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Original Research Article

Studies on the Effect of Propolis from Meghalaya, India on the Biochemical and Ultrastructural Changes in Murine Ascites Dalton's lymphoma

Surya Bali Prasad* and Ibanrikynti Turnia
Cell and Tumor Biology Laboratory, Department of Zoology, School of life Sciences,
North-Eastern Hill University, Umshing-Mawkynroh, Shillong - 793022, India
***Corresponding author**: sbnehu@gmail.com
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Abstract: Propolis is a resinous mixture that honeybees produce by mixing saliva and beeswax with exudates gathered from tree buds, sap flows or other botanical sources. Despite its many biological activities, scientific research on propolis is still limited. The present study was undertaken to examine the effect of propolis from Meghalaya on the biochemical changes and ultrastructural features in Dalton's lymphoma (DL) cells so as to understand the possible involvement of these changes in its antitumor activity. Methanol extract of propolis (MeOH-propolis), as well as cisplatin treatment, caused a significant decrease in reduced glutathione (GSH), sialic acid and an increase in lipid peroxidation in DL cells. MeOH-propolis-mediated development of chromatin condensation, nuclear fragmentation, and irregularities in mitochondrial cristae was also observed in tumor cells. Cisplatin treatment caused the appearance of small fine microvilli like processes and disruptions in the mitochondrial membrane and cristae. It is suggested that these ultrastructural changes together with the biochemical changes could assist in its anticancer activity. The findings show that MeOH-propolis induced changes in DL cells were quite comparable to that of reference anticancer drug cisplatin. It is suggested that molecular exploration may assist to understand more insight into the MeOH-propolis-mediated biochemical changes and anticancer activity.
Key words: Dalton's lymphoma, propolis, Meghalaya, glutathione, sialic acid

Introduction

The etiology of cancer and its treatment has been one of the main global health-care concerns for human beings. Chemotherapy is an important way of cancer treatment that can be used either singly or in combination with surgery and/ or radiotherapy. However, the efficacy of most of the anticancer drugs is often hampered by the development of various side effects (McDonnell, 2016) and acquired resistance by cancer cells (Siddik, 2003). Natural products derived from plants, animals, microbes, fungi have attracted the attention of researchers so as to develop least side effects while maintaining therapeutic efficacy (Harvey, 2008; Bhanot *et al*., 2011; Khazir *et al*., 2014; Lichota and Gwozdzinski, 2018).

Plants have a long history of use in the treatment of cancer and some of the naturally occurring plant derivatives that have been used as anticancer drugs are vincristine, vinblastine, paclitaxel, docetaxel, topotecan, and irinotecan (Iqbal *et al*., 2017). Like plants, the identification of animal resources for medical cures is also gaining importance in human health care (Alves and Rosa, 2005; Verma and Prasad, 2013). Several workers have reported a number of animal products that exhibit antitumor properties such as snake venom protein (Lipps, 1994), cantharidin isolated from red-headed blister beetles, *Epicauta hirticornis* (Verma and Prasad, 2013), anti-cancer peptide (ANTP) isolated from *Buthusmartensii karsch*

(Kapoor, 2010; Gomes *et al*., 2010), the skin extract (TSE) from common Indian toad (*Bufo melanostictus*, Schneider) (Giri *et al*., 2006, 2018).

One such natural animal product is propolis which is obtained from apiculture and has important nutritional and medicinal properties. Propolis or 'bee glue' is a resinous substance collected by worker-bees from numerous plant resinous secretions. It is used by honey bees as a sealant to protect their hives by blocking the cracks and also as an antiseptic agent (Anjum *et al*., 2018). Propolis has become a subject of increasing interest among the researchers worldwide because of its versatile biological activities such as antibacterial, antiviral, immunomodulating, anticancer (Banskota *et al*., 2002; Kapare *et al*., 2017), anti-inflammatory, antidiabetic, antiulcer, antifungal effects (Sforcin and Bankova, 2011; Wagh, 2013; Choudhari *et al*., 2013; Anjum *et al*., 2018). Propolis is as old as honey and generally named with its country/region such as Argentinean propolis (Aguero *et al*., 2010), Chinese and Brazilian propolis (Ishihara *et al*., 2009), Netherland propolis (Banskota *et al*., 2002), Sydney propolis (Cole *et al*., 2010), Portuguese propolis (Valente *et al*., 2011) and Indian propolis (Kumar *et al*., 2008; Lyyam *et al*., 2010).

