Synopsis of the thesis submitted for the award of PhD degree (Biochemistry) of the University of Mysore, India.

Title: Studies on antioxidants and antimetastatic components from spices against cancer

Candidate: M. A. Harish Nayaka

Cancer has been identified as a major devastating disease among populations of both developing and developed countries. Diet and tobacco are two major factors contributing 35% each to the total disease incidence of 70%. Tobacco induced cancers have been predicted to come down due to awareness in the public. However, the same is not possible in diet causing cancers - i.e., lacunae in appropriate diet, due to socio-economic problems. The exogenous supply of antioxidants has been the choice to manage the disease. However, the dietary supplementation trials with vitamin antioxidant and antioxidants from green tea showed only a partial success. Recently, the use of dietary antimetastatic components has elicited promises in cancer disease management. Although several antimetastatic components have been reported, galactose rich polysaccharides appear to inhibit the metastasis effectively by blocking the initial stage of galectin mediated cancer and normal cell interaction, a crucial event in metastasis and tumor spread. Presence of such components in spices and the role of spices antioxidants, the efficiency of combinational supplementation of antioxidants and antimetastatic components against cancer are to be elucidated.

The main objective of the proposed research is to understand the efficacy of combinational supplementation of antioxidants and antimetastatic components from
spices against cancer. Following objectives have been proposed.

Objectives

1. Isolation and characterization of newer antioxidants from black cumin and swallow root.

2. Isolation and characterization of antimetastatic components from spices

3. Determination of efficacy and mechanism of action of antioxidants and antimetastatic components using in vitro biochemical and cell culture assay model systems

4. Efficacy of combinational supplementation of antioxidants and antimetastatic components against cancer employing in vivo cancer animal models

The research work carried towards achieving these objectives makes the subject matter of the thesis. The thesis is divided in to 5 chapters, and contents provided in each chapter is highlighted as follows:

Chapter I

This chapter begins with a general account on the cancer and cancer incidences as well as the role of dietary agents, in particular spices in cancer prevention. The role of dietary antioxidants, their source and mechanism of anticancer effect has been mentioned in the chapter. A brief introduction to the biochemical aspects starting from free radical generation, their effect on biomolecules, ultimately leading to cancer initiation, progression and metastasis has been depicted. The problem of metastasis and the necessity for dietary antimetastatic compounds also has been highlighted.

Further, various cancer treatments mainly surgery, radiation therapy and chemotherapy and their drawbacks have been emphasized in order to justify for the
need for the alternatives which is the main goal of the thesis. The chapter also provides an overview of current literature on black cumin (Nigella sativa) and swallow root (Decalepis hamiltonii), the spice sources selected for the proposed investigation. End of this chapter also highlights the needs-aims and scope of the current study.

Subsequent chapters - chapter II to V deals objectives 1 - 4, respectively. These chapters have a uniform format depicting hypothesis underlying the specific objective, work concept adapted, a brief introduction pertaining to the respective objectives, materials and methods, results, discussion and a relevant summary and conclusions highlighting the important outcome of the chapter. Literatures cited, references for methodologies are provided as references at the end of the thesis.

**Chapter II deals with Objective-1 - Isolation and characterization of newer antioxidants from black cumin (Nigella sativa) and swallow root (Decalepis hamiltonii).**

Black cumin (BC) and swallow root (SR) sources were selected to look for newer water soluble antioxidants (AOX). During screening of some of the commonly consumed spices for antioxidant activity, BC and SR were two sources reportedly showing more antioxidant activity in aqueous extract than that of solvent extracts. Plenty of reports are available on lipid soluble antioxidants from spices. The biopotencies against health disorders are also reported. However the current need is to adjunct the lipid soluble with water soluble AOX since majority of the diseases are caused by oxidative stress (OS) and successful inhibition or prevention of the same in vivo necessitates both water and lipid soluble antioxidants. BC and SR with higher activity in aqueous extracts were selected. Also these are the two major spices used successfully in Ayurveda medicine particularly in the form of aqueous extracts. Studies were therefore initiated with the above objective - 1.

Results indicated higher antioxidant potency in both black cumin (BCAE) and swallow root (SRAE) water extracts as evident from various antioxidant assays in comparison with known standard antioxidants. Further, swallow root extracts
showed 30-100 fold higher antioxidant activity in various assays including cytoprotective assay on NIH 3T3 fibroblasts. Attempts made to investigate potential bioactive compound by HPLC analysis indicated tannic acid (77 %) and protocatechuic acid (23 %) in BC. In SR, however, since 30-fold higher phenolic content was noticed than BC and since the phenolics were also associated with polysaccharides, efforts were made to isolate free (SRFP), hydrolyzed (SRHP) and bound (SRBP) fractions of SR and activity was determined. Among the phenolic extracts of SR, SRHP exhibited higher activity followed by SRBP and SRFP.

Swallow root phenolic extracts contained both hydroxybenzoic and cinnamic acid derivatives as antioxidant molecules to different extent. Although HMBA was found to be a predominant component in SRAE, the bulk of the activity was contributed mainly from gentisic, gallic, protocatechuic, vanillic and p-coumaric acids. Earlier studies have shown the presence of HMBA as a major constituent in methanol extracts and in the volatile oil fraction of swallow root. However, the present investigation demonstrates HMBA also in aqueous extracts, which was further confirmed by NMR spectral analysis.

Health beneficial properties reportedly exhibited upon Ayurveda medications of consuming aqueous extracts of BC and SR could be from phenolic acids-tannic acid in BC, gentisic, gallic, protocatechuic and vanillic acids in SR, respectively. Although, these antioxidants are reported in other sources, it is newer in the selected sources, particularly in understanding their attributes to health beneficial properties.

