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Assessment of cytotoxic and apoptotic activities of Assam propolis on Dalton's lymphoma cell line

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Abstract
Propolis, a resinous substance collected by honeybees, has gained significant attention for its wide range of biological activities. These include antimicrobial, antioxidant, anti-inflammatory, immune-modulatory, neuroprotective, and anticancer properties. In this study, we investigated the cytotoxic effects of propolis collected from Assam, India, on Dalton's lymphoma (DL) cells over a 24-hour period. The findings revealed that Assam propolis exhibits a dose-dependent cytotoxic effect on DL cells. As the concentration of propolis increased, the cytotoxicity towards the DL cells also escalated. The half-maximal inhibitory concentration (IC_{50}) value, which indicates the concentration required to inhibit 50% of the cell population, was calculated to be 8.8±0.01 mg/ml. This suggests that Assam propolis has a potent cytotoxic effect on DL cells at relatively low concentrations, making it a promising candidate for further exploration as an anticancer agent. Given its range of biological activities, including its newly confirmed cytotoxic potential against DL cells, Assam propolis could serve as a valuable natural product for developing cancer therapies. Further studies may be undertaken to investigate its mechanism of action and its potential synergistic effects with other anticancer agents.

Keywords: Cancer, cell death, MTT assay, trypan blue, tumor

Introduction
Propolis is a natural and sticky material, also known as bee glue, that honey bees (*Apis mellifera*) produce from saps, resins, and mucilages collected from various parts of the plant, such as leaves, flower buds, and tree barks, then mixing them with beeswax and several bee enzymes. The word propolis originates from ancient Greek, in which "pro" stands for "at the entrance to" and "polis" for "community" or "city", indicating that this natural product is used in hive protection and defense. (Forma & Brys, 2021) [9]. Propolis has garnered attention for its diverse biological activities, including antimicrobial, antioxidant, anti-inflammatory, immune-modulatory, neuro-protective, and anti-cancer properties (Bhatti *et.al*, 2024; Lesmana *et.al*, 2024) [4, 14]. It is synthesized by honey bees from products collected in the buds, saps and resins, mucilage, lattices and other plant sources.(Forma & Brys, 2021; Zabaiou *et al*., 2017) [9, 28]. The collections are mixed with bee wax generated from bee metabolism. Additionally, propolis has been documented to seal crevices and open spaces within the beehive structure. Propolis samples collected from diverse sources showcase distinct physical properties (Zabaiou *et al*., 2017) [28] Propolis is ambiguous in terms of source and derived from plants and animals (Iqbal *et al*., 2019) [11]. Based on the plant source and location, propolis has been classified into seven different types. They are – Poplar, Birch, Mediterranean, Greek, Red, Clusian and Pacific (Forma & Bryś, 2021) [9].
Propolis exhibits significant anti-cancerous properties due to its rich composition of bioactive compounds. These compounds include polyphenols, flavonoids, and other phytochemicals that contribute to its therapeutic effects (Zullkiflee *et.al*, 2022; Orsolic *et.al*, 2022) [30]. Propolis has been shown to inhibit cancer progression by targeting multiple signaling pathways, such as phosphoinositide 3-kinases (PI3K)/Akt and mitogen-activated protein kinase (MAPK), which are crucial for cell survival and proliferation (Elumalai *et.al*, 2022) [8].
The anti-cancer potential of propolis is largely attributed to its ability to induce apoptosis, a form of programmed cell death, in cancer cells. This is achieved through both extrinsic and intrinsic apoptotic pathways, involving the activation of proapoptotic proteins like Bax and the inhibition of antiapoptotic proteins such as Bcl-2 (Masoud *et.al*, 2022) [16]. Furthermore, specific compounds in propolis, such as caffeic acid phenethyl ester (CAPE), chrysin, and artepillin C, have demonstrated the ability to suppress tumor growth and angiogenesis, which is the formation of new blood vessels that supply nutrients to tumors. These compounds also enhance the oxidative stress in cancer cells, leading to their death while sparing normal cells (Elumalai *et.al*, 2022; Zhang *et.al*, 2014) [8, 29].
Overall, propolis and its constituents offer a promising natural alternative for cancer treatment, with the potential to be developed into target-specific, cost-effective anticancer drugs for various cancer cell lines with minimal side effects. Our current study explores the cytotoxic potential of propolis collected from the state of Assam, India on Dalton's Lymphoma (DL) cells.

Materials and methods
1. Site of collection
In January 2021, *Apis indica* propolis was obtained from beekeepers in the village of Rani-Garbhanga Reserve Forest situated near the Ahomgaon (Betkuchi) region of the Kamrup Metropolitan district in Assam.

