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Goniothalamin Isolated from *Goniothalamus andersonii* Improves Hematological and Biochemical Markers in Induced leukemia BALB/c Mice

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ABSTRACT

The efficacy of goniothalamin, a styryl-lactone derivative as an alternative drug for leukemia treatment has generated interest among natural products scientists. However, the studies on goniothalamin treatment effects on serum biochemicals in relation to haematology have been lacking. This study evaluated the effect of goniothalamin (GTN) isolated from *Goniothalamus andersonii* supplemented into induced leukemia BALB/c mice model to determine the serum biochemicals and hematological markers changes post supplementation. The mice were categorized into normal mice (NM), untreated leukemia (LM) and goniothalamin-treated leukemia (GTN-LM) groups. Treated mice were supplemented with 40 mg goniothalamin per kg b.w. from day-14 and every alternate day until day-28. The present study showed that the enlarged spleen of leukemia mice was reduced toward normal dimension following GTN treatment. Besides, the serum biochemicals related to kidney and liver functions including urea (8.43 ± 0.85 mmol/L), creatinine (20.33 ± 0.67 µmol/L), total bilirubin (1.78 ± 0.17 µmol/L), alanine transaminase (ALT) (65.33 ± 23.51 U/L) and aspartate aminotransferase (AST) (245.00 ± 36.17 U/L) of the GTN-LM group were found to be significantly different than LM group. Conversely, the insignificant difference of these biomarkers between GTN-LM and NM groups had indicated the improvement of leukemia toxicity to normal condition. The GTN-LM peripheral blood containing lower immature granulocytes and monocytes as well as had higher apoptotic index (35%) as compared to LM signified that goniothalamin had induced the apoptotic cell death. Thus, this finding highlighted the potential of goniothalamin as an alternative medicine against leukemia.

***Keywords*:** Goniothalamin, *Goniothalamus andersonii*, serum biomarkers, hematological biomarkers, induced leukemia BALB/c mice

Introduction

Cancer is a significant global health issue, leading to millions of deaths worldwide annually.[1] Cancer as a multifaceted cluster of genetic diseases, is hallmarked by the uncontrolled growth and spread of abnormal cells in the body.[2] Leukemia is a malignancy starting in blood-forming tissues, including bone marrow and lymphatic system, which causes abnormal production of numbers of white blood cells. In people with leukemia, the bone marrow produces an extreme amount of abnormal white blood cells (leukocytes) involving granulocytes (neutrophils, eosinophils and basophils), monocytes and lymphocytes.[3] The current chemotherapeutic drugs against leukemia like cytarabine and methotrexate are reported to affect the spleen, liver and kidneys of the patients,[4] while acute myeloid leukemia (AML) patients treated with drugs, such as midostaurin and clofarabine were reported to exhibit adverse drug reactions (ADRs).[5]

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Thus, the efficacy of goniothalamin as an alternative drug for leukemia treatment has generated interest among natural products scientists.
Medicinal plants have long been known to improve human health.[6] *Goniothalamus andersonii* is one such medicinal plants. Several parts of *Goniothalamus* spp. such as the leaves have been used externally to allay fever[7] while the seeds have been used to treat scabies.[8] Goniothalamin (GTN) is a natural bioactive styryl-lactone, isolated from *Goniothalamus* species which are vastly distributed in Indochina, Sumatra, Peninsular Malaysia and Borneo.[9,10] This compound has shown cytotoxic effects against various cancer cell lines.[11, 12, 13, 14, 15, 16] Previous *in vivo* study showed that GTN treatment had led to cancer delay in the prostate anterior lobe.[17] This compound also reduced the oral tumor size in the treated Sprague Dawley rats and had induced apoptosis in the cancer cells as compared to the untreated animals.[18] Besides, the efficient release of *rac*-GTN from pH-responsive acetalated dextran (Ac-Dex) nanoparticles which resulted in the inhibition of prostate cancer progression in transgenic adenocarcinoma of the mouse prostate (TRAMP) model was also confirmed.[19] Moreover, GTN was found to be safe at doses up to 200 mg/kg in the acute and subacute exposure in Sprague Dawley rats models.[20] Previous study in authors' laboratory revealed that GTN was effective in the treatment of induced myelomonocytic leukemia in BALB/c with a significant at 40 mg/kg b.w. To complement these findings, the current study reported the effect of GTN treatment on biochemical parameters, morphology, peripheral blood and spleen dimension of induced BALB/c leukemia mice.

