SYNOPSIS

Introduction

Cancer is a disease of misguided cells which have high potential of proliferation in an excessive and inappropriate way and have the ability to escape surveillance of the immune system and invade to distant tissues. It is the world’s second foremost cause of death after cardiovascular disease. According to WHO, an estimated 14.1 million new cancer cases and 8.2 million cancer related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008. The most common causes of cancer death were cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%). It is estimated that there will be approximately 19.3 million new cancer cases per year by 2025, due to growth, ageing and life style of the global population (WHO, 2013).

Cancer is caused by both exogenous (eg. tobacco, infectious organisms, chemicals, and radiation) and endogenous factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). Cancer development (carcinogenesis) is a multistep process and recognized three important phases - initiation, promotion and progression. Initiation involves one or more stable cellular changes in a cell, arising spontaneously or induced by carcinogen. In next step, termed promotion, the initiated cells undergo a clonal expansion under the influence of promoting agents that act as mitogens mediated through the direct interaction with DNA or signal transduction pathway Usually, the promotion phase is a slow, gradual process and requires more prolonged exposure to the promoting agent, which is reversible, and can be arrested by anti-carcinogenic agents (Ruddon, 2007).

Progression is the process through which successive changes in the neoplasm give rise to a clinically detectable tumour and is accelerated by additional exposure to genotoxic agents inducing genetic instability, nonrandom sequential chromosomal aberrations and malignant conversion. As the tumor progression proceeds, the cells lose their adherence property, detach from the tumor mass, invade the neighboring tissues and form distant metastases, resulting in widely spread cancers (Hanahan and Weinberg, 2000).
Oncogenes and tumour suppressor genes are directly involved in carcinogenesis. Gain of functional mutation in proto-oncogenes leading to oncogene results abnormal cell proliferation and loss of functional mutations in tumour suppressor genes leading to suppression of cell differentiation and apoptosis, are considered the major genetic events in cancer development. A large number of mutations in specific oncogenes—eg. ras, src, MAP kinase, NF-kB, Bcl-2, c-myc, cyclin D raf, etc. and tumor suppressor genes are P53, Rb, PTEN have been found to be closely associated with different types of cancers (Vogelstein and Kinzler, 1998).

Thus the carcinogenesis is a multistage molecular events induced by genetic and epigenetic mechanisms that upset pathways controlling cell proliferation, apoptosis, differentiation, and senescence. Therefore, several diverse approaches are necessary for prevention, treatment and management of cancer. Various chemotherapeutic agents in single, in combination and in conjunction with surgery, radiotherapy and immunotherapy are used widely for the treatment of cancers. The major obstacle of cancer treatment is the recurrence of tumor and the side effects of chemotherapy drugs are multi drug resistance. Common toxicities encountered are hematological, gastrointestinal, skin and hair follicle toxicity, nervous system toxicity, local toxicity, metabolic abnormalities, hepatic toxicity, urinary tract toxicity, cardiac toxicity, pulmonary toxicity, gonadal toxicity etc (George and Craig, 2010). Due to the toxicity associated with conventional chemotherapeutic drugs, scientists are searching for selective anti-cancer agents that target specific molecules to eliminate cancer cells while sparing normal cells (Sawyers, 2004).

Consistent with the old English proverb “Prevention is better than cure”, one of the multifactorial approaches to our fight against cancer is based on prevention of the disease through the use of non-toxic dietary supplements, micronutrients and natural compounds. This approach is generally referred to as “chemoprevention” that is defined as the use of natural or synthetic agents that reverse, inhibit, or prevent the development of cancer. Thus the major goal of chemoprevention is to delay the onset of cancer as well as decrease its incidence. An effective chemoprevention requires the use of non-toxic agents that inhibit specific molecular steps in the carcinogenic pathway. The active components of dietary phytochemicals that can act as chemopreventive agents are curcumin, genistein, resveratrol, diallyl sulfide, S-allyl cysteine, allicin, lycopene, capsaicin, diosgenin, 6-gingerol, ellagic...
acid, ursolic acid, silymarin, anethol, catechins, eugenol, isoeugenol, dithiolthiones, isothiocyanates, indole-3-carbinol, isoflavones, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, vitamin C, D-limonene, lutein, folic acid, beta carotene, selenium, vitamin E, flavonoids, and dietary fiber (Aggarwal and Shishodia, 2006).