2. Preparation of crude extract
Methanolic crude extract of propolis was prepared by dissolving 100 grams of dried propolis in 500 ml of 70% methanol. The mixture was stirred moderately at room temperature overnight. The propolis was filtered three times, with each filtration followed by 24 hours of incubation at room temperature. The combined filtrate was concentrated under reduced pressure using a rotary evaporator, forming the crude extract. The final extract was stored in a dry, cool place for future use.

3. Animal maintainance

Inbred Swiss albino mice were maintained in the lab under standard conditions. They were kept in well-ventilated polypropylene cages at a temperature of 20 ± 2°C, with free access to food pellets (Amrut Laboratory, New Delhi) and water. Both male and female mice, aged 10-12 weeks and weighing 25-30 grams, were selected for the experiments. The study was conducted in compliance with approval from the Animal House Facility of Cotton University, Guwahati, Assam (CU/TB/2018), adhering strictly to CPCSEA guidelines. Animals were routinely monitored for any signs of infection. Dalton's Lymphoma ascites was induced intraperitoneally, with each transplant containing $1x10^6$ cells (0.25 ml in 1X phosphate buffer saline, pH 7.8). Tumor development was observed within 4-6 days post-transplant, with each DL-induced mouse having an approximate life span of 20 ± 3 days within 4-6 days post-transplant, with each DL-induced mouse having an approximate life span of 20 ± 3 days.

4. Cell culture

Dalton's Lymphoma (DL) cell line was obtained from NEHU, Shillong. Cells were cultured in RPMI-1640 medium containing 10% FBS (Hyclone, Logan, UT), 2 mM glutamine, and penicillin-streptomycin antibiotic (Mediatech, Herndon, VA). The cell culture was incubated in a CO2 incubator having 37°C temperature, and 5% CO2 until the cells reached a confluency of 80%. The cells, thereafter was subjected to sub-culturing for further experiments.

5. Cell viability study using trypan blue exclusion assay

The cytotoxic effects of the crude propolis extract on DL cells were evaluated using Trypan Blue staining at varying concentrations. The assay is based on the principle that the dye cannot pass through the membrane of viable cells (Strober *et.al*, 2015) [25]. DL cell suspensions were treated with different doses of the extract (2 mg/ml, 5 mg/ml, 10 mg/ml, and 15 mg/ml) for 24 hours, and cytotoxicity was assessed across different doses. In brief, 50 µL of DL cells were placed in a Neubauer chamber and mixed with an equal volume of Trypan Blue solution (0.4% in PBS). The cells were observed under a microscope at 40X magnification, while viable cells were counted at 10X magnification using ImageJ software. Cisplatin was used as a reference drug, and cells treated with sterile 1X PBS served as the control. Non-viable cells appeared blue, whereas viable cells remained unstained.

The percentage viability of the cells was calculated as follows:

Percentage Viability = 1-(No. of non-viable cells/ Total number of cells) X 100

6. MTT assay

The viability of cells can be assessed with the help of MTT assay. The assay relies on the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide), a yellow water-soluble tetrazolium dye, primarily by the mitochondrial dehydrogenases, to purple colored formazan crystals (Patravale *et.al*, 2012) [19]. Viable cells are able to metabolize MTT formazan crystal whereas non-viable cells are incapable of this conversion by the action of mitochondrial reductase (Kumar *et.al* 2018; Patravale *et.al*, 2012) [12, 19]. The viability of the cells can be directly co-related to the optical density (OD) of the formazan crystal. In brief, DL cells were seeded in 24-well plates with RPMI-1640, 10%FBS and anti-biotic solution (5000 units/ml each of Penicillin and Streptomycin). The cells were treated with different doses and incubated for 24 hours in a CO2 incubator, 37°C and 5%CO2. Cisplatin was used as reference drug. After the completion of 24 hours, cells were treated with 20µL MTT (5mg/ml) with further incubation for 2 hours. The dye was removed and 100 µL DMSO was added for 10 mins at 37°C to dissolve the formazan crystals. The cells were transferred into a 96 well microtiter plate and absorbance was taken in a microplate reader at 570nm. The IC_{50} obtained was selected as dose for apoptosis assay of DL cells.

