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Table (3) Effect of different concentrations of methanol extract of *Rubus ideaus* and exposure time (2 , 4 hours) on lymphocytes IL-4 concentrations.

Time exposure	Concentration of extract	Concentration of IL-4 pg/ml
2 Hours	50	9.162
	150	12.00
	250	20.212
	Control	14.275
4 Hours	50	50.212
	150	302.712
	250	303.337
	Control	62.712

Treatment of human lymphocytes with different methanol extract of *Rubus idaeus*concentrations for 4 hour exposure, showed difference in their effects on both cytokine levels. As shown in Table (3) a large increase in IL-4 level was dependent on increasing in methanol extract of *Rubus idaeus*concentration while in contrast, at the concentrations (250mg/ml) that was (303.337pg/ml) in comparison with control (62.712pg/ml).While IL-4 level was (50.212pg/ml) and (302.712pg/ml), as lymphocytes treated with (50 mg/ml) and (150mg/ml) methanol extract of Rubusideaus respectively. A study was conducted effects of water extract from red raspberries on the in vitro and in vivo on tumor cell viability and immune parameters. According to the study the extract exhibited a potent cytotoxic effect on variety of cancer cells in vitro by comparison to an ascorbic acid control and including immune cell proliferation[23]. However, result from which assay indicate that the concentrations prepared of methanol extract of Rubusideaus used in this study were sufficient to kill tumor cells of Lymphocyte cell lines in vitro.

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Table (2) Effect of different concentrations of methanol extract of *Rubus ideaus* and exposure time (2 , 4 hours) on lymphocytes IL-2 concentrations.

Time exposure	Concentration of extract	Concentration of IL-2 (pg/ml)
2 Hours	50	157.500
	150	636.870
	250	736.875
	Control	576.875
4 Hours	50	718.750
	150	951.250
	250	1070
	Control	887.000

Treatment of human lymphocytes with different methanol extract of *Rubus idaeus*concentrations for 4 hour exposure, showed difference in their effects in Table (2) a large increase in IL-2 level was dependent on increasing in methanol extract of *Rubus idaeus*concentration while in contrast, at the concentrations (250mg/ml) that was (1070pg/ml) in comparison with control (887pg/ml).While IL-2 level was (718.750 pg/ml) and (951.250 pg/ml), as lymphocytes treated with (50 mg/ml) and (150mg/ml) respectively.

The effect of different concentrations of methanol extract of *Rubus idaeus*on cytokine level produced by treated lymphocytes after (2,4 hours), was shown in Table (3). Methanol extract of *Rubus idaeus*and exposure time 2 hours, increased significantly IL-4 level on ($p \leq 0.05$) at the concentrations (250 mg/ml) that was (20.2 pg/ml) in comparison with control (14.275pg/ml).While IL-4 level was (9.162pg/ml and 12.00 pg/ml) as lymphocytes treated (with 50 mg/ml),(150mg/ml) methanol extract of *Rubus idaeus*respectively.