Meghalaya is one of the eight States of Northeast India, known for its clouds and the highest rainfall in the world (Fig. 1). Meghalaya is situated between 25 ^{0} 47'-26^{0} 10' N latitude and 89^{0} 45'-92^{0} 47' E longitude covering an area of approximately 22,430 sq.km. comprising of eleven districts.
In view of the variety of reports on the properties of propolis from different parts of the world, the antibacterial and antitumor potential of propolis from Meghalaya was assessed earlier for the first time by us (Turnia *et al*., 2015). The broad chemical composition of methanol-extract of propolis (MeOH-propolis) from Meghalaya has revealed the presence of various compounds mainly 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-tetraethyl-2-hexadecene-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-dihydroxypropoly ester (Turnia, 2018). Consequently, present studies were undertaken to further examine the effect of methanolic extract of this propolis on the glutathione, glutathione-related enzymes, sialic acid and other biochemical and ultrastructural changes in Dalton's lymphoma cells so as to know the possible involvement of these changes in its anticancer activity. Cisplatin (*cis*-diamminedichloroplatinum-II), a well established anticancer drug, was used as the reference drug for a comparative analysis of the propolis-mediated findings.

Materials and methods

Chemicals

Reduced glutathione (GSH), 5, 5 N-dithiobis-2-nitrobenzoic acids (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, Mo, USA. Ethylenediamine tetra-acetic acid (EDTA), and other chemicals used in the experiments were of analytical grade and purchased from SRL Pvt. Ltd., Mumbai, India. Cisplatin solution (1mg per mL of 0.9%, NaCl) was obtained from Biochem Pharmaceutical Industries, Mumbai.

Experimental animals and tumor maintenance

Swiss albino mice colony is being maintained under conventional laboratory conditions at room temperature of 22±2°C keeping 5-6 animals per propylene cage using paddy husk as the bed with food pellets (Amrut Laboratory animal feeds, New Delhi) and drinking water *ad libitum*. Inbred mice of both sexes in the age group of about 10-12 weeks weighing about 28-30 g were used for the experiments. Ascites Dalton's lymphoma is a transplantable and highly invasive T cell lymphoma that develops an ascitic tumor in murines (Goldie and Felix, 1951). Ascites Dalton's lymphoma (DL) has been widely used by the researchers for evaluating the anticancer potentials of a variety of drugs. Ascites Dalton's lymphoma is being maintained *in vivo* in mice by serial intraperitoneal (i.p.) transplantation of approximately 1x10^{7} viable tumor cells per animal (0.25 ml in phosphate-buffered saline (PBS), pH 7.4). Tumor transplanted hosts usually survive for 19-21 days.

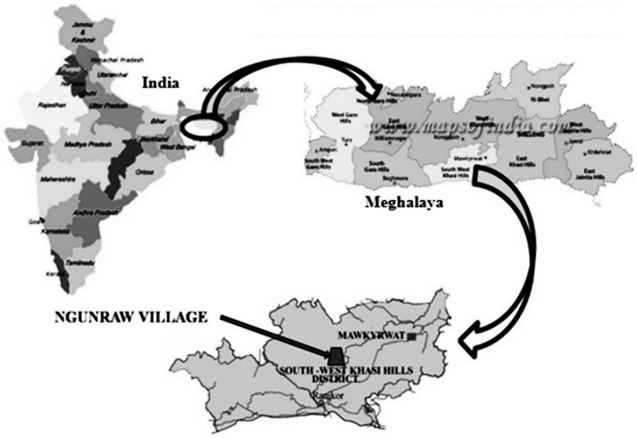

Fig. 1. The maps showing propolis collection site, Ngunraw village in South-West Khasi Hills district of Meghalaya, India (Maps not at scale)

All experimental protocols in this study using animals have been performed according to ethical standards and guidelines of the North-Eastern Hill University (Institutional ethics committee (animal models), North-Eastern Hill University, Shillong, India having approval reference IEC/NEHU/ December 4/2014.

Propolis collection and preparation of methanolic extract

In present studies, the propolis was collected from the beehives in Ngunraw village of Meghalaya State, so it is being referred here as Meghalaya propolis (Fig. 1). Ngunraw village is approximately 135 Km away from Shillong, situated between 25°17'52.5"N latitude and 91°19'23.2"E longitude located under Mawkyrwat block of South West Khasi Hills district, Meghalaya.

Unprocessed wild honey has a certain amount of propolis when extracted. Raw propolis was collected from Ngunraw village during spring and winter seasons. It was cleaned, freed from wax and its methanolic extract was prepared as described earlier (Turnia *et al.*, 2015). Briefly, thirty grams of raw propolis was taken and dissolved in 300 ml of 70% methanol with continuous stirring for 48 hours. After the removal of insoluble materials by filtration with Whatman filter paper the filtrates were centrifuged and the supernatants were collected. The supernatants were evaporated to dryness in an oven under the temperature of 50^0-55^0C. The crude extract obtained was dissolved in phosphate buffer saline (pH 7.4). Stock solutions were used to prepare the requisite dilutions. The methanol extract of propolis has been abbreviated as MeOH-propolis in the text, tables, and figures.

