Effectiveness of Fermented Papaya on Colon Cancer

Research Proposal
submitted to
Department of Biotechnology
Government of India
New Delhi

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Vittal Mallya Scientific Research Foundation
93/3 & 94/5, 23rd cross, 29th main
BTM II Stage, Bangalore-560076
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PROFORMA –I

PROFORMA FOR SUBMISSION OF PROJECT PROPOSALS ON RESEARCH AND DEVELOPMENT, PROGRAMME SUPPORT

(To be filled by the applicant)

PART I: GENERAL INFORMATION

1. Name of the Institute/University/Organisation submitting the Project Proposal:

Vittal Mallya Scientific Research Foundation, Bangalore.

2. State: Karnataka

3. Status of the Institute: Nonprofit R&D

(Please see Annexure-I)

4. Name and designation of the Executive Authority of the Institute/University forwarding the application:

Dr. Anil Kush, Research Director, Vittal Mallya Scientific Research Foundation,

94/3 & 94/5, 23rd cross, 29th Main, BTM II Stage, Bangalore-560076

5. Project Title: "Effectiveness of Fermented Papaya on Colon Cancer"

6. **Category of the Project** (Please tick): R&D√/
Programme Support

7. Specific Area *(Please see Annexure - II):* CANCER BIOLOGY

9. Total Cost (Rs.) 25, 00,000/-

10. Is the project Single Institutional or Multiple-Institutional (S/M): Single

11. If the project is multi-institutional, please furnish the following:

Name of Project Coordinator:
Affiliation: Not applicable
Address:

12. Scope of application indicating anticipated product and processes.

1. The present work is focused on evaluating probiotic enriched fermented papaya product (FPP) in preventing colon cancer.
2. The proposal emphasizes to find out the role of papaya fermented with *L. plantarum* on various biomarkers of colon cancer. Important serum and tissue markers of colon cancer that will be studied are Carcinoembryonic antigen (CEA), β-catenin and Aberrant Crypt Foci (ACF).
3. Fermented product from papaya has improved anti oxidant activity and is rich in short chain fatty acids SCFA's. SCFA production mainly butyrate decreases the luminal pH, and may thereby stimulate mineral absorption and reduce secondary bile acid formation in the colon. This can play a vital role in reducing tumor formation in the azoxymethane (AOM) treated rats and that these data support a potential protective role of butyrate in colon cancer.
4. The studies can lead to development of novel scientific ways and products to manage the Colon Cancer.

13. Project Summary (Not to exceed one page. Please use separate sheet)

Colon cancer is one of the leading causes of cancer death both in men and women. Prognosis for advanced colon cancer is poor, and hence prevention is required to control the incidence of disease. Epidemiological studies show that diet plays a role in the etiology of most large bowel cancers, implying that it is potentially preventable disease. Among various diet, Papaya is known to promote immune system and is a potent cancer fighter that is highly effective against hormone related to cancer as well as other cancer. Papaya can stop the growth of cancer cell halt metastasis and normalized cell cycle[1]. Antioxidants like beta-carotene, ascorbic acid and alpha-tocopherol are proven to prevent oxidation caused by free radicals in *in vitro* and *in vivo* studies. These antioxidants must be supplied from natural sources like from fruits and vegetables rather than from supplements.

It has been reported that the growth of putrefactive bacteria that liberate carcinogenic enzymes is inhibited in low pH and probiotics on long-term administration has been found to reduce faecal pH along with lower proliferative activity in the upper colonic crypts. Fermented foods are known to deliver beneficial probiotics or bacteria into the body. They help us to absorb vitamins and nutrients from food, help the body to detoxify, boost the immune system and help to keep bad bacteria or microorganisms at bay.

Fermented Papaya Product (FPP) is rich in antioxidants, phenolic content (scavengers of free radicals) and SCFAs. Short chain fatty acids (SCFAs; acetic, propionic and butyric acid) formed in the colon due to fermentation decreases the luminal pH, and may thereby stimulate mineral absorption and reduce secondary bile acid formation in the colon. The growth of colonic tumour cell lines has been reported to be slowed down by butyrate. Butyrate is a major metabolite in colonic lumen arising from bacterial fermentation of dietary fiber. Butyrate also appears to reduce cell differentiation and stimulate apoptosis. Most supplements are prepared chemically, unlike FPP, which is naturally produced. Also the intake of vitamins and supplements, especially in high doses, is not without possible toxicities.

In the present work we propose to use FPP developed in-house at VMSRF using known probiotics like *L. plantarum* to investigate their efficiency in animal model of colon cancer. We already have taken some lead in terms of evaluating the immunomodulatory effect of fermented papaya, and we are successful in controlling paw edema. The animals were tested for humoral and cell mediated immune response against sheep Red Blood Cells (R.B.C). The immune

response was stimulated in the animals. In view of the above facts we propose to study the anti carcinogenic effects of fermented papaya product and its effect on various cancer markers. These hold promise to assess in more detail how fermented papaya may contribute to Colon Cancer risk reductions.

PART II: PARTICULARS OF INVESTIGATORS

(*One or more co-investigators are preferred in every project. Inclusion of co-investigator(s) is mandatory for investigators retiring before completion of the project*)

Principal Investigator:

14. Name: Dr. Anaxee Barman

Date of Birth: 30th September, 1984 Sex (M/F): Female

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Number of research projects being handled at present: NIL

Co-Investigator

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Designation: Scientist

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E-mail: latha@vmsrf.org

Number of Research projects being handled at present: one

PART III: TECHNICAL DETAILS OF PROJECT

(Under the following heads on separate sheet)

16. Introduction (not to exceed 2 pages or 1000 words)

Introduction

Colon cancer is a disease originating from the epithelium that is lining the colon and rectum. Most colorectal cancers should be preventable, through increased surveillance, improved lifestyle, and, probably, the use of dietary chemo preventative agents. Epidemiological and animal studies suggest that diets low in animal fat and high in fruits, vegetables, grains, and legumes may protect against colon cancer. Consumption of papaya may cut risk of certain cancers. The fruit is an excellent source of beta carotene that prevents damage caused by free radicals that may cause some forms of cancer.

Authors have demonstrated that those fibers which promotes a stable butyrate-producing colonic ecosystem decreases the rate of aberrant crypt foci (ACF) in rats, thus adding to the line of evidence that a stable butyrate-producing colonic ecosystem, as related to selected fibers, reduces risks of developing colon cancer[2]. Aberrant crypt foci (ACF) have been identified and defined as putative precancerous lesions of the colon in both experimental models and human[3] . And the fiber of papaya is able to bind cancer-causing toxins in the colon and keep them away from the healthy colon cells. These nutrients provide synergistic protection for colon cells from free radical damage to their DNA.

About 80% of our immune system is located in the tissues in and around the digestive tract. The decline of immune system function in aging persons and cancer patients may be attributed to increasing levels of free radicals.