Materials and Methods

Drug and Cell Culture Condition

Goniothalamus andersonii bark was supplied by the Department of Chemistry, Universiti Kebangsaan Malaysia (UKM) and submitted to Herbarium Faculty Bioresources and Food Industry, Universiti Sultan Zainal Abidin (Voucher no: UniSZA/A/000000042). Goniothalamin (Figure 1S) extraction, purification and characterization were conducted according to the previous optimized method.[21] Goniothalamin was dissolved in DMSO (0.1% in PBS pH 7.4). The murine BALB/c myelomonocytic leukemia (WEHI-3B) cells (American Type Culture Collection, Manassas, VA) were cultivated in RPMI-1640 culture media containing L-Glutamine supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (10,000 U/mL) in a humidified atmosphere (5% CO_2 at 37 °C). After 80% confluency was reached, the cells were detached by using trypsin (1.0 mL) and centrifuged (300 ×g, 4 °C for 10 min) before the media was discarded. The cells were subcultured once every three days. The cells were diluted to a concentration of 1.5×10^5 cells/mL in 100 µL PBS before induction of leukemia into the mice.

Mice Model, Leukemia Induction and Goniothalamin Treatment

All the experimental procedures were carried out in strict accordance and approved by Universiti Sultan Zainal Abidin Animal Ethics Committee (Ethic approval no.: UAPREC/04/048). Healthy male BALB/c mice were purchased from established facility (Universiti Sains Malaysia, Kubang Kerian), housed in individually ventilated cages under standard laboratory conditions (12:12 h light: dark cycle, constant humidity 60 ± 5%, 25 ± 2°C) and fed with standard rodent food pellets and drinking water *ad libitum*.

Male mice at age eight weeks (25-30 g) were randomly grouped into normal (NM), untreated leukemia (LM) and GTN-treated leukemia (GTN-LM) groups. WEHI-3B cells (1.5×10^5cells/mL) were induced via intraperitoneal route (I.P.) injections and inoculated in mice of LM and GTN-LM groups at day-0. Goniothalamin (40 mg/kg b.w.) was administrated into GTN-LM, while the NM and LM groups were supplemented with PBS, via I.P. route from day-14 and every alternate day until day-28. At the end of the experiment, all mice were anesthetized via carbon dioxide inhalation, and the blood samples were collected via cardiac puncture into non-heparinized tubes for biochemicals and hematological studies. Then, the mice were sacrificed for organs weighing.

Mortality, Body Mass, and Organs Weights Analysis

The mortality was monitored daily until day-28 (day-0 to day-28) and reported in the form of Kaplan-Meier survival curve. The body weight was recorded weekly. The rodents were then sacrificed for organs (liver, spleen and kidneys) weighing prior to fixing in 10% buffered neutral formalin. The spleen width, length, and thickness were measured by using a Vernier calliper. The spleen ellipsoid volume was calculated according to formula.[22]

$$0.524 \times L \times W \times T$$

where;