Propolis treatment schedule

Tumor-transplanted mice were randomly divided into three groups consisting of seven mice in each group. Group-I mice serving as tumor-bearing control received normal saline only. Group-II mice were administered with the reference drug, cisplatin (i.p., 2 mg/kg body weight/ day) for five consecutive days starting from 6^{th} day post-tumor transplantation. Group-III mice were administered with methanol extract of propolis (MeOH-propolis) (i.p., 50 mg/kg body weight) for 5 consecutive days starting from the 6^{th} day post-tumor transplantation. The dose and schedule of cisplatin and MeOH-propolis treatment of tumor-bearing mice were selected after Verma and Prasad (2013) and Turnia *et al.* (2015). After 24, 48 and 96 h of treatment i.e., on the 11^{th}, 12^{th} and 14^{th} day post tumor transplantation, the ascites DL were collected and used for the biochemical and ultrastructural study.

Reduced glutathione (GSH) estimation

Total reduced glutathione (GSH) in DL cells was determined using the method of Sedlak and Lindsay (1968). Ascites tumor was collected from the mice in different groups at 24, 48 and 96 h of treatment and centrifuged. The 5% tissue homogenates of DL cells pellets were prepared in 0.02 M EDTA (pH 4.7). Total GSH was determined by adding the tissue homogenate or pure reduced form of glutathione (100µl) to 0.9 ml of 0.02 mol/L EDTA, pH 4.7 and 1 ml of 0.2 mol/L Tris–EDTA buffer, pH 8.2, and followed by 20µl of Ellman's reagent (10 mmol/L DTNB in methanol). After 30 min of incubation at room temperature, the reaction mixture was centrifuged at 3000g and the absorbance of the clear supernatant was read against a reagent blank at 412 nm in a Varian Carey-50 spectrophotometer.

GSH-related enzymatic study

DL cells from different treatment groups were collected and washed in ice-cold physiological saline. 10% tissue homogenate was prepared in a motor-driven Teflon-pestle homogenizer in

cold condition in the specific buffer solutions for specific enzyme assays i.e. for glutathione S-transferase (GST) assay, 0.1 mol/L sodium phosphate-1 mmol/L Na_2EDTA buffer (pH6.5) and for glutathione reductase (GR) assay, 0.2 mol/L potassium phosphate-1 mmol/L Na_2EDTA buffer (pH7.5). The tissue homogenates were centrifuged at 27000g for 20 min at 4ºC and the supernatants were used as the respective enzyme source.

Glutathione S-transferase (GST; EC 2.5.1.18) assay: Glutathione S-transferase activity was assayed following the method of Habig *et al*., (1974). The assay volume (1.0 mL) contained 850 µL of 0.1 mol/L potassium phosphate^{-1} mmol/L Na_2 EDTA buffer (pH 6.5), 50 µl of 20 mmol/L CDNB in 95% ethanol and 50 µl of 20 mmol/L reduced glutathione in deionised water. The overall reaction (maintained at 30°C) was started by adding 50 µl tissue supernatant as the enzyme source and the increase in absorbance was monitored at 340 nm for 3 min in a spectrophotometer. The enzyme activity was calculated using the extinction coefficient (E_{340} = 9.6 L mmol^{-1} cm^{-1}). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conjugation of 1 ìmole of CDNB per min.

Glutathione reductase (GR; EC 1.6.4.2) assay: GR activity was assayed according to Smith *et al*., (1988). Total assay volume (1.05ml) contained 500µl of 0.2M potassium phosphate-1mM Na_2EDTA buffer (pH 7.5), 250 µl of 3 mmol/ L DTNB in 0.01M phosphate buffer, 175 µl water, 50µl of 2mM NADPH in 10mM Tris-HCl buffer (pH 7.0) and 50µl of GSSH (maintained at 24^{0}C). The reaction was initiated by the addition of 25µl of the supernatant as enzyme source to the reaction mixture and the decrease in the absorbance at 340nm was monitored at 37^{0}C for about 3 min. The enzyme activity was calculated using the extinction coefficient (E_{340} = 6.22mM^{-1}cm^{-1}). One unit of glutathione reductase activity is defined as the amount of enzymes that catalyze the reduction of 1µmol of NADPH per min. Specific activity was expressed as units per mg of protein.

Lipid peroxidation (LPO) determination

LPO was determined in the form of malondialdehyde concentration in DL cells following the method of Buege and Aust (1978). Ascites tumor was collected from the mice in different groups and centrifuged. Briefly, 5% homogenate of DL cells pellet was prepared in 0.15 M KCl. To 1 ml of the homogenate, 2 ml of TCA-TBA-HCl reagent (15% trichloroacetic acid and 0.375% thiobarbituric acid dissolved in 0.25 N HCl) was added and mixed thoroughly. The sample was heated in a boiling water bath for 15 min and then cooled at room temperature. The precipitate was removed by centrifugation at 1000g for 10 min. The absorbance of the clear supernatant was read at 535 nm. The malondialdehyde (MDA) concentration of the tissue sample was calculated using an extinction coefficient of 1.56 x 105 M^{-1}cm^{-1} and expressed as nmol of MDA/mg protein.