Research shows that fermented foods can restore the balance of intestinal flora, and this leads to vastly improved intestinal health and digestion because of the introduction of probiotics into the digestive tract.

Fermented Papaya Products, a papaya dietary supplement is made with papaya, utilizing traditional fermentation methods. FPPs main function is its physiologic ability to restore normal cellular functions in all the body by fighting against imbalance of the oxidant/pro-oxidant status and to improve overall immunity by delivering healthy bacteria/probiotics in the body. Probiotics are the health enhancing functional food ingredients used therapeutically to prevent diarrhea,

improve lactose tolerance and modulate immunity. They may also have potential to prevent cancer and lower serum cholesterol levels.

Several mechanisms could explain the preventive action of probiotics delivered by fermented food against colorectal cancer onset. They include: alteration of the intestinal micro flora; inactivation of carcinogenic compounds; competition with putrefactive and pathogenic micro biota; improvement of the host's immune response; anti-proliferative effects via regulation of apoptosis and cell differentiation; fermentation of undigested food. Lactic acid producing bacteria (LAB) play an important role in retarding colon carcinogenesis by possibly influencing metabolic, immunologic, and protective functions in the colon .Concentrations of LAB may increase in the colon after the consumption of foods containing probiotics. However little is known about the role of FPP in colon cancer.

Benefits of Using FPP are many:

- Chronic or debilitating illnesses like liver diseases, cancer, AIDS and others take a toll in the body's energy and defense reserves. The body fights against the infection but in so doing, exhausts much of its energy. FPP replenishes the needed energy to enhance the body's defense systems and reverses the symptoms associated with these diseases.
- Constant stress exerts a cumulative harmful effect of free radicals that contributes to general body weakness which in turn lowers our immune competence. This condition may trigger the onset of disease if allowed to continue unabated. FPP has been observed and reported to reduce the effects of stress.

The present study is conducted to confer if fermented papaya can efficiently deliver healthy bacteria in the colon which may interact and have an impact on host intestinal flora differently than single probiotic preparations.

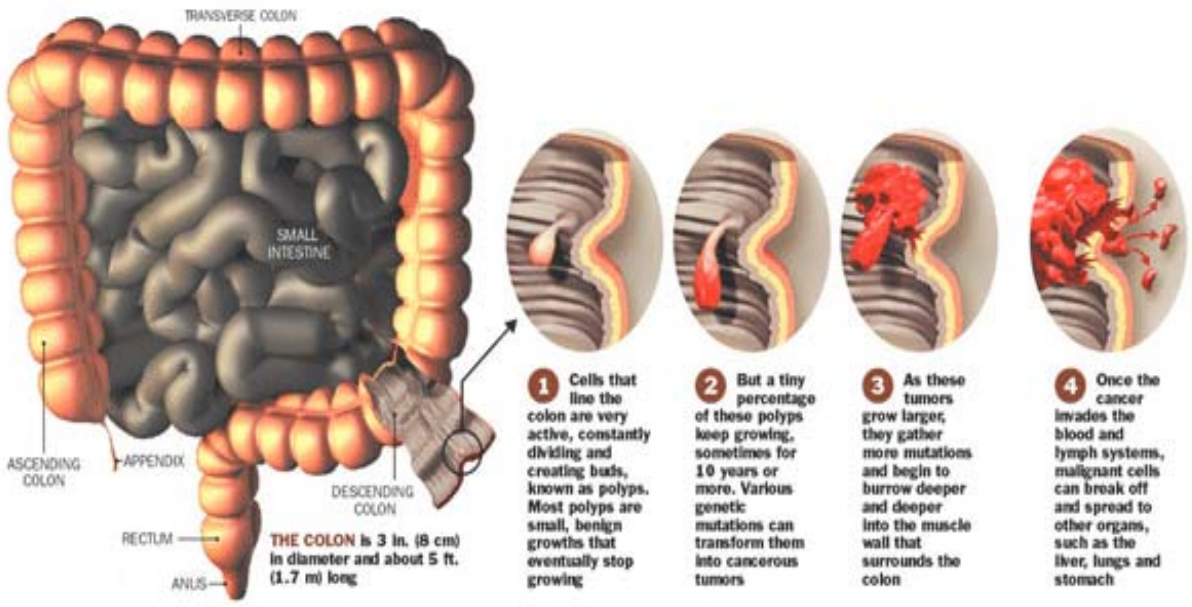

Fig 1: Stages of colon cancer (Source : Mezhal Ulao *in* Labels: Cancer, Health Facts, wellness. http://emptystreets27.blogspot.in/2009/08/wellness-wednesday-on-empty-streets.html)

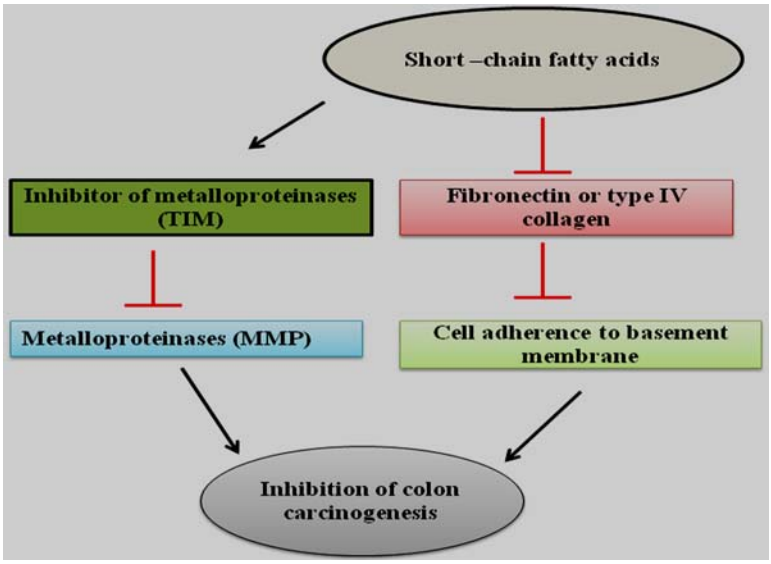

Fig 2: Mechanism of action of SCFA **(**Source: Djamilatou Adom and Daotai Nie: Regulation of Autophagy by Short Chain Fatty Acids in Colon Cancer Cells. Department of Medical Microbiology, Immunology, and Cell Biology, Southern Illinois University School of Medicine and Simmons Cancer Institute, Springfield, IL, USA**)**

16.1 Origin of the proposal:

Fermented papaya product can act as cleansers of free radicals, optimize the functions of endogenous antioxidants in the body, protecting cell membranes and DNA against oxidative stress**.** We have developed the fermented food from Papaya fruits in VMSRF and the findings suggest that fermented product from papaya has improved anti oxidant activity and is rich in SCFAs (table 1) and is a scavenger of free radicals. The immunomodulatory potential was also studied and fermented papaya has shown potent immunomodulatory effect.

