preparation of extracts

(a) Procurement: Euphoria longana were bought from Local Fruit House, Ahmedabad 380001, Gujarat, India.

(b) Defatting of Euphoria longana seeds: The seeds of *Euphoria longana* were crushed and grinded to obtain were powdered using a grinder and then passed through sieve no. 60. The fine powder obtained was defattened by subjecting petroleum ether into it for 30 min. The fraction was filtered, removed and then dried to obtain the product.

(c) Preparation of Euphoria longana methanolic extract: The defatting was followed by extraction of 500mg of longana powder where 70% methanol in a ratio of 1:2 was added to the powder and heated for 3 to four hours at 90 °C. It was then filtered, and the process was repeated two to three times with the obtained residue. After collecting all the three extract, it was kept for drying. The dried product was dissolved in acetone and and filtered. The acetone fraction contains the polyphenols dissolved in it which was dried and obtained as the yield.

4.1.2 Preliminary Test of longana seed extract

The yield of acetone fraction was further confirmed for the presence of various phytochemical moieties. The tests test performed by the method described by Harborne, 1973

Following was the preliminary test performed:

(a) Test for Flavonoids:

1) Shinoda Test: Sample extract was dissolved in 1ml of water to which 5ml of 95% ethanol was added and the solution was heated. Further magnesium turning and then few drops of conc. HCL were added. The presence of red or orange colour showed the presence of flavonoids.

(b) Test for carbohydrates:

Presence of glycoside was determined by procedure described by:

1) Molish's test: To the extract, α-naphthol solution was added and shaken vigorously. The insoluble particles were allowed to settle down and further conc.HCL was added from the edges of the test tube. The presence of violet coloured ring at the junction of mixture proved presence of carbohydrate.

2) Fehling's test: To the extract, equal quantities of fehling's solution A and B were added and heated. Formation of a brick red precipitate indicated the presence of carbohydrates.

3) Benedict's test: 5ml of Benedict's reagent was added to extract which was boiled for two minutes and then cooled. The formation of a red precipitate showed the presence of carbohydrates.

(c) Test of Tannins:

1) Ferric chloride solution was added to the extract, the presence of dark blue or greenish black color showed the presence of tannins.

2) KOH test: 1mL freshly prepared 10% KOH was added to 1mL of extract. Appearance of dirty white precipitate indicated the presence of tannins.

(d) Test for proteins:

Biuret Test: The extract was dissolved in 1ml of 40% sodium hydroxide solution, followed by addition of two drops of 1% copper sulphate solution. The formation of violet color indicated the presence of proteins.

(e) Test for Steroids:

Lieberman Burchardt test: Chloroform was added to the extract, and to this solution, few drops of acetic acid and 1mL concentrated sulphuric acid was added. This gave deep red color at the junction of 2 layers.

(f) Tests for Saponins:

Foam test: A little quantity of water was added to small amount of extract. On shaking, the foam is produced persisting for 10 min. This confirmed the presence of saponin.

(g) Test for Alkaloids:

1) Mayer's test (Potassium Mercuric Iodide): To the extract, few drops of Mayer's reagent was added. A creamy white precipitate confirms the presence of alkaloid.

2) Wagner's Tests (Solution of Iodine in Potassium Iodide): Extract was added with few drops of Wagner's reagent. The formation of reddish brown colourd precipitate shows presence of alkaloids.

3) Hager's Test (Saturated solution of picric acid): Hager's reagent was added to the extract. The presence of yellow precipitate shows the presence of alkaloids.

(h) Test for phenolic compound:

On addition of the following solutions to the extract, a specific change in the solution shows the presence of phenols:

1) 5% Fecl3 solution: Deep black blue color appears.

2) Lead acetate solution: White precipitate.

3) Gelatin solution: White precipitate.

4) Bromine water: Discoloration of bromine water.

5) Dilute potassium permanganate test: Disappearance of precipitate.

6) Potassium dichromate: Red precipitate.

4.1.3 Qualitative Test of longana extract

Thin layer chromatography:

Qualitative test for presence of polyphenols was performed using TLC by method. Merck silica gel 60 F254 on an aluminum support (10 _ 10 cm) was used to confirm the presence of polyphenols in the given extract. Solution was prepared by dissolving 10mg in 5ml (2mg/ml concentration. The spots of standard gallic acid, ellagic acid and corilagin were placed on the TLC plate with the help of capillary and then, the TLC plate was kept in a solvent system. The solvent system used for gallic acid and corilagin was toluene: ethyl acetate: formic acid: methanol (5: 5: 2.5: 2.5) and for ellagic acid was toluene: ethyl acetate: formic acid (5: 5: 2.5). The solvent system was saturated for 30 min and ran upto a distance of 8 cm.

4.2 In vivo Pharmacological Studies:

4.2.1 Drugs and chemicals

Euphoria longana seed were bought from Aflatoon fruit house, Manek chowk, Ahmedabad 380001, Gujarat, India, 4-NQO was purchased from TCI.

4.2.2 Animal Approval for experimentation

Protocol of the experiment was approved by Institutional Animal Ethics Committee in accordance with the guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), held under Ministry of social justice and Empowerment, Government of India. Protocol number: IP/PCOL/MPH/17/004 approved on date 21st July 2015.

4.2.3 Study Protocol

After 5 days of acclimatization, 4-8 weeks old Wistar rats were randomly assigned to 6 and 7 groups according to study plan I and II respectively. Normal control group was common for both the study plans.

(a) 4- NQO induced Oral Cancer in rats

• Group I (NC) Normal control administered with 0.9% saline only.
• Group II (OC) Disease control animals induced with Oral Cancer by applying 4-nitroquinoline-1-oxide (0.5% 4-NQO in propylene glycol) to the rat tongue thrice a week for 24 weeks.
• Group III (OCE0) Oral cancer induced animals treated with *Euphoria longana* extract (280mg/kg p.o.) from day 0.
• Group IV (OCE60) Oral cancer induced animals treated with *Euphoria longana* extract (280mg/kg p.o.) from week 24.
• Group V (OCF) Oral cancer induced animals treated with 5-FU (24.5mg/kg p.o.) from week 24.
• Group VI (OCEF) Oral cancer induced animals treated with *Euphoria longana* (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.) from week 24 respectively.

(b) 4- NQO induced oral cancer in Diabetes mellitus induced rats

- Group I (NC) Normal control administered with 0.9% saline only.
- Group II (DM II) Animals were fed with High fat diet HFD containing 58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal *ad libitum*, for 14 days followed by a single low dose streptozotocin (STZ 35mg/kg i.p.)
- Group III (DMOC) Animals were induced with Diabetes (HFD + STZ) as well as oral cancer (0.5% 4-NQO)
- Group IV (DME) Animals induced with only Diabetes treated with *Euphoria longana* (280mg/kg p.o.)
- Group V (DMM) Animals induced with only Diabetes with *Euphoria longana* (280mg/kg) treated with Metformin (400 mg/kg p.o.)

- Group VI (DMOCE) Animals induced with both Diabetes and Oral cancer treated with *Euphoria longana* (280mg/kg p.o.)
- Group VII (DMOCMF) Animals induced with both Diabetes and Oral cancer treated with *Euphoria longana* (280mg/kg p.o.) and Metformin (400 mg/kg p.o.)
- Group VIII (DMOCMFE) Animals induced with both Diabetes and Oral cancer treated with *Euphoria longana* (280mg/kg p.o.), Metformin (400 mg/kg p.o.) and 5-fluorouracil (24.5mg/kg p.o.)

4.2.4. Induction of Diabetes Mellitus type II and Oral Cancer

Oral Cancer was induced in the animals using 0.5% 4-NQO in propylene glycol thrice a week for a period of 24 weeks. After the disease induction of 24 weeks, the animals were treated with *Euphoria longana* and 5- fluorouracil for the duration of 8 weeks after 24th week.

Type II Diabetes Mellitus was induced by giving high fat diet containing 58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal *ad libitum*. The animals were treated with this high fat diet for 2 weeks followed by a single low dose streptozotocin (35mg/kg i.p.) injection. After 6-8 weeks, diabetes was confirmed by hyperglycemia, then 0.5% 4-NQO in propylene glycol was applied to the tongue of animals thrice a week upto 12 weeks. The animals were treated with *Euphoria longana*, metformin and 5- fluorouracil for the duration of 8 weeks after the 12th week of disease induction.

4.3 In vivo parameters

a) Blood collection

At the end of experimental period i.e. 20 weeks, blood samples were collected in clean dry centrifuge tubes from retro orbital plexuses under light ether anesthesia. The blood samples collected were centrifuged at 6000 rpm for 8 min at 20ºC and the serum was separated which was then stored at -20 ºC for the further biochemical analysis. The serum was analysed for fasting serum glucose level and oral glucose tolerance test with the help of glucose kit procured by Accucare using auto bio analyzer. Glycosylated hemoglobin and mean blood glucose was measured from whole freshly collected blood samples by ion exchange resin method procured by Accucare using UV spectrophotometer (SHIMADZU 2450 instrument) and the spectra was obtained using software (UV probe).

b) Preparation of tissue homogenate

Animals were sacrificed by spinal dislocation technique and the tongues were excised out, rinsed with ice cold saline. Again rinsed with distilled water and immediately stored at -20 ºC till further biochemical analysis. 10% tongue tissue was homogenized in 10 ml ice cold Tris hydrochloride buffer. The prepared homogenates were centrifuged and used for the determination of antioxidant and tissue specific parameters.

4.4 SERUM BIOCHEMICAL PARAMETERS ESTIMATION

4.4.1 GLYCEMIC PARAMETERS

a) Estimation of Glucose:

Principle

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoeimine dye as indicator.

Procedure:

Reagents and samples were pipette out as follows:

	Blank	Standard	Sample
Sample	-	-	10µl
Standard	-	10µl	-
Reagent	1000µl	1000µl	1000µl

Mix & Incubate for 5 min. at 37°C or 15 min. at R.T. Measure absorbance of Sample (AT) and Standard (AS) against Reagent Blank at 505 nm. The colour is stable for 30 min. at R.T.

Calculation:

Total Glucose (mg/dl) = AT/AS x conc. Standard

b) Glycosylated Haemoglobin (HbA1c):

Principle:

Glycosylated Hemoglobin (GHb) is normal adult haemoglobin (HbA1) which is covalently bonded to a glucose molecule. GHb concentration is dependent on the average blood glucose concentration. It is formed progressively and irreversibly over a period of time and is stable till the life of the RBC. Whole blood is mixed with lysing reagent to prepare hemolysate. This is then mixed with a weakly binding cation exchange resin. The non- Glycosylated Haemoglobin bind to the resin leaving GHb free in the supernatant. The GHb percentage is determined by measuring the absorbance of the GHb fraction and of the total Hb. GHb kit is based upon the property of non-Glycosylated Hemoglobin to bind with a weak cation exchange resin leaving GHb free in the supernatant.

Procedure:

Step I - Hemolysate preparation:

- Pipette 0.25 ml of lysing reagent (2) in a test tube.
- Add to it 0.05 ml of well mixed whole Blood/control.
- Mix well and allow to stand at room temperature for 5 minutes.

Step II - GHb seperation and assay

- Bring Resin Tube (1) to assay temperature by incubating the tube in a water bath.
- Add to it 0.1 ml of haemolysate (from step1)
- Position a Resin Separator in the tube, so that the rubber sleeve is approximately 3 cm above the resin level.
- Mix the contents on vortex mixer continuously for 5 minutes.
- Allow the resin to settle at assay temperature for 5 minutes, push down the Resin separator in the tube until the Resin is firmly packed.
- Pour the supernatant directly into a cuvette and measure the absorbance against deionized water.

Step III - Total Haemoglobin (THb) assay

- Pipette 5.0 ml of deionized water into a test tube.
- Add to it 0.02 ml of hemolysate (from step 1).
- Mix and read absorbance against deionized water.

Calculation:

GHb% = A of GHb/A of THb x 7.2 x Temperature factor (Tf)

For assay at 23°C Tf = 1.0; at 30°C Tf = 0.9.

c) Oral Glucose Tolerance Test (OGTT)

The animals were fasted for 16 hours. Then glucose load (3g/kg) was administerd orally to each rat with an oral feeding needle before performing the OGTT. Blood samples were collected from retro orbital plexuses, at 0 (just before the oral administration of glucose), 30,

60, 90, 120 and 180 min after glucose load for the assay of glucose. The blood samples were centrifuged and the serum was separated. The glucose concentration was measured with a Glucose kit (Accucare laboratory, India) using autobioanalyzer.