Sialic acid estimation

The ascites Dalton's lymphoma collected from mice in different experimental groups were centrifuged (1000g, 10 min, 4ºC) to obtain tumor cell pellets. The sialic acid concentration was determined using the method of Warren (1959). The 5% homogenate of tumor cell pellets were prepared in 0.1 N H_2SO_4 and incubated it for 1 h at 80ºC with intermittent shaking in a water bath. The homogenates were then centrifuged at 2000g for 15 min and the clear supernatant was used for sialic acid estimation.

To 0.2 ml of the sample supernatant, 0.1 ml of periodate solution (sodium periodate, 0.2M in 9M phosphoric acid) was added. This was followed by the addition of 1 ml of arsenite solution (sodium arsenite, 10% in a solution of 0.5M sodium sulfate in 0.1N H_2SO_4) and 3 ml of thiobarbituric acid, and kept in a boiling water bath for 15 min. After cooling, the chromophore was extracted in an equal volume of cyclohexanone by shaking and centrifuged at 2000g for 10 min. The clear upper cyclohexanone phase containing the chromophore was removed and the optical density was recorded at 532 nm and 549 nm against the blank. The sialic acid concentrations were determined from the extinction coefficient using the formula:

$$\text{Sialic acid (µmol)} = 0.090 \times O.D_{549} - 0.033 \times O.D_{532}$$

Protein estimation

Protein estimation in DL cells was determined following the method of Bradford (1976).

Transmission electron microscopy (TEM)

Dalton's lymphoma ascites collected from the mice under different experimental conditions were centrifuged (1000g, 10 min at 4º C) and the cell pellets were processed for TEM as described earlier by Martha *et al.*, (2013).

Statistical analysis

The results were expressed as mean± S.D. The data were statistically analyzed using one-way analysis of variance (ANOVA) to compare the level of significance between control and treated groups. *P*-value <0.05 was considered statistically significant in all cases.

Results

Reduced glutathione (GSH) content

MeOH-propolis treatment of tumor-bearing mice caused a significant decrease in GSH levels in DL cells. In the case of reference drug cisplatin treatment also, a similar trend of a decrease in GSH level was observed (Fig. 2).

Glutathione S-transferase (GST; EC 2.5.1.18) and glutathione reductase (GR; EC 1.6.4.2) activity

As compared to the corresponding control, MeOH-propolis, as well as cisplatin treatment, caused an overall decrease in GST and GR activity in DL cells (Table 1).

Lipid peroxidation (LPO)

Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells and LPO was measured in terms of MDA concentration in DL cells from mice under different treatment conditions. As compared to the corresponding control, MeOH-propolis, as well as cisplatin treatment, caused an overall increase in LPO in DL cells (Fig. 3).

Sialic acid content

As compared to the control, MeOH-propolis treatment of tumor-bearing mice resulted in an overall decrease in the sialic acid content in DL cells. In the case of the reference drug, cisplatin also a decrease in sialic acid content in DL cells was noted after treatment (Fig. 4).

Ultrastructure of DL cells

Control DL cells showed normal organelles' structural features with small processes uniformly distributed over the cells. MeOH-propolis treatment developed deformities in mitochondria with more pronounced vacuoles. There was the appearance of fragmented nuclei and thickened mitochondrial irregular cristae (Fig. 5). Cisplatin treatment also caused the appearance of small fine microvilli like processes and disruptions in the mitochondrial membrane and cristae (Fig. 5)

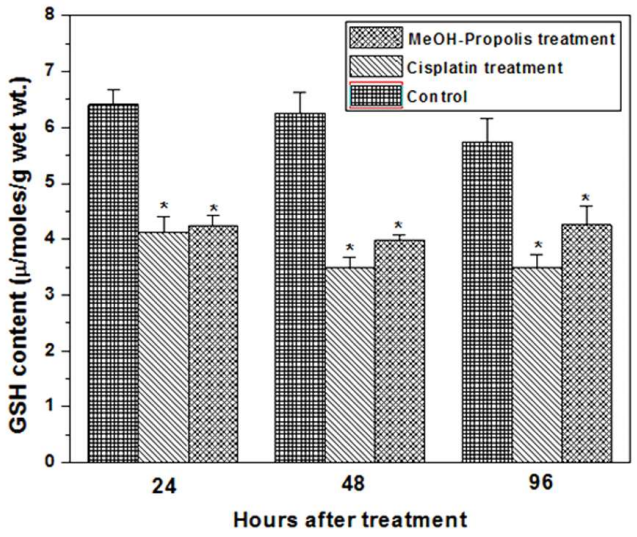

Fig. 2. Changes in total GSH content in Dalton's lymphoma (DL) cells from tumor-bearing mice under different treatment conditions. Control= DL cells from untreated tumor-bearing hosts receiving extract vehicle alone. The results are expressed as mean ± S.D., n=4. The significance of the changes between control and the treated group was tested by ANOVA, **p* d" 0.05.