7. Study of cell apoptosis using acridine orange(ao)/ethidium bromide dual staining method

Cancer cells possess the ability to evade apoptosis by upregulation of anti-apoptotic proteins which ensures a continuous proliferation of the cells (Pfeffer *et.al* 2018) [20]. Acridine Orange/Ethidium Bromide apoptotic assay is a simple yet efficient assay which allows simultaneous assessment of apoptotic index and membrane integrity. Acridine Orange permeates all cells and stains the cells green. Ethidium Bromide only enters the cells with a compromised membrane integrity and intercalates with its DNA, and stains the nucleus red. (Ribble *et.al* 2005, Renvoizé *et.al* 1998) [22, 23]. Briefly, 50µl DL cells were treated with propolis crude extract with the The apoptotic activity was monitored for 24. The cells were incubated at 37°C, with 5% CO. Cisplatin was used as positive control and PBS was used as vehicle. The cells were treated with 10µl AO/EtBr (100µg/ml in PBS). The cells were later observed by fluorescent microscope using blue filter. The total percentage of apoptotic cells were determined by calculating the apoptotic index apoptotic index as was done by Prasad and Koch (2014) [21].

Results

1. Cell viability study using trypan blue exclusion assay

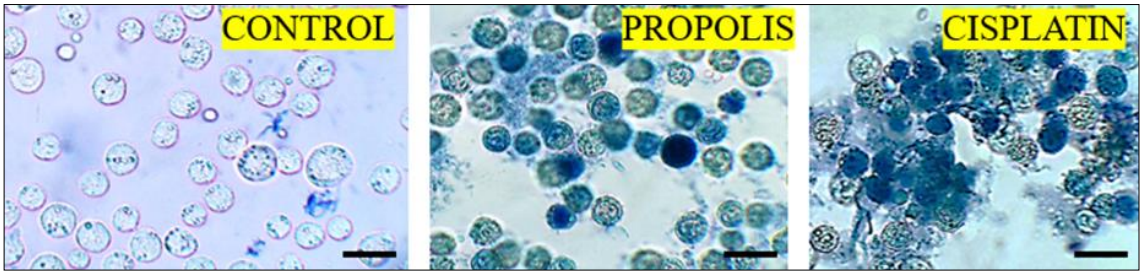

Fig 1: Trypan blue assay on DL shows distinct staining patterns. The intensity of stain of Trypan blue dye increases with increase of DL cell cytotoxicity. Scale bar 20 µm.

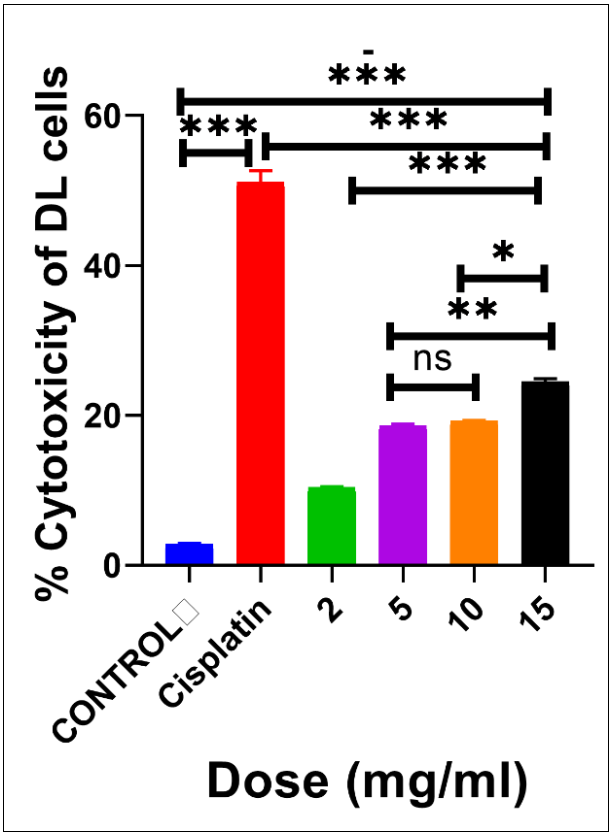

Fig 2: One way ANOVA in the 24-hour Trypan Blue assay across doses (2,5,10,15) mg/ml F (5,12) = 614.9, $p \leq 0.0001$, followed by post-hoc Bonferroni test; n=5; p value ≤0.001. Significant differences represent ***p<0.0001; **p<0.001; *p<0.01; ns p>0.01

One way ANOVA in the 24-hour Trypan Blue assay found that highly significant differences in doses F (5,12) = 614.9,($p \leq 0.0001$) was observed between control and all treatment groups (2,5,10,15) mg/ml and positive control cisplatin. This shows that propolis exhibits dose dependent cytotoxicity. The control group showed significant difference ($p < 0.0001$) in DL cell cytotoxicity as compared to all doses (2-15) mg/ml. Cisplatin showed significantly higher cytotoxicity ($p < 0.0001$) as compared to propolis across all doses. Propolis showed highest cytotoxicity was observed in 15 mg/ml at 24.53±0.371. (Mean ± SEM). This was significantly lower ($p < 0.0001$) than the cytotoxicity of cisplatin which was observed to be 51.1± 1.557 (Mean ± SEM). The changes in the staining intensity of the DL is illustrated in Fig 1.