4.3.2 Oxidative stress parameter

a) Total Protein estimation

Total Protein was estimated by the method of Lowry et al., 1951.

Principle:

The phenolic group of tyrosine and tryptophan residues (amino acid) in a protein will produce a blue purple color complex, with maximum absorption in the region of 660 nm wavelength, with Folin Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins.

Reagents:

1. Reagent A- 2% sodium carbonate in 0.1 N NaOH.
2. Reagent B- 0.5% copper sulphate ($CuSO_4$. $5H_2O$) in 1% potassium sodium tartrate.
3. Reagent C- Alkaline copper solution (50 ml of solution A & 1 ml of solution B were mixed prior to use).
4. Reagent D-Folin-Ciocalteau reagent (1 part of reagent: 2 parts of distilled water).

Procedure:

Blank	**Test**
0.2 ml of D.W.	0.2 ml of supernatant
Diluted upto 1 ml with Tris HCL	Diluted upto 1 ml with Tris HCL
5 ml Reagent C	5 ml Reagent C
Allowed it for 10 minutes	
0.5 ml Reagent D	0.5 ml Reagent D

All reagents were mixed well and kept at room temperature for 30 min. in dark place and absorbance was read against blank at 600 nm. The protein level was calculated using standard curve which was plotted using standard albumin.

(b) Malondialdehyde (MDA) level:

Malondialdehyde formation (MDA) was estimated by the method of Ohkawa et al., 1979.

Principle:

The method estimates MDA, a product of lipid peroxidation process. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) under mildly acidic conditions to form a pink colouredchromogen, whose intensity was measured colorimetrically at 535 nm. The MDA content, a measure of lipid peroxidation, was assayed in the tissue homogenate in the form of thiobarbituric acid-reactive substances (TBARS) and expressed as nano moles of MDA per milligram of protein.

Reagents:

1. Sodium lauryl sulphate (SLS) (8%) - 8 gm of SLS in 100 ml of distilled water.

2. Acetic acid (20%) - Prepared in 0.27 M hydrochloric acid (2.29mL HCL in 100 ml water)
3. Thiobarbituric acid (TBA) (1% in Tris hydrochloride, pH 7): (Freshly prepared) - 1gm of thiobarbituric acid in 100 ml of Tris hydrochloride buffer pH 7.
Note: Thiobarbituric acid solubilized in Tris-HCl by exposing the solution to hot steam under the water bath for 5-10 min or by constant sonication for 30 min.

Procedure:

Blank	**Test**
0.2 ml of distilled water	0.2 ml of homogenate
0.2 ml of SLS	0.2 ml of SLS
1.5 ml acetic acid in HCl	1.5 ml acetic acid in HCl
1.5 ml TBA	1.5 ml TBA
0.6 ml distilled water	0.6 ml distilled water
Heated for 45 min in water bath at 95ºC and cool	Heated for 45 min in water bath at 95ºC and cool
1 ml of distilled water	1 ml of distilled water
5 ml mixture of n- butanol + pyridine (15:1)	5 ml mixture of n- butanol + pyridine (15:1)
Centrifuge at 4000 rpm for 10 min	Centrifuge at 4000 rpm for 10 min

All reagents were mixed well and pink color developed in upper organic layer, the absorbance of which was read against blank at 532 nm. MDA level was calculated using molar extinction coefficient of malonaldehyde.

(c) Glutathione (GSH) level:

A GSH level was estimated by the method of Moron et al, 1979.

Principle:

Glutathione consists of sulfhydryl groups and 5, 5- dithiobis 2- nitro benzoic acid (DTNB) is a disulphide compound which gets readily attacked by these sulfhydryl groups and forms a yellow colored anion (5-thio 2-nitrobenzoic acid) which is measured colorimetrically at 412 nm.

Reagents:

1. Trichloroacetic acid (TCA) (10%) - 10 gm of TCA in 100 ml of distilled water.
2. 0.3 M Na_2HPO_4 - 4.26 gm of Na_2HPO_4 in distilled water.
3. DTNB (Fresh) - 40 mg in 100 ml of 1% sodium citrate and covered with aluminium foil.

Procedure:

Blank	**Test**
0.2 ml of distilled water	0.2 ml of supernatant

1 ml of TCA (10%)	1 ml of TCA (10%)
Kept in ice bath for 30 min & centrifuged for 10 min at 4 ˚C at 3000 rpm, 0.5 ml of supernatant taken	Kept in ice bath for 30 min & centrifuged for 10 min at 4 ˚C at 3000 rpm, 0.5 ml of supernatant taken
0.5 ml of Supernatant	0.5 ml of Supernatant
2 ml di-sodium hydrogen phosphate	2 ml di-sodium hydrogen phosphate
0.25 ml DTNB (covered with aluminium foil)	0.25 ml DTNB (covered with aluminium foil)

All reagents were mixed well and absorbance was read against blank at 412 nm. The GSH level was calculated using standard curve which was plotted using standard GSH.

(**d) Superoxide dismutase (SOD) level:**

SOD was estimated by the method of Mishra and Fridovich, 1972.

Principle:

The O2- substrate for SOD is generated indirectly during the oxidation of epinephrine at alkaline pH. Oxygen is build in the solution, the formation of adrenochrome occurs because O2- reacts with epinephrine to form adrenochrome. Toward the end of reaction, when the epinephrine is consumed, the adrenochrome formation slows down. When observed for long times, the adrenochrome disappears and insoluble brown products form in the solution. SOD reacts with the O2- formed during the oxidation of epinephrine and therefore it slows down the rate of formation of the adrenochrome as well as its amount. At this last step which is a slow process, SOD is said to inhibit the oxidation of epinephrine.

Reagents:

1. EDTA – 0.0001 M (9.3 mg/250 ml)
2. Carbonate buffer – pH 9.7 (8.4 gm NaHCO3 + 10.6 gm Na2CO3 in 500 ml)
3. Epinephrine – 0.003 M (50 mg/100 ml in HCL (pH 2) and covered with aluminium foil.

Procedure:

Blank	**Test**
0.2 ml of distilled water	0.2 ml of supernatant
0.1 ml EDTA	0.1 ml EDTA
0.5 ml carbonate buffer	0.5 ml carbonate buffer
1 ml epinephrine	1 ml epinephrine

All reagents were mixed well and absorbance was read against blank at an interval of 30 sec for 3 min at 480 nm. The SOD level was calculated using standard curve which was plotted using standard SOD.

4.3.3 Inflammatory Markers

(a) Interlukin-6

Principle

The RayBio® Rat IL-6 ELISA kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of rat IL-6 in serum (rat IL-6 concentration is low in normal serum/plasma and may not be detectable in this assay), plasma, and cell culture supernatants. This assay employs an antibody specific for rat IL-6 coated on a 96-well plate. Standards and samples are pipetted into the wells and IL-6 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-rat IL- 6 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and colour develops in proportion to the amount of IL-6 bound. The Stop Solution changes the colour from blue to yellow, and the intensity of the color is measured at 450 nm.

Reagent preparation

- Bring all reagents and samples to room temperature (18 - 25ºC) before use.
- Assay Diluent B should be diluted 5-fold with deionized or distilled water before use.
- Preparation of standard: Briefly spin a vial of standard protein. Add 500 µl Assay Diluent C into Item C vial to prepare a 10,000 pg/ml standard solution. Dissolve the powder thoroughly by a gentle mix. Pipette 300 µl Assay Diluent C into each tube. Use the 10,000 pg/ml standard solution to produce a dilution series. Mix each tube thoroughly before the next transfer. Assay Diluent C serves as the zero standard (0 pg/ml).

10000pg/ml	Standard No 1	500µl standard
4000pg/ml	Standard No 2	300µl Assay diluent C+200µl Standard No 1
1600pg/ml	Standard No 3	300µl Assay diluent C+200µl Standard No 2
640pg/ml	Standard No 4	300µl Assay diluent C+200µl Standard No 3
256pg/ml	Standard No 5	300µl Assay diluent C+200µl Standard No 4
102.4pg/ml	Standard No 6	300µl Assay diluent C+200µl Standard No 5
40.96pg/ml	Standard No 7	300µl Assay diluent C+200µl Standard No 6
0pg/ml	Standard No 8	300µl Assay diluent C

- If the Wash Concentrate (20X) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
- Briefly spin the Detection Antibody vial before use. Add 100 µl of 1X Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4ºC for 5 days). The detection antibody concentrate should be diluted 80-fold with 1X Assay Diluent B and used in step 5 of Part VI Assay Procedure.
- Briefly spin the HRP-Streptavidin concentrate vial and pipette up and down to mix gently before use, as precipitates may form during storage. HRP-Streptavidin concentrate should be diluted 400-fold with 1X Assay Diluent B.
-

Procedure

- Bring all reagents and samples to room temperature (18 - 25^{o}C) before use. It is recommended that all standards and samples be run at least in duplicate.
- Label removable 8-well strips as appropriate for your experiment.
- Add 100 µl of each standard and sample into appropriate wells. Cover wells and incubate for 2.5 hours at room temperature with gentle shaking.
- Discard the solution and wash 4 times with 1X Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel Pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
- Add 100 µl of 1X prepared biotinylated antibody to each well. Incubate for 1 hour at room temperature with gentle shaking.
- Discard the solution. Repeat the wash as in step 4.
- Add 100 µl of prepared Streptavidin solution to each well. Incubate for 45 minutes at room temperature with gentle shaking.
- Discard the solution. Repeat the wash as in step 4.
- Add 100 µl of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

- Add 50 µl of Stop Solution to each well. Read at 450 nm immediately.

4.5 Fixation and Processing of Tissues for Histological Studies.
Fixation and Processing of Tissue

1. Caudal region of tongue was excised from the rat and was stored in 10% formaldehyde until the processing for histopathological analysis.

2. The tongue sample was then kept in following solutions for 2 hours each i.e. 40% formalin, xylene, 60% alcohol, 70% alcohol, 80% alcohol and twice in 100% (Absolute) alcohol.

3. Later the tissues were kept in melted paraffin twice before drying. After they were dried, the blocks of paraffinized tissues were cut according to the size of tissue.

4. 5mm thick sections of tissues were serially cut on a leica microtome in horizontal plane and mounted on glass slide with help of egg albumin in glycerin solution glycerin solution.

5. The sections were deparaffinized in xylene and downgraded through descending grades of alcohol and finally water.

6. They were then stained with 10% hematoxyline for 3-5 minutes and staining was intensified by placing in running water.

7. These sections were stained with 10% eosin for 2 minutes and quickly passed through ascending grades of alcohol.

8. Finally they were treated with xylene followed by mounting and analyzing under OLYMPUS (trinocular-CX21FS1) microscope with 10X magnification setting.

5. RESULTS

5.1 Phytochemical Analysis

5.1.1 Preliminary Test for Identification of components of polyphenol rich extract

Phytochemical tests were carried out using polyphenol rich fraction for presence of various chemical moieties. Following are the list of chemical test performed.

Table 5.1.1 Preliminary tests for Identification of Chemical Constituents

Sr. No	Detection of chemical	Chemical test performed	Result
1	Test for Flavonoid	Shinoda Test	**Positive**
2	Test for Carbohydrates	Molish's Test	**Positive**
		Fehling's Test	**Positive**
		Benedict's Test	**Positive**
3	Test for Tannins	Ferric chloride Test	**Positive**
		KOH test	**Positive**
4	Test for Saponins	Foam Test	**Positive**
5	Test for phenolic compound	5% FeCl3	**Positive**
		Lead acetate	**Positive**
		Gelatine solution	**Positive**
		Bromine water	**Positive**
		Potassium dichromate	**Positive**
		HNO3	**Positive**
6	Test for Proteins	Biuret test5	Negative
7	Test for Alkaloids	Wagner's Test	Negative
		Mayer's Test	Negative
		Hager's Test	Negative
8	Test for Steroids	Lieberman Burchard test	Negative

5.2 Qualitative test:

Thin layer chromatography:

- Thin layer chromatography of the sample showed dark spots at Rf 0.38, 0.56 and 0.64 for corilagin, ellagic acid and gallic acid repectively. The mobile phase used for gallic acid and corilagin was toluene: ethyl acetate: formic acid: methanol (5: 5: 2.5: 2.5) and for ellagic acid, it was toluene: ethyl acetate: formic acid (5: 5: 2.5).