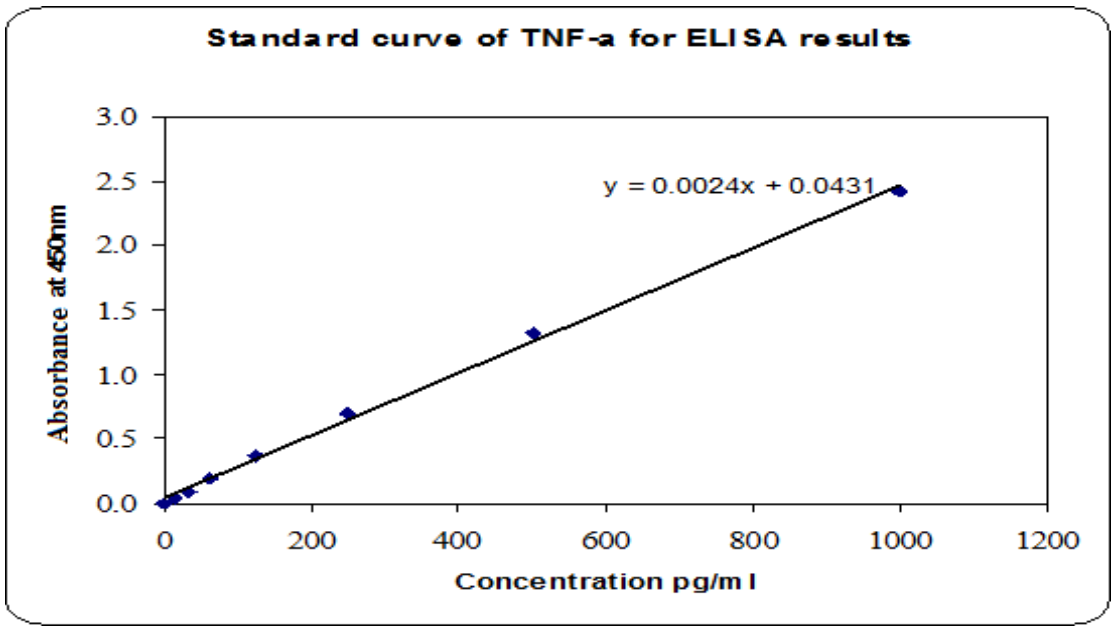

Figure (2)

The effect of different concentrations of methanol extract of *Rubus idaeus*on cytokine level produced by treated lymphocytes after (2,4 hours), was shown in Table (2). The greatest effect of methanol extract of *Rubus idaeus*treated lymphocytes for 2 hours exposure caused, it increased significantly IL-2 level on ($p \leq 0.05$) at the (736.875pg/ml, 636.870pg/ml), at methanol extract concentration (250mg/ml and 150mg/ml) in comparison to control (576.875pg/ml), at the lowest concentration of (50mg/ml) significantly IL-2 level was (157.500 Pg/ml).

the plant cytotoxicity were differ from one cell culture to another depend on whether whole plant extract was used or any of the plant component, in fact many nutritive and nonnutritive phytochemicals with diversified pharmacological properties have shown promising responses for the prevention and treatment of various cancers, including different types of cancer[21]. The benefit derived from raspberry consumption have been attribute to their content of polyphenol, flavonoid, anthocyanin, ellagitannin, and vitamin, it is the phytochemicals that are responsible for many of the biological activities of raspberries, including antioxidant, anti-inflammatory and anticancer properties[22].

Effect of methanol extract of *Rubus idaeus*on Cytokine Levels (IL- 2 and IL-4)

In order to trace cytokines (IL- 2 and IL-4) level in the supernatant of the treated lymphocytes with alcohol Extract of *Rubus idaeus*at different concentrations and exposure time 2 and 4 hours as well as control culture and standard solutions, ELISA was used and standard curve for both interleukins was plotted separately Figure (1 and 2)

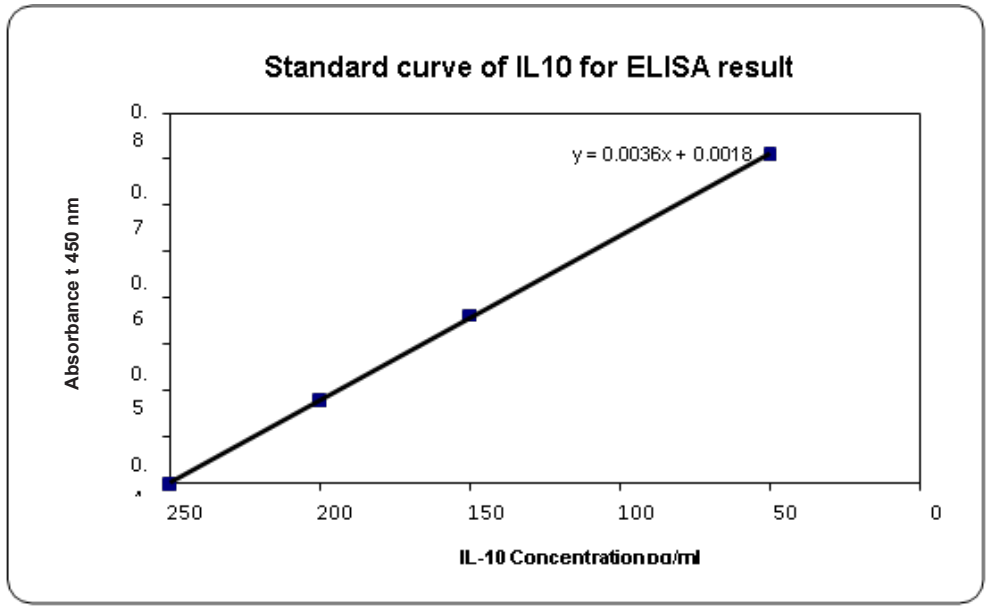

Figure (1)