W = Width; L = Length; T = Thickness

White Blood Cells (WBC) Hematological Study

The hematological parameters including total white blood cells (WBC), lymphocytes and neutrophils count in NM, LM and GTN-LM groups were measured. The blood was collected into 0.5 mL lithium heparin micro blood tube at day-28 via cardiac puncture by using 26-gauge (12 mm, ½ inch) needle. The peripheral blood (20 µL) smear were prepared and stained with Leishman staining method according to.[23] Briefly, a single drop of blood (20 µL) was added near one end of clean microscopic glass slide. The smear was made according to slide wedge method and allowed to dry for three min. Leishman stain was applied with a dropper to cover the well-dried smear and left for three min before fixing with methanol (1 mL). Next, the slide was rinsed with PBS (pH 6.8) and vertically air dried to obtain a purple-pinkish tinge blood smear. The slides were mounted with Richard-Allan Scientific Cytoseal 60 mounting media DPX (dibutylphthalate polystyrene xylene) and left to dry. The cells were counted under the light microscope with 400× and 1000× magnification (Leica Microsystems, Model DM750, Singapore) by using differential blood cell counter-9 windows 8 keys (M.R.C. LTD, United Kingdom).[23] The apoptotic percentage[24] and the apoptotic index[25] of WBC in peripheral blood were calculated by using the following respective formula.

$$\% \text{ Apoptotic} = \frac{\text{Number of Apoptotic Cells}}{\text{Total Cells Counted}} \times 100$$

$$\text{Apoptotic Index} = \frac{\text{Number of Apoptotic Nuclei}}{\text{Number of Observed Nuclei}} \times 100$$

Serum Biomarkers Analysis

The markers investigated were kidney function (sodium, potassium, chloride, urea, uric acid, and creatinine), heart function (creatine kinase (CK)), and liver function (total protein, albumin, globulin, albumin globulin ratio, total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT)) parameters. The blood of NM, LM and GTN-LM (n=3) were collected, left at room temperature for 3 h before centrifuged (300 ×g, 4 °C, 15 min). The serum was collected, kept in 2.0 mL micro-centrifuge tubes, sealed with parafilm, and analyzed by using Cobas 4000 series Cobs c3111Clinical Chemistry Analyser (Roche, Basel, Switzerland) performed at Gribbles Pathology Laboratory (Putrajaya, Malaysia).

Statistical Analysis

Data (mean values ± standard error means (SEM)) were calculated by using Microsoft Excel (2019). The mortality curve was determined by using XLSTAT 2014 (Addinsoft Inc., New York, United States). One-way ANOVA was performed to compare within the group and Kruskal-Wallis analysis was performed with Dunn multiple comparison tests to compare experimental groups with controls by using Statistical Analysis for Social Science (SPSS) or SPSS Statistics version 17.0. (IBM Inc., New York, United States).

Results and Discussion

Cumulative Survival, Weights and Dimension

Leukemia was assessed by weight loss and leukocyte count, the presence of malignant blasts and atypical lymphocytes in the peripheral blood. The cumulative survival pattern of mice was investigated following WEHI-3B cells inoculation at day-0 and GTN treatment at day-14. GTN-LM presented a significant prolonged survival rate at 95% as compared to LM at 90% after day-20. Upon reaching day-24, GTN-LM showed a higher survival rate at 90% as compared to LM group at only 80%. By day-28, the cumulative survival of GTN-LM was at 80% which was higher than the LM group at only 60%. Meanwhile, 100% of NM group survived until the experiment ended. The overall survival rate is illustrated in Figure 1.

From day-14 to day-28, the LM showed slight weight increment as compared to NM, while GTN-LM looked fatigue and loosed their appetite after each treatment, resulting a sequential weight loss from day-21 (Figure 2A). Following the inoculation of WEHI-3B cells, the cells travelled through the bloodstream and into organs, especially spleen. The spleen microenvironment not only helps in promoting the growth of leukemia cells but also stimulated the expression of CCR7 and at the same time amplifies the migration ability of leukemia cells.[26,27,28,29] Thus, the significantly higher spleen weight of the LM as compared to GTN-LM and NM was expected (Figure 2D). The LM spleen enlargement or splenomegaly occurred because more immature leukemia cells migrated into the spleen over time and the cells rapid growth cause the leukocytes build-up in the form of immature blasts.[30] The liver weight of LM was also found to be the highest, followed by GTN-LM and NM (Figure 2B). In addition, the splenic volume of the LM (0.25 ± 0.03 cm^3) was larger than GTN-LM (0.17 ± 0.02 cm^3) and NM (0.16 ± 0.02 cm^3). The LM had the longest length and the largest width (length: 2.13 ± 0.06 cm; width: 1.00 ± 0.05 cm), followed by GTN-LM (length: 1.89 ± 0.08 cm; width: 0.81 ± 0.07 cm) and NM (length: 1.77 ± 0.07 cm; width: 0.62 ± 0.04 cm). The spleen dimension of all animals is shown in Figure 3. The spleen enlargement or

splenomegaly in the LM group was expected because more leukemia cells migrated into the spleen over time and the immature leukemia cells rapid growth would cause the build-up of leukocytes in the form of immature blasts.[30]