Table 5.2.1 Polyphenols obtained on TLC plates

Plate used	Merck silica gel 60 F254 on an aluminum support (10cm)
Concentration of the extract used	2 mg/ml
Mobile Phase used for gallic acid and corilagin	toluene: ethyl acetate: formic acid: methanol (5: 5: 2.5: 2.5)
Mobile Phase used for ellagic acid	toluene: ethyl acetate: formic acid (5: 5: 2.5)
Spot measurement	283 nm
Rf obtained	• Corilagin- 0.38 • Ellagic acid- 0.563 • Gallic acid- 0.64

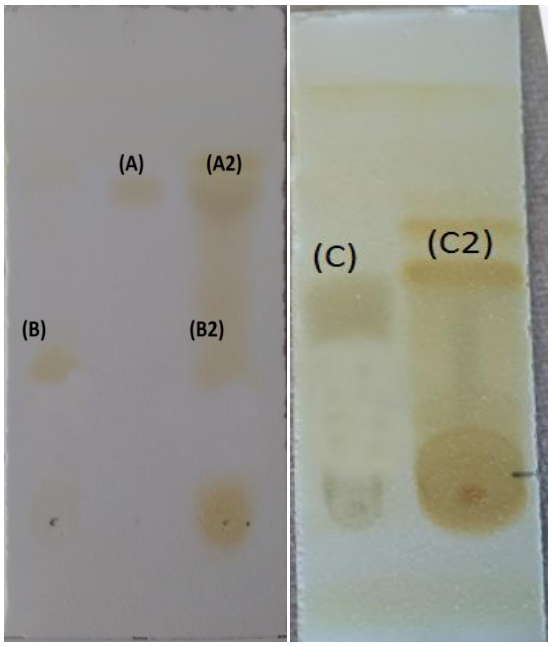


Figure 5.2.2 Spots obtained on TLC plate

Standard: (A)- Gallic acid, (B)- Corilagin, (C)- Ellagic acid

Extract: (A2)- Gallic acid, (B2)- Corilagin, (C2)- Ellagic acid

5.3 4- NQO induced Oral Cancer in rats

5.3.1 General Parameters

At the end of 24 weeks, 4-Nitroquinoline N-oxide (4-NQO) produced a significant decrease in body weight, food and water intake in oral cancer diseased animals as compared to the normal control group. The disease treated groups with *Euphoria longana* from day 0 of induction of oral cancer showed constant body weight of animals. There was reduction in body weight, food and water intake of diseased animals treated with *Euphoria longana,* 5- fluorouracil (5-FU, 24.5mg/kg) and combined treatment of both *Euphoria longana* and 5-FU after 24 weeks of induction which was less than that of the oral cancer control group. However no significant improvement in the body weight, food and water intake of diseased animals was seen after the treatment. (figure 5.3.1.a, 5.3.1.b, 5.3.1.c)

(a) Body weight

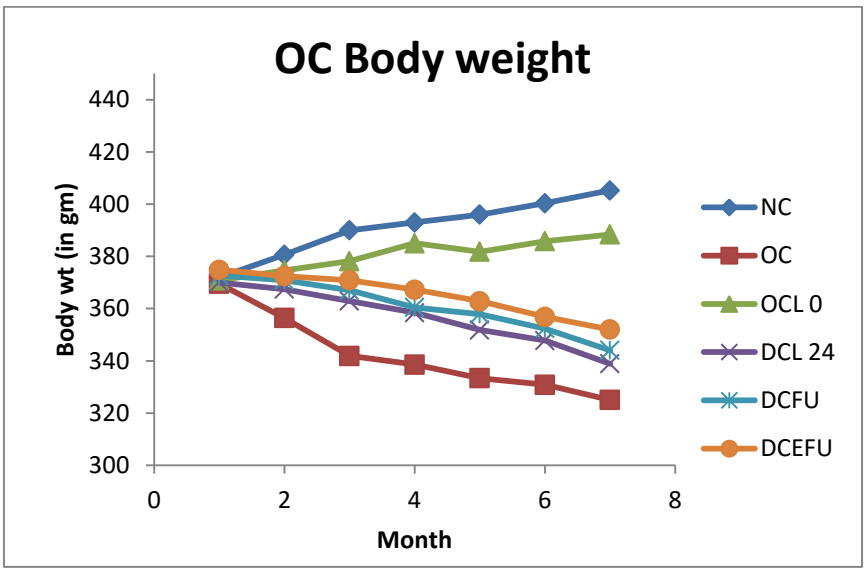


Figure 5.3.1 (a) The effect on body weight of 4-NQO induced oral cancer rats.

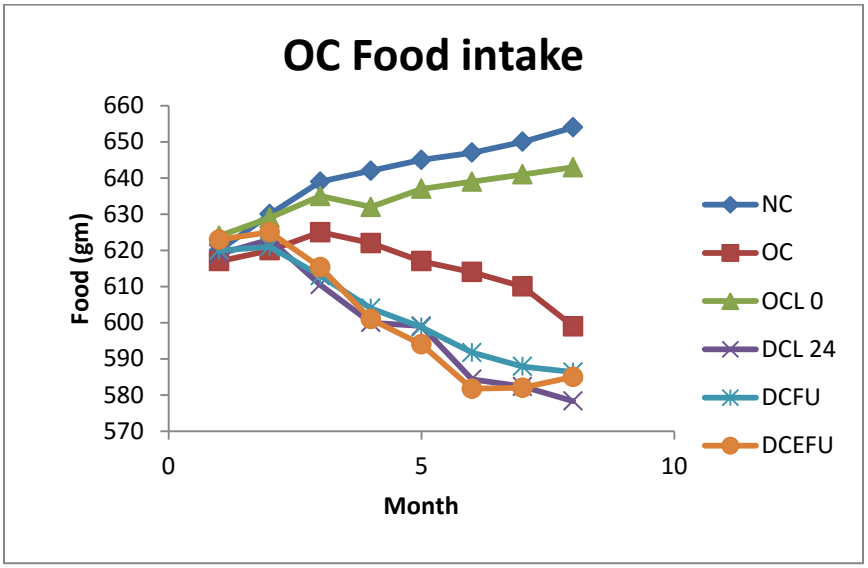


Figure 5.3.1 (b) The effect on food intake of 4-NQO induced oral cancer rats.

NC Normal control, OC -Oral cancer disease control
OCE- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from day 0
OCE24- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from week 24
OCF- OC treated with 5-FU (24.5mg/kg p.o.) from week 24
OCEF- OC treated with *Euphoria longana* (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.) from week 24 respectively.

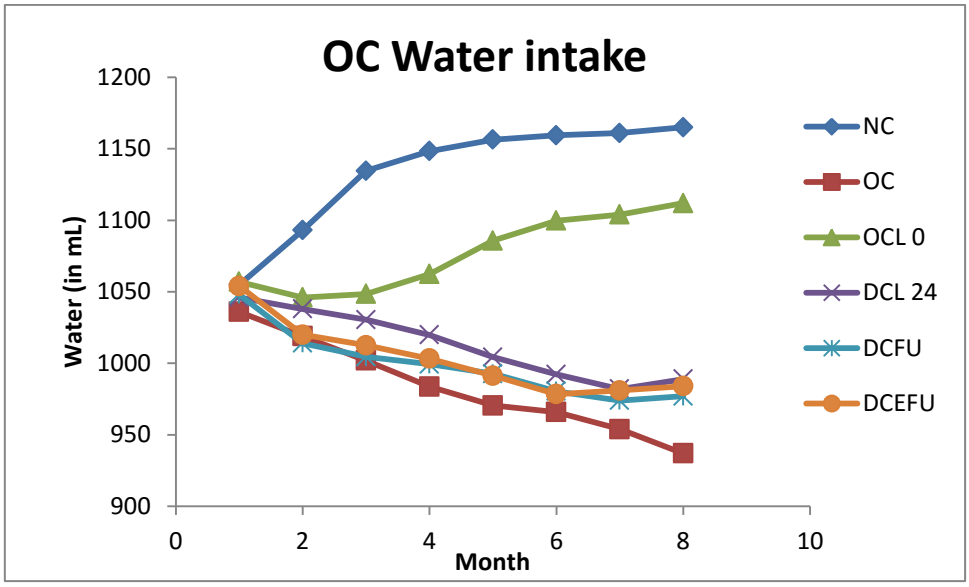


Figure 5.3.1 (c) The effect on water intake of 4-NQO induced oral cancer rats.

NC Normal control
OC -Oral cancer disease control
OCE- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from day 0
OCE24- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from week 24
OCF- OC treated with 5-FU (24.5mg/kg p.o.) from week 24
OCEF- OC treated with *Euphoria longana* (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.) from week 24 respectively

5.3.2 Oxidative stress parameter

Effect on Malondialdehyde (MDA), Glutathione (GSH) and Superoxide dismutase (SOD) levels on 4-NQO induced oral cancer rats.

The MDA levels were significantly increased in the diseased oral cancer control groups ($p < 0.001$) as compared to the normal control group (NC). But in comparision to the diseased oral cancer groups (OC), the level was significantly decreased ($p < 0.001$) in the group of animals treated with *Euphoria longana* 280mg/kg p.o (from 0 day of induction as a preventive therapy for oral cancer), 5- fluorouracil (5-FU, 24.5mg/kg) and with combination of the treatment with both *Euphoria longana* (EL) and 5-FU after 24 weeks of induction. Also there was significant decrease ($p < 0.01$) in the MDA level of the animals treated with *Euphoria longana* after 24th week of induction in correspondence to diseased oral cancer control groups.

GSH level was significantly decreased ($p < 0.001$) in oral cancer control group as compared to normal control group where as the level was found to be significantly increased ($p<0.05$) in oral cancer groups treated with *Euphoria longana* 280mg/kg p.o (from 0 day of induction), 5- fluorouracil (5-FU, 24.5mg/kg) and combined treatment of

both *Euphoria longana* and 5-FU after 24 weeks of induction. There was decrease in the GSH level of group treated with Euphoria longana after 24 weeks of induction but was not found significant to the level of oral cancer control group.

The level of Superoxide dismutase (SOD) enzyme was decreased significantly ($p < 0.001$) in oral cancer control group when compared with normal control group and the same was found significantly increased in the groups treated with *Euphoria longana* 280mg/kg p.o. (from 0 day of induction), 5- fluorouracil (5-FU, 24.5mg/kg) and combined treatment with both *Euphoria longana* and 5-FU after 24 weeks of induction. Also the level of SOD was found to be increased in oral cancer treated with *Euphoria longana* only after 24 weeks of induction but was less significant ($p < 0.01$) as compared to the other treatment groups. Though there was significant increase in the SOD level in 5- FU alone treated group as well as both EL and FU combined treated group but on the contrary the level was found more increased in FU treated group as compared to the combination therapy.

Table 5.3.2: The effect on MDA, GSH and SOD levels in 4-NQO induced oral cancer rats.

Parameters	NC	OC	OCE0	OCE24	OCFU	OCEFU
MDA levels (nmoles/mg of protein)	51.23 ± 1.317	63.55 ± 1.213***	57.96 ± 1.309###	59.77 ± .7726##	57.10 ± .5311###	57.18 ± .8571###
GSH levels (µg/ mg of protein)	2.648 ±0.1755	1.708 ±1.794***	2.069 ±0.1149 #	2.029 ±0.02	2.087 ±0.01#	2.130 ±0.09774#

SOD activity (Units/ mg of protein)	0.04333 ±0.007073	0.01187 ±0.00211***	0.03349 ±0.005041###	0.02843 ±0.0008402###	0.03945 ±0.004636###	0.03328 ±0.003473###

Each group consists of nine animals. Values are expressed as Mean ± SEM.
* Significantly different from the Normal control group (p<0.05), ** (p<0.01), *** (p<0.001), # Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001), NC- Normal control, OC- Oral cancer disease control, OCE- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from day 0, OCE24- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from week 24, OCF- OC treated with 5-FU (24.5mg/kg p.o.) from week 24, OCEF- OC treated with *Euphoria longana* (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.) from week 24 respectively.

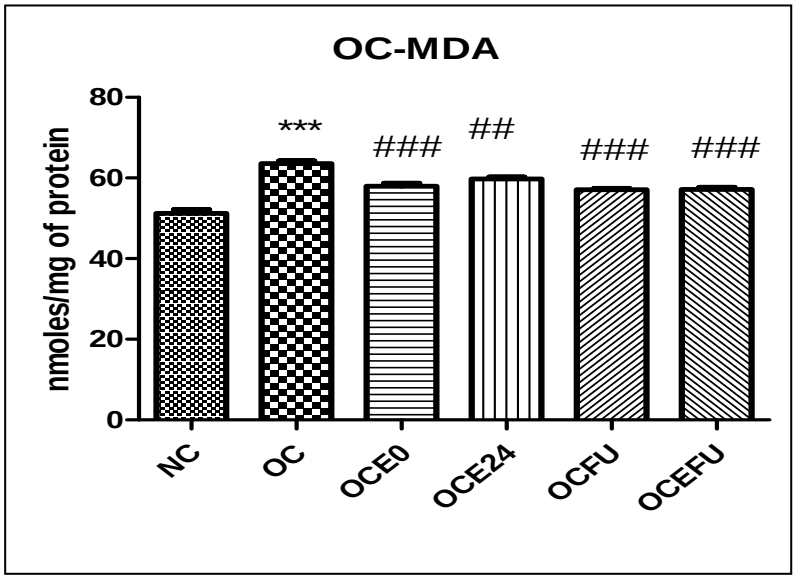


Figure 5.3.2 (a) Effect on MDA levels in 4-NQO induced oral cancer rats.