Table(1) effect of methanol extract of *Rubus ideaus* on normal human lymphocytes using MTT assay treated for 24 hours

Concentration of extract mg/ml	Viability%	Toxicity%
50	122±	88
75	127±	87
100	185±	85
150	212±	88
200	240±	60
250	295±	5
control	69±	31

Results indicated that a significant differences($P \leq 0.05$) among the methanol extract of *Rubus idaeus*at different concentrations as compared with results of the untreated lymphocytes as negative control. As shown in Table (1), the methanol extract of *Rubus idaeus*stimulating effect for the immune –system by increasing lymphocytes proliferation , especially at high concentrations (250,200,150 mg/ml) in respect to control result. Numerous studies have demonstrated the anticancer properties of raspberries on tumor cell proliferation in vitro[17]. And studies from this laboratory support these finding [18]. Which needed to be interpreted, raspberries have been shown to protect against free radical-induced cell damage and reduce oxidative stress, prevent the growth of cancer cells in vitro and in animal. The results attributed to their capacity to stimulate or inhibit protein phosphorelation that regulate cell function or by counter balancing the effect of cellular protein tyrosine phosphatases [19]. As a result of the relatively poor prognosis and response to conventional chemo radiotherapy, there is a great need for new effective agents, renewed attention in recent years to natural therapies has stimulated a new research interest in traditional practices, herbs have become a target for the search for new anticancer drugs, about half of the drugs used in clinical practice come from natural products[20]. Various in vitro studies about the mechanism of

-IL-10 Standard vial: a recombinant human IL-10in a buffered protein base with preservative, lyophilized----(2vials).

-Calibrator Diluents II (5X):cell culture medium-------(22ml).

-Wash Buffer (20X): concentrated buffered surfactant solution------(60)ml.

-Substrate A:buffered solution with H 2O2---------(10ml).

-Substrate B:buffered solution with TMB----------(10ml).

-Stop Solution include H2SO4----------(14ml).

Procedure:

Practical work was done following the instructions of US Biological IL-10 kit protocol/Biochemical &Biological Reagents, United State Biological. Catalog No(18432-05).The supernatants of treated lymphocytes with different concentrations of extracted polysaccharide (previous steps) were applied with IL-10 ELISA protocol Briefly:

Aliquot of 100µl from each standard and samples were added (in duplicate) into the antiboby pre-coated microtiter plate, then incubated for 1 hour at room temperature. Without discarding standards or sample solutions about 50 µl Pab (biotin) was added to each well, incubated for 1 hour at room temperature then the plate was washed to remove any unconjugated antibodies. The Avid in attached with HRP enzyme was added to all wells in quantity of 100 µl the plate was incubated in dark at room temperature for another 1 hour followed by washing step, finally 100 µl substrate mixture was added for 15 minutes stand period in dark at room temperature then to stop their reaction, 100µl stop solution was added. At the end of experiment a standard curve for different standard concentrations versus their absorbance at 450nm were plotted, then each IL-10concentration was calculated and then evaluated statistically.

Results and Discussion:

Effect methanol extract of Rudus ideaus on normal human Lymphocyte proliferation:

Lymphocyte proliferation was determined using MTT method. Results of the effect of different concentrations of extract of *Rubus idaeus*on proliferation of normal human lymphocyte are shown in Table (1).

from extracted polysaccharides were chosen, these are:(1000, 500, 250) µg/ml as they showed the potent proliferative effect for the normal lymphocytes. they were prepared from polysaccharides stock solution, then all solutions were sterilized with 0.22 µm Millipore filter.

-To each plate one milliliter/well was added from each polysaccharides concentration in triplicate. Negative control represented by untreated cells suspended in growth medium was included.

-One plate was incubated for 2 hours, the other incubated four 4hours in CO2 incubator at 37C0.

-At the end of each interval times all wells were aspirated and transferred in separated vacuum tubes and centrifuged for 20 minutes at 2000 rpm.