White Blood Cells (WBC) Hematological Markers

Figure 4 shows the photomicrographs of peripheral blood smears of the mice model. The LM group contained abundant level of leukocytes in the form of reactive lymphocytes (blast cells) and neutrophils with single small Döhle inclusion as well as disintegrated cell bodies due to necrosis (Figure 4B). In leukemia patient, WBC formed by bone marrow are anomalous. Neoplastic cells containing large, irregular, doughnut-shaped and cuplike nuclei, as well as immature granulocytes and blast cells were detected.[32] Meanwhile, the apoptotic cells could be detected in GTN-LM cells as illustrated in Figure 4C with considerably higher apoptotic index at 35.00 ± 0.65%, as compared to LM mice as depicted in Figure 5. Apoptosis is the process of programmed cell death. During apoptosis, cell fragmentation occurs without leakage of cellular contents to the adjacent tissue and does not cause inflammatory responses.[33] The apoptotic cells have distinct morphological features, including chromatic margination, nuclear condensation, membrane blebbing, fragmentation and cell condensation of the cell with preservation of organelles.[31,32] The apoptotic index less than or equal to 10% was considered as weak.[25]

The current findings correlated with the previous studies indicating the goniothalamin promotion of apoptosis cell death of pro-monocytic, human myeloid leukemia (U937) cell lines by mitochondrial (caspase-9 and Smac) and death receptor (caspase-8) pathways.[13] The WBC hematological markers in LM was the highest and significantly different as compared to GTN-LM and NM groups as shown in Table 1. This result aligned with the previous study stating that mice with AML had a higher peripheral WBC in which more than 40% of leukocytes were in the form of immature myeloid and blasts replacing the normal components in the blood.[34] The high number of circulating monocytes signify the inflammation condition of the cells due to leukemia.[35] The monocytes content (31.86%) in control corresponded to the previous study reporting the percentage of monocytes subtype b (AML-M5b) in the range of 30% to 80% of total WBC count.[36]

Serum Biochemical Markers

The different markers exist, predominately in blood, can be used as biomarkers for the organs, including kidney and liver functions or injury.[37] The present serum biochemicals analysis revealed the biomarkers affected by both leukemia inoculation and goniothalamin supplementation (Table 2). The kidney function parameters involving creatinine and urea in LM were significantly lower than GTN-LM, while no significant difference between GTN-LM and NM were found. Lower amount of urea in LM corresponded to condition when the rodents suffered from acute myeloid leukemia in which the renal damage had caused the reduction of blood urea nitrogen (BUN) reabsorption in the renal tubules.[38,39,40]

Furthermore, heart and liver functions involving total bilirubin, ALT and AST levels of LM were significantly higher than GTN-LM and NM, while no significant difference was found between GTN-LM and NM. The increase of bilirubin, AST and ALT were known to be associated with the mice development of hepatic steatosis.[37] The elevated AST and ALT levels is the common indicator of hepatocyte injury from leukemia stream because when the hepatocytes membrane is injured, the cytosol enzymes including AST and ALT will be released into the blood.[41,42] In this study, the highest level of ALT and AST in LM in comparison to GTN-LM and NM showed that there was a possibility for liver injury to occur in LM group which corresponded with previous statements regarding the elevated AST and ALT. Besides, the LM had the lowest and significantly different total protein as compared to the GTN-LM and NM groups. This correlated with earlier research reported that leukocytes affected with leukemia would become larger, more watery and contain less apparent protein. As the leukocytes size increases, the apparent protein declines with the disease progression.[43]