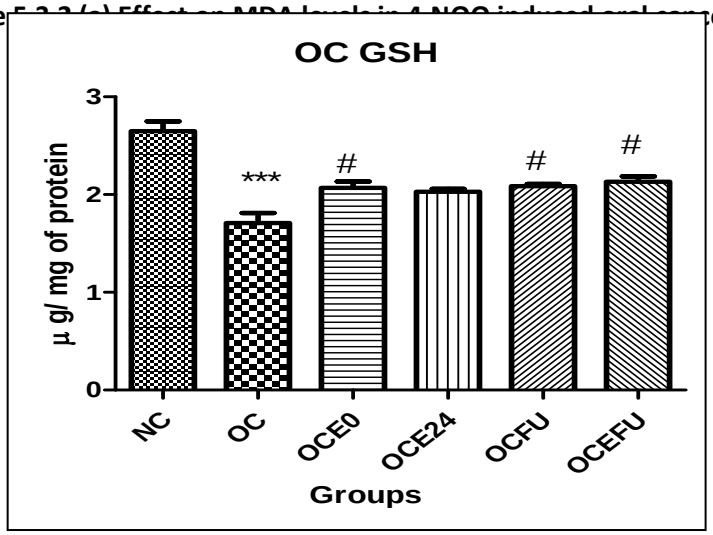


Figure 5.3.2 (b) Effect on GSH levels in 4-NQO induced oral cancer rats.

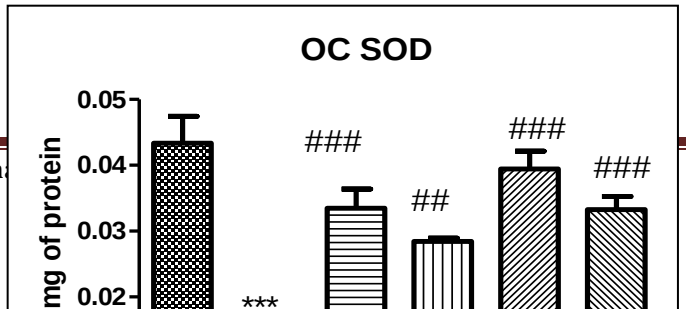


Figure 5.3.2 (c) Effect on SOD levels in 4-NQO induced oral cancer rats.

Each group consists of nine animals. Values are expressed as Mean ± SEM.
* Significantly different from the Normal control group ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), # Significantly different from the Disease control group ($p<0.05$), ## ($p<0.01$), ### ($p<0.001$), NC- Normal control, OC- Oral cancer disease control, OCE- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from day 0, OCE24- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from week 24, OCF- OC treated with 5-FU (24.5mg/kg p.o.) from week 24, OCEF- OC treated with *Euphoria longana* (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.) from week 24 respectively.

5.3.3. Tumour Specific Parameters

(a) Interlukin-6 (IL-6)

The level of pro inflammatory marker IL-6 was found significantly ($p<0.05$) increased in oral cancer disease group than normal control group and there was seen reduction of IL-6 level in the groups treated with *Euphoria longana* 280mg/kg p.o (from 0 day of induction), *Euphoria longana* 280mg/kg p.o, 5-fluorouracil (5-FU, 24.5mg/kg) and combined therapy of both after 24 weeks of induction. But the reduction was not significant as compared to the diseased group. However, the IL-6 level was found more reduced in group treated with *Euphoria longana* from 0 day of induction but that reduction was less as compared to the oral cancer treated with 5- fluorouracil (5-FU, 24.5mg/kg) and combination of both. The combination of both *Euphoria longana* 280mg/kg p.o, 5- fluorouracil (5-FU, 24.5mg/kg) was found to reduce the level more effectively as compared to other treatment groups.

Table 5.3.3: Effect on IL-6 levels in 4-NQO induced oral cancer rats.

Parameter	NC	OC	OCE0	OCE24	OCFU	OCEFU

IL- 6 levels (pg/mL)	1650 ± 56.57***	5390 ± 1212	2780 ±381.8	4238 ±1382	3223 ± 1500	2813 ± 313.4

Each group consists of nine animals. Values are expressed as Mean ± SEM.
* Significantly different from the Normal control group (p<0.05), ** (p<0.01), *** (p<0.001), # Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001)
NC- Normal control, OC- Oral cancer disease control, OCE- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from day 0, OCE24- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from week 24, OCF- OC treated with 5-FU (24.5mg/kg p.o.) from week 24, OCEF- OC treated with *Euphoria longana* (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.) from week 24 respectively.

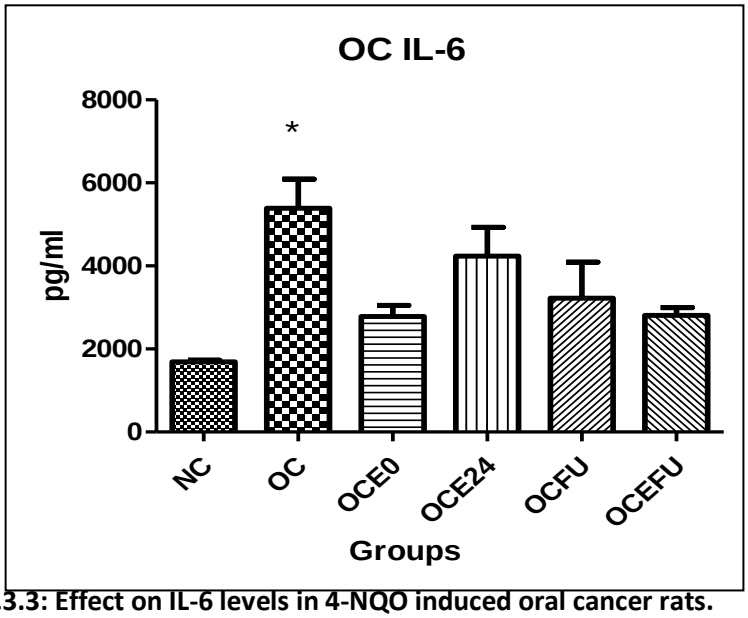


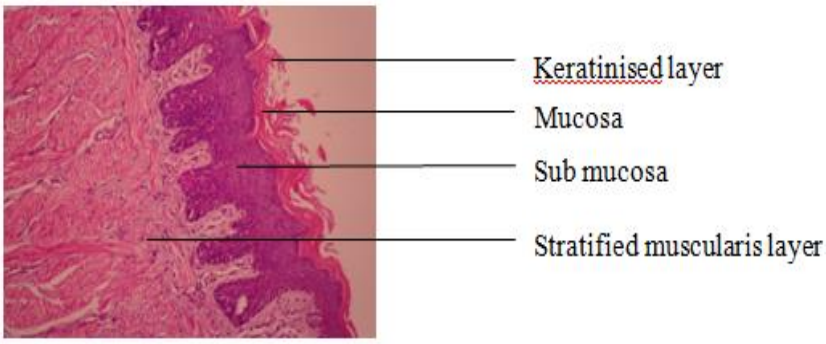
Figure 5.3.3: Effect on IL-6 levels in 4-NQO induced oral cancer rats.

Each group consists of nine animals. Values are expressed as Mean ± SEM.
* Significantly different from the Normal control group (p<0.05), ** (p<0.01), *** (p<0.001), # Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001), NC- Normal control, OC- Oral cancer disease control, OCE- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from day 0, OCE24- OC treated with *Euphoria longana* extract (280mg/kg p.o.) from week 24, OCF- OC treated with 5-FU (24.5mg/kg p.o.) from week 24, OCEF- OC treated with *Euphoria longana* (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.) from week 24 respectively.

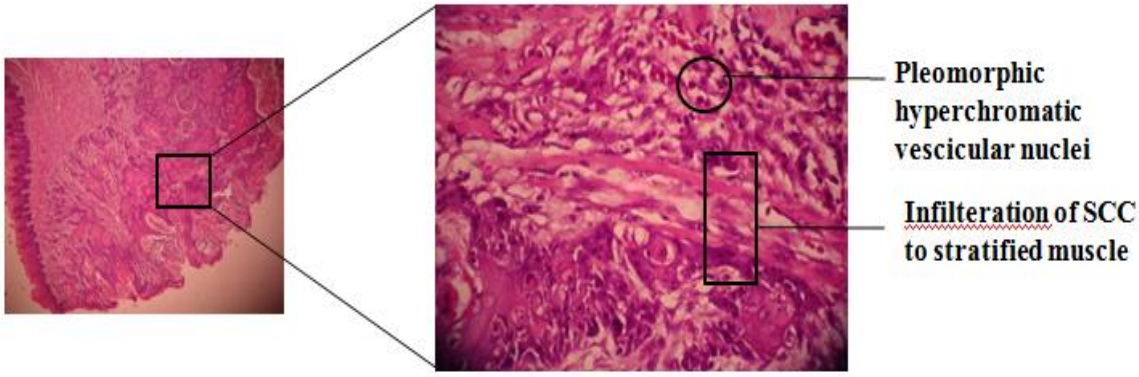
5.3.4 Histopathological study

The section of excised tongue of normal control group animal shows normal mucosa, sub mucosa, stratified muscles and normal papillae. The normal tongue has a keratin layer and normal nucleus present in squamous epithelium. The section of a disease control group (OC) reveals stratified squamous epithelium with squamous cells having pleomorphic

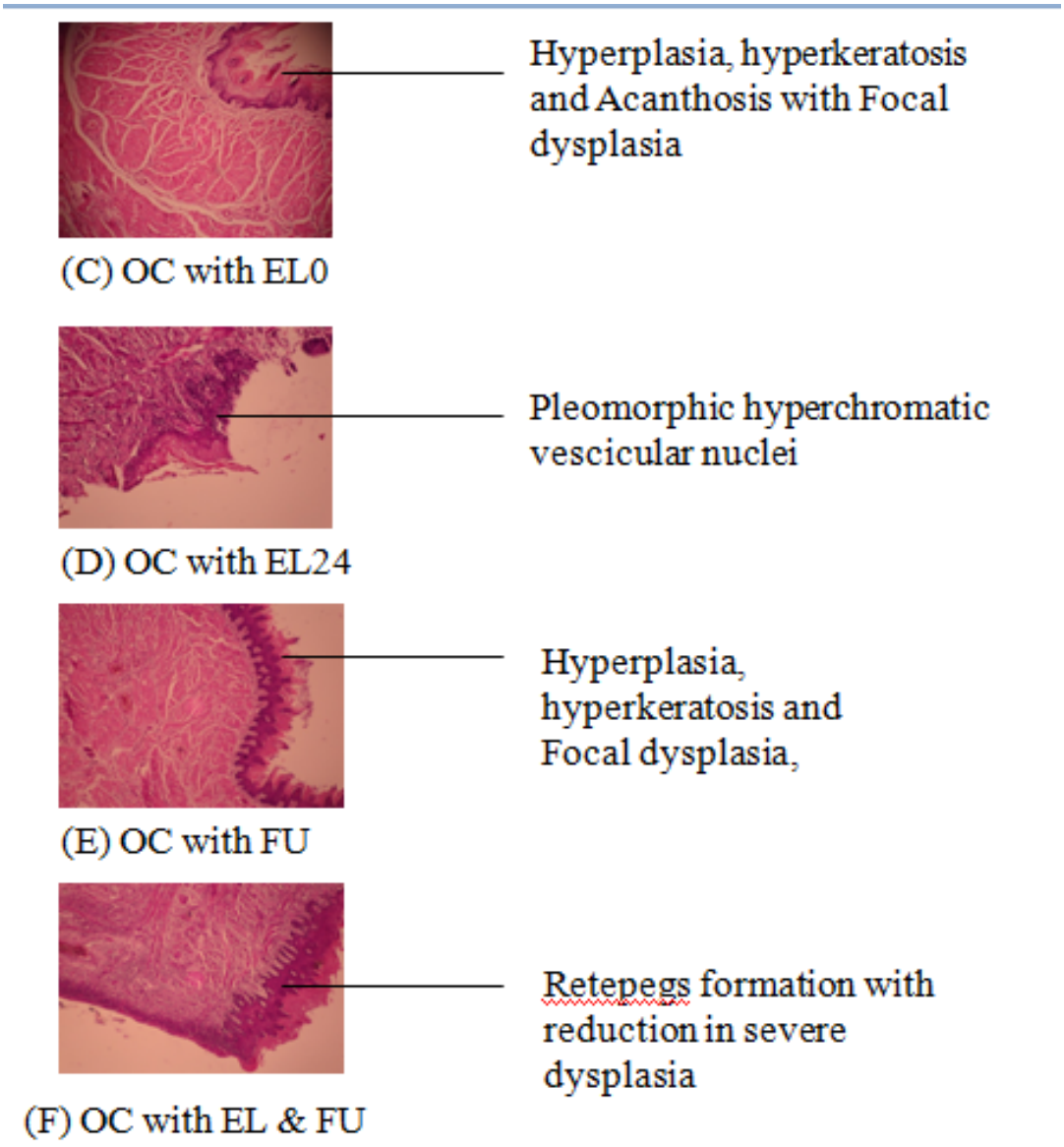
hyperchromatic vescicular and prominent nuclei, and pinkish cytoplasm. Mitosis is present with no invasions seen into the deeper tissues with squamous cells infilterating the stratified muscles. But these cancerous conditions were not observed in the treatment groups with *Euphoria longana,* 5-FU and combination of both treatment in oral cancer group from day 0 (OCEL0). But only hyperplasia, hyperkeratosis and acanthosis were observed in treatment groups. There was formation of retepegs in *Euphoria longana* treatment group after 24th week (OCEL24). The oral cancer groups treated with standard (5- FU) and with the combination of both (EL and FU) showed less of the cancerous conditions and with reduced pleomorphic and prominent nuclei.