-The supernatants of each tube were separated and kept at -20C0 to be estimated by ELISA kit, while the pellets were immediately used for CD markers determination and molecular expression detection for cytokines.

The supernatants of treated lymphocytes with different concentrations of extracted polysaccharide (previous steps) were applied with IL-10 and TNF- α ELISA protocol. At the end of experiment a standard curve for different standard concentrations verses absorbance at 450nm was plotted, then a calculation of each IL-10 concentration for all test readings were applied according to straight line equation. Results were also evaluated statistically.

The Cytokine IL-10 Level

Human IL-10 ELISA kit contain the key components required for the quantitative measurement of natural and/or recombinant HIL-10 in a sandwich ELISA format within the range of 50-1600pg/ml. The kit exhibits no significant crosser activity with human IL-1a, IL-2, IL-3, IL-4, IL-6, IL-7, -IL-8, TNF-α,TNF-β, and IFN-γ.

The Kit Components:

-IL-10 Microtiter plate, 1x96 wells Pre-coated with anti-human IL-10 monoclonal antibody.

-Pab (Biotin) :Anti-human IL-10 polyclonal antibody conjugated to Biotin ---(7ml).

-HRP (Avidin): Avidin conjugated to the HRP enzyme(Horse Radish Peroxidase)-----(14ml).

at 2000 rpm. The step was repeated twice.

-The isolated lymphocyte cells were collected again and suspended in RPMI-1640 medium supplemented with 10% fetal calf serum, containing100 units/ml penicillin, and 100µg/ml streptomycin, then transferred into appropriate tissue culture flask and incubated for 18 hours at 370C in 5% CO2 incubator.

Determination of cell viable counting for the isolated lymphocytes

The cell count and viability were determined according to[9] procedure. Try pan blue 1% solution freshly prepared in PBS was used. Dead cells unlike viable cells took up the dye within seconds which could be easily distinguished under light microscope.

-Aliquot of 10µl from both Try pan blue stain and lymphocyte cell suspension were mixed for 30 seconds, then 10 µl from the mixture was applied gently into both grooves edge at the two sides of a heaemocytometer chamber, underneath the cover slip.

-Under light microscope 40X objective lens all cells were counted in 1mm2

,then a separate counting of viable (transparence) and non-viable (blue) cells was done.

-Cell concentration (cell/ml), total cell count and %viable cell count were calculated as follow:

-Cell concentration (cell/ml)= number of counted cells X dilution factorX104

-Total cell count=cell concentration (cell/ml) X the original fluid volume

-% viable cells= number of living cells/ total number of cells X 100

-The viable counting with Try pan blue result should be more than 90% viable cells count. These steps were done before any of the following immune tests[15].

Determination of the Cytokine Level by ELISA Technique following [16]

-Measuring 1000µl of the suspended cells were seeded in each of the 24 well tissue culture plate (1X106cell/well), two plates were needed, one plate incubated for 2 hours intervals and the other incubated for 4 hours.

-Both plates were incubated at least for 2 hours in a CO2 incubator before treatments.

-From MTT results done in the previous step, three effective concentrations

well. The plates were then incubated at 37 °C in an incubator supplemented with 5% CO2 for 24 h [11]. After incubation, *Rubus idaeus* extract was added to each well at different concentrations and incubated for 24 h[10].

Cytotoxicity assay using the 3-[4,5-dimethylthiazoyl] -2,5- diphenyltetrazolium bromide (MTT) test

This test was performed by dissolving 3-[4,5-dimethylthiazoyl]-2, 5-diphenyltetrazolium bromide in phosphate buffered saline (PBS) at 2 mg/ml, filtrated by a 0.22 µm millipore filter. 50 µl of the MTT dye was added to each of the microtiter plate wells containing human lymphocytes treated with different concentrations of *Rubus idaeus* extract for 24 h. The MTT-formazan crystals, which are formed only by live cells, were dissolved in 100 µl Dimethyl sulphoxide (DMSO), enabling the optical density of each well to be measured using an ELISA reader at a transmitting wavelength of 620 nm [12]. The inhibitory rate was measured according to[13] as follows:

$$\text{Cytotoxicity viability} = \frac{\text{O.D. of control} - \text{O.D. of Sample}}{\text{O.D. of control}} \times 100$$

Lymphocyte Culturing and Viable Counting by [14],[10]

-From eight healthy volunteers five milliliters of venous blood were taken from each, their ages in the range of (25-35) years old, never taken medication at least 10 days ago.

