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The Effects of the Crude and the Active Component of *Dendrostellera lessertii* on the Co-cultures of Human Monocytes with CCRF-CEM or K562 Cell Lines

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Abstract

The effects of an alcoholic extract ($EtOH/H_2O$, 1:1 v/v) of *Dendrostellera* (Thymelaeceae) and one of its purified component (MW=663) was evaluated on the growth behavior of two human leukemia cell lines (CCRF-CEM and K562) in the presence and absence of isolated human monocytes. A 50% growth inhibition was observed in both cell lines using 160 μL of the 1:100 dilution of the crude extract (containing 0.5 g plant powder per mili-liter) or 1.4μM of the purified component. In the co-culture of the isolated human monocytes and each of the leukemia cell lines, however, a four fold of increase in growth inhibition was observed, possibly due to the secretion of TNF-α by the cultured monocytes, under the effect of *D.lessertii*. The plant extract or its purified component were not only capable of enhancing TNF-α secretion but also they were capable of quenching the number of TNF-α receptors in the cultured monocytes, in less than two hours, in a dose dependent manner. These data, with similarity to *Taxol* effects on human monocytes and macrophages, may suggest a similar mechanism, down regulation of TNF-α receptors, for the *Dendrostellera* extract and its purified component.

Keywords: *Dendrostellera, human TNF ?¦ Leukemia, LPS, monocyte, Taxol, Thymelaeaceae, TNF ?Þ receptor.*

Introduction

Various species of Tymelaeaceae family have been used to treat cancer type disorders since 200 A.D(Abe et al., 1977; Baba et al., 1986; Kasai et al., 1981; Kuphen and Boxter, 1975) and references to their use in folk medicine have appeared in the herbal literature of many countries (Harwell, 1971) Additionally, the active components of many species of thymelaeaceae have also been structurally characterized and shown to have antineoplastic activities. For example, Daphnoretin from wikstroemia Indica C.A. mey (Hall *et al.,* 1982); Mezerein from Daphne mezereum (Kupchan and Baxter, 1974) and Genkwadaphnin and yuanhuacine from Daphne genkua (Hall *et al.,* 1982), among many others, have been shown to posses strong biological anti-tumor activities. Despite these reports, there is no report concerning the medicinal application of *Dendrostellera* in Iranian folk medicine. Therefore, as part of our studies of plants for new anticancer agents with emphasis on Thymelaeaceae family, we examined the cyto-toxicity and anti-tumor activity of an alcohol: water (1:1, v/v) extract of *Dendrostellera* leaves and one of it's purified component against two types of human leukemia cell lines. After exploring the high anti