Table. 1. Glutathione s-transferase (GST) and glutathione reductase (GR) activity (µmoles/min/mg protein) in Dalton's Lymphoma (DL) cells under different treatment conditions

Treatment groups	GST	GR
Control 24 h	0.303 ± 0.061	0.216 ± 0.014
48 h	0.313 ± 0.017	0.191 ± 0.010
96 h	0.360 ± 0.034	0.188 ± 0.022
MeOH-Propolis 24 h	0.242 ± 0.002*	0.169 ± 0.014*
48 h	0.273 ± 0.011*	0.152 ± 0.021*
96 h	0.246 ± 0.013*	0.131 ± 0.021*
Cisplatin 24 h	0.253± 0.008*	0.172 ± 0.012*
48 h	0.242± 0.004*	0.146 ± 0.022*
96 h	0.259± 0.016*	0.128 ± 0.023*

Control= DL cells from untreated tumor-bearing hosts receiving extract vehicle alone; DL= Dalton's Lymphoma. The results are expressed as mean ± S.D., n=4. The significance of the changes between control and the respective treated group was tested by ANOVA, **p* ≤0.05.

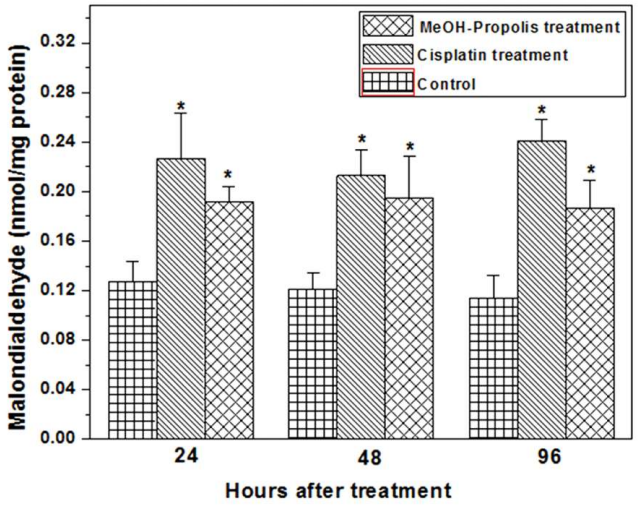

Fig. 3. Changes in lipid peroxidation (LPO) in the form of malondialdehyde concentration (nmol/mg protein) in Dalton's lymphoma (DL) cells of tumor-bearing mice under different treatment conditions. Control= DL cells from untreated tumor-bearing hosts receiving extract vehicle alone. The methanol extract of propolis (MeOH-propolis) and cisplatin treatment caused a significant increase in LPO in DL cells. The results are expressed as mean ± S.D., n=4. The significance of the changes between control and the treated group was tested by ANOVA, $^{*}p \leq 0.05$.

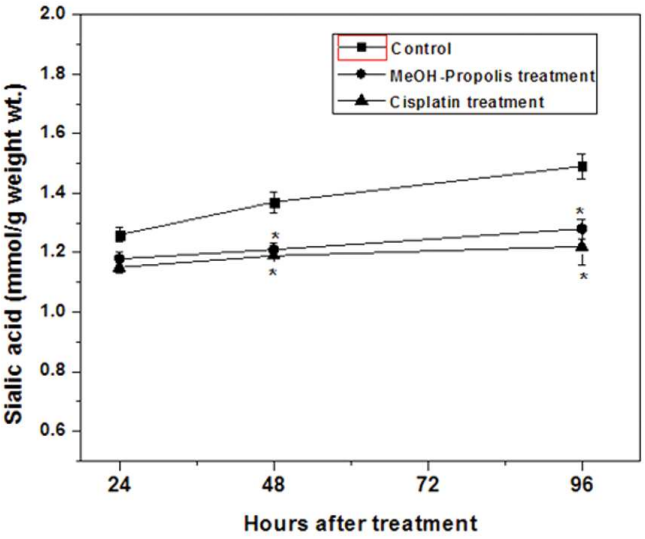

Fig. 4. Changes in total sialic acid content in Dalton's lymphoma (DL) cells of tumor-bearing mice under different treatment conditions. Control= DL cells from untreated tumor-bearing hosts receiving extract vehicle alone. The methanol extract of propolis (MeOH-propolis) and cisplatin treatment caused a significant decrease in sialic acid in DL cells. The results are expressed as mean ± S.D., n=4. The significance of the changes between control and the treated group was tested by ANOVA, $^{*}p \leq 0.05$.