Ingestion of probiotic bacteria via fermented products may promote desirable changes in the gastrointestinal tracts of humans and other animals in gut. They should provide a means of promoting physiological improvements and providing the fermentative substrate to the manipulation of the colonic micro flora, the production of beneficial metabolites such as short-chain fatty acids, and the reduction in the numbers and activity of pathogenic bacteria by acting on various colon markers. In addition there seems to be an immunological angle so far the gut defense to various oncological triggers is concerned.

Current treatments of colon cancer including chemotherapy, radiotherapy, and surgery are all associated with a high risk of complications and are not always successful. FPP may present a novel, economically feasible nutraceutical supplement for the management and prevention of colon cancer by delivering beneficial probiotic to the colon.

Table 1: Comparison on the quantity of SCFA's in raw and fermented papaya

Components (ng/100 mL of sample)	**Sample description**	
	Raw	**Fermented**
Butyric acid - C4	1.51	382.14
Valeric acid - C5	0	1.125
Caproic Acid - C6	5.32	719.95
Caprylic Acid - C8	6.71	244.27
Capric Acid - C10	20.77	114.66

16.2 (a) Rationale of the study supported by cited literature

Free radicals are known to be associated with aging and many diseases, such as cancer. Among the free radicals, hydroxyl radicals are the most reactive ones, which damage proteins,

deoxyribonucleic acids (DNA) and promote lipid per oxidation. For these reasons, it is expected to take food rich in antioxidant ordinarily for prevention of such diseases.

The long natural bio-fermentation process preserves the antioxidant property of papaya and also enhances the immune-modulating features. The fermentation process modifies the ratio between complex carbohydrates and proteins and increases it by 30 times in the fermented product. The final fermented product contains much new class of oligosaccharides which exhibit a wide spectrum of immune modulating activity.

Evidence has also been reported for a protective effect of F.P.P. supplementation against oxidative damage in the brain with different model systems, improves the physical condition of the rats and increases life span. FPP delivers healthy bacteria/probiotics to fight against pathogenic bacteria. The capability of modulating fecal enzymes bacteria activity is a strain-specific characteristic for probiotics. Putrefactive intestinal microbiota such as Bacteroides spp. and Clostridium spp. has been implicated in the pathogenesis of (colon rectal cancer) CRC while numerous lactic acid bacteria (LAB) have been shown to possess cancer-preventing attributes[4]. The beneficial probiotic bacteria have been found to interact with the gut epithelial cells, the M cells in the Payer's patches and allied immune cells and start the immune signals. LAB have been shown to increase the production of (hepatic) enzymes involved in the metabolism of carcinogens absorbed by ileal and colonic mucosa of the host[5.]

The alarming increase in inappropriate use of antibiotics and development of bacterial resistance makes probiotics a very interesting field for research. At present, these agents have shown several beneficial effects in a variety of gastrointestinal and non-gastrointestinal disorders including colon cancer. They offer dietary means to support the balance of the intestinal flora. As altered balance of the intestinal flora is an important cause of several gastrointestinal diseases, they may be used to correct such disorders like local immunological dysfunction, destabilize intestinal function, prevention of infections caused by pathogenic microorganisms and disturbed intestinal metabolism. Thus, these agents hold immense potential for delivering novel therapies in different diseases in future.

These observations seem to be very interesting as it would make possible an effective strategy for CRC primary prevention. Scientific research has also shown that the papaya's foliate, vitamin C, beta-carotene, and vitamin E protects the colon cells and lowers the risk of colon

cancer. So we aim to find out if the nutritional value of this fruit and immunomodulatory effect of probiotics can be a good choice for decreasing the risk of colon cancer. And using fermented papaya may serve as a beneficial complement to traditional medicine. Our proposed work is one such effort to develop fermented papaya by using probiotic *L.plantarum* as nutritional therapy against disease condition like colon cancer.

(b) Hypothesis

- Colon cancer outcomes may benefit from FPP supplementation by specifically causing a reduction on various colon markers by its antioxidant and immunomodulatory property.
- Our hypothesis is that administration of papaya fermented with *Lactobacillus plantarum* is enriched with antioxidants, SCFA's which can minimize the numbers of aberrant crypts in the distal colon of rats that develop in response to the carcinogen azoxymethane.
- The protective effect of L. *plantarum* and nutritionally rich papaya when used in combination as FPP can restore the colonic damage induced by Azoxymethane then using single probiotic.

(c) Key questions.

Following are the vital questions this project would like to address:

1. The ideal combination of papaya and probiotics so that they can be ideally delivered in the colon knowing that are supposed to pass through varied (often hostile) micro eco and bio-system in gut?
2. Molecular markers as tools to understand cancer biology and their response in FPP fed animals?
3. Histological response in the colon and other vital organs if any in the FPP fed animals?

16.5 Current status of research and development in the subject (both international and national status)

Papaya fruits have a juicy taste rich in antioxidant nutrients like carotene, vitamin C, vitamin B, flavonoids, foliate, panthotenic acids and minerals such as potassium and magnesium,

the fruit is also a good source of fiber all these are reported to promote the functions of cardiovascular system and provide protection against colon cancer.

The involvement of oxidative stress mechanisms in several biological and pathological processes including ageing, cancer, cardiovascular and neurodegenerative diseases has continued to fuel suggestions that processes can potentially be modulated by treatment with free-radical scavengers and antioxidant. The high mortality of colon cancer is to a large extent caused by the frequent occurrence of liver metastasis. Risk factors for colon cancer include both hereditary and environmental factors. Dietary patterns represent controllable risk factors for the development of colon cancer. Much attention has focused on decreasing colon cancer risk through increasing intake of dietary fiber; recently, this has included interest in the consumption of prebiotics and probiotics[6]. The term "probiotics" was first introduced in 1953 by Werner Kollath. Contrasting antibiotics, probiotics were defined as microbially derived factors that stimulate the growth of other microorganisms. Early studies examined the effects of milk fermented with lactobacilli and *Candida* on tumor formation. The investigators found that colon tumorigenesis induced by 1,2 dimethylhydrazine (DMH) was reduced in rats given the fermented milk. The influence of lactoferrin on azoyxymethane-induced aberrant crypts was studied where *Bifidobacterium longum* (3% of diet) was used as a positive control in their studies. Both lactoferrin and *B. longum* reduced aberrant crypt foci (ACF)[7]. It was also found that consumption of bifidobacteria or inulin or both together inhibited AOM-induced small ACF. It was also reported that the consumption of the lyophilized cultures of bifidobacteria inhibited the development of ACF in the colon of Azoxymethane induced cancer rats by 50%. The fermented papaya fruit is a promising nutraceutical as an antioxidant. It improves the antioxidant defense in elderly patients even without any overt antioxidant deficiency state at the dose of 9g/day orally[8]. The bacterial fermentation of indigestible carbohydrates generates short-chain fatty acids [SCFA] and gas; while the gas is eliminated in the feces, SCFA [mainly acetate, propionate and butyrate] represent nutrients and growth signals for the intestinal mucosa and may play a role in CRC prevention. A wide spectrum of positive effects exerted by butyrate is suggested, with a high potential for a therapeutic use in human medicine[9].