-Each five milliliters was transferred into vacuumed tubes containing 0.2% EDTA as anticoagulant with continuous gentle shaking.

-The human peripheral blood was diluted with PBS (pH=7.2) in 1:1 ratio.

-About 5 milliliters of the diluted cell suspension was layered onto three milliters of Ficoll separation fluid (lymphprep; specific gravity=1.077g/l, placed into vacuumed tube (10 ml capacity).

-The tubes were centrifuged at 2000 rpm for 30 minutes.

-The lymphocyte cells were collected with sterile Pasteur pipette, transferred into 10ml vacuumed tubes, suspended with 5ml RPMI 1640, and centrifuged for 10minutes

in aging and various disease processes[5].

Materials and methods

Plant material and preparation of the crude extract

Sample preparation of ripe blackberries *Rubus idaeus* were collected from the farmlands of Tarmia city in the north of Baghdad . Identified and authenticated as *Rubus idaeus* by Professor Dr.Ali Al Musawy Biology departments, college of science, University of Baghdad. *Rubus idaeus* fruit were then washed, cleaned, fresh weighed, and preserved at -20 C0 for using it in extraction step. methanol extract prepared from blackberry juice according to[6]. After preparation of extract , the solvent alcohol was evaporated by oven at 4000C, and the prepared dry extract was weighed and dissolved by Dimethylsulfoxide DMSO in order to prepare the concentrations for cytotoxic study [7]. *Rubus idaeus* extract stock solution was made by mixing 400µl of extract with 10µl DMSO and complete the volume up to 1ml using serum free medium to get the concentration of 400µl extracts /1ml medium[8]. Then nine crud extract concentration starting with 50 mg/ml till 3.9 mg/ml in a twofold dilution manner were applied to the microliter plate containing 200µl/ weel of the mono confluent layer. The first weel concentration became 250mg / ml for the extract and the last concentration treated weel was 3.9 mg /ml.

Preparation of toxin concentration

Both standard *Rubus idaeus* extract were dissolved in methanol at 1 mg/ml and used to prepare concentrations of 25, 50, 100 and 200 ng/ml in complete culture media (RPMI-1640 medium supplemented with 10% fetal calf serum, containing a solution of penicillin 100 units/ml and streptomycin 100 µg/ml)[9].

Collection and processing of human lymphocytes

This work was held at Al-Nahrain Biotechnology center Laboratories. Peripheral venous blood was taken from a healthy 26 year old male donor and lymphocytes were extracted following [10]. The suspension of cultured human lymphocytes was adjusted until the number of cells was about 1 × 104 cells/ml. 100 µl of the cell suspension was then dispensed into each of the 96 wells of a microtiter plate to give a final cell count of 1000 cells/

Introduction

Raspberry, *Rubus idaeus* L., plant belonging to the Rosaceae family for genus Rubus. The geographical distribution of *Rubus idaeus* L. cover a wide area Europe to northern Asia and most temperate areas. As a well-known fruit crop, raspberry fruits were used not only for nutritional purpose, it is also used in many countries in folk medicine to treat wounds, colic pain and some other disease like diarrhea and renal disease [1]. Many of the health benefits derived from blackberry *Rubus idaeus* consumption are due to their content of polyphenols, which are responsible for many of the biological activities of *Rubus idaeus* L, including antioxidant, anti-inflammatory and anticancer properties[2]. In recent times, dietary antioxidants have received increasing attention because of their important function in mitigating the damaging effects of oxidative stress on cells and tissues [3]. Raspberries have significantly high levels of phenolic flavonoid phytochemicals such as anthocyanins, ellagic acid (tannin), quercetin, gallic acid, cyanidins, pelargonidins, catechins, kaempferol and salicylic acid [4]. Anthocyanins are the major contributors to the red colour pigment in berry fruits. The synthesis of anthocyanin depends on many ecological and physiological factors, as well as on berry species and cultivars, fresh raspberries are an excellent source of vitamin C, which is also a powerful natural antioxidant, berries weighing 100g provides about 47% of Daily Recommended Intake of vitamin C, consumption of fruits rich in vitamin C helps the body develop resistance against infectious agents, counter inflammation, and scavenge harmful free radicals, large numbers of bioactive compounds contribute to the antioxidant capacity , but the total activity is mainly reflected in their total phenolic and ascorbic acid concentrations, raspberry contains antioxidant vitamins like vitamin A, and vitamin E, in addition to the above antioxidants is also rich in several other health promoting flavonoid polyphenol antioxidants such as lutein, zea-xanthin, and ß-carotene in small amounts, altogether, these compounds act as protective scavengers against oxygen-derived free radicals and Reactive Oxigen Species that play a role