Discussion

Natural products have played a vital role in treating and preventing human diseases including cancer. Propolis is one such natural product that has attracted researchers' interest because of its broad spectrum of biological activities (Burdock, 1998; Choudhari *et al.*, 2013; Ahangari *et al.*, 2018; Anjum *et al.*, 2018). The chemical composition and biological properties of propolis can vary depending on the bee species, seasonality, vegetation, and flowers from which the bees collect the exudates (Ahangari *et al.*, 2018). In present studies, it was noted that the Indian hive-bee, *Apis cerana indica* (Apidae) is the common honey bee species reared by the people in Ngunraw village, South West Khasi Hills district, Meghalaya, India (Fig. 1). MeOH-propolis has earlier been reported to induce apoptosis in murine Dalton's lymphoma cells (Turnia *et al.*, 2015). Other studies have shown that ethanolic extract of propolis augmented TRAIL-induced apoptotic death in prostate cancer cells (Szliszka *et al.*, 2011). Various studies have demonstrated anticancer properties of propolis and its phenolic components by different mechanisms such as cell cycle arrest, induction of apoptosis (Sawichka *et al.*, 2012; Choudhari *et al.*, 2013), inhibition of cancer cell proliferation and tumor growth (Szliszka *et al.*, 2011; Turnia and Prasad, 2015).

Reduced glutathione (GSH, γ-l-glutamyl-L-cysteinyl-glycine) is the most abundant antioxidant in the cells and plays an important role in a multitude of cellular processes such as cell differentiation, proliferation, and apoptosis, and disturbances in GSH homeostasis are involved in the etiology and progression of many diseases including cancer (Traverso *et al.*, 2013). The modulation of the GSH-based antioxidant redox system has been suggested as a promising therapeutic strategy for overcoming cancer cell progression and drug resistance. The observed significant decrease in GSH levels (Fig. 2), as well as GST and GR activities in DL cells after MeOH-propolis treatment (Table 1), may reflect alterations in the antioxidant ability accompanied with decreased proliferation/survival of DL cells. The reference drug, cisplatin treatment also showed a decrease in GSH level in DL cells (Fig. 2). A direct correlation between GSH levels and cellular