There has been a large increase in complementary and alternative medicines (CAM) for cancer treatment worldwide with the prevalence as high as 80%. Herbal medicine is based on the use of plants or plant extracts to treat diseases and promote health and has been offered especially for cancer treatment over the last century. Therefore, medicinal plants have become important and reliable sources for anticancer agents and worldwide efforts are ongoing to find new plants with biological activity (Newman et al., 2003). Nowadays, plant derived natural products such as flavonoids, terpenes, alkaloids etc have received considerable attention due to their diverse pharmacological properties including chemotherapeutic and chemopreventive effects (Osawa et al., 1990).

Among various CAM modalities, Traditional Chinese Medicine (TCM) has been widely practiced for thousands of years in China and Western countries (Xu et al., 2006). The root of Scutellaria baicalensis commonly referred to as 'Baikal skullcap', Huang qin' or the 'Golden Root, is probably one of the most widely used herbs in TCM preparations. The flavonoid rich elements of root are considered to impart anti-inflammatory, anti-viral, anti-bacterial and anti-neoplastic activities. It is also one of the most widely studied species in Scutellaria genus, being used either alone or more often in combination with other plants for a wide range of cancers in TCM (Zhang et al., 2003).

Although, S. baicalensis has beneficial effects, the availability of the plant is reported to be declining in the wild. On this context, China has listed S. baicalensis as a nationally protected plant (Jiangsu New Medical College, 1977). This has led the exploration of other Scutellaria species with similar biological efficacy worldwide. Five species of Scutellaria have been recorded in the Western Ghats of Peninsular India, a major hotspot of biodiversity. Scutellaria plants are herbs growing 900–1000 m above sea level in semi-evergreen forest or grass lands. No reports are available on the biological properties of the species. In the present study, we
investigated the biological properties of root of two native species, *S. colebrookiana* Benth and *S. violacea* (Heyne ex Benth) Don. collected from Western Ghats of Kerala with special emphasis on anti-cancer activity.

**OBJECTIVES OF THE PRESENT STUDY**

1. To determine the *in vitro* and *in vivo* antioxidant property analysis of *S. colebrookiana* and *S. violacea*.
2. To determine the anti-inflammatory activity
3. To assess the anti-mutagenic and anti-carcinogenic effect of *S. colebrookiana* and *S. violacea*
4. To determine the cytotoxic and antitumour activity
5. Phytochemical screening of *S. colebrookiana* and *S. violacea* using various analytical techniques.

**METHODS**

*S. colebrookiana* and *S. violacea* were collected between October 2009 and February 2010 from Nelliyampathy and Wayanad regions of Western Ghats of Kerala, India, respectively. The voucher specimens of *S. colebrookiana* (No. KFRI 30832) and *S. violacea* (No. KFRI 30833) have been identified and deposited in the Herbarium of Kerala Forest Research Institute by Dr. N. Sasidharan, Taxonomist, KFRI, Thrissur, Kerala, India. Roots of the plants were used for the study.

**In vitro and in vivo antioxidant activity and toxicity analysis of *S. colebrookiana* and *S. violacea**

Experiments:

1) *In vitro* antioxidant activity: Superoxide, hydroxyl, 2, 2-diphenyl-1-picryl hydrazyl (DPPH$^+$), 2,2-azo bis–3-ethylbenzthiazoline-6-sulphonic acid (ABTS$^+$) radicals scavenging activity, FRAP assay and inhibition of lipid peroxidation. Also analyzed prevention of H$_2$O$_2$ induced RBC hemolysis and membrane lipid peroxidation.

2) Prevention of AAPH (2, 2’-Azobis (2-amidinopropane) dihydrochloride) induced hemolysis, membrane lipid peroxidation and protection of membrane protein by SDS-PAGE.
3) *In vivo* antioxidant activity of extracts against NaF induced oxidative stress.

Parameters assessed: Superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase activities and glutathione content in blood and liver.

4) Toxicity studies of chloroform extract of *S. colebrookiana* and *S. violacea*.

Parameters assessed: Body weight, hemoglobin, organ weight, WBC count, ALP, SGPT, SGOT, urea and creatine.

**Determination of anti-inflammatory activity of S. colebrookiana and S. violacea**

Experiments:

1) Inhibition of lipoygenase activity by *S. colebrookiana* and *S. violacea*
2) Determination of anti-inflammatory activity using mouse acute (carrageenan and dextran) and chronic (formalin) inflammatory models

**Antimutagenic and anticarcinogenic potential of S. colebrookiana and S. violacea**

Experiments:

1) Effect of *Scutellaria* on the mutagenicity induced by mutagens in *Salmonella typhimurium* strains.
2) Anti-mutagenic assay using direct acting mutagens sodium azide, MNNG (1-methyl-2-nitro-1-nitrosoguanidine) and NPDA (4-nitro-o-phenylenediamine).
3) Determination of anti-mutagenicity against mutagens needing activation.
4) Determination of anti-mutagenicity of *S. colebrookiana* and *S. violacea* against tobacco.
5) Determination of anti-carcinogenic potential of *S. colebrookiana* and *S. violacea* using a two-stage mouse skin carcinogenesis.