2. MTT assay

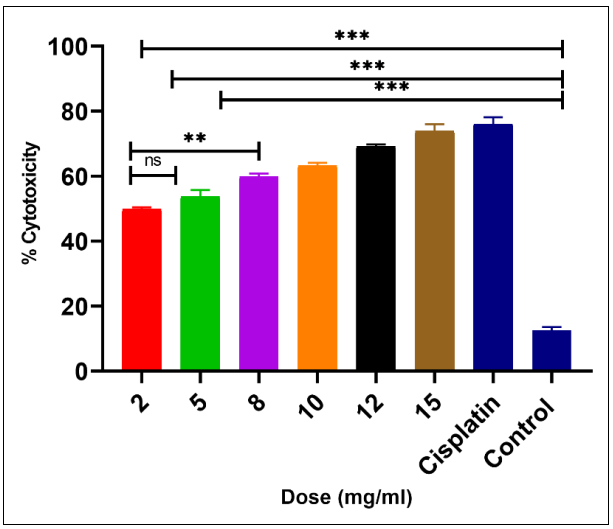

Fig 3: One way ANOVA in the 24-hour Trypan Blue assay across doses (2,5,8,10,12,15) mg/ml F (5,12) = 614.9, ($p \leq 0.0001$), followed by post-hoc Bonferroni test; n=5; p value ≤0.001. Significant differences represent ***p<0.0001; **p<0.001; *p<0.01; ns p>0.01

A one-way ANOVA of the 24-hour MTT assay revealed highly significant differences in cytotoxicity across doses F(7, 23) = 222.7, ($p \leq 0.0001$) between the control and all treatment groups (2, 5, 10, and 15 mg/ml) as well as the positive control, cisplatin. Based on absorbance values, the percentage of cytotoxicity was calculated, showing that propolis induces dose-dependent cytotoxicity. The control group exhibited a significant difference in DL cell cytotoxicity ($p < 0.0001$) compared to all propolis doses (2–15 mg/ml). While cisplatin demonstrated significantly higher cytotoxicity ($p < 0.0001$) than propolis at all tested doses, the highest cytotoxicity for propolis was recorded at 15 mg/ml, with a value of 73.90 ± 2.098 (Mean ± SEM). However, this was still significantly lower ($p < 0.0001$) than cisplatin's cytotoxicity, which was 75.99 ± 2.135 (Mean ± SEM). The IC_{50} obtained was 8.8±0.01 mg/ml

3. Study of cell apoptosis using acridine orange(ao)/ethidium bromide dual staining method

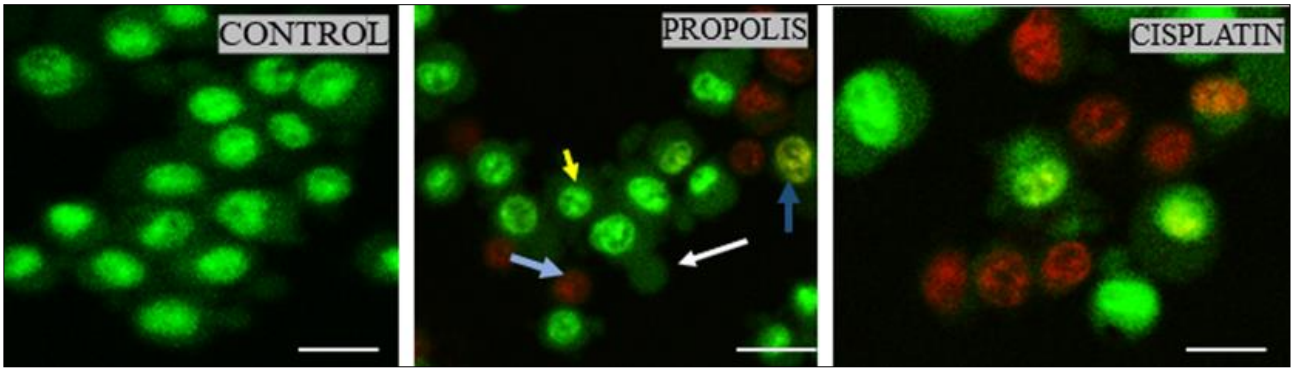

Fig 4: Study of the apoptotic features of DL cells in three different groups namely – control, propolis and cisplatin. Distinct apoptotic features like membrane blebbing (white arrow) and chromosome condensation (yellow arrow) were seen. The study also helped to differentiate between early and late apoptotic cells (dark blue and light blue arrows respectively). Scale bar 20 µm.