Butyrate possesses both preventive and therapeutic potential to counteract inflammation-mediated ulcerative colitis (UC) and colorectal cancer. It is thus suggested that Butyrate

enhances apoptosis of T cells in the colonic tissue and thereby eliminates the source of inflammation.

In our previous work at VMSRF, raw Papaya (*Carica papaya* L.) was fermented with a papaya endophyte strain (PE-LR-3). The endophyte fermented product exhibited 61.2% free radical scavenging activity where as the fermented product of microbial cocktail (*S. cerevisiae*: PE-LR-3) exhibited 75.7% free radical scavenging activity[10].

References:

1. Sheikh, F. and Krishnamurthy, R. Papaya (Carica Papaya): Source Material for Anticancer. *J of Pharmaceutical Sciences* **2013** ; 2: 25-34
2. Perrin P, Pierrea, F.; Patrya,Y,; Champc , M. and Menanteaua J. Only fibers promoting a stable butyrate producing colonic ecosystem decrease the rate of aberrant crypt foci in rats. *Gut* **2001**;48:53–61
3. Bird, R.P. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett* **1987**; **37**: 147-151
4. Uccello**,** M.; Malaguarnera**,** G.; Basile, F. and Biondi , A. Potential role of probiotics on colorectal cancer prevention. *BMC Surgery* 2012; 12(Suppl 1):S35
5. Chakraborti, C.K. The Status of Synbiotics in Colorectal Cancer. *Life Sciences and Medicine Research* **2010**; 2011: 20
6. Brady, L.J.; Gallaher, DD. and Busta, F. The role of probiotic cultures in the prevention of colon cancer**.** *J nutr* **2000**;130(2S Suppl):410S-414S
7. Linda, J. B.; Daniel, D. G. and Frank, F. B. The Role of Probiotic Cultures in the Prevention of Colon Cancer. *The J of Nutrition* **2000:** 411s-414s
8. Krishna, K.L.; Paridhavi, M. and Jagruti, A. Patel Review on nutritional, medicinal and pharmacological properties of Papaya (Carica papaya Linn.). *Natural Product Radiance,* **2008**;7(4):364-373
9. Canani, R.B.; Costanzo, M.; Leone, L.; Pedata, M.; Meli, R. and Calignano, A. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J Gastroenterol* **2011**; 17(12): 1519-1528
10. Krishnan,P.; Bhat,R.; Kush,A.; Ravikumar,P. Isolation and functional characterization of bacterial endophytes from Carica papaya frui ts. *J Appl Microbiol.* **2012**; 113(2):308-17

16.6 The relevance and expected outcome of the proposed study

- Current treatments of colon cancer including chemotherapy, radiotherapy, and surgery are all associated with a high risk of complications and are not always successful, highlighting the need to develop new treatment strategies. Papaya has more carotene compared to other fruits which help to prevent damage by free radicals. Fermented papaya is a promising nutraceutical as an oxidant. In this background, it's worthwhile to design fermented food from papaya, rich in nutrition and having immunomodulatory potency which can help preventing cancer.
- At present there is no clear understanding of fermented food and colon cancer risk prevention. In this context we have aimed in using fermented papaya considering the nutritional properties of papaya suggesting that it can decrease the expression of biomarkers for colorectal cancer, in view of the fact that these parameters have been recognized as potential risk factors for colon carcinogenesis.

16.7 Preliminary work done so far

The antioxidant content in fermented product was assessed by scavenging the stable free radicals such as DPPH (2, 2'-Diphenyl-1-picryl hydrazyl) and ABTS (2,2-azinobis 3-ethylbenzothiazolin 6-sulfonic acid) using filtrate of fermented sample (**fig 3**) . The fermented product was analyzed by GC-FID for short and medium chain fatty acids (Table1)

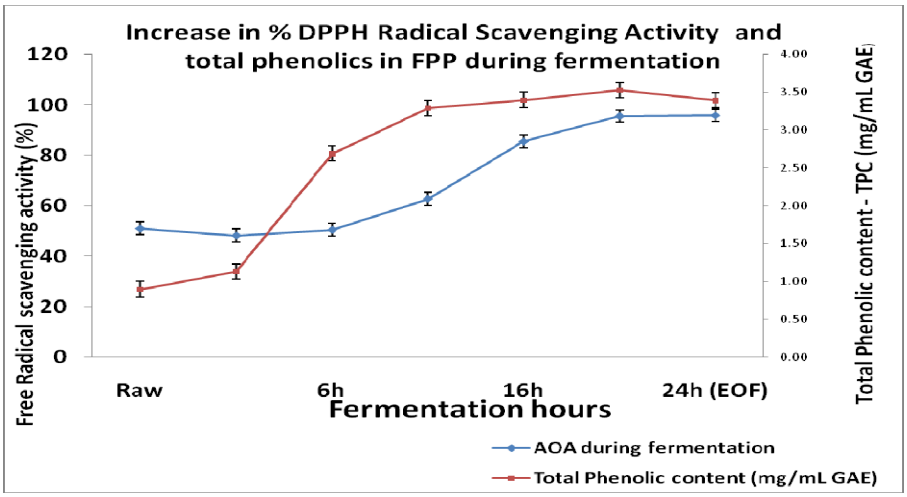

Fig 3: % Free radical scavenging activity and total phenols in unfermented and fermented papaya

This was evaluated earlier for Immunomodulatory effect in rats. The heam agglutinating (HA) antibody titers in the experimental rats are represented in (Table 2).The animals were tested for humoral and cell mediated immune response against sheep R.B.C. Paw edema was induced in Wistar rats using caragennan and fermented papaya was fed in different doses to evaluate its immunomodulatory effect. A dose of 500mg was found to have beneficial effect in reducing edema (Fig 4).

Table 2: HA titres in rats

Sr.No.	Treatment	Primary immune response/Hg titer
1	Control	64
2	PFP 500mg/kg	1024
3	Cyp treated	32

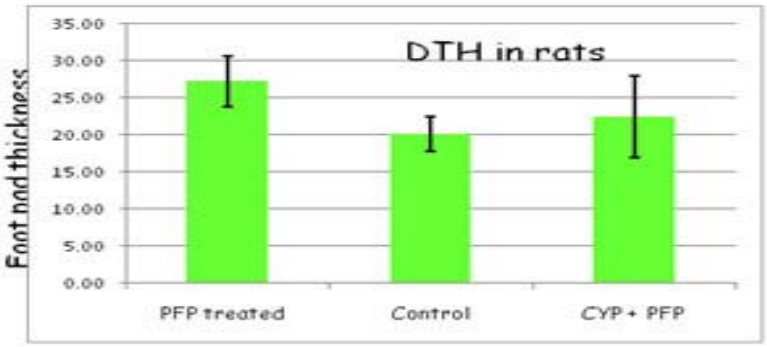

Fig 4: Graphical representation of foot pad thickness in control and treated rats-Delayed Type Hypersensitivity (DTH).