(A) Normal control



(B) Disease oral cancer control



(A) NC Normal control, (B) OC Oral cancer disease control, (C) OC treated with *Euphoria longana* extract (280mg/kg p.o.) from day 0, (D) OCE24 OC treated with *Euphoria longana* extract (280mg/kg p.o.) from day 24, (E) OCF OC treated with 5-FU (24.5mg/kg p.o.) from day 24 and (F) OCEF OC treated with *Euphoria longana* (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.) from week 24 respectively.

5.4. 4- NQO induced oral cancer in Diabetic rats

5.4.1 General Parameters

The body weight of animals induced with diabetes was increased after high fat diet and was found increasing till the end of the study as compared to the normal control groups. The animals treated with Metformin and *Euphoria longana* resulted in slightly reduced weight of animals. But the animals induced with both diabetes and oral cancers were found to have reduced body weight throughout the study. Also in the treatment groups of diabetic oral cancer induced animals, there was reduction in the body weight from the time oral cancer was induced.

Though, the food intake as well as the body weight during the induction of diabetes was increased but after the cancer induction, the food intake was reduced as compared to the normal and only diabetes induced animals. The water intake was normal in the animals which was observed sometimes decreasing and sometimes increasing but only in the groups induced with diabetes and oral cancer both the water intake was found reduced as compared to other groups. This suggests that the decrease in water intake was due to the induction of oral cancer. Even in the treatment groups of animals there was not seen any changes in water intake.

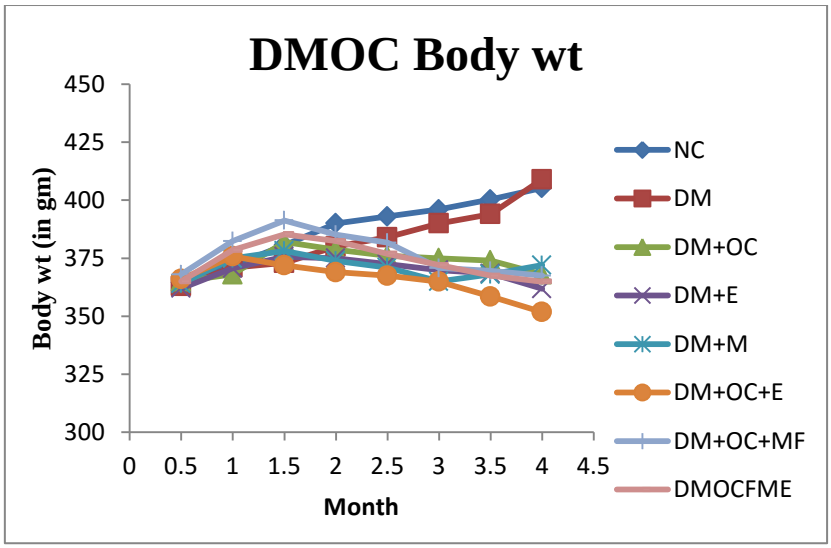


Figure 5.4.1 (a) The effect on body weight in 4-NQO induced oral cancer in Diabetic rats

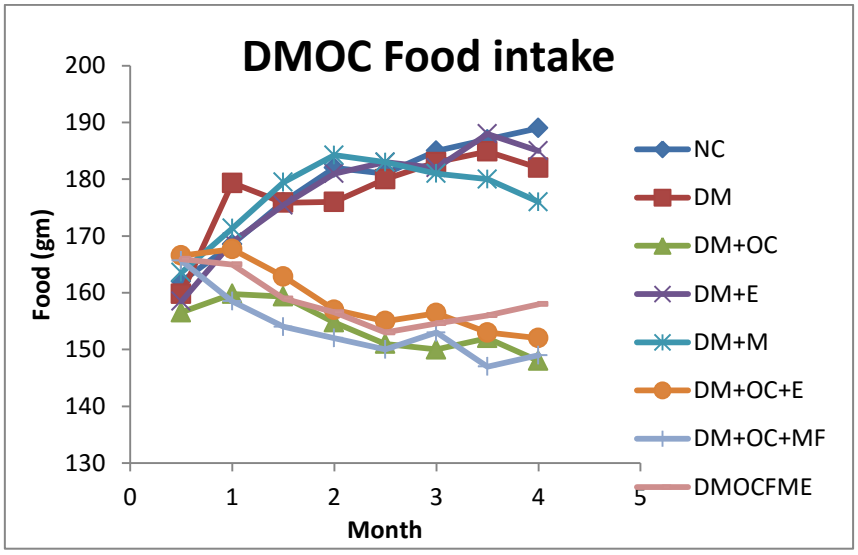


Figure 5.4.1 (b) The effect on food intake in 4-NQO induced oral cancer in Diabetic rats

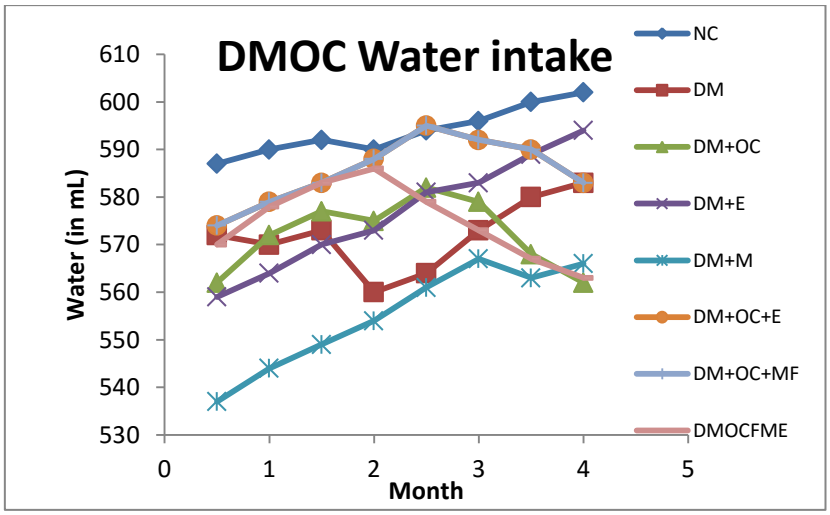


Figure 5.4.1 (c) The effect on water intake in 4-NQO induced oral cancer in Diabetic rats

NC: Normal control, DM: Diabetic disease control, DMOC: Diabetic Oral cancer disease control, DME: Diabetes treated with *Euphoria longana* extract (280mg/kg p.o.), DMM: Diabetes treated with Metformin (400mg/kg p.o.), DMOCE: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.)
DMOCFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.), DMOCEFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), Metformin (400mg/kg p.o.) and 5-FU (24.5mg/kg p.o.)

5.4.2. Glycemic parameters

(a) Glucose

Glucose was found normal in normal control group but was elevated significantly ($p<0.001$) in diabetic disease control and diabetic oral cancer control groups. The diabetic group treated with metformin (400mg/kg p.o.) alone have been found with significantly reduced glucose levels significantly ($p<0.001$) as compared to diabetes mellitus control groups (DM). However, the level was also found reduced in the diabetic groups treated with *Euphoria longana* (280mg/kg p.o.) but was not significant as compared to the diabetic control groups. Moreover, the glucose levels were reduced significantly ($p<0.001$) in diabetic oral cancer groups treated with both standard drugs, Metformin (400mg/kg p.o.) and 5- fluorouracil (24.5mg/kg p.o.) and also in the groups treated with combination of Metformin (400mg/kg p.o.), 5- fluorouracil (24.5mg/kg p.o.) and Euphoria longana (280mg/kg p.o.). Also *Euphoria longana* (280mg/kg p.o.) alone reduced the glucose level in diabetic oral cancer groups but the reduction was not significant as compared to the disease control groups. Only the standard treatment was found to be effective in reducing the blood glucose level.

(b) Glycosylated Hemoglobin (HbA1c)

The glycated Hb was significantly ($p<0.001$) increased in the diabetic and diabetic oral cancer control groups compared to the normal control group but was slightly found higher when compared to diabetic oral cancer control group. The diabetic animals treated with only Metformin (400mg/kg p.o.) and only *Euphoria longana* (280mg/kg p.o.) were found with significantly reduced level of HbA1c with $p< 0.001$ and $p< 0.05$ respectively indicating the effectiveness of standard therapy with metformin. There was comparatively significant reduction of the levels in the diabetic oral cancer control group which were treated with *Euphoria longana* ($p<0.01$) and with combination of both standard drugs metformin and 5-fluorouracil alongwith *Euphoria longana* ($p<0.001$). But there was not seen any reduction in HbA1c in the animals treated with only standard drugs metformin and 5-fluorouracil.

(c) Oral Glucose Tolerance Test (OGTT)

The tolerance for glucose loaded orally in the diabetic rats was measured where there was significantly ($p<0.01$) less tolerability in Diabetic oral cancer control group (DMOC) than normal control group (NC). However the tolerability was also reduced in diabetic group (DM) but was not significant as compared to DMOC. The treated groups of DMOC with standard combination i.e metformin and 5- FU and the other combination of EL, metformin and 5- FU have shown significantly improved glucose tolerance ($p<0.005$).

Table 5.4.2 The effect on Glucose, Glycosylated Hb and OGTT levels in diabetic oral cancer rats

Parameter	NC	DM	DMOC	DME	DMM	DMOCE	DMOCFM	DMOCEFM

Glucose	93.06± 11.27	152.8± 8.182***	210± 8.272*** ###	133± 5.798	92.18±6.774 ###	129.6±8.180 $$$	101.5±14.24 $$$	114.8± 2.616 $$$
HbA1c	7.685± 0.09192	14.28 ± 0.3182***	13.230± 0.3748***	12.06± 0.7778#	8.005± 0.04950###	10.21± 0.2609$$	12.80± 0.9263	8.238± 0.1584$$$
OGTT	134.4±38.69	188.9± 43.35	267.5± 64.61**	192±58.22#	125.7± 18.35###	188± 52.03	152.9± 47.45$	151.6± 42.92$

Each group consists of 8 animals. Values are expressed as Mean ± SEM.
* Significantly different from the Normal control group (p<0.05), ** (p<0.01), *** (p<0.001), # Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001), $ Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001)

NC: Normal control, DM: Diabetic disease control, DMOC: Diabetic Oral cancer disease control, DME: Diabetes treated with *Euphoria longana* extract (280mg/kg p.o.), DMM: Diabetes treated with Metformin (400mg/kg p.o.), DMOCE: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), DMOCFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.), DMOCEFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), Metformin (400mg/kg p.o.) and 5-FU (24.5mg/kg p.o.)