التأثير المضاد للتكاثر لمستخلص الميثانول من ثمار نبات (*Rubus idaeus* L) على الخلايا الخلوية اللمفاوية خارج الجسم

محمد محمود فرحان الحلبوسي ..رغد كاظم اللهيبي ..ميثاق نزهان محمود.. بتول عمران ذيب

الخلاصة

تشير الدراسة الحالية وجود تأثيرات لنبات *Rubus idaeus* من النوع البري العراقي تعمل كعامل مضاد للسرطان وعامل مناعي، واجريت دراسة تأثير سمية مستخلص كحول الميثانول المحضر من نبات التوت الاسود البري *Rubus idaeus* الذي جمع من شمال بغداد وحضر من مستخلص كحول الميثانول الخام عدة تراكيز لدراسة سميته الخلوية في خط الخلايا اللمفاوية الدموية، وكذلك اختبار تحفيز انتشار الخلايا اللمفاوية بطريقة صبغة MTT.

واظهرت النتائج إن المستخلص الكحولي الخام من نبات *Rubus idaeus* له تأثير تحفيزي لنظام المناعة من خلال زيادة الانتشار للخلايا اللمفاوية الطبيعية وخاصة عند التراكيز العالية (250، 200، 150 ملغم / مل) مقارنتا بنتائج مجموعة السيطرة غير المعاملة بالمستخلص. كما تم اختبار تراكيز مختلفة من المستخلص الكحولي الخام على حركيات خلوية من (4، 2-IL) ولمدة تعرض 2 و4 ساعة . اظهرت النتائج تأثير سمي ومعدل تثبيط بزيادة معنوية ((P≤0.05 وخاصة عند التركيز العالية (150و250 ملغم/ مل) وبوقت تعرض 4 ساعات، اذ إن جميع التراكيز المحضرة من المستخلص الكحولي المحضر من التوت الاسود البري اظهرت تأثير سمي ضد الخلايا المسرطنة اللمفاوية خارج الجسم ويختلف تأثيرها السمي ما بين تلك التراكيز ويزداد بزيادة التركيز والوقت

In vitro anti-proliferative effect of Methanol Extract from (*Rubus idaeus* L) on Lymphocyte cell lines

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Abstract

This study demonstrates the favorable effects of Iraqi wild type *Rubus idaeus* as anticancer and immunomodulating agent. The investigated of *Rubus idaeus* methanol extract prepared from ripe blackberries that collected from the farmlands north of Baghdad, the crud extract prepared at the several concentrations for cytotoxic study, and investigated stimulation on lymphocytes proliferation by MTT method. Results showed methanol extract of *Rubus idaeus*stimulating effect for the immune–system by increasing lymphocytes proliferation, especially at high concentrations (250,200,150 mg/ml) as compared with the control result. Also this study investigated in different concentrations of methanol extract of *Rubus idaeus* on Cytokine Levels (IL-2,4) and exposure time (2 and 4 hours) as well as control culture and standard solutions ELISA standard curve, cytotoxic effect showed a dose dependent inhibition rate increased with the increase of *Rubus idaeus*extracts concentration (P□0.05). Results showed that there were potent toxic effects on Lymphocytes IL-2,4 and exposure time 4 hours specially when treated with the concentration (150 and 250mg/ml). All the concentration prepared from methanol extract of *Rubus idaeus*showed cytotoxic effect against lymphocytes cancer cell lines in vitro with a significant difference between all concentrations as cytotoxic agent with increased concentrations and time.

Keywords: *Rubus idaeus* L., Lymphocyte cell., MTT assay