17. Specific objectives (should be written in bulleted form, a short paragraph indicating the methods to be followed for achieving the objective and verifiable indicators of progress should follow each specific objective)

We plan to focus in multi-facet manner on the following theme objectives:

1. **Induction of colorectal cancer in rats by (AOM) Azoxymethane**: Azoxymethane (AOM) is a potent carcinogen used to induce colon cancer in rats and mice. It has been used in studies evaluating efficacy of preventative treatment for azoxymethane-induced carcinogenesis. Azoxymethane 15 mg/kg body weight will be injected intraperitoneally once a week for 2 weeks. The colon cancer will be confirmed by the appearance of aberrant crypt foci (ACF) in the colons of the experimental mice

2. **Alteration of physicochemical conditions in the colon and estimation of faecal butyrate**: We will test the faecal P^H of the experimental animals. The fermented papaya rich in butyrate can lower the luminal P^H and thus prevent Cancer of colon. It is expected that the concentration of secondary soluble bile acids in the faeces will decrease considerably by administration of fermented papaya. Hence, cancer protective effect of FPP may be due to their colonic pH lowering property as well as secondary bile acid formation reducing property.
3. **Decrease in the expression of Biomarkers for colorectal cancer**: The study aims to find out if consumption of fermented papaya can effectively reduce the concentration of various colon cancer biomarkers. The various CRC markers are:
   - **Carcino Embryonic antigen (CEA)**
   - **β-catenin**
   - **Aberrant Crypt Foci (ACF)**

     Colon cancer usually metastasize to liver so we will also assay the liver enzyme test as SGPT, SGOT.
4. **Histopathological changes and Immunohistochemistry of intestine:** The various changes in the colon and other vital organs as liver, spleen, lymph nodes will be examined in the treated and controlled animals. Immunohistochemistry is used to characterize various surface and intracellular proteins from cells of all tissues.

18. Work Plan: should not exceed 3-4 pages (the section can be divided according to the specific aims and under each specific aim, the following should be stated clearly as sub headings)

18.1 Methodology/experimental design to accomplish the stated aim

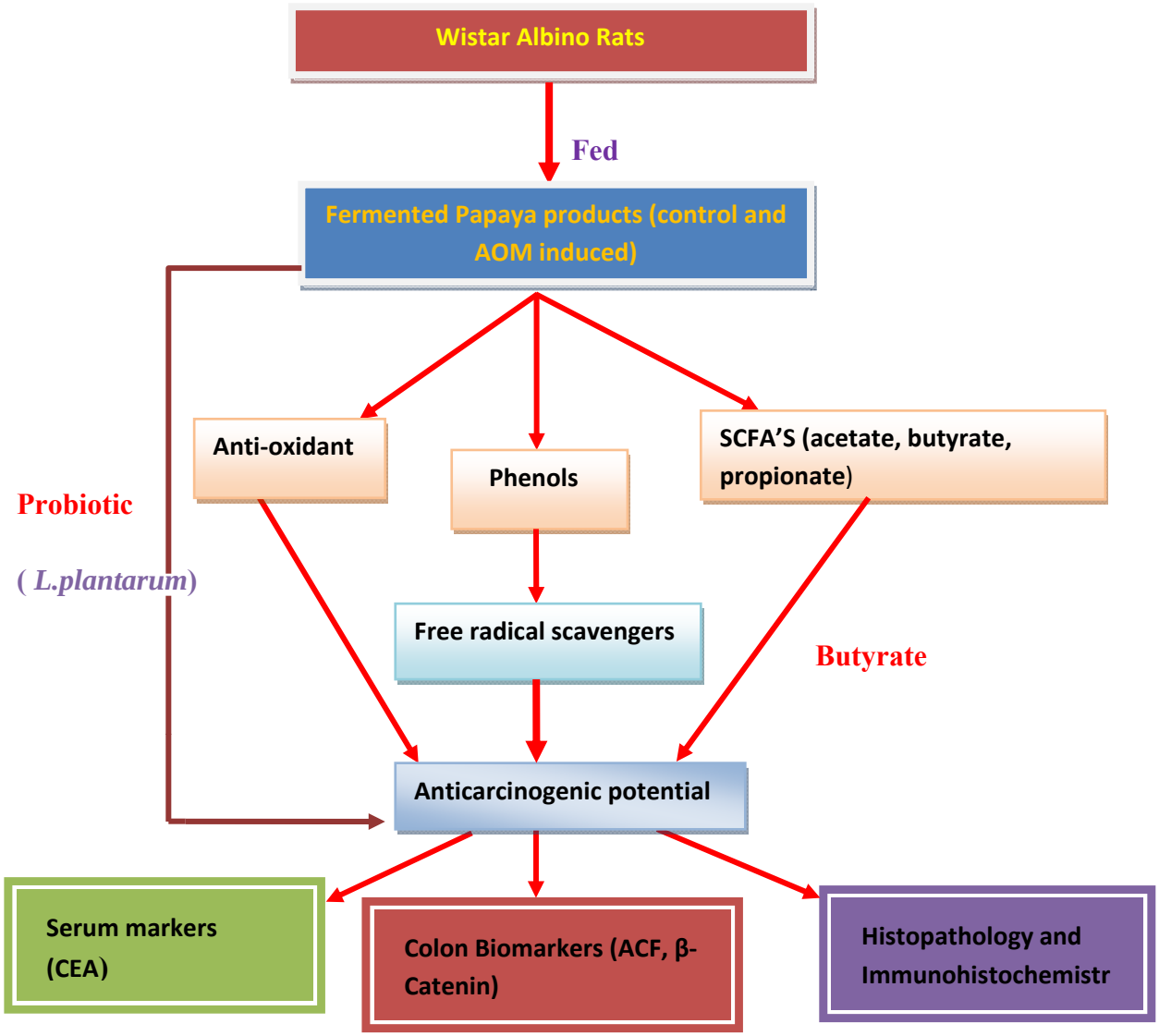

Fig 5: Work flow chart

Male albino Wistar rat will be used for the experiment. They will be housed in polypropylene cages (3 rats/cage). The temperature and relative humidity will be maintained at 27°C ± 2 and

50% respectively. Light and dark cycles will be 12 h each. Feed and water will be provided ad libitum. After a 2 week period of acclimatization, the animals will be divided into various groups and fed the experimental diets. During this time weekly body weights and daily feed intake will be recorded. Animals will be maintained as per the principle and guidelines of the Ethics Committee.