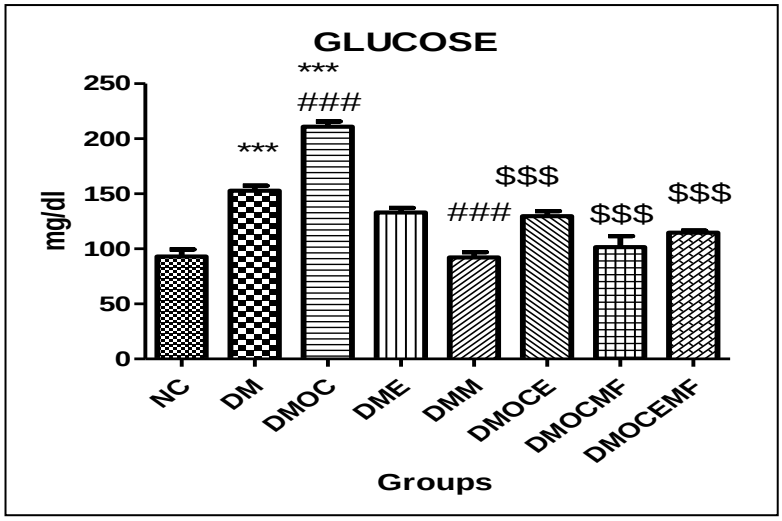


Figure 5.4.2 (a) The effect on Glucose levels of diabetic oral cancer rats

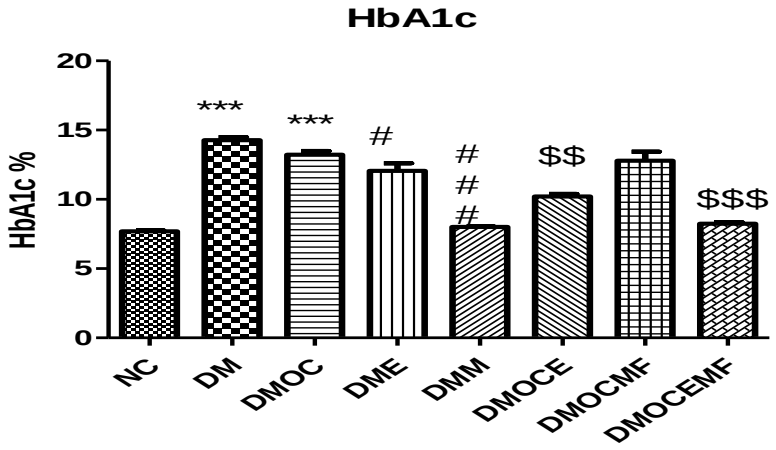


Figure 5.4.2 (b) The effect on HbA1c levels of diabetic oral cancer rats

Each group consists of 8 animals. Values are expressed as Mean ± SEM.
* Significantly different from the Normal control group (p<0.05), ** (p<0.01), *** (p<0.001), # Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001), $ Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001)

NC: Normal control, DM: Diabetic disease control, DMOC: Diabetic Oral cancer disease control, DME: Diabetes treated with *Euphoria longana* extract (280mg/kg p.o.), DMM: Diabetes treated with Metformin (400mg/kg p.o.), DMOCE: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), DMOCFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.), DMOCEFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), Metformin (400mg/kg p.o.) and 5-FU (24.5mg/kg p.o.)

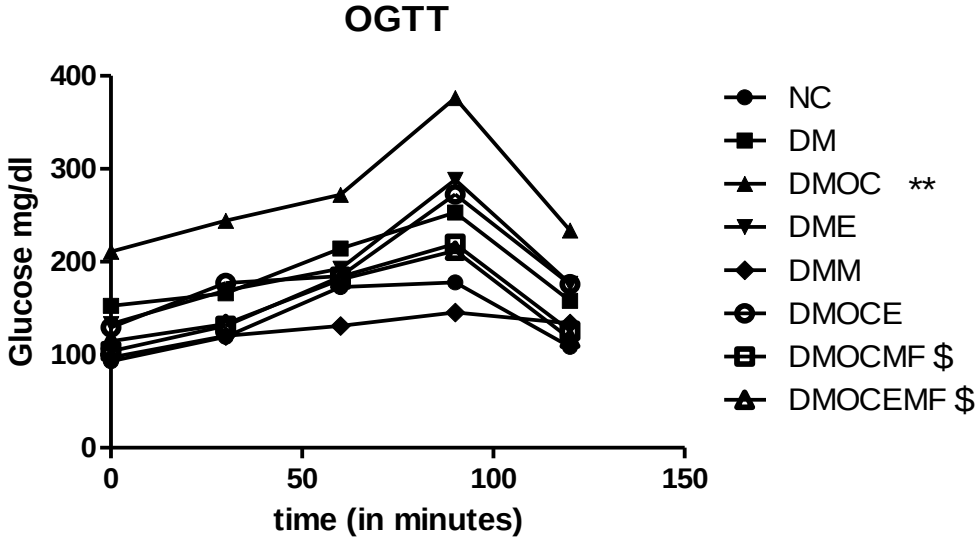


Figure 5.4.2 (c) The effect on OGTT levels of diabetic oral cancer rats

Each group consists of 8 animals. Values are expressed as Mean ± SEM.
* Significantly different from the Normal control group (p<0.05), ** (p<0.01), *** (p<0.001), # Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001), $ Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001)

NC: Normal control, DM: Diabetic disease control, DMOC: Diabetic Oral cancer disease control, DME: Diabetes treated with *Euphoria longana* extract (280mg/kg p.o.), DMM: Diabetes treated with Metformin (400mg/kg p.o.), DMOCE: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), DMOCFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.), DMOCEFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), Metformin (400mg/kg p.o.) and 5-FU (24.5mg/kg p.o.)

5.4.3 Oxidative stress parameters

Malonaldehyde (MDA) levels were significantly increased in diabetic control ($p<0.001$) and diabetic oral cancer control ($p<0.01$) groups as compared to the normal control group (NC). But in the treatment groups of diabetes mellitus with metformin (DMM) and *Euphoria longana* (DME) the MDA level was reduced but was not significant to that of DM group with more reduction of MDA seen in metformin treated animals. Moreover, there was significant reduction of MDA levels in the animals treated with *Euphoria longana* ($p<0.05$), with both Metformin and 5- FU ($p<0.01$) and with combination of *Euphoria longana*, metformin and 5-FU ($p<0.001$). However more reduction was seen in both metformin and 5- FU treated groups as well as in EL, metformin and 5- FU combined treated groups as compared to the other treated groups.

GSH level was significantly ($p<0.001$) reduced in diabetic disease control (DM) and diabetic oral cancer control groups (DMOC) as compared to the normal control group (NC). But *Euphoria longana* (EL) and metformin treated diabetic groups were found with slight increase in the GSH levels. Though the elevation in the level was not significant as compared to diabetic disease control group (DM) but metformin treated group were found with higher level of GSH than that of EL comparatively. There was significant increase in GSH level of treatment groups with both metformin and 5-FU (($p<0.01$) DMOCMF) and in groups with combined treatment of EL, Metformin and 5-FU (($p<0.001$) DMOCEMF) while combination treatment group (DMOCEMF) having higher levels than that of only Metformin and 5-FU (DMOCMF) treated group. Also there was increased GSH level in diabetic oral cancer group treated with only EL but was not significant to DMOC.

The level of Superoxide dismutase activity was found reduced significantly in diabetic disease control ($p<0.05$) and diabetic oral cancer control groups ($p<0.001$) as compared to normal control group (NC). But *Euphoria longana* (EL) and metformin treated diabetic groups were found with slight increase in the SOD levels. Though the elevation in the level was not significant as compared to diabetic disease control group but metformin treated group have higher level of enzyme than EL. There was significant increase of SOD level in treatment groups with both metformin and 5-FU ($p<0.05$) and in groups with combined treatment of EL, Metformin and 5-FU ($p<0.01$) with DMOCEMF group having higher levels than DMOCMF group. Also there was increased SOD level in diabetic oral cancer group treated with only EL but was not significant to DMOC.

Table 5.4.2: The effect on MDA, GSH and SOD levels in diabetic 4-NQO induced oral cancer rats.

Parameter	NC	DM	DMOC	DME	DMM	DMOCF	DMOCFM	DMOCEFM
MDA	49.57± 11.260	54.95± 2.234***	65.24±1.546 #	58.52±1.088	52.73±.2291	59.08 ± 1.661 $	55.52 ±51.54 $$	53.99± 1.979 $$$
GSH	2.648± 0.1755	1.991 ± 0.06548 ***	1.649 ± 0.01819 ***	2.076± 0.09822	2.3 ±0.08039	1.971 ± 0.05843	2.318 ± 0.1200 $$	2.560 ± 0.04001 $$$
SOD	0.04333±0.007073	0.02424 ± 0.004083*	0.01530 ± 0.005145 ***	0.02622 ±0.004998	0.03110 ± 0.000441 0	0.02372 ± 0.003146	0.03620 ± 0.001764 $	0.03900 ± 0.001029 $$

Each group consists of 8 animals. Values are expressed as Mean ± SEM.
* Significantly different from the Normal control group (p<0.05), ** (p<0.01), *** (p<0.001), # Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001), $ Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001)

NC: Normal control, DM: Diabetic disease control, DMOC: Diabetic Oral cancer disease control, DME: Diabetes treated with *Euphoria longana* extract (280mg/kg p.o.), DMM: Diabetes treated with Metformin (400mg/kg p.o.), DMOCE: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), DMOCFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.), DMOCEFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), Metformin (400mg/kg p.o.) and 5-FU (24.5mg/kg p.o.)

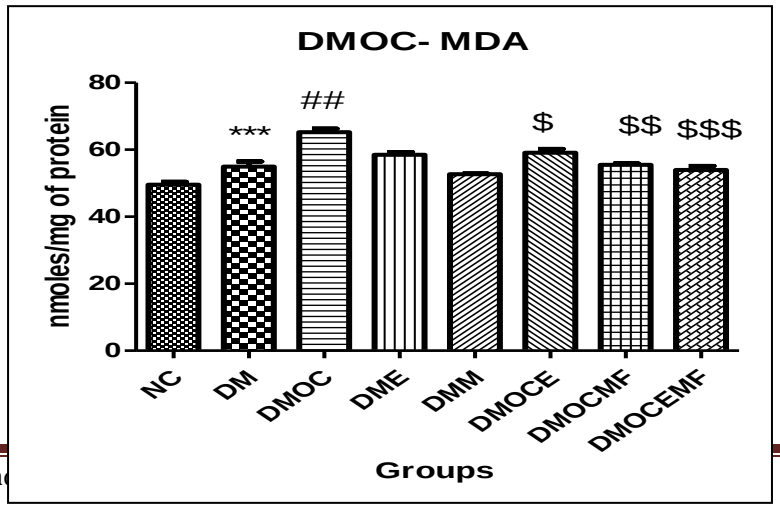


Figure 5.4.3 (a) The effect on MDA levels of diabetic oral cancer rats

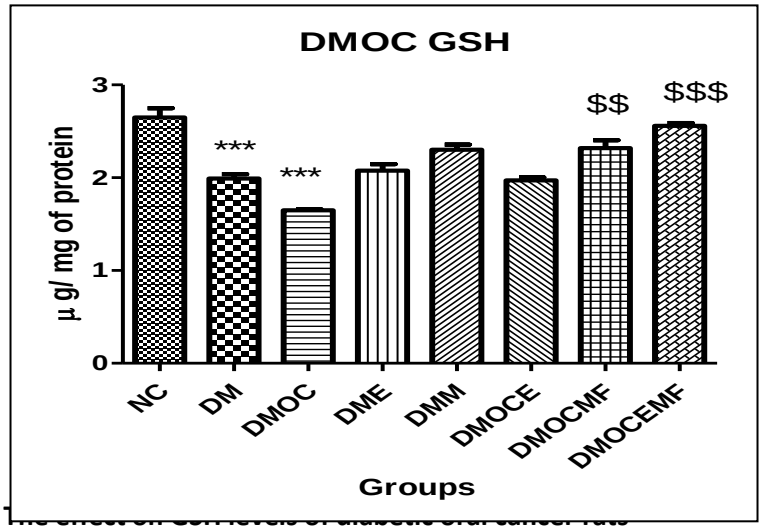


Figure 5.4.2 (b) T

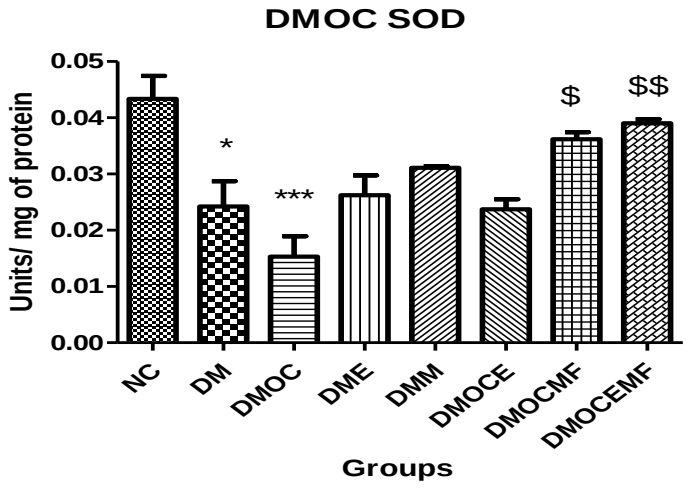


Figure 5.4.2 (c) The effect on SOD levels of diabetic oral cancer rats

Each group consists of 8 animals. Values are expressed as Mean ± SEM.
* Significantly different from the Normal control group ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), # Significantly different from the Disease control group ($p<0.05$), ## ($p<0.01$), ### ($p<0.001$), $ Significantly different from the Disease control group ($p<0.05$), ## ($p<0.01$), ### ($p<0.001$)

NC: Normal control, DM: Diabetic disease control, DMOC: Diabetic Oral cancer disease control, DME: Diabetes treated with *Euphoria longana* extract (280mg/kg p.o.), DMM: Diabetes treated with Metformin (400mg/kg p.o.), DMOCE: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), DMOCFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.), DMOCEFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), Metformin (400mg/kg p.o.) and 5-FU (24.5mg/kg p.o.)