**Anti-cancer activities of chloroform extract of S. colebrookiana and S. violacea**
1) *In vitro* anti-cancer activity of extract was determined against DLA (Dalton’s lymphoma ascites) and EAC (Ehrlich’s ascites carcinoma) cells by short term cytotoxic assay.

2) *In vitro* anti-proliferative activity of extract was determined by MTT assay using cancer cell lines - HeLa, Hep-2, Kato, Caco-2, MCF-7, AGS, HT-29, Jurkat, THP-1, MOLT-4, L929, SKBR3 and a normal cell line, Vero.

3) Morphological analysis of treated cells using an inverted, phase contrast and fluorescent microscopes.

4) DNA fragmentation assay by agarose gel electrophoresis.

5) Anti-tumour activity of *Scutellaria* was determined by DLA induced solid and EAC induced ascites mice models.

**Phytochemical screening of S. colebrookiana and S. violacea**

Phytochemical screening of chloroform extract of *S. colebrookiana* and *S. violacea* were done by

1) Phytochemical analysis
2) UV-Vis spectrophotometric analysis
3) HPLC
4) HPTLC
5) FT-IR analysis

**RESULTS**

Even though, the species of *Scutellaria* are well known and multipurpose herbs worldwide, no reports are available on the species of *Scutellaria* native to Western Ghats of Kerala. Hence, the present study was aimed to explore the biological properties of two species, *S. colebrookiana* and *S. violacea* collected from Western Ghats region.

Among the four extracts (petroleum benzene, chloroform, acetone and methanol) of *S. colebrookiana* and *S. violacea* root obtained by soxhlet extraction, the chloroform extract showed considerable free radical scavenging activity. The chloroform extract of *Scutellaria* species showed significant reduction of Fe$^{3+}$, ABTS$^+$ and DPPH radicals, scavenging of superoxide and hydroxyl radicals and inhibition of lipid
peroxidation. Extracts were found to be effective against RBC hemolysis and RBC membrane lipid peroxidation induced by 2’-azobis (2-amidinopropane) dihydrochloride (AAPH) and hydrogen peroxide. Oral administration of *S. colebrookiana* and *S. violacea* extracts significantly increased the activity of catalase, superoxide dismutase, glutathione reductase and glutathione in blood and liver in NaF challenged mice. In acute and sub acute toxicity study, chloroform extracts of both species did not produce toxic symptoms up to 1000 mg/kg b. wt. *Scutellaria* extracts were also found to be effective against carrageenan or dextran induced acute and formalin induced chronic inflammation in mice in a dose dependent manner. Lipoxygenase enzyme, a mediator of inflammation was found to be inhibited by the extracts. These *in vitro* and *in vivo* studies revealed the antioxidant and anti-inflammatory potentials of *Scutellaria* species studied.

Chloroform extract showed significant cytotoxicity towards Dalton’s Lymphoma Ascites (DLA) and Ehrlich’s Ascites Carcinoma cells (EAC). Oral administration of extracts showed significant reduction on DLA induced solid tumor and increased the life span of EAC induced ascite’s tumour bearing mice. The anti-proliferative activity of *S. colebrookiana* and *S. violacea* was determined using various cancer cell lines such as L929, Kato, HeLa, MCF-7, SKBR-3, Hep-2, Jurkat, AGS, HT-29, CaCo-2, MOLT-4, THP-1 and normal cell line, Vero. *S. colebrookiana* and *S. violacea* extracts showed strong anti-proliferative activity towards Jurkat and HeLa cells. Both plants also showed good anti-proliferative activity towards SKBR-3 and MCF-7 and found to be moderately cytotoxic to Hep-2, L929 and THP-1 cells in a dose dependent manner. Other cancer cell lines such as AGS, MOLT-4, Caco-2, HT-29 and Kato were found to be less sensitive. Vero cell line was found to be less susceptible to *S. colebrookiana* and *S. violacea* extracts.