a) Azoxymethane induced (AOM) colorectal cancer model in rats

Male rats will be used in this study. They will be randomly divided into various groups and will be fed the same experimental diet for the whole experimental period. The rats in the control group will be given 1 ml intra peritoneal injection of 0.9% physiological saline solution once a week for 2 weeks and the rats in the AOM-injected group will be given 2 intra peritoneal injections of AOM dissolved in physiological saline once a week (15 mg/kg body weight) for 2 weeks. Body weight and food intake will be recorded weekly for whole duration of the experiment. The animals will also be maintained in metabolic cage for monitoring the colonic damage by examining the faecal and urinary output like bile salts.

b) Treatment groups fed with fermented papaya

The treatment groups will be fed the experimental diet plus FPP (standardized dose) via oral route with a standardized dose with or without AOM injections. After the last AOM injection, the rats will be continuously fed ad-libitum their specific diets till the end of the experiment. Faecal pH has to be measured by homogenizing in 3 volumes of saline further faecal samples will be diluted with internal standard and stored for metabolic analysis.

c) Preparation of colon in experimental rats

On final day of experimentation the animals will be sacrificed by decapitation under diethyl ether anesthesia after an overnight fast and the colon tissues will be removed for subsequent analysis. A known weight of digesta mixed with saline after pH measurement will be stored in -20^{o}C for metabolic analysis. The colons will be carefully removed from rats and will be kept on a glass plate in ice jackets. They will be opened longitudinally and the caecal and colonic contents collected for SCFA measurements. The small intestine and colon will be examined for intestinal tumors.

d) Aberrant Crypt Foci (ACF) Enumeration

ACF are commonly accepted precursor lesions for colonic tumors. Fixed colons will be stained with 0.2% methylene blue in Kreb's ringer bicarbonate buffer for 20 minutes in a Petri dish and rinsed with physiological saline solution. After staining, the colons will be placed with the mucosal surfaces up on a slide, to be examined with a light microscope under 40X magnification and scored for ACF. In brief, the ACF can be distinguished from normal crypts by their darker stain, enlarged and slightly elongated size, thick epithelial lining, slightly elongated cryptal opening and often slit shaped. The total number of ACF will be recorded for all examined colons.

e) Analysis of serum and tissue markers

Serum samples will be collected in regular intervals to estimate the various colon cancer biomarkers. The blood samples can be collected once in four weeks to know the levels of tumor marker carcinoembryonic antigen (CEA), to establish animal model of colon cancer. Although the oldest, CEA remains the most widely used serum marker in patients with CRC. β-catenin labeling index will be used as markers for adenocarcinomas in the colon. At its earliest stage (Stage 0), colon cancer is limited to the inner lining of the colon. As colon cancer progresses, it can grow through the colon and extend to nearby structures. The most advanced stage of colon cancer (Stage IV) indicates cancer has spread to other areas of the body such as the liver **(**Fig: 5), so liver enzymes will also be tested as SGPT, SGOT.

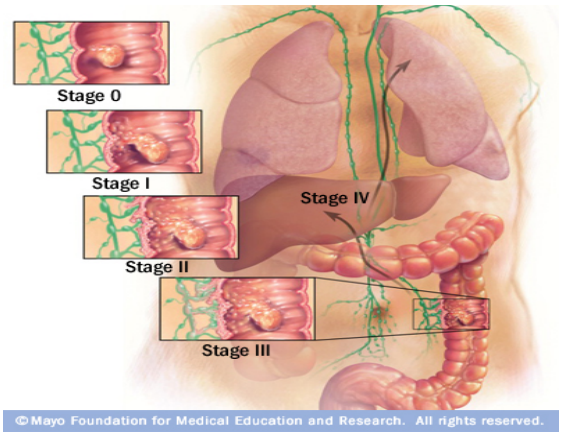

Fig: **6** Colon Cancer Metastatize to Liver (source: Mayo Foundation for Medical Education and Research)

f) Histopathology and Immunohistochemistry

At the end of the experiment animals will be sacrificed under ether anaesthesia and various organs as colon, liver, spleen, will be collected. The tumor tissues will be fixed in 10% formalin and paraffin-embedded. Histological sections will be cut at 3-μm thickness and stained with Hematoxylin and Eosin for Histopathological examination and Immunohistochemistry will also be performed to check for the colon markers.

18.2 Connectivity of the participating institutions and investigators (in case of multi- institutional projects only)

Not applicable

18.3 Alternate strategies (if the proposed experimental design or method does not work what is the alternate strategy)

Not applicable

19. Timelines: (Please provide quantifiable outputs)

Period of study	**Achievable targets**
6 Months	➢ Breeding and raising the colony of animals for experimentation ➢ Acclimatization of the experimental animals to the environment
12 Months	➢ Standardization of dose of fermented papaya ➢ Induction of colon cancer by intraperitoneal injection of azoxymethane and establishing of the model

18 Months	➢ *In vitro* analysis of fermented papaya. ➢ Feeding the normal rats with normal diets
24 Months	➢ Feeding the cancer induced rats with experimental diet. ➢ Analysis of feces and urine for P^H and bile salt
30 Months	➢ Collection of blood samples from different experimental groups of animals fed with fermented papaya diet, estimation of various markers for colon cancer
36 Months	➢ Collection of organs of interest from all the experimental groups of animals, histological analysis, analysis of metabolites in cecum content and assaying the serum samples

20. Name and address of 5 experts in the field

Sr.No.	Name	Designation	Address
1	Dr Suryanarayana	Principal Scientist	Indian Veterinary Research Institute, Department of foot and mouth disease, Hebbal, Bangalore-560024.
2	Dr Ramesh Agarwal	Principal Scientist	Centre for Cellular & Molecular Biology,Uppal Road, Hyderabad - 500 007
3	Dr J Kumar	Professor	Dean, GB Pant University, Udampur, Pantnagar Dehradun.
4	Dr Mohan Singh	Principal Scientist	National Research center on pulses, Kalyanpur, Kanpur-208024
5	Dr BL Umapati	Head of the department	Departmentof Microbiology KIMS Bangalore -4

PART IV: BUDGET PARTICULARS

Budget (In Rupees)

A. Non-Recurring (e.g. equipments, accessories, etc.)

S. No.	Item	Year 1	Year 2	Year 3	Total
1	Faecal p^H meter	4,00,000	--	--	4,00,000

Sub Total = 4, 00,000

B. Recurring

B.1 Manpower (See guidelines at Annexure-III)

S. No	Position No.	Consolidated Emolument	Year 1	Year 2	Year 3	Total
1.	Research Associate	22,000+HRA	3,43,200	3,43,200	3,43,200	10,29,600

Sub-Total (B.1) = 10, 29, 600

B.2 Consumables

S. No.	Item	Quantity	Year 1	Year 2	Year 3	Total
1.	CEA	9	1,00,000	1,00,000	1,00,000	3,00,000
2.	β-catanen	9	1,00,000	1,00,000	1,00,000	3,00,000
3.	SGPT, SGOT kits	9	50,000	50,000	50,000	1,50,000
4.	Fine chemicals		25,000	25,000	25,000	75,000
5.	Glass wares &Accessories		25,000	25,000	25,000	75,000

Sub-Total (B.2) = 9, 00,000

Other items	Consolidated Emolument	Year 1	Year 2	Year 3	Total
B.3 Travel		25,000	25,000	25,000	75,000
B.4 Contingency		30,000	30,000	30,000	90,000
B.5 Overhead (If applicable)		NA	NA	NA	NA
Sub-total of B (B.1+B.2+B.3+B.4+B.5)					24,94,600
Grand Total (A + B)					25,00,000

Note: Please give justification for each head and sub-head separately mentioned in the above table.