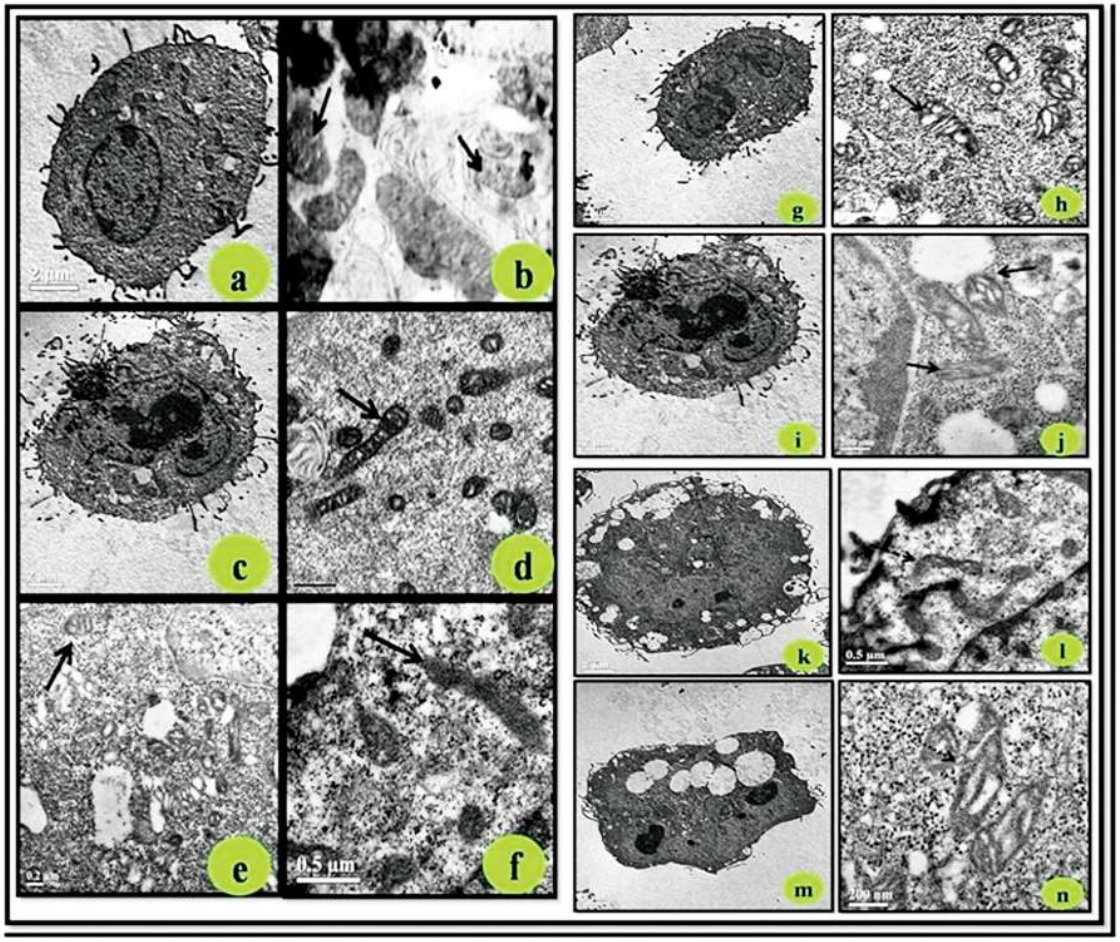

Fig. 5. Electron micrographs of Dalton's lymphoma (DL) cells from tumor-bearing mice under different treatment conditions. Control= DL cells from untreated tumor-bearing hosts receiving extract vehicle alone. Control DL cells (a), with distinct mitochondria cristae (b). DL cells showing nuclear fragmentation (c) and mitochondrial deformities in the arrangement of mitochondrial cristae (d), after cisplatin treatment for 24 hrs. Further deformities in mitochondria with thickened cristae (e, f), were observed after cisplatin treatment for 96 hrs. MeOH-propolis treatment for 24-96 h mice showing changes in DL cells (g, i, k and m) and mitochondrial changes at the corresponding time point (h, j, l and n). The MeOH-propolis treatment developed pronounced vacuoles, thickening, reduction and disruption/swelling in the mitochondrial cristae.

proliferation, cancer metastatic activity as well as a decrease in the rate of cancer cells proliferation with a decrease in GSH level in cancer cells has been reported (Estrela *et al*., 1995; Carretero *et al*., 1999; Khynriam and Prasad, 2003). GSH plays a vital role in cell proliferation and apoptosis, and disturbances in GSH homeostasis are involved in the etiology and progression of cancer (Traverso *et al*., 2013). Cancer cell lines containing low GSH levels have been demonstrated to be much more sensitive than control cells to the effect of irradiation (Meister, 1991). Glutathione S-transferases (GSTs) function as detoxification enzymes that catalyze the conjugation of a wide variety of endogenous and exogenous electrophilic compounds including chemotherapeutic agents with GSH thereby decreasing their reactivity with cellular macromolecules (Eaton and Bammler, 1999). GST is one of the several factors that are proposed to affect tumor sensitivity to anticancer drugs and cancer cells can acquire resistance by overexpressing GSTs that may increase detoxification and circumvent the cytotoxic action of antitumor drugs (Kodera *et al*., 1994; Traverso *et al*., 2013). An elevated expression of GSTs, combined with high GSH levels, can increase the rate of conjugation and detoxification of chemotherapy agents, thus reducing their effectiveness (McLellan and Wolf 1999). The decrease in GST activity in DL cells after MeOH-propolis as

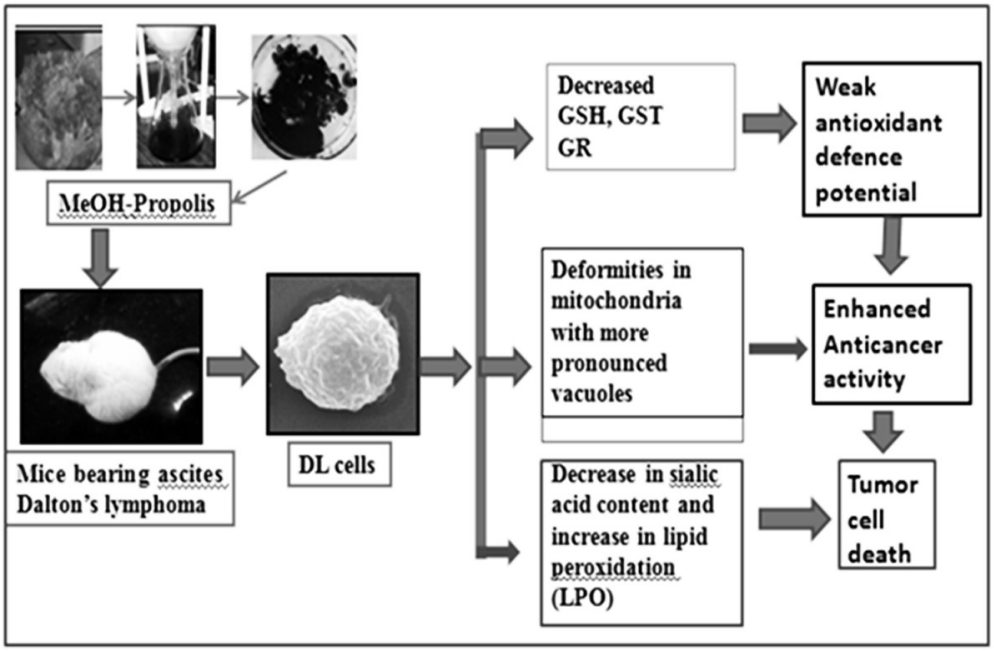

Fig. 6. Summary of MeOH-propolis–mediated various changes leading to cytotoxicity in DL cells.

well as cisplatin treatment may involve a lesser conjugation of GSH with different electrophilic agents in DL cells, thus, increasing their tumoricidal activity. Glutathione reductase (GR) catalyzes the NADPH-dependent reduction of glutathione disulfide (GSSG) to GSH and cellular GSH : GSSG ratio is maintained. It has been previously reported that in other cases also such as the methanol extract of the stem bark of *Dillenia pentagyna* (Rosangkima*et al.*, 2008) and cantharidin (Verma and Prasad, 2013) caused a significant decrease in GSH level and GR activity in DL cells as a mode of its antitumor activity. MeOH-propolis or cisplatin-mediated decrease in GR activity in DL cells may hamper its ability to convert GSSG to GSH and result in a decrease in cellular GSH concentration. Thus, the decrease in GSH level could involve/relate with a decrease in GST and GR activities in DL cells by MeOH-propolis and it could be a noteworthy step in its anticancer activity.