5.4.4 Inflammatory Markers

(a) Interlukin-6

The IL-6 is an inflammatory marker which was increased in the diabetic control (DM) and diabetic oral cancer control (DMOC) groups as compared to the normal control group (NC) but there was no significant difference between them. The IL-6 level was reduced in the diabetic group treated with metformin (DMM) and Euphoria longana (DML) but no significant difference was found. However, the metformin treatment was more effective than *Euphoria longana*. Also in the diabetic oral cancer group treated with both Metformin and 5- FU and with combination of *Euphoria longana*, metformin and 5- FU (DMOCEMF), IL-6 levels were reduced but without any significant difference.

Table 5.4.3 (a) The effect on IL-6 levels of diabetic oral cancer rats

Parameter	NC	DM	DMOC	DME	DMM	DMOCE	DMOCFM	DMOCEFM
IL-6	1690± 56.57	3223 ±1500	5705 ± 2029	2735 ±502	2500 ±1103	3295 ± 247.5	2925 ± 233.3	1725± 35.36

Each group consists of 8 animals. Values are expressed as Mean ± SEM.
* Significantly different from the Normal control group (p<0.05), ** (p<0.01), *** (p<0.001), # Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001), $ Significantly different from the Disease control group (p<0.05), ## (p<0.01), ### (p<0.001)

NC: Normal control, DM: Diabetic disease control, DMOC: Diabetic Oral cancer disease control, DME: Diabetes treated with *Euphoria longana* extract (280mg/kg p.o.), DMM: Diabetes treated with Metformin (400mg/kg p.o.), DMOCE: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), DMOCFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.), DMOCEFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), Metformin (400mg/kg p.o.) and 5-FU (24.5mg/kg p.o.)

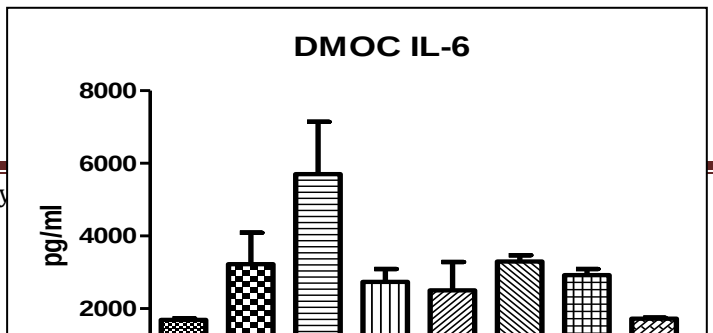


Table 5.4.4 (a) The effect on SOD levels in diabetic oral cancer rats

Each group consists of 8 animals. Values are expressed as Mean ± SEM.
* Significantly different from the Normal control group ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), # Significantly different from the Disease control group ($p<0.05$), ## ($p<0.01$), ### ($p<0.001$), $ Significantly different from the Disease control group ($p<0.05$), ## ($p<0.01$), ### ($p<0.001$)

NC: Normal control, DM: Diabetic disease control, DMOC: Diabetic Oral cancer disease control, DME: Diabetes treated with *Euphoria longana* extract (280mg/kg p.o.), DMM: Diabetes treated with Metformin (400mg/kg p.o.), DMOCE: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), DMOCFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.) and 5-FU (24.5mg/kg p.o.), DMOCEFM: Diabetic oral cancer treated with *Euphoria longana* extract (280mg/kg p.o.), Metformin (400mg/kg p.o.) and 5-FU (24.5mg/kg p.o.)

5.4.5 Histopathological study

The section of excised tongue of normal control group animal shows normal mucosa, sub mucosa, stratified muscles and normal papillae. The normal tongue has a keratin layer and normal nucleus present in squamous epithelium. No changes were observed in the diabetes mellitus induced group of animals and in the treated groups of diabetes mellitus. Only hyperkeratinization on the epithelium was observed. But in the animals induced with diabetes mellitus and oral cancer (DMOC), there were elongated retepegs observed with hyperkeratinization, keratohyaline granules and inflammatory cells in the sub epithelial layer. The keratohyaline granules might be present due to some viral etiology. The histopathological section of diabetic oral cancer animals treated with *Euphoria longana* (DMOCEL), Metformin and FU (DMOCMF) revealed the presence of keratohyaline granules but were seen reduced as compared to the diabetic oral cancer control groups. In the diabetic oral cancer groups treated with the combination of EL, Metformin and FU (DMOCEMF), only hyperkeratosis was observed with inflammatory cells present in the cytoplasm and with the reduction in the keratohyaline granules.

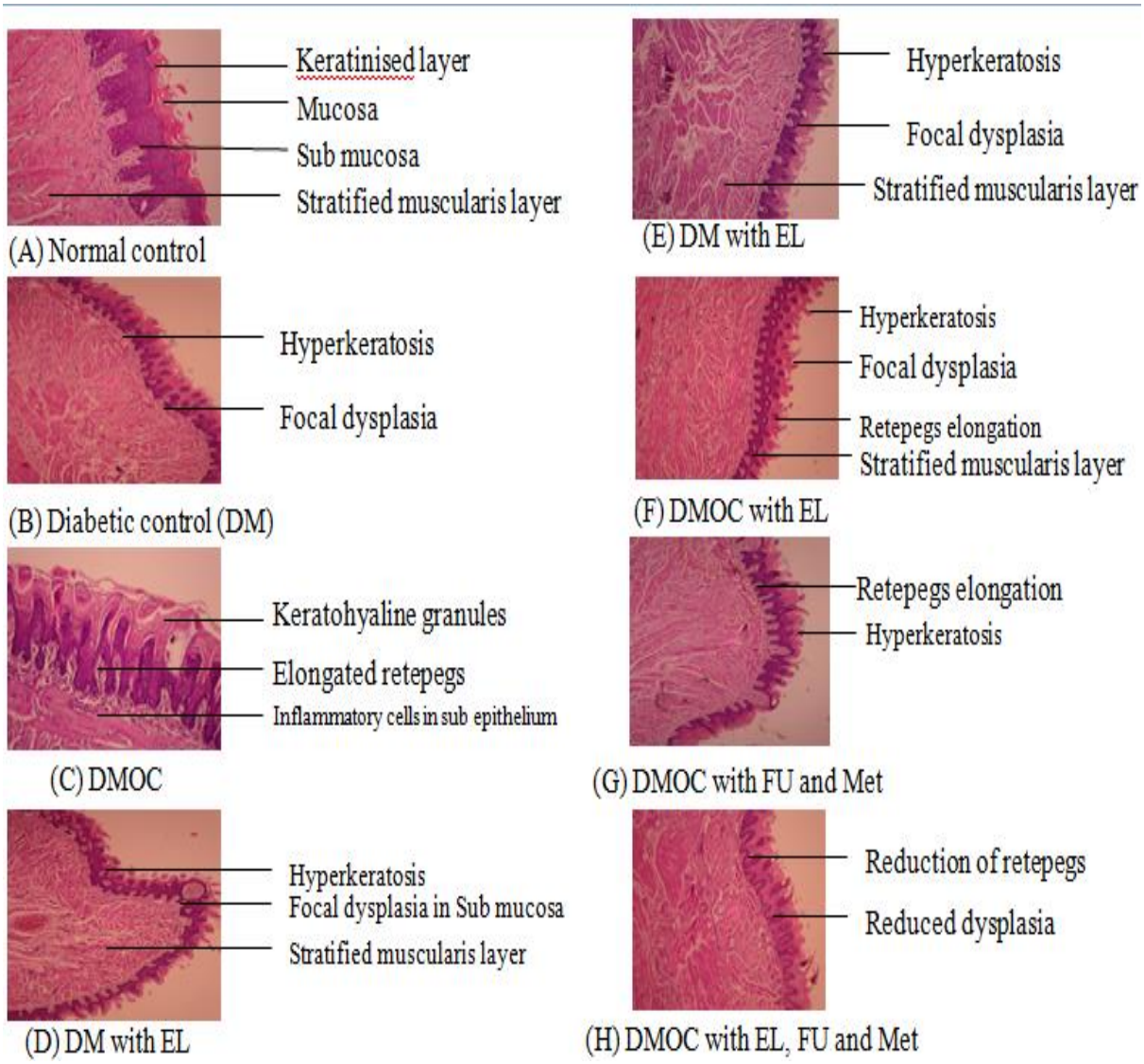


Figure 5.4.5 (A) NC Normal control, (B) DM Diabetic control animals, C) DMOC Diabetes and Oral cancer control, (D) DME treated with *Euphoria longana* extract (280mg/kg p.o.), (E) DMM treated with Metformin (400mg/kg p.o.), (F)DMOCE treated with *Euphoria longana* extract (280mg/kg p.o.), (G) DMOCMF treated with Metformin (400mg/kg p.o.) and 5-FU (24.5mg/kg p.o.), (H) DMOCEMF treated with *Euphoria longana* extract (280mg/kg p.o.), 5-FU (24.5mg/kg p.o.) and Met.

6. Discussion

Oral carcinogenesis involves multiple stages for its progression. There are various oncogenes and tumor suppressor genes which if over expressed, altered or mutated, lead to their enhanced function, deactivation or functions opposite to its actual. So there are multiple pathways which get altered in precancerous condition leading to cancerous condition causing changes in cell proliferation, angiogenesis[5]. The major risk factors are smoke or smokeless tobacco use, excessive alcohol consumption and human papillomavirus (HPV) infection[3]. The alcohol consumed is converted into acetaldehyde by an enzyme alcohol dehydrogenase, this acetaldehyde is cytotoxic as it produces free radicals and hydroxylated bases of DNA. The tobacco smoke creates free radicals which interacts with acive metals present in saliva and thus the anti oxidant activity of saliva is lost (29)

Most invasive oral carcinomas are preceded by a preinvasive stage, which may last for many years. Tumor progression in epithelia has been classified as normal, hyperplastic (non-dysplastic), dysplastic carcinoma in situ and invasive carcinoma. There are genetic alterations in oncogene or the tumour suppressor genes, or genomic instability, or epigenetic modifications in the genes which are responsible for altered physiology of the body cells. The cells become hypoxic and in that stress condition they release various inflammatory cytokines and pro- inflammatory mediators like TNF-1α, IL-1β, IL-6, HIF-1α, and COX-2, hence causes chornic inflammation in the tissues. These mediators cause degradation of extracellular matrix (ECM) which is the microenvironment of the cell and is regulated by Matrix Metalloproteinase (MMPs). These mediators are responsible for the overexpression of MMP due to which the ECM is degraded. Also due to chronic inflammation, neoplasia occurs that causes increased levels of VEGF, cell proliferation and angiogenesis. When the levels of these inflammatory mediators are high, the transcription factors like Nuclear factor kappa-B (NF-κB) are activated which are responsible for MMP production. The overexpression of this transcription factor and NF-κB kinase are related to metastasis, invasiveness and antiapoptotic activity. Thus the dysregulation of NF-κB lead to inflammation, cancer and autoimmune diseases. The growth factors are increased due to cell proliferation and tumour formation[6]. The tumour growth factors (TGF) are over expressed where Epidermal Growth factors (EGF), VEGF and TGF-β are very specific to Oral cancer.