**Chapter III deals with Objective – 2 - Isolation and characterization of antimetastatic components from spices**

Majority of cancer mortalities are due to metastasis, a process of cancer spread and secondary tumor formation. Galectin-3, a β-galactoside binding lectin present on the cancer cell surface has been implicated in tumor spread and metastasis, from studies of our laboratory and other investigators. Further, higher levels of galectin-3 correlated with the advancement of the cancer disease and it is believed that
galectin-3 of cancer cells bind to normal cells and establishes secondary tumors. Recently the role of pectic polysaccharides has gained importance due to their key participation in controlling cancer metastasis through the blockade of galectin-3 present on the metastatic cancer cells. In this chapter therefore, efforts have been made to isolate galectin-3 inhibitory pectic polysaccharides from black cumin and swallow root. Galectin-3 agglutinates red blood cells due to cross-linking by binding to galactose residues present on the red blood cells. Components that inhibit this agglutination or galectin-3 inhibitor therefore can be a potential antimetastatic compound. Results indicated 6.2 % yield of a pectic polysaccharide from SR with significant activity. A minimum inhibitory concentration (MIC) of 1.85 µg equivalent carbohydrate/mL with ~ 70 and 30 fold higher activity was observed for swallow root pectic polysaccharide - SRPP when compared to that of black cumin pectic polysaccharide - BCPP (130 µg equivalent carbohydrate/mL) and standard galactose (25 µg/mL). Further, SRPP was subjected to purification and separated into 4 fractions on DEAE cellulose column chromatography, of which 0.15 M ammonium carbonate eluted fraction showed higher antimetastatic activity. Characterization studies of this fraction revealed the presence of arabinose (66 %) and galactose (27 %) in its polysaccharide chain. The molecular weight of this pectic polysaccharide was found to be ~ 250 kDa. Comparison of sugar composition analysis with antimetastatic potency revealed that arabinose and galactose residues are important for galectin-3 blocking effect.

**Chapter IV deals with Objective - 3 - Determination of efficacy and mechanism of action of antioxidants and antimetastatic components using in vitro biochemical and cell culture assay model systems**

Chapter II and III provides potent antioxidant and antimetastatic compounds from black cumin and swallow root. In order for them to act, they need to interact with the host protein (human serum albumin) or DNA, in case of antioxidants and β-galactosides, galactose containing extra cellular matrix components of cells in case of antimetastatic polysaccharides. Binding of antioxidants to plasma proteins is an important pharmacological
parameter, since it frequently affects the distribution and elimination of these compounds, which dictates the duration and intensity of physiological action. Results presented in chapter IV suggest the different degree binding of BCAE and SRAE due to their constituted phenolic acids to both protein and DNA indicating their probable mechanism of protection a) by shielding from free radicals in vivo; b) by enhancing their bioavailability and c) by limiting their degradation. Also inhibition of invasion of highly metastatic MDA-MB-231 cells and triggering of apoptosis was observed upon interaction of SRPP with galectin-3. Studies in chapter IV therefore substantiate the results of chapter II and III and provide additional evidence for their potential bioactivity.

**Chapter V deals with the Objective - study of the efficacy of combinational supplementation of antioxidants and antimetastatic components against cancer employing in vivo cancer animal models**

This chapter deals with the antioxidant extracts of BC and SR and their in vivo efficacy using CCL₄ induced hepatotoxicity and 20 - methylcholanthrene induced cervical cancer models in female albino Wistar rats and Swiss albino mice, respectively. Also, the chapter deals with the in vivo antimetastatic potency of SRPP in addition to SR - antioxidants.

Results of CCl₄ toxicity studies indicated significant protection by BCAE and SRAE. About 50 - 62 % of antioxidant enzymes were modulated when compared to that of the CCl₄ induced group. No significant increases of liver function enzymes (SGPT, SGOT, ALP) were observed upon treatment with the extracts suggesting non-toxic nature of BC and SR extracts. BC and SR extracts also indicated protection against 20-methylcholanthrene induced cervical cancer.

Results from in vivo antimetastatic studies showed significant reduction (88 %) of lung metastases induced by B16F10 melanoma cells, upon treatment with SRPP. SRPP was effective in reducing superficial tumor nodules, implantation percentage,
growth index and penetrability of cancer cells into the lung tissue indicating the effective inhibition of metastasis and invasion. Down regulation of galectin-3 levels - a marker molecule for metastasis, suggest that SRPP is effective in blocking galectin-3 and galectin-3 mediated cell invasion and metastasis. SRPP treated animals also safeguarded antioxidant enzymes that could be attributed to the presence of bound phenolics in them. HMBA an antioxidant of SR did not show antimetastatic activity. Also antioxidant rich SR extract – SRAE did not potentiate the activity of SRPP suggesting their poor efficacy in inhibiting metastasis in vivo.

Overall research results of the thesis highlight the importance of antioxidants of BC and SR, more so the SR, in inhibiting oxidant mediated molecular and cellular damages that may lead to cancer. Besides, it also contained a potent antimetastatic polysaccharide that is effective in modulating galectin-3 mediated metastasis in vitro and in vivo. Less toxic nature of these dietary sources and specific targeting of SRPP to only cancer cell via their binding ability to galectin - 3 further emphasizes their potential role as alternatives in the management of cancer.

Candidate

M.A. Harish Nayaka

Guide

Dr. Shylaja M Dharmesh