Evaluation of anticancer activity and antioxidant properties of propolis from Meghalaya

Ph.D THESIS ABSTRACT
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Cancer is a disease involving heritable defects in cellular control mechanism(s) on cell division that result in the formation of invasive malignant tumors which is commonly referred to as cancers. Carcinogenesis or the development of cancer, from normal cell to a cancerous cells is a multistep process of clonal progression driven by a series of somatic mutations that progressively convert the cell from normal growth to precancerous state and finally to cancerous state. The fundamental goal of cancer research is to understand how a normal cell undergoes neoplastic transformation, so as to develop cancer treatment that can cure the disease and secondly to palliate i.e., life prolongation and relief of sufferings due to advancement of the disease. Chemotherapy is an effective treatment against cancers, and hundreds of different types of drugs have been used either singly or in combination with surgery and other therapies. However, full use of these drugs have been limited due to the development of various side effects such as nephrotoxicity by cisplatin (Yao et al., 2007), doxorubicin induced cardiac myopathy (Batist et al., 2001), gonadal toxicity after cyclophosphamide administration (Haubitz, 2007) or drug resistance by cancer cells. In an attempt to lessen such problems and maintaining the therapeutic efficacy, the use of natural sources such as products obtained from plants and animals’ preparations have attracted the scientists all over the world.

Plants have a long history of use in the treatment of cancer. Some of the naturally occurring plant derivatives that have been used as anticancer drugs are vincristine, vinblastine, paclitaxel, docetaxel, topotecan and irinotecan (Bhanot et al., 2011). However, like plants, identification of animal resources for medical cures is also gaining importance in human health care (Alves et al., 2005). The healing of human diseases by the use of therapeutics obtained or ultimately derived from animals is known as zootherapy. Animal-based medicines can be prepared from the entire animal, parts of the animal’s body or products of metabolism (body secretions and excrements, or from other materials related to animals (nest and cocoons) (Costa-Neto, 2005). In India, since time immemorial, immense work in the field of zootherapy and traditional medicines has been done and documented in books like Ayurveda and Charaka Samhita. The Hindu religion in India have been using five
animal products viz. milk, urine, dung, curd and ghee for purification and other medicinal purposes.

Honeybees are social insects which belong to the class: Insecta, order: Hymenoptera, super-order: Apocrita, super-family: Apoidea, family: Apidae, subfamily: Apinae. There are more than 11 species of *Apis* existing worldwide. *Apis cerana indica* is the most common species found in India. The important activity of bees, in terms of benefits to humans, is their ability to pollinate, something which is rarely observed by non-specialists and goes unappreciated. Honeybees products viz., honey, bee-bread, brood, royal jelly, and propolis, are of immense importance to mankind.

Propolis is one of the important substances derived from honeybees. The word propolis comes from the Greek word, pro = in/for defense, and polis = city, referring to the defense of the city or the hive (*Santos et al.*, 2002). It is a resinous sticky dark-colored material that honeybees collect from living plants, mix with wax and use in construction and adaptation of their nests. Propolis is used by honeybees in the construction and maintenance of their hives, so, it is commonly known as bee glue. It is also used in blocking holes and cracks, to repair combs, to strengthen thin borders of the comb, and for making the entrance of the hive weather tight. The precise composition and constituents of propolis varies depending on the geographical vegetation and plants sources from where they collect it. Khalil, 2006, reported many compounds among which polyphenols, terpenoids, steroids, sugars and amino acids have been identified as the main constituents. Biological and therapeutic actions of propolis are generally attributed to its constituents of plant origin, mainly phenolics (*Burdock, 1998*). Different ecosystems, plant exudates and secretions cause variability in propolis composition and thus, could serve as varying types of propolis. Propolis has been reported to show diverse biological activities such as antibacterial, antiviral, anti-inflammatory, anticarcinogenic and immune-modulatory activities (*Sforcin et al.*, 2000; *Vynograd et al.*, 2000)