Conclusion

In conclusion, the survivability for GTN-LM was higher than LM groups. GTN also helped in reducing the spleen enlargement by maintaining the length, width and the volume of the spleen. GTN was also found to induce apoptosis of the leukemia cells. Besides, the kidney function parameters involving creatinine and urea, and heart-liver functions involving total bilirubin, ALT and AST were determined to be the biomarkers affected by the treatment. This was concluded because the biomarkers levels detected in leukemia mice treated with GTN were statistically insignificant difference with normal mice, while significantly different with untreated leukemia mice. These findings signified the effectiveness of GTN in reducing splenomegaly, inhibiting the leukemia cells progression via apoptosis and the biochemical markers adaptation toward the normal condition level post treatment. Thus, this current study has highlighted goniothalamin to be a potential alternative of antileukemic agent.

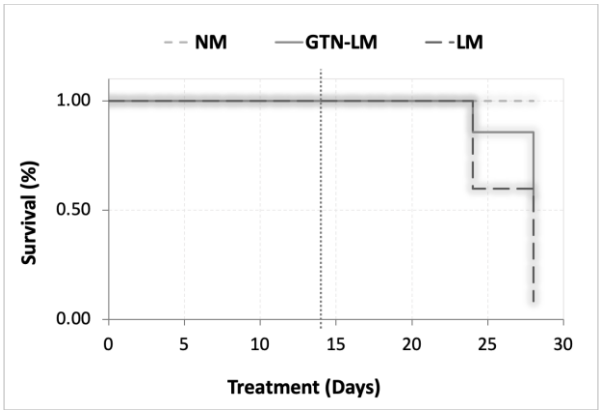

Figure 1: Kaplan-Meier survival curve shows of BALB/c mice post leukemia inoculation. The vertical grey dotted line indicates goniothalamin therapy start point at day-14. Goniothalamin (40 mg/kg b.w.) was supplemented via intraperitoneal. NM: normal mice; LM: untreated leukemia; GTN-LM: goniothalamin-treated leukemia mice.

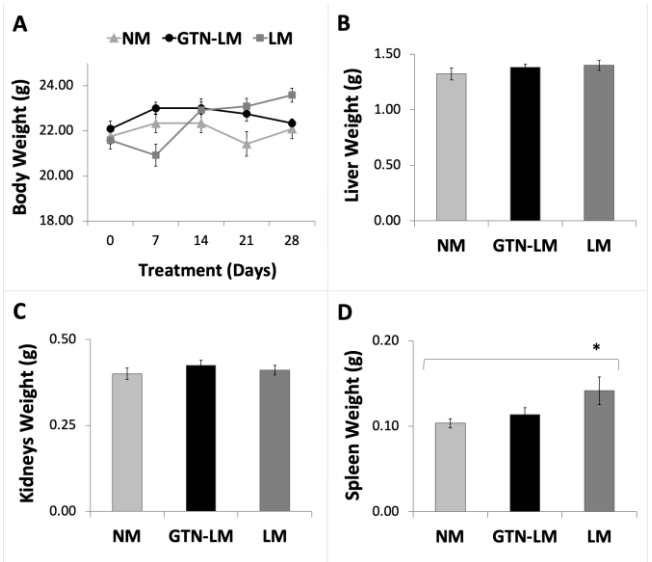

Figure 2: The effect of goniothalamin treatment on the body and organs weights (g) of leukemia BALB/c mice. (A) Body, (B) Liver, (C) Kidneys, (D) Spleen. Data are presented as the mean ± standard error (SEM) (n=12). Asterisk (*) indicates the significant difference between groups (p<0.05) according to one-way ANOVA. NM: normal mice; LM: untreated leukemia; GTN-LM: goniothalamin-treated leukemia mice.

Table 1: White blood cells (WBC) hematological markers of normal (NM), untreated leukemia (LM) and goniothalamin-treated leukemia mice (GTN-LM)

Mice Group	WBC (10^3/µl)	Lymph (10^3/µl)	Lymph (%)	Mono (10^3/µl)	Mono (%)	Neut (10^3/µl)	Neut (%)
NM	10.92 ± 0.28[a]	2.02 ± 0.19	18.57 ± 0.19	2.04 ± 0.18[a]	18.67 ± 0.18	4.29 ± 0.54	39.32 ± 0.54
GTN-LM	16.89 ± 1.98[a]	4.98 ± 0.48	29.51 ± 0.48	5.78 ± 0.38[c]	34.22 ± 0.38	2.84 ± 0.04	16.84 ± 0.04
LM	29.50 ± 4.78[b]	9.25 ± 0.37	31.39 ± 0.37	9.40 ± 0.32[c]	31.86 ± 0.32	5.06 ± 0.47	17.14 ± 0.47

*Data are presented as the mean ± standard error (SEM) (n=3). Different superscript letters signify significant mean differences between groups (p<0.05). Goniothalamin (40 mg/kg b.w.) was supplemented via intraperitoneal route. WBC: White blood cell; Lymph: lymphocytes; Mono: monocytes; Neut: neutrophils.

Table 2: Serum biochemical of kidney, heart, and liver function parameters of normal mice (NM), untreated leukemia (LM) and goniothalamin-treated leukemia mice (GTN-LM)

No.	Biochemical (unit)	NM	GTN-LM	LM
1	Sodium (mmol/L)	151.67 ± 4.18	148.67 ± 0.33	150.67 ± 1.20
2	Potassium (mmol/L)	8.70 ± 0.87	8.23 ± 0.54	7.80 ± 0.55
3	Chloride (mmol/L)	111.67 ± 2.19	110.00 ± 1.00	111.33 ± 1.45
4	Urea (mmol/L)	9.37 ± 0.32[a]	8.43 ± 0.85[a]	7.73 ± 0.46[b]
5	Uric acid (mmol/L)	0.34 ± 0.02	0.29 ± 0.02	0.26 ± 0.04
6	Creatinine (µmol/L)	23.33 ± 1.20[a]	20.33 ± 0.67[a,b]	16.10 ± 0.90[b]
7	Total Protein (g/L)	53.00 ± 1.73[b]	49.33 ± 0.67[a]	45.33 ± 1.20[a]
8	Albumin (g/L)	36.33 ± 1.33	33.67 ± 0.67	32.00 ± 1.53
9	Globulin (g/L)	16.67 ± 0.88	15.67 ± 0.67	15.33 ± 1.33
10	Albumin/Globulin Ratio	2.17 ± 0.13	2.13 ± 0.12	2.10 ± 0.17
11	Total Bilirubin (µmol/L)	2.00 ± 0.07[a]	1.78 ± 0.17[a]	3.15 ± 0.46[b]
12	Creatine kinase (CK) (U/L)	2910.67 ± 480.44	2414.00 ± 463.66	2477.00 ± 937.18
13	Gamma glutamyl-transferase (GGT) (U/L)	2.36 ± 0.02	2.29 ± 0.04	2.13 ± 0.03
14	Alkaline phosphatase (ALP) (U/L)	198.67 ± 9.87	159.00 ± 9.61	131.33 ± 15.84
15	Aspartate aminotransferase (AST) (U/L)	221.67 ± 30.33[a]	245.00 ± 36.17[a]	319.33 ± 47.56[b]
16	Alanine transaminase (ALT) (U/L)	53.33 ± 8.17[a]	65.33 ± 23.51[a]	88.67 ± 11.14[b]

*Data are presented as the mean ± standard error (SEM) (n=3). Different superscript letters signify significant mean differences between groups (p<0.05). Goniothalamin (40 mg/kg b.w.) was supplemented via intraperitoneal route.

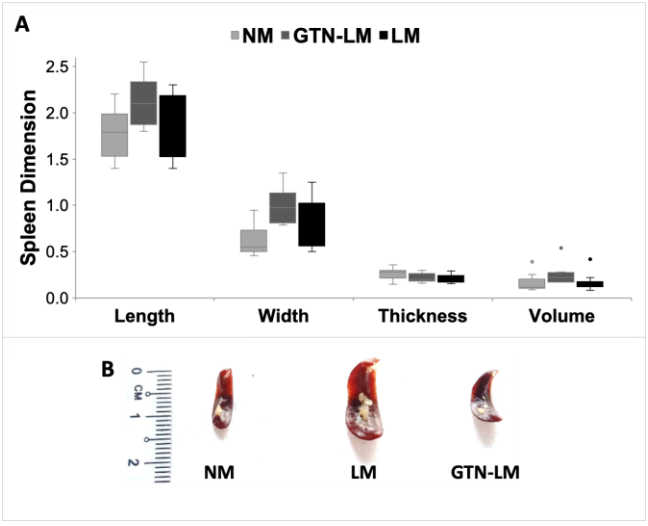

Figure 3: The effect of goniothalamin treatment on the spleen dimension of leukemia BALB/c mice. (A) The dimension (length (cm), width (cm), thickness (cm) and volume (cm^3)), (B) The representative images of the spleens. NM: normal mice; LM: untreated leukemia; GTN-LM: goniothalamin-treated leukemia mice.

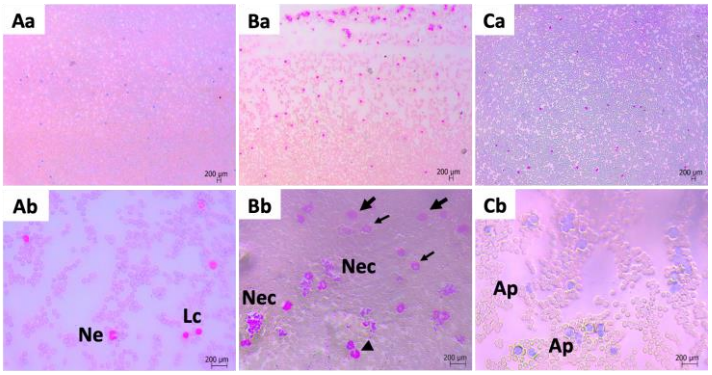

Figure 4: Photomicrographs show the peripheral blood smears prepared using Leishman's staining of different mice groups observed at day-28 under 100× magnification (a; upper panel) and 400× magnification (b; lower panel). (A) Normal mice (NM), (B) Untreated leukemia (LM), (C) Goniothalamin-treated leukemia mice (GTN-LM). Arrowhead; doughnut-shaped and cuplike nuclei; Thin arrow; neutrophils with single small Döhle inclusion body; Thick arrow; reactive lymphocytes cells; Lc; Lymphocyte, Ne: Neutrophil; Ap: Apoptosis; Nec: Necrosis.

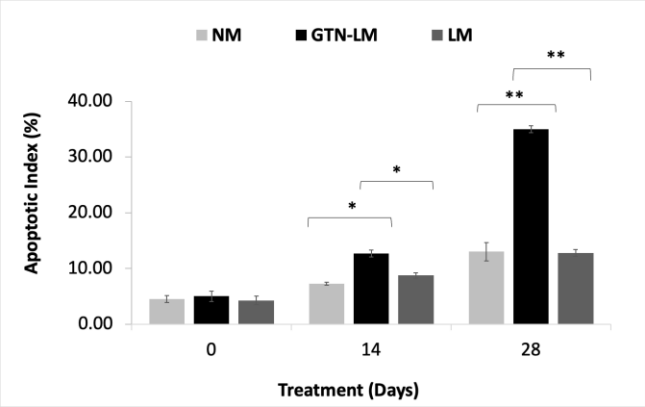

Figure 5: The apoptotic index of the immature white blood cells of goniothalamin-treated leukemia mice. Data are presented as the mean ± standard error (SEM) (n=3). Asterisk (*) indicates the significant difference between groups (p<0.05) according to one-way ANOVA. NM: normal mice; LM: untreated leukemia; GTN-LM: goniothalamin-treated leukemia mice.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the works presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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