Study of apoptosis assay revealed that propolis induced distinct apoptotic features in DL cells. This is evident from the apparent morphology of cells showing features like membrane blebbing and chromosome condesnation. The intensitiy of Ethdium bromide staining was highest in cisplatin which had the highest % apoptotic cells. Propolis had a significantly lower % apoptotic cell count of 8.50 ± 1.55 (Mean ± SEM) which was lower than cisplatin which had %apoptotic cells of 50.86 ±2.262.

Discussion

Propolis is a resinous substance which has a wide array of biological activity. The biological properties of propolis is governed by the site and season of collection due to the variation in the surrounding flora (Wozniak *et.al* 2019; Valencia *et.al* 2012) [26]. This makes propolis an interesting candidate as a source of drug for different disease. Propolis collected from different parts of world have shown anti-cancerous, anti-oxidant and a wide array of medicinal values. Our current study was intended to explore its cytotoxic effects on Dalton's Lymphoma (DL). cells. Since the North-East of India is a biodiversity hotspot (Saikia and Kalita *et.al*, 2009 [24].

In-vitro cytotoxic cell study was also conducted to assess the cytotoxic potential of methanolic extract of Assam propolis. Cisplatin was taken as reference drug. Cell viability assays like Trypan blue exclusion dye assay and MTT were conducted and it revealed dose dependent cytotoxicity with the greatest cytotoxicity occurring at 15mg/ml in a 24 hour study. Similar cytotoxicity was seen in methanolic extract of Brazilian propolis in pancreatic cell line PANC-1 (Awale *et.al*, 2008) [2]. Cell viability study of propolis collected from Maharashtra showed far greater potency. MTT study showed IC50=250µg/ml for 24 hours. Ethanolic extract of propolis collected from Iran extremely potent cytotoxic activity having IC50=50 µg/ml in AGS gastric cancer cell lines (Amini-Sarteshnizi *et.al*, 2015) [1]. Mendonca *et.al* revealed ethanolic extracts of propolis from Brazil showed high cytotoxicity against multiple human cell lines SF-295 (glioblastoma), OVCAR-8 (ovary) and HCT-116 (colon). Turkish propolis ethanolic extract also exhibited high cytotoxic activity against human epithelial lung cancer cell line A549 (Demir *et.al*, 2016) [6]. Moroccan propolis in ethanol and ethyl acetate showed cytotoxic effects in BSR (hamster renal adenocarcinoma), Hep-2 (human laryngeal carcinoma) and P815 (murine mastocytoma) (Mouse *et.al*, 2011) [17].

The apoptotic inducing potential was of the propolis extract was also explored using Acridine Orange/Ethidium Bromide dual staining assay. The dose taken was the IC50 of the 24-hour study of MTT assay. Cisplatin was used as reference drug. Characteristic apoptotic features like Morphological features such as chromatin condensation, membrane blebbing, and cell shrinkage (Elmore 2007) [7] were observed. These results are in agreement with the findings of Garcia *et.al* (2024) [10] where time dependent apoptotic activity was observed over a 7-day study period of red Propolis collected from Alagoas, Brazil. Turnia *et.al*, had conducted a a study of methanolic extract of North-East Indian Propolis, and had found a similar pattern. The study by Li *et.al*, (2007) [15] showed a similar decline in cell viability in prostate cancer cell lines. Kuo *et.al* (2023) [13] showed that propolis can be used in conjunction with thermal cycling hyperthermia and low intensity ultrasound in a a therapy called triple treatment on PANC-1 cells.

Conclusion

This study highlights the cytotoxic potential of propolis collected from Assam, a region known for its rich biodiversity, against Dalton's lymphoma (DL) cells. The dose-dependent cytotoxicity observed, with an IC_{50} value of 8.8±0.01 mg/ml, confirms the significant anticancer properties of Assam propolis. These findings support the therapeutic potential of propolis in cancer treatment, particularly in regions like North-East India, where unique flora contribute to the bioactive components of propolis. Given its demonstrated cytotoxic and apoptotic effects, Assam propolis emerges as a promising natural candidate for cancer therapeutics.

Future studies should focus on testing the efficacy of Assam propolis on other cancer cell lines and in *in-vivo* models to further validate its anticancer properties. Additionally, understanding its mechanism of action and exploring its possible synergistic effects with other treatments could pave the way for developing effective cancer therapies based on natural products.

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