The main mechanism of action of propolis and its compounds, regarding their antitumoral action, are related to apoptosis, cell cycle arrest and interference on
metabolic pathways. El- Khawaga et al. (2003) reported that Egyptian propolis inhibited the growth and proliferation of Ehrlich Ascites Carcinoma tumor volume in mice. Brazilian green propolis showed a remarkable activity against different tumor cells \textit{in vitro} (Bassani-Silva et al., 2007; Bufalo et al., 2009b), and the main mechanisms by which propolis affects tumor cells are related to the inhibition of cell growth and apoptosis (Sforcin, 2007). \textit{In vivo}, treatment of Brazilian green propolis significantly increases the cytotoxic activity of natural killer cells against murine lymphoma (Sforcin et al., 2002).

The North-east region of India represents an extremely unique eco-system rich in medicinal plants and animals associated with traditional medicine and medical system. North-east region comprises of the eight sister states viz. Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Tripura and Sikkim. Meghalaya is situated between $25^\circ 47'-26^\circ 10' \text{N}\text{ latitude and } 89^\circ 45'-92^\circ 47' \text{E}\text{ longitude covering an area of } 22,429 \text{ sq.km. comprising of 11 districts. The indigenous people of Meghalaya include Khasis, Garos and Jaintias who have their respective dialect, distinct ways of life, belief, traditions and cultural heritage (Kharkongor and Joseph, 1981). Ngunraw is a medium size village located under tehsil Mawkyrwat of South West Khasi Hills district, Meghalaya with total 400- 450 families residing. Ngunraw village has population of approximately 1737 of which 836 are males while 901 are females as per population Census 2011. The village is approximately 135 Km away from the main city Shillong and it covers about 10,000 square metres. Agriculture is their main source of livelihood. However, apart from agriculture the local inhabitants also practice apiculture, as the different products obtained from apiculture have been used by them for different pharmacological and medicinal therapeutic activities.

Based on preliminary survey during January 2013 to March 2014 involving group discussion and personal interviews with the ethnic inhabitants of Ngunraw village, it has come to our knowledge that the people of this region frequently use many products of animal origin for the treatment of different ailments such as wound healing, stomach ulcer and illness of intestines including cancer suspected cases. Preliminary survey also revealed that propolis is one of the traditional medicines used
by them. However, the systematic evaluation of biological activities particularly in relation to anticancer activities of propolis from Meghalaya has not been evaluated.

Therefore, considering the importance of propolis in general and an unexplored study in relation to anticancer activities of propolis from Meghalaya, the present research study was undertaken for the Ph.D. degree with the following main objectives:

- To evaluate the antitumor effect of propolis extract in tumor bearing mice.

- To explore the antioxidant property and protective activity of Propolis extract on the host animal.

For the assessment of antitumor activity, ascites Dalton’s lymphoma has been regularly used as an important murine experimental tumor model. Methanol and aqueous extracts of propolis were prepared following the method of Nagai et al. (2003) with slight modifications, and these were used for screening of antitumor activity in tumor-bearing mice. The results from the screening of the doses for antitumor activity indicate that MeOH crude extract of propolis at 50 mg/kg body wt. has comparatively better antitumor activity. Thus, based on the ILS results obtained, methanol crude extract of propolis at a dose of 50mg/kg body weight was selected for antitumor, antioxidant activity and biochemical studies. Henceforth, methanol extract of propolis will be expressed as MeOH-propolis in the text.

Surface topographical and internal structural changes in tumor cells collected from the mice under the above treatment conditions were done by Light microscopy, Scanning electron microscopy and Transmission electron microscopy. Liver, kidney, spleen and DL cells were collected from control and treated mice and used for the analysis of protein, sialic acid, glutathione (reduced form), glutathione related enzymes and lipid peroxidation using standard protocols. Cytotoxicity and apoptosis study in DL cells was done by trypan blue exclusion test and AO/EB staining method respectively. Blood samples were collected from control and treated mice and used for different haematological determinations.
Viability analysis of splenocytes and DL cells in vivo, after MeOH-propolis treatment revealed that there was less cytotoxicity in the spleen cells as compared to that of tumor cells. This indicates that normal cells were less sensitive to MeOH-propolis as compared to DL cells. Apoptosis plays a critical role in the pathogenesis of tumor disease. Uncontrolled proliferation and a defect in the process of apoptosis constitute crucial elements in the development and progression of malignant tumors. The study based on AO/EB showed that MeOH-propolis treatment caused severe membrane blebbing and cell shrinkage, cell membrane abnormalities, fragmented nuclei, chromatin condensation, cell membrane disintegration with scattered fragmented nuclei and appearance of cytoplasmic vacuoles in a time-dependant manner.