Financial Year: April - March

In case of multi-institutional project, the budget estimate to be given separately for each institution.

Justification A:

The animals will be housed in metabolic cages separately to monitor fecal and urine output for analysis of metabolites. Fecal pH meter is necessary as it indicates the gut microflora inhabitating the experimental animals..

Justification B1:

Project involves microbial work and preparation of different feed formulations as well as developing animal model for colon cancer. These techniques needs skilled person hence one RA has been requested. The RA will be involved in critical skills like molecular techniques and analytical techniques to execute the project on time.

Justification B2:

The kits CEA, CRP are very are essential as they are the indicators of colon cancer at different stages and these kits are expensive. SGPT, SGOT are necessary to assay the function of liver, as colon cancer usually metastasizes to liver. The molecular biology reagents, antibodies and enzymes for PCR are expensive hence more budget for consumables are needed.

Justification B3:

The travel budget is mainly meant for some advanced training in some Centers of Excellence in probiotics and for attending National level meetings in the area of Colon cancer and Probiotics.

PART V: EXISTING FACILITIES

Resources and additional information

1. Laboratory:
    a. Equipments

Sl. No.	Name of equipment	Make	Funding agency
1	Laminar flow Hood	Alpha	DBT
2	Table top centrifuge	Sigma	DBT
3	Inverted microscope	Olympus	DBT
4	MilliQ water system	Millipore	Self
5	PCR machine	Techne	Self
6	Analytical HPLC	Waters	Self
7	CO_2 Incubator	Nuair	DBT
8	Preperative LC	Waters	Self
9	Lyophilizer	Virtis	Self
10	NBS Bioflow2000	New Brunswick	Self
11	HPLC	Shimadzu	Self
12	GC-MS	Shimadzu	Self

2. Other resources such as clinical material, animal house facility, glass house. Experimental garden, pilot plant facility etc.

Vittal Mallya Scientific Research Foundation, a nonprofit organization is equipped with well maintained animal house facility required for basic research studies. The animal house registered under CPCSEA, Government of India and all experimental design and research plan procedures will be approved by Institutional Animal Ethics Committee (IAEC).

PART VI: DECLARATION/CERTIFICATION

It is certified that

a) The research work proposed in the scheme/project does not in any way duplicate the work already done or being carried out elsewhere on the subject.
b) The same project proposal has not been submitted to any other agency for financial support.
c) The emoluments for the manpower proposed are those admissible to persons of corresponding status employed in the institute/university or as per the Ministry of Science & Technology guidelines (Annexure-III)
d) Necessary provision for the scheme/project will be made in the Institute/University/State budget in anticipation of the sanction of the scheme/project.
e) If the project involves the utilization of genetically engineered organisms, we agree to submit an application through our Institutional Biosafety Committee. We also declare that while conducting experiments, the Biosafety Guidelines of the Department of Biotechnology would be followed in to.
f) If the project involves field trials/experiments/exchange of specimens, etc. we will ensure that ethical clearances would be taken from concerned ethical Committees/Competent authorities and the same would be conveyed to the Department of Biotechnology before implementing the project.
g) It is agreed that any research outcome or intellectual property right(s) on the invention(s) arising out of the project shall be taken in accordance with the instructions issued with the approval of the Ministry of Finance, Department of Expenditure, as contained in Annexure-V.
h) We agree to accept the terms and conditions as enclosed in Annexure-IV. The same is signed and enclosed.
i) The institute/university agrees that the equipment, other basic facilities and such other administrative facilities as per terms and conditions of the grant will be extended to investigator(s) throughout the duration of the project.
j) The Institute assumes to undertake the financial and other management responsibilities of the project.

Signature of Principal Investigator
Date:

Signature of Executive Authority
of Institute/University with seal
Date:

Signature of Co-Investigator

Date:

PART VII: PROFORMA FOR BIOGRAPHICAL SKETCH OF INVESTIGATORS

Provide the following information for the key personnel in the order listed on PART II. Follow this format for each person. **DO NOT EXCEED THREE PAGES**

Name: Dr. Anaxee Barman

Designation: Research Scientist

Department/Institute/University: Vittal Mallya Scientific Research Foundation

Date of Birth: 30th September 1984 Sex (M/F) Female SC/ST: NA

Education (Post-Graduation onwards & Professional Career)

Sl No.	Institution Place	Degree Awarded	Year	Field of Study
1	Veterinary College Bangalore	M.V.Sc	2009-2011	Veterinary Physiology

Position and Honors

Position and Employment (Starting with the most recent employment)

Sl No.	Institution Place	Position	From (Date)	To (date)
1	Vittal Mallya Scientific Research Foundation	Research Associate	2012	Till date
2	College Of Veterinary Sciences	Teaching associate	2011	2012

Honors/Awards

Awarded **Gold Medal** from KVAFSU, Bangalore Veterinary College for scoring the highest G.P. of **9.4**

Professional Experience and Training relevant to the Project

- Dr. Anaxee Barman has Performed gavaging of rats involving **blood collection** for estimation of various biochemical parameters, **dissecting**, **examining**, **weighing organs** and collecting tissue specimens for **histophysiology**, as a part of **post graduate course work** in **Dept. of Vety. Physiology** for a period of two years.
- Experience in ELISA, involving **thyroid hormone estimation** i.e **tri-iodothyronine** and **thyroxine** during the research work.
- Experience of six months in handling laboratory animals during research work.

Publications (Numbers only).

Books: Nil Research Papers: Nil Reports: Nil General articles: Nil

Patents: Nil Others (Please specify): Nil

Research Support

Ongoing Research Projects

Sl No.	Title of Project	Funding Agency	Amount	Date of sanction and Duration
	Not applicable			

Completed Research Projects (State only major projects of last 3 years)

Sl No.	Title of Project	Funding Agency	Amount	Date of completion
	Not applicable			

Place: Signature of Investigator
Date:

PART VII: PROFORMA FOR BIOGRAPHICAL SKETCH OF INVESTIGATORS

Provide the following information for the key personnel in the order listed on PART II.
Follow this format for each person. **DO NOT EXCEED THREE PAGES**

Name: Dr. Latha Diwakar
Designation: Scientist

Department/Institute/University: Vittal Mallya Scientific Research Foundation

Date of Birth: 30th November 1973 Sex (M/F) Female SC/ST: NA

Education (Post-Graduation onwards & Professional Career)

Sl No.	Institution Place	Degree Awarded	Year	Field of Study
1	**National Brain Research Center**	**Ph.D**	**2004**	**SRF, National Brain Research Center**
2	**Mysore University**	**M.Sc.**	**1996**	

Position and Honors

Position and Employment *(Starting with the most recent employment)*

Sl No.	Institution Place	Position
1	Vittal Mallya Scientific Research Foundation	Scientist
2	National Institute of Mental Health & Neurosciences	Postdoctoral Fellow
3	National Brain Research Center, Gurgaon & Indian Institute of Science, Bangalore	Senior Research Fellow
4	National Institute of Mental Health & Neurosciences	Junior Research Fellow

5	Adichunchanagiri Cancer Research Center	Junior Research Fellow
6	Central Food Technological Research Institute, Mysore	Project Assistant

Honors/Awards

Awarded Travel grant to attend International Brain Research Organization School at Hong Kong in the year December 2006.