The oxidation products of ROS in biological samples are of great interest because they are more stable and reflect the magnitude of oxidative stress (Erejuwa*et al.*, 2013). One of such oxidation products is lipid peroxidation (LPO) products which are formed when ROS attack polyunsaturated fatty acids (PUFAs) leading to membrane structural and/or functional damage. Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. MeOH-propolis or cisplatin-mediated increase in lipid peroxidation in DL cells (Fig. 3) may facilitate the cellular membrane damages in DL cells. Lipid peroxidation products have been suggested to serve as a therapeutic tool to induce death of proliferating cancer cells (Erejuwa *et al.*, 2013). The observed decrease in GSH level could be coupled with an increase in LPO in DL cells after MeOH-propolis treatment.

The term sialic acid is used to describe derivatives of neuraminic acid, where the amino group of neuraminic acid is substituted by either an acetyl or glycolyl group. Changes in glycosylation involving the upregulation of sialyltransferases and alteration of terminal sialic acid residues are a hallmark of cancer (Pearce and Läubli, 2016). The high sensitivity of sialic acid as a tumor marker has been reported in a variety of cancerous conditions (Narayanan,1994; Achalli *et al.*, 2017) and increased levels of sialic acid on the cell surface and in serum in different cancers (Prasad, 1986; Dadhich*et al.*, 2014; Zhang *et al.*, 2019). Therefore, in view of the importance of sialic acids in the manifestation of the biological properties of

malignant cells, the elucidation of sialic acid changes in DL cells after MeOH-propolis treatment could be very helpful to understand its antitumor activity. MeOH-propolis treatment of tumor-bearing mice resulted in an overall decrease in the sialic acid content in DL cells (Fig. 4). The anticancer drugs cyclophosphamide and cisplatin have also been reported to decrease the sialic acid content in fibrosarcoma (Sarna *et al*., 1988) and DL cells (Nicol and Prasad, 2002). Sialic acid bears a negative charge owing to a carboxyl group and plays a role in cellular functions, such as transport of positively charged compounds, cell-to-cell repulsion on cell membranes and masking cell membrane antigenic determinants (Narayanan, 1994; Achalli *et al*., 2017). It has been suggested that sialic acid may control/mask the expression of surface antigens and sialic acid release may cause the possible exposure of certain antigenic sites to increase the immunogenicity of tumor cells (Sarna *et al*., 1988). Therefore, it is suggested that here also MeOH-propolis mediated decrease in sialic acid in DL cells may expose antigenic sites on DL cells and should be an important step in its anticancer activity.

Transmission electron microscopy of DL cells showed that MeOH-propolis or cisplatin treatment developed chromatin condensation, and nuclear fragmentation., irregularities in cristae and disruptions in the mitochondrial membrane (Fig. 5). The anticancer drugs have been reported to depolarize mitochondrial membrane potential (MMP) and increase the reactive oxygen species (ROS) in mitochondria in cancer cells as one of the imperative steps in the anticancer activity (Costantini *et al*. 2000; Verma and Prasad, 2013; Peng *et al*., 2017). Intracellularly, the majority of GSH is found in the cytosol (about 90%), while mitochondria contain nearly 10% and the endoplasmic reticulum contains a very small percentage (Lu, 2009). The MeOH-propolis or cisplatin treatment caused a decrease in total GSH level in DL cells (Fig. 2) which should also include a decrease in mitochondrial GSH. An increase in ROS has also been correlated with increased LPO in cells (Ayala et al. 2014). A decrease in GSH level in DL cells should be involved with increased production of ROS in mitochondria developing mitochondrial dysfunction and increased LPO in Dalton's lymphoma (DL) cells which together should be involved in its anticancer activity.

Conclusion

The antitumor activity of the methanolic extract of propolis (MeOH-propolis) against murine ascites Dalton's lymphoma involves different biochemical changes such as decrease in glutathione (GSH) level, glutathione S-transferase (GST), glutathione reductase (GR) activities, sialic acid content and increase in lipid peroxidation (LPO) in tumor Dalton's lymphoma cells. MeOH-propolis-mediated development of chromatin condensation, nuclear fragmentation, and irregularities in mitochondrial cristae, etc in tumor cells was also observed which together with the decrease in reduced glutathione, sialic acid and increase in lipid peroxidation should assist in its anticancer activity as summarized in Fig. 6. However, molecular exploration may assist to understand more insight into the MeOH-propolis-mediated biochemical changes and anticancer activity.

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