The functional and structural properties of malignant cells are influenced by its membrane or surface changes. Light microscopical study of DL cells after MeOH-propolis treatment caused appearance of membrane blebbing, folding and cytoplasmic vacuoles indicating the occurrence of various membrane disruptions, appearance of apoptotic bodies and tumor cells lysis/death. The appearance of cell surface blebs and membrane rupture resulting after treatment should be considered as an important step towards the cell death.

Surface morphological alterations are important signs of cell injury which may be considered as specific markers of apoptosis. The Scanning electron microscopy observations revealed a series of surface changes in DL cells following different treatment. MeOH-propolis treatment for 24-96 h revealed membrane blebs, loss in microvilli, cell membrane fusion, shrinkage and deformities in comparison to the control DL cells. The interactions of tumor cells with the phagocytic cells through fine membrane projections in MeOH-propolis treatment were also found.

A distinctive feature of the early stages of programmed cell death is the disruption of active mitochondria. Control DL cells showed distinct mitochondrial features with regular cristae. Treatment of tumor-bearing mice with MeOH-propolis showed irregularities in the structure of mitochondria acquiring a roundish shape, more thickened, irregular arrangement of cristae and mitochondrial membrane
disruption with more pronounced vacuoles, which was quite comparable to that observed in CDDP treatment.

The evaluation of the antibacterial properties revealed that MeOH-propolis from Meghalaya has more pronounced effect on gram positive bacteria (*Staphylococcus aureus and Bacillus cereus*) as compared to that on gram negative bacteria (*Salmonella enterica and Escherichia coli*).

MeOH-propolis treatment resulted in an overall decrease in the sialic acid content in kidney, spleen and in DL cells. Decrease in glutathione (GSH) level in DL cells and tissues of tumor-bearing mice were observed after CDDP treatment. GSH level in DL cells was also noted to decrease after MeOH-propolis treatment.

Studies on GSH-related enzymes showed that MeOH-propolis treatment of tumor bearing mice caused a decrease in glutathione-s-transferase (GST) activity in liver and kidney. In spleen and DL cells GST activity showed no significant changes. MeOH-propolis treated tumor-bearing mice showed a decrease in glutathione reductase (GR) activity in liver, kidney, and DL cells at (24-48 h) as compared to control. Decrease in glutathione peroxidase (GPx) activity in liver, kidney and spleen was noted in MeOH-propolis treated tumor bearing mice as compared to control.

The lipid peroxidation (LPO) was measured in terms of malondialdehyde (MDA) concentrations in different tissues of mice. As compared to control, MeOH-propolis treated tumor-bearing mice resulted in an increase level of LPO in spleen and DL cells at 48 -96 h and a decrease in LPO level was noted in liver and kidney.

The haematological findings showed that after MeOH-propolis treatment of tumor bearing mice, the haemoglobin content, RBCs and WBCs count was found to be significantly increasing, suggesting the immunotherapeutic effect of propolis in cancer cells. Major RBC’s abnormalities were observed after CDDP treatments. On the other hand MeOH-propolis treated tumor-bearing mice showed significantly less frequency of abnormalities in the RBCs as compared to CDDP which may suggest that MeOH-propolis has minimal or no hematotoxicity.
Sperm abnormality analysis in mice showed that CDDP induced various types of sperm abnormalities in DL bearing mice with amorphous head and hooks less sperm occurring more frequently. However, MeOH-propolis treatment showed less frequency of sperm abnormalities in the tumor bearing hosts as compared to CDDP treatment.