Diabetes is a chronic, metabolic disorder in which the blood glucose levels get elevated which causes to serious damage to the heart, blood vessels, eyes, kidneys, and nerves as the disease progresses. Type II diabete mellitus is due to the resistance towards the insulin and impaired response to insulin by the body[7]. Several immunologic and metabolic changes occur in the oral mucosa due to Diabetes mellitus. There is a relationship between diabetes and oral mucosa like immunological and periodontal disease. The diabetic patients have precancerous lesions like erythroplakia and leukoplakia more than non diabetic patients. Almost 90% of systemic metabolic disorders lead to oral mucosal damage. Diabetes also has the influence on microenvironment of the oral cavity and creates complications of oral mucosa like lesions in the mucosa and tongue, change in saliva composition, Xerostomia, osteolytic lesions, odontogenic abscess, granulomas, gingivitis and periodontitis and loss of teeth. Diabetes is

characterized by hyperglycemia which leads to production of reactive oxygen species thus increasing the oxidative stress. This causes the modification in the lipids, proteins and DNA. Along with these mechanisms, in type II diabetes there is a condition of Hyperinsulinemia due to which there is increased production of Insulin like receptor, also insulin signaling and insulin mitogenic pathway remains unregulated. These all mechanisms are responsible for initiation and progression of the cancer.[7] The hypothesized biological mechanism is related to the effect of insulin and insulin-like growth factors (IGFs) axis, which would trigger intracellular signaling cascades with mitogenic and antiapoptotic effects. The insulin levels are elevated in diabetes mellitus type II which increases concentrations of insulin-like growth factor-1(IGF-1). Elevation of these both stimulates the growth of cell by proliferation of cells, apoptosis inhibition and increasing the mitogenesis in cell lines. High level of insulin in the body also triggers the release of several pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). These cytokines ultimately leads to inflammation and leads to malignancies of oral cavity.[1]

Dimocarpus Longan (Lour.) also known as *Euphoria longana* is a fruit of subtropical climate and belongs to the lychee family, Sapindaceae. The pulp is used to produce canned syrup and juice in food and beverage industry where the seeds are discarded as waste material, so these seeds can be utilized to develop beneficial health products. The seeds of longana contain high amount of polyphenols as compared to the fruit. There are 11 known phenolic components present in the seed, among which gallic acid; ellagic acid and corilagin are the major ones. The aqueous extract of seeds of *Euphoria longana* contains higher content of polyphenol as compared to the ethanolic extract [21]. In our present study, the longana seeds were extracted in hot water and the preliminary tests for the same have showed the presence of flavonoids, carbohydrates, tannins, saponins and phenolic compounds in the aqueous extract of *Euphoria longana*. The qualitative analysis by Thin Layer Chromatography have showed the presence of the three major polyphenols, gallic acid, ellagic acid and corilagin with Rf values 0.64, 0.56 and 0.38 respectively.

The longana is recognized for various pharmacological activities due to the huge amount of polyphenols present in the fruit that exhibit antioxidant properties. The gallic acid and ellagic acid possess activities such as antiplasmodial, antimicrobial, antioxidant and anticancer by inhibiting nuclear factor kappa-B activation[22]. Corilagin have been reported to exert its action on TGF-β/AKT/ERK/Smad signaling pathway and provide the therapeutic benefit by reducing the growth of ovarian cancer cells. Some in-vitro cell line studies have suggested that corilagin arrest the growth of cell at G2/M phase [10]. Also it has a potential anti-inflammatory activity as it inhibits the NF-kappa B nuclear translocation at genetic and protein levels resulting in reduction of pro-inflammatory cytokines and mediators like TNF-α, IL-1 β, IL-6, NO (iNOS) and COX-2 [23].

The exposure to smoke or smokeless tobacco creates free radicals which interacts with active metals present in saliva and thus the anti oxidant activity of saliva is lost (29). A carcinogen, 4-nitroquinoline-1-oxide (4-NQO) is able to induce cancer in rat tongue and has become a model

for developing chemo preventive therapies of oral cancer. The 4-NQO produces oral lesions in rats that are similar to human lesions. Oral tissues develop hyperplasia, dysplasia, and cancer examined histologically[24]. 4-NQO exerts potent intracellular oxidative stress and its metabolic product binds to DNA predominantly at guanine residues. The damage caused by 4-NQO appears similar to the damage as imposed by other carcinogens present in tobacco, which is the main risk factor for oral cancer. In addition 4-NQO exhibits similar histological as well as molecular changes which are produced in human oral carcinogenesis [25]. The model for oral cancer induction in rats includes the 4-NQO dissolved in water for 22 weeks [24] or 0.5% of 4-NQO in propylene glycol can be applied using a paint brush on rat tongue thrice a week[26]. In present study, the model was slightly modified to induce oral cancer by applying 0.5% of 4-NQO in propylene glycol on rat tongue thrice a week for a duration of 24 weeks.

High fat diet (HFD) with single low dose streptozotocin (STZ) is used to induce diabetes mellitus type II where high fat diet produces insulin resistance by increasing the fatty acid and their oxidation whereas streptozotocin causes the initial β cell dysfunction and hyperglycemia[27]. High blood glucose was found in HFD and STZ treated rats. Hyperglycemia and hyperinsulinemia leads to production of reactive oxygen species thus increasing the oxidative stress. In our present study, diabetes was induced using HFD and STZ model followed by oral cancer induction using 4- NQO model where the precancerous conditions were observed in a short duration of induction which indicates that the diabetic conditions increases the incidence of oral cancer. Also the precancerous conditions of oral cancer were induced earlier in diabetes induced animals as compared to the animals only induced with oral cancer for 12 weeks of application of 4- NQO. The *Euphoria longana* flower extract has an anti diabetic activity as it decreases the glucose level, percent of glycated Hb and also improves the glucose tolerance in the diabetic rats due to the presence of polyphenols and polysaccharides[28]. In present study the *Euphoria longana* seed extract has reduced glucose and glycosylated Hb; and also improved the glucose tolerance. The diabetic oral cancer control groups were found to have more disease progression with the disease as compared to only diabetic control. The combination treatment with EL, metformin and 5-FU reduced the glucose levels more effectively than the treatment with only EL.

In cancer, reactive oxygen species (ROS) plays an important role in cancer development phase which results in increased radical formation and favors the formation of tumor. This also leads to an increased MDA level which is due to application of carcinogens like 4-NQO. Different defensive antioxidant mechanisms are involved in the radical generation like GSH and SOD levels which are decreased, and MDA levels that is increased in cancerous condition. These mechanisms are more involved in depressing the radical formation. Longana seed extract also showed protective action against focal cerebral ischemia/reperfusion injury by significantly reducing the MDA levels and increasing SOD and GSH levels, GSH-Px, MPO activity, concentrations of TNF and IL-1 in the brain tissue. These anti-oxidant and anti-inflammatory properties of longana are mainly observed because of presence of gallic acid and ellagic acid. Longana showed significant inhibition of NO production by inhibiting histamine release and pro inflammatory cytokine production in mast cells [17].

At the end of 24 weeks, 4-Nitroquinoline N-oxide (4-NQO) produced a significant decrease in body weight, food and water intake in oral cancer diseased animals as compared to the normal control group. The disease treated groups with *Euphoria longana* from day 0 of induction of oral cancer showed constant body weight of animals. There was reduction in body weight, food and water intake of diseased animals treated with *Euphoria longana,* 5- fluorouracil (5-FU, 24.5mg/kg) and combined treatment of both *Euphoria longana* and 5-FU after 24 weeks of induction which was less than that of the oral cancer control group. However after the treatment no significant improvement in the body weight, food and water intake of diseased animals was seen which may be because to short duration of treatment.

In evidence to this, our study showed decrease in GSH and SOD activity and increase in MDA levels in 4-NQO induced oral cancer in rats which suggests the poor antioxidant status. The treatment in the animals induced with only oral cancer was found effective. The EL treatment given from the 0 day showed less progression of the disease as compared to the other treated groups especially with EL administered after 24 weeks of induction of oral cancer. This may be attributed to the protective effect of longana due to the presence of gallic acid and ellagic acid which have antioxidant activity. So this may be suggestive of use of *Euphoria longana* as a preventive therapy for oral cancer. Moreover, the combined therapy proved more effective when compared to the treatments given alone. Also in the diabetic oral cancer groups, GSH and SOD were reduced with MDA elevated suggesting the oxidative status of the animals. But the treatment showed reduction in the oxidative stress with combination treatment with EL, 5-FU and metformin being more effective. Levels of GSH and SOD are significantly increased and level of MDA was significanctly decreased in animals treated with longana alone as well as in combination with 5-FU alone and with metformin as compared to disease control group. Hence it is suggestive due to the protective role of longana against free radical generation.

The EL seed extract consists of gallic acid, ellagic acid and corilagin in major amount among all the polyphenols. Corilagin present in the seeds have ability to arrest the growth of cell at G2/M phase [10]. Also it has a potential anti-inflammatory activity as it inhibits the NF-kappa B nuclear translocation at genetic and protein levels resulting in reduction of pro-inflammatory cytokines and mediators like TNF-α, IL-1 β, IL-6, NO (iNOS) and COX-2 [23]. In our study, the level of IL-6 was found increased in the oral cancer control group significantly as well as in diabetic oral cancer control group. The treatment with *Euphoria longana* (from 0 day of induction), *Euphoria longana*, 5- fluorouracil (5-FU) and combined therapy of both after 24 weeks of induction of oral cancer, reduced the IL- 6 level. But the reduction was not significant as compared to the diseased group. However, the IL-6 level was found more reduced in group treated with *Euphoria longana* from 0 day of induction but the combination of both EL and FU reduces the level more effectively. Also in the diabetic control (DM) and diabetic oral cancer control (DMOC) groups the level was increased but was not significant as compared to the normal control group (NC). The IL-6 level was insignificantly reduced in the treatment groups, though it was not significant but it was effective. Also the Histopathological section revealed

there was presence of inflammatory cells in the sub mucosal layer of the tongue of the diseased animals with diabetes and oral cancer.

The section of excised tongue of normal control group animal shows normal mucosa, sub mucosa, stratified muscles and normal papillae with a keratin layer and nucleus present in squamous epithelium. The section of a disease control group (OC) revealed stratified squamous epithelium with squamous cells having pleomorphic hyperchromatic vescicular and prominent nuclei, and pinkish cytoplasm. Mitosis is present with no invasions seen into the deeper tissues with squamous cells infilterating the stratified muscles. But these cancerous conditions were not observed in the treatment groups with *Euphoria longana,* 5-FU and combination of both treatment in oral cancer group from day 0 (OCEL0). But only hyperplasia, hyperkeratosis and acanthosis were observed in treatment groups. There was formation of retepegs in *Euphoria longana* treatment group after 24th week (OCEL24). The oral cancer groups treated with standard (5- FU) and with the combination of both (EL and FU) showed less of the cancerous conditions and with reduced pleomorphic and prominent nuclei.

No changes were observed in the diabetes mellitus induced group of animals and in the treated groups of diabetes mellitus. Only hyperkeratinization on the epithelium was observed. But in the animals induced with diabetes mellitus and oral cancer (DMOC), there were elongated retepegs observed with hyperkeratinization, keratohyaline granules and inflammatory cells in the sub epithelial layer. The keratohyaline granules might be present due to some viral etiology. The histopathological section of diabetic oral cancer animals treated with *Euphoria longana* (DMOCEL), Metformin and FU (DMOCMF) revealed the presence of keratohyaline granules but were seen reduced as compared to the diabetic oral cancer control groups. In the diabetic oral cancer groups treated with the combination of EL, Metformin and FU (DMOCEMF), only hyperkeratosis was observed with inflammatory cells present in the cytoplasm and with the reduction in the keratohyaline granules.

Further studies are warranted to elucidate the mechanism of action of *Euphoria longana* in diabetic oral cancer with special reference to NFκB, TNF-α and IGF-1.

CONCLUSION

- Our study reveals that diabetes mellitus type II is a risk factor for development of oral cancer which might be due to the enhanced oxidative stress in diabetic condition that leads to the progression of cancer.

- The treatment with *Euphoria longana* showed protective effect in oral cancer and the combination of *Euphoria longana* with other chemotherapeutic agent might be beneficial to improve the diseased condition than using a chemotherapeutic agent alone.

- Also in the animals induced with diabetes as well as oral cancer, the combined treatment has reduced the progression of disease as compared to the treatment given alone. Hence this might prove to contribute for beneficial and therapeutic approach in the treatment of oral cancer associated with diabetes mellitus type II.

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