The morphology of treated cells was observed by fluorescent microscopy after staining with Acridine Orange/Ethidium Bromide or Hoechst stain. Condensed chromatin, nuclear fragments and apoptotic bodies were observed in susceptible cell lines, Jurkat and HeLa cells. Presence of DNA ladders indicated the double strand break in treated cells which is characteristic of apoptosis.
Ames test is widely accepted test to identify the chemicals and drugs which can produce mutation and is a high predictive tool for in vivo mutagenicity (Michaud et al., 2000). Chloroform extract of S. colebrookiana and S. violacea did not exhibit mutagenic activity or toxicity in the range of tested doses (0.5–2.5 mg/plate), either in the presence (+S9) or absence of (−S9) microsomal activation. Anti-mutagenic activity of chloroform extract was screened by direct and indirect acting mutagens and tobacco. Both extracts showed inhibitory action on mutagenicity induced by direct acting mutagens MNNG, NaN₃ and NPDA in a dose dependent manner. Extracts inhibited the mutagenicity of AAF and tobacco in a concentration dependent manner. These results suggest that the extracts have antimutagenic potential.

To assess the anticarcinogenic potential of Scutellaria, two stage skin papilloma model was used and the onset of papilloma was induced by the application of DMBA and croton oil. Compared to DMBA + Croton oil (carcinogen control) treated mice, topical application of Scutellaria extracts reduced the number of papillomas on mouse skin in a dose dependent manner. Anticarcinogenic property of S. colebrookiana and S. violacea is possibly due to its antioxidant, anti-inflammatory and antimutagenic effects.

Upon preliminary phytochemical screening, chloroform extract of S. colebrookiana and S. violacea showed the presence of phytoconstituents like alkaloids, flavonoids, carbohydrates, phenols, proteins, phytosterols and diterpenes. The spectrophotometric scanning within wavelength range 200-900 nm showed three peaks (210, 276, and 360 λ) which are similar to standard baicalein (Sigma Aldrich, USA). HPLC chromatogram obtained for chloroform extract of both Scutellaria species showed similar profiling to that of standard baicalein with a major peak retention time of 3.4 min. Presence of baicalein in extracts was also confirmed by HPTLC analysis.

FTIR spectral analysis was utilized to identify the functional group of the active ingredients on the basis of peak value in the vicinity of infrared radiation. Chloroform extract of S. colebrookiana showed characteristic absorption bands at 3460 cm⁻¹(for a hydroxyl group), 2962 cm⁻¹ (for C-H stretching), 1728 cm⁻¹ (for a carbonyl group (C=O), 1668 and 1490 cm⁻¹ (C=C ring stretch) and at 1261 cm⁻¹ (C-O
stretching vibration), The characteristic absorption band were exhibited at 3446 cm\(^{-1}\) (for a hydroxyl group), 1726 cm\(^{-1}\) (for a carbonyl group, C=O), 1261 cm\(^{-1}\) (C-O stretching vibration), 1606 cm\(^{-1}\),1508 cm\(^{-1}\),1450 cm\(^{-1}\) and 1415 cm\(^{-1}\) (C=C stretching) by S. violacea extract.

To summarize, the present study revealed a wide spectrum of biological properties with Scutellaria species collected from Western Ghats of Kerala. Anti-proliferative properties of extracts against various cancer cell lines and induction of apoptosis was also noticed. Spectrophotometric, HPLC and HPTLC analysis confirmed the presence of baicalein, a flavonoid already reported from Scutellaria baicalensis. Presence of baicalein might be responsible for the biological properties shown by S. colebrookiana and S. violacea. Due to the rich source of various bioflavonoids, Scutellaria species are being exploited worldwide for developing chemotherapeutic/chemopreventive drugs. Some countries including China listed S. baicalensis as nationally protected plant (class III conserved plant) due to the marked decrease of wild resources (Yuan et al., 2010). To meet the increasing demand for Skullcaps root, large-scale cultivation programs are essential (Ran & Zhou, 1999). Hence, the exploration of Scutellaria species with biological potential is thus important. On this context, the study suggests that S. colebrookiana and S. violacea and other Scutellaria species seen in Western Ghats could be a source for the important bioactive flavonoids including baicalein.

The thesis has been divided into 7 chapters as follows:

Chapter 1: Review of literature.

Chapter 2: Materials and methods.

Chapter 3: In vitro and in vivo antioxidant analysis of S. colebrookiana and S. violacea.

Chapter 4: Anti-inflammatory activities of S. colebrookiana and S. violacea.

Chapter 5: Anti-mutagenic and anti-carcinogenic potential of S. colebrookiana and S. violacea.

Chapter 6: Cytotoxic and antitumour activities of S. colebrookiana and S. violacea.