Histological examination of liver, kidney and spleen from tumor-bearing mice after CDDP treatment showed damages in renal tubular cells represented by glomerular atrophy, infiltration of cells and tubular congestions, destruction of the renal tubular cells in kidney. CDDP treatment showed sinusoidal distortion and remarkable locational hepatocytes damage in liver. However, mild to moderate damage of hepatic cells was observed after MeOH-propolis treatment indicating the non-toxic effect of MeOH-propolis in the liver. Reduced destruction of renal tubular cells with lesser glomerular damage in kidney and normal structural organization of spleen was observed in MeOH-propolis treated mice respectively.

The antioxidant activity of MeOH-propolis is comparable to that of ascorbic acid showing increased scavenging and inhibition activity with increasing sample concentrations as seen in DPPH assay, super oxide anion activity, hydroxyl radical scavenging activity and metal chelating activity.

The GC-MS analysis revealed various compounds present in MeOH-propolis extract which were identified as 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, 2-furancarboxyldehyde,5-(hydroxymethyl), Beta-D-glucopyranose, 16-anhydrous, tetradecanoic acid, 3,7,11,15-tetranethyl-2-hexadecen-1-ol, N-hexadecanoic acid, 6-octadecanoic acid, methyl ester, Oleic acid, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, palmitoyl chloride and 9-octadecanoic acid (Z)-, 2,3-dyhydroxy propoly ester. These compounds may be involved in causing different biological activities such as antitumor, antioxidant and antibacterial etc by MeOH-propolis extract from Meghalaya.

Based on the various aspects of studies undertaken and their results, following important conclusions may be derived:
This is the first report on the antitumor activity of propolis extracts from Meghalaya. The crude aqueous and methanol extract of propolis from Ngunraw village, South West Khasi hills district of Meghalaya, India depicted potential antitumor activity against murine ascites Dalton’s lymphoma, with MeOH-propolis showing better therapeutic efficacy than aqueous extracts. This could be due to the various compounds and constituents that are solubilised in MeOH-propolis extract. Further, MeOH-propolis has higher cytotoxicity, towards DL cells as compared to normal spleen cells.

The induction of apoptosis and development of mitochondrial abnormalities in DL cells could be one of the factors involved in the antitumor activity of MeOH-propolis.

The decrease in the cellular GSH levels and increase in LPO in DL cells after MeOH-propolis treatment may reduce the protective ability in the tumor cells which may show more cytotoxic effect under these conditions. At the same time the decrease in GSH related enzymes in DL cells could be one of the possible steps to cause decrease in the GSH level in DL cells, thus, weakening the cellular antioxidant system.

As most of the sialic acid moieties are mainly confined as the mucopolysaccharides component of the plasma membrane. The observed decrease of sialic acid moieties in DL cells after propolis treatment may lead to increase in the antigenecity of DL cells.

No change or betterment in the haematological values noted after MeOH-propolis treatment may suggest that propolis may have minimal/no hematotoxicity.

Sperm abnormality (SA) study revealed that MeOH-propolis treatment of the host showed less mutagenic effects as compared to that of CDDP suggesting that MeOH-propolis is very less mutagenic in the hosts.

Histological observations of liver, kidney and spleen depicted very less histological damages after MeOH-propolis treatment, thus suggesting no hepatotoxicity, nephrotoxicity etc in the hosts.

The antioxidant activity of MeOH-propolis was noted which showed increased scavenging and inhibition activity with increase sample concentrations.
The GC-MS analysis of MeOH-propolis revealed different phytoconstituents/compound with biological and pharmacological properties. The observed effective therapeutic activities of propolis could be mainly due to the presence of flavonoids in MeOH-propolis.

From the study it is evident that propolis from Meghalaya could be a useful antitumor agent. However, further details on the molecular mechanisms behind the therapeutic efficacy of propolis and their bio-components need to be explored.
REFERENCES