Awarded Travel grant to attend Neurodegeneration workshop at Melbourne, Australia organized by society for neuroscience (SFN), USA, from 10th -12th July 2007.

Professional Experience and Training relevant to the Project

The Co-investigator **Dr. Latha Diwakar** has experience in cell biology, drug metabolism and enzyme kinetics at NBRC (National Brain Research Centre) Gurgaon, IISc (Indian Institute of Science) and NIMHANS (National Institute for Mental Health and Neurosciences) Bangalore. She has worked on animal model of Parkinson's, motor neuron disease and ischemia. She has also experience in molecular techniques like transfection and transformation. She has got a paper in Faseb journal on role of ASK (Apoptosis Signaling Kinase) in animal model of Parkinson's. The project on antioxidant and anticancerous effect of flavonoids has been recommended for financial support from DST from young scientist scheme.

Publications (Numbers only).

Books: .Nil Research Papers, 12 Reports: Nil General articles: Nil

Patents: Nil Others (Please specify) : Nil

Selected peer-reviewed publications (Ten best publications in chronological order)

1. Hatti KS, Diwakar L, Rao GV, Kush A, Reddy GC 2009 Abssiyone and related flavonoids as potential steriodogenesis modulators. Bioinformation.
2. Diwakar L, Ray A, Ravindranath V, 2008. Complex I assay in mitochondrial preparations from CNS. Curr.Protoc. Toxicol 38:17.10. 1-17.
3. Diwakar L, Rudresh Kumar KJ, Bachnalkar A, Ravindranath V, Christopher R, Nagaraja D. 2008. The influence of MTR A2756G polymorphism on plasma homocysteine in young south Indians. Clin Chim Acta. [Epub ahead of print] No abstract available.
4. Diwakar L, Ravindranath V, 2007. Inhibition of cystathionine-gamma-lyase leads to loss of glutathione and aggravation of mitochondrial dysfunction mediated by excitatory amino acid in the CNS. Neurochem Int. 50, 418-426.
5. Diwakar L, Kenchappa RS, Annepu J, Ravindranath V, 2007 Downregulation of Glutaredoxin but not glutathione loss leads to mitochondrial dysfunction in female mice CNS: Implications in excitotoxicity. Neurochem Int.51, 37-46.
6. Diwakar L, Kenchappa RS, Annepu J, Saeed U, Sujanitha R, Ravindranath V, 2006. Down-regulation of glutaredoxin by estrogen receptor antagonist renders female mice susceptible to excitatory amino acid mediated complex I inhibition in CNS. Brain Res. 1125(1):176-184.
7. Karunakaran S, Diwakar L, Saeed U, Agarwal V, Ramakrishnan S, Iyengar S, and Ravindranath V. 2007 Activation of apoptosis signal regulating kinase 1(ASK1) and translocation of death-associated protein, Daxx, in substantia nigra pars compacta in a mouse model of Parkinson's disease: protection by alpha-lipoic acid. FASEB J. 21:2226-36. (Co- first author).
8. Kenchappa RS, Diwakar L, Annepu J, Ravindranath V, 2004. Estrogen and neuroprotection: higher constitutive expression of glutaredoxin in female mice offers protection against MPTP-mediated neurodegeneration. FASEB J. 18:1102-1114. (Co-first author).
9. Devi L, Diwakar L, Raju TR, Kutty BM, 2003. Selective neurodegeneration of hippocampus and entorhinal cortex correlates with spatial learning impairments in rats with bilateral ibotenate lesions of ventral subiculum. Brain Res. 960:9-15.
10. Kenchappa RS, Diwakar L, Boyd MR, Ravindranath V, 2002. Thioltransferase (glutaredoxin) mediates recovery of motor neurons from excitotoxic mitochondrial injury. J Neurosci. 22:8402-8410.

List maximum of five recent publications relevant to the proposed area of work.

1. Diwakar L, Ravindranath V, 2007. Inhibition of cystathionine-gamma-lyase leads to loss of glutathione and aggravation of mitochondrial dysfunction mediated by excitatory amino acid in the CNS. Neurochem Int. 50, 418-426.
2. Diwakar L, Kenchappa RS, Annepu J, Saeed U, Sujanitha R, Ravindranath V, 2006. Down-regulation of glutaredoxin by estrogen receptor antagonist renders female mice susceptible to excitatory amino acid mediated complex I inhibition in CNS. Brain Res. 1125(1):176-184.
3. Karunakaran S, Diwakar L, Saeed U, Agarwal V, Ramakrishnan S, Iyengar S, and Ravindranath V. 2007 Activation of apoptosis signal regulating kinase 1(ASK1) and translocation of death-associated protein, Daxx, in substantia nigra pars compacta in a

mouse model of Parkinson's disease: protection by alpha-lipoic acid. FASEB J. 21:2226-36. (Co- first author).

4. Kenchappa RS, Diwakar L, Annepu J, Ravindranath V, 2004. Estrogen and neuroprotection: higher constitutive expression of glutaredoxin in female mice offers protection against MPTP-mediated neurodegeneration. FASEB J. 18:1102-1114. (Co-first author).
5. Devi L, Diwakar L, Raju TR, Kutty BM, 2003. Selective neurodegeneration of hippocampus and entorhinal cortex correlates with spatial learning impairments in rats with bilateral ibotenate lesions of ventral subiculum. Brain Res. 960:9-15.

Research Support

Ongoing Research Projects

Sl No.	Title of Project	Funding Agency	Amount	Date of sanction and Duration
1.	"Chemically synthesized derivatives of Boswellic Acid as modulators of cytokine mediated cell signaling: implications in chronic inflammatory diseases"	DBT	35,00,000	Dec 2010 – Dec 2013

Completed Research Projects (State only major projects of last 3 years)

Sl No.	Title of Project	Funding Agency	Amount	Date of completion
1	**"**Deciphering cytotoxic, antioxidant and anti cancerous activity of chemically synthesized prenylated and non prenylated flavonoids".	DST	19,00,000	October 2009- September 2012

Place: Signature of Investigator
Date: