

Ex-Vivo Anticancer Evaluation of Tongkat Ali Roots Extract Against Lymphocyte Cell Line of Human CML Patient

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ABSTRACT

This study was designed to assess the anticancer efficacy of TA root extract against human lymphocytic cell-lines of CML and the possible mechanisms for such cytotoxic activity. *Eurycoma longifolia* Jack is an herbal plant that identified locally as Tongkat Ali (TA) or Pasak Bumbi in Malaysia and Indonesia, respectively. TA has a long documented ethnobotanical history as a health invention. One of the pathogenic malignancy of human hematopoietic stem cells that display an obvious elevation of granulocytes is a chronic myelocytic leukemia (CML) which also manifested with splenomegaly and bone marrow hyperplasia and cause 15-20% of all leukemias. Serial concentrations of 2.5, 5, 10, 20 and 40 µg/mL were prepared from the dried root extracts using dimethyl sulfoxide (DMSO) as a solvent. The potential of cytotoxic effect of TA roots extract against lymphocytes from CML patient reveals steep raise of lymphocytes growth inhibition rate (IR) with TA concentrations. Determination of MDA and SOD activity as well as evaluation of genotoxicity of TA by comet assay, the results confirm that TA has cytotoxic and antiproliferative activity and no genotoxicity at concentration of 10 µg/ml. This gives a promising antileukemic activity with different mechanisms of action.

Keywords: Cytotoxicity, Lymphocytes, tongkat ali, SOD, MDA and Comet.

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INTRODUCTION

Eurycoma longifolia Jack is a herbal plant identified locally as Tongkat Ali (TA) or Pasak Bumbi in Malaysia and Indonesia, respectively. TA has a long documented ethnobotanical history as a health invention⁽¹⁾, TA root extract has a pleiotropic activities like energy booster, anti-ulcer, anti-malarial, anti-viral, anti-pyretic, anti-proliferative and anti-cancer. Root extract of *E. longifolia* is legendary for its effective role in improving male sexuality (aphrodisiac) also called Malaysian ginseng, *in-vivo* testing stated an elevation of testosterone levels two weeks consequent to administration of TA aqueous root extract⁽²⁾. Besides that, progressive and total mortality and vitality as well as sperm concentration were similarly increased. Male rats treated with root extract of TA were also reported a sexual behavior improvement. Pleiotropic medicinal uses of TA attributed to a phytochemical as alkaloids, squalene and quassinoid⁽³⁾. An alkaloid 9-methoxycanthin-6-one, exhibited cytotoxic efficiency against human lung and breast cancer cell lines (respectively, MCF-7 and A-549)⁽⁴⁾. Phytochemical production in large-scale would amplified as the medicinal uses of the plant increase, both the intact plant and the hairy root culture are capable to accumulate phytochemical to same extent⁽⁵⁾. Recent concern was to enhance the hairy root's yields through nutrient guidance, culture situation or application of any external aspects that can participate to the high construction of the useful phytochemicals⁽⁶⁾. A plentiful studies showed that the butanolic, ethanolic, methanolic or aqueous extract of TA and a different phytochemicals have presented hopeful cytotoxic and anti-proliferative effects versus different human cancer cell-lines encompassing cervical carcinoma (Hela), breast cancers (MCF-7), lung carcinoma (A-549

cells), liver carcinoma (HepG2), human ovarian carcinoma (CaOV-3 and A-2780) and gastric carcinoma (MGC-803 and BGC-823 cells)⁽⁷⁾. *Eurycomanone* from root extract revealed the highest anticancer activity among the different phytochemicals, and the possible cytotoxic mechanism is the stimulation of programmed cell death (apoptosis) through tumor suppressor gene (p53) upregulated expression in addition to the antimicrobial and antiplasmodial efficiencies⁽⁸⁾.

E. longifolia pharmacokinetic profile revealed that TA and its phytochemicals exhibited bad absorption when administered orally and low bioavailability. Conversely, the *E. longifolia* plasma volume of distribution and its phytochemicals is significantly high which advocates their well-distribution in different tissues⁽⁹⁾.

One of the malignant disease of human hematopoietic stem cells that characterized by an obvious elevation of granulocytes is a chronic myelocytic leukemia (CML) which also manifested with splenomegaly and bone marrow hyperplasia and cause 15-20% of all leukemias⁽¹⁰⁾. This study was designed to evaluate the anticancer activity of TA root extract against human lymphocytic cell-lines of CML and the possible mechanisms for such cytotoxic activity.

MATERIALS AND METHODS

Permission from patient of CML was taken to collect blood sample. The root extract of *E. longifolia* was obtained from University Kebangsaan Malaysia (UKM).

Plant material

Roots of *E. longifolia* Jack extraction at 60°C with 95% methanol (MeOH) along 6 hours/day then the yield was filtered and the deposit extracted repeatedly for 5 days with two fresh parts of solvent. At room temperature

Ex-Vivo Anticancer Evaluation of Tongkat Ali Roots Extract Against Lymphocyte Cell Line of Human CML Patient

ranged from 24-27°C and under partial vacuum the filtrate was evaporated to dryness to produce dark brown deposit⁽¹¹⁾.

Serial concentrations of 2.5, 5, 10, 20 and 40 µg/mL were prepared from the dried root extracts using dimethyl sulfoxide (DMSO) as a solvent.

Cells and medium

Lymphocytes from CML human cancer patient aged 40 years old was obtained by using ficoll method depending on difference in the densities between blood cells (whole blood without serum). Lymphocytes separate from other blood components like granulocytes, erythrocytes and dead cells depend on density centrifugation utilizing Ficoll-Paque with a density of 1.007 g/ml. On test tube containing 3ml of blood sample add phosphate buffer saline (PBS) mixing gently then 5ml taken and added to another test tube and add 3ml of Ficoll, Lymphocytes which has lower density will gather at the plasma-gradient margin while permit the passage of the higher density erythrocytes, granulocytes and dead cells through the Ficoll layer⁽¹²⁾. This method is compatible with the technique for isolation of peripheral blood mononuclear cells PBMC, established by Bøyum in 1968.

Lymphocytes were kept in Rosswell Park Memorial Institute (RPMI)-1640 medium (ABI) having fetal bovine serum 10% (Capricorn), L-glutamine 2 mM, and penicillin and streptomycin 100 U/ml (Sigma). In humidified incubator holding 5% CO₂ at 37°C the cells were cultured to produce a monolayer cells⁽¹³⁾.

Ex-vivo assessment of cytotoxicity

Lymphocytes were placed into Falcon® 96-well plates (Corning, USA) at a rate of 100 µl of cells and 100 µl of media per well (each well-adjusted to have 1*10⁴ cells via hemocytometer) and incubated overnight for attachment⁽¹⁴⁾. The culture medium was then substituted by the plant extracts in serial Serial concentrations of 2.5, 5, 10, 20 and 40 µg/mL, triplicates were used for each extract concentration as well as the negative control which is DMSO and cells were incubated for 48 hours. After that 10 µL 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) solution (5 mg/ml, from Sigma-Aldrich) was supplemented to each well and the culture re-incubated for additional 2 h at 37°C. Then, the medium was cautiously taken away and 100 µL of MTT solvent solution (1:1 Dimethyl sulfoxide and isopropanol) was inserted to each well. Another

incubation of plate for 10 min at room temperature before measuring of optical density at 570 nm in ELISA reader. DMSO without cells was utilized as a control for absorbance reading, the gained result were used to determine the IC₅₀ which describes the inhibitory concentration of TA root extract necessary to decrease 50% in cell viability⁽¹⁵⁾.

Cell viability% = (Mean optical density/ control optical density) x 100%

Calculation of IC₅₀ values by plotting % growth inhibition rate of cells versus concentrations of TA extract. Appropriate calibration curve was obtained after drawing of the trend line, correlation coefficient (R²) > 0.90 and the results described as mean ± standard deviation (SD), n=3.

Malondialdehyde (MDA) assay

Sample preparation

Collect cells into centrifuge tube, after centrifugation get rid the supernatant, add 1 ml of assay buffer for 5 × 10⁶ cell, sonicate (with power 20%, sonication 3s, interval 10s, 30 times replication); centrifuged for 10 minutes at 8000g 4 °C, placed the supernatant into a new centrifuge tube and keep it on freeze for finding⁽¹⁶⁾.

Assay procedure

Place 100µl of cells into microcentrifuge tube, add 200µl of dye reagent and mix, placed it in the oven, for 30 minutes at 90 °C, after that put it on ice, centrifuged for 10 minutes at 10000g, 25 °C. Then add 200 µl of the supernatant into the microplate. Record OD at 532 and 600 nm, negative control is DMSO.

$$\text{MDA (nmol/ml)} = \frac{[(\text{OD}_{532} - \text{OD}_{600}) \times V_{\text{Total}}] / (\epsilon \times d) \times 10^9}{V_{\text{Sample}}}$$

$$= 32.25 \times (\text{OD}_{532} - \text{OD}_{600})$$

Superoxide Dismutase (SOD) assay

Sample preparation

Cells placed in centrifuge tube, remove the supernatant after centrifugation, add 1 ml of assay buffer for 5×10⁶ cell, sonicate (with sonication 3s, power 20%, interval 10s, 30 times repetition); centrifuged for 10 minutes at 8000g 4 °C, the supernatant placed into a new centrifuge tube and preserved it on ice for assessment⁽¹⁷⁾.

Assay procedure

Into the microplate add the following reagent as illustrated table 1:

Reagent	Sample	Standard	Blank
Reaction Buffer	30 µl	30 µl	30 µl
Substrate	100 µl	100 µl	100 µl
Enzyme	1 µl	1 µl	1 µl
Sample	19 µl	--	--
Standard	--	19 µl	--
Distilled water	--	--	19 µl
Dye Reagent	50 µl	50 µl	50 µl

$$\text{SOD (U/mg)} = \frac{(C \text{ Standard} \times V \text{ Standard}) \times (\text{OD Blank} - \text{OD Sample})}{(\text{OD Blank} - \text{OD Standard}) \times (V \text{ Sample} \times C \text{ Protein})}$$

$$= 30 \times (\text{OD Blank} - \text{OD Sample}) / (\text{OD Blank} - \text{OD Standard}) / C \text{ Protein}$$

Comet assay

Single-cell gel electrophoresis (SCGE) or comet assay is a sensitive, not complicated and fast technique for

determining the breaks of DNA strands in eukaryotic cells. Geno-toxicity of drugs, environmental and chemical contaminations can be evaluated via comet assay.

Ex-Vivo Anticancer Evaluation of Tongkat Ali Roots Extract Against Lymphocyte Cell Line of Human CML Patient

The cells are implanted in agarose after lysis by detergent to produce a supercoiled DNA coupled with the nuclear matrix. Assemblies like comets as a consequence of alkaline electrophoresis can be detected by fluorescent microscope⁽¹⁸⁾.

Extending of breaks of DNA strand toward anode is the concept of comet assay.

Preparation of reagents

Comet agarose

The agarose bottle was placed in water bath for 20 minutes at 90-95°C, then the agarose bottle the bottle was relocated in water bath for 20 minutes at 37°C and kept until used.

DNA dye

1µl from vista Green DNA staining stock solution diluted with 9ml TE buffer (Tris 10mM, EDTA 1mM at pH 7.5).

Lysis buffer

14.6 gm NaCl, 20 ml EDTA solution and 10ml lysis solution were dissolved in distilled water, pH adjusted to 10 by NaOH 10N and complete the volume by distilled water to 100ml and kept for using at 4 °C.

Alkaline solution

1.2gm of NaCl and 0.2ml of EDTA solution dissolved in 90ml of distilled water. pH was corrected to 10 by using 10N NaOH and add distilled water up to 100 ml then stored at 4 °C for using.

Statistical analysis

Statistical packages for social sciences (SPSS) version 16 was used for data analysis. Mean ± standard deviation (SD) stated for the data analysis description.

Paired T-test used to differentiate between two means of all studied parameters. P-value < 0.05 was considered as statistically.

RESULTS

Ex-vivo assessment of cytotoxicity

The potential of cytotoxic effect of TA roots extract against lymphocytes from CML patient reveals steep raise of IR with TA concentrations as shown in fig-1, the highest IR of (54.49±4.25) was detected at concentration 10µg/ml. Which then slightly declined at higher concentrations (20 and 40µg/ml).

The IC₅₀ value was 8.84µg/ml indicating a strong anti-proliferative activity against lymphocytes.

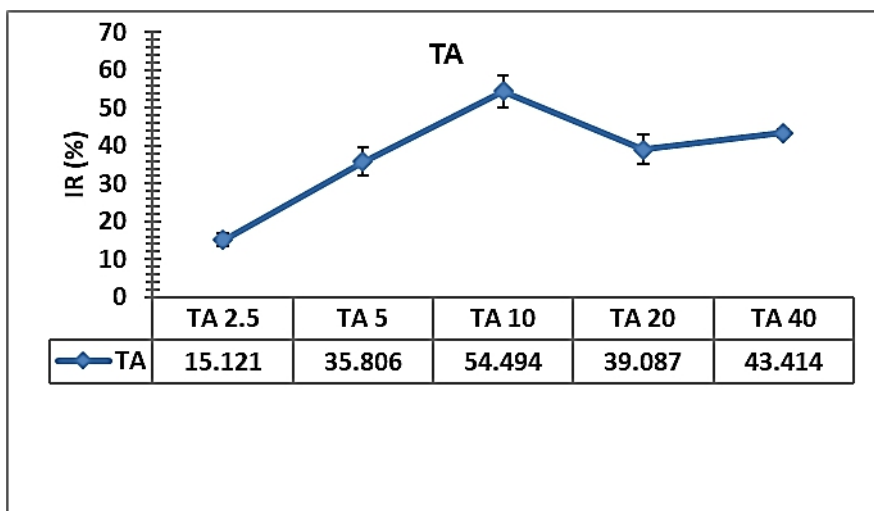


Figure-1: IR% of lymphocytes treated with different concentrations of TA, data expressed as Mean± SD.

Malondialdehyde (MDA)

Fig-2 represent the status of MDA in lymphocytes treated with TA at concentration 10 µg/ml and lymphocytes treated only with DMSO (control) without treatment of TA root extract. MDA levels in TA treated and untreated lymphocytes were 6.42 ± 0.78 nmol/ml and 10.55± 1.58 nmol/ml respectively. A significant changes (p<0.05) in

MDA levels between TA treated and untreated lymphocytes.

Superoxide Dismutase (SOD)

Activities of SOD in control and TA treated lymphocytes were represented in fig-3, SOD activities were 1.62± 0.12 U/mg protein and 2.55 ± 0.31 U/mg protein. Significant increase in SOD activity (p<0.05) in TA treated lymphocytes when compared with control lymphocytes.

Ex-Vivo Anticancer Evaluation of Tongkat Ali Roots Extract Against Lymphocyte Cell Line of Human CML Patient

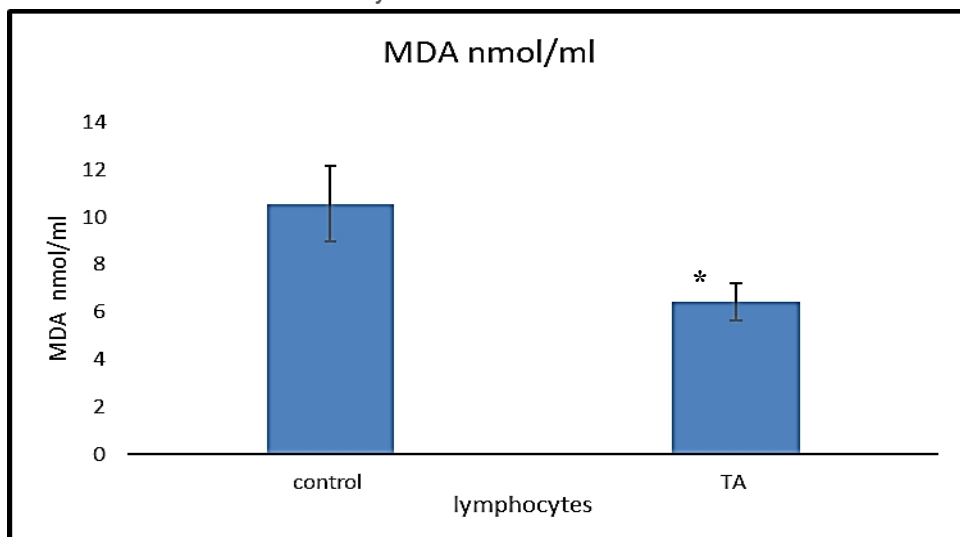


Figure-2: MDA levels in control and TA treated lymphocytes, data represented as Mean \pm SD, * indicates significant difference ($p < 0.05$).

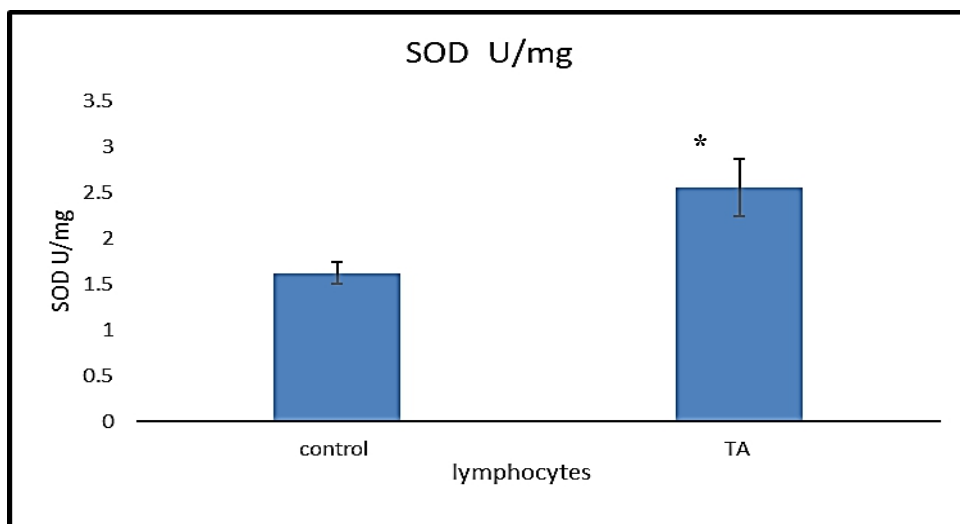


Figure-3: SOD activities in control and TA treated lymphocytes, data represented as Mean \pm SD, * indicates significant difference ($p < 0.05$).

Comet assay

The DNA damage in lymphocytes was determined as the percentage of tail formed which was significantly more in H_2O_2 treated lymphocytes ($P < 0.05$) compared with low percentage of tail was measured in lymphocytes treated with $10 \mu\text{g/ml}$ of TA as shown in fig-4 (a, b and c).

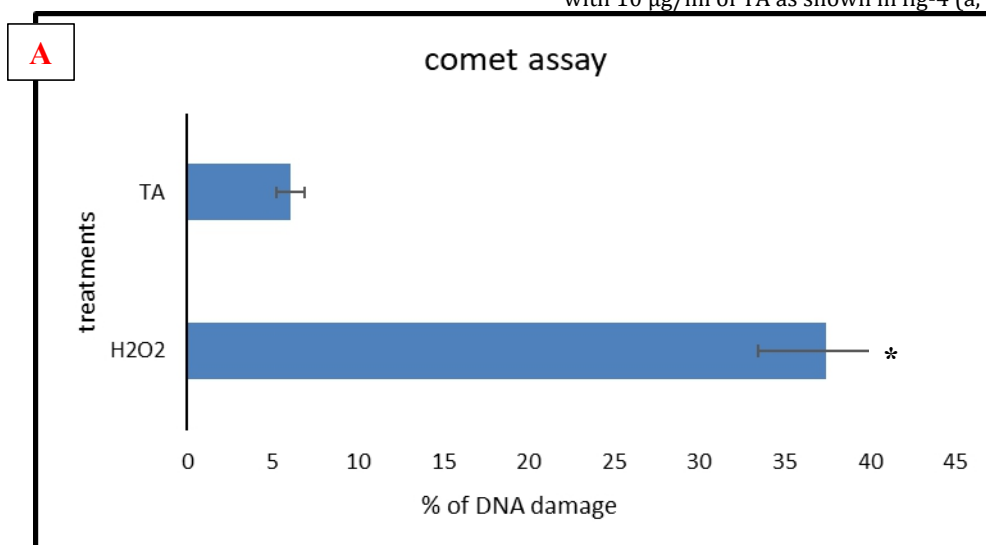


Figure-4 a: percentage of DNA damage in lymphocytes treated with TA compared with that treated with H_2O_2 . The results represented as Mean \pm SD, *

Significant difference ($P < 0.05$).

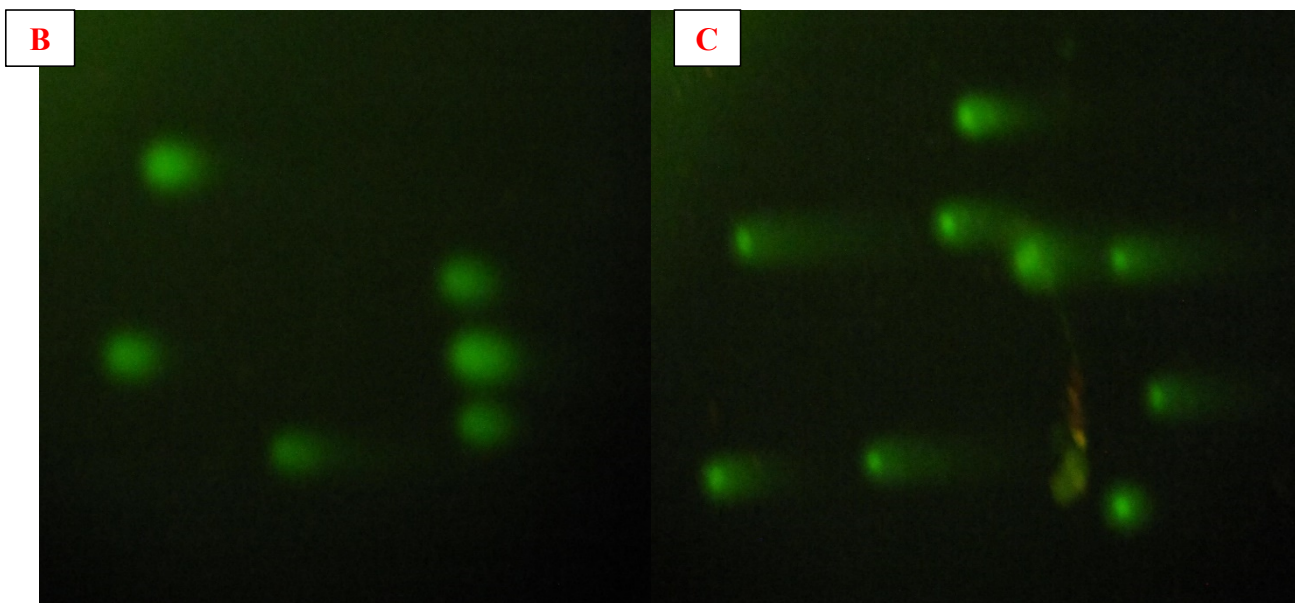


Figure-4 b: represent lymphocytes treated with TA, c: represent lymphocytes treated with H_2O_2 and show DNA damage.

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DISCUSSION

The current study was designed to evaluate the anticancer activity of *Eurycoma longifolia* (Tongkat Ali) Root Extract against Lymphocyte cell line of Human Chronic Myeloid Leukemia (CML) patient, through measuring the percent of growth inhibition rate. TA anticancer activity may attributed to the active constituents of the extract that induce a programmed cell death (apoptosis) through upregulation of pro-apoptotic protein (Bax) and tumor suppressor protein (p53) expression, and down regulation of anti-apoptotic (Bcl-2) genes expression of cervical cancer Hela cells⁽¹⁹⁾. Prevention of oxidative damage of lymphocytes through the increase in SOD activity may play an important role as an antioxidant with the reduction of lipid peroxidation mediated by MDA level declining to reach the normal balance between peroxidant /antioxidant mechanism⁽²⁰⁾. This study illustrate the genotoxicity of TA roots extract via comet assay and this reveal that TA roots extract has a meager geneotoxic efficacy on lymphocytes at concentration $10\mu\text{g/ml}$ this will be due to eurycomanone one of the most active constituent of TA roots extract that has antiproliferative effect on cancer cells via induction of DNA fragmentation at a low concentration⁽²¹⁾.

CONCLUSION

Togkat Ali root extract has a potent cytotoxic activity on lymphocytes obtained from CML patient, which occurs through induction of SOD activity as antioxidant and reduction of lipid peroxidation and mutagenicity through declining of MDA levels. Despite this entire TA roots extract has meager genotoxic activity.

CONFLICT OF INTEREST

Authors declare no conflict presented.

ACKNOWLEDGMENT

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Ex-Vivo Anticancer Evaluation of Tongkat Ali Roots Extract Against Lymphocyte Cell Line of Human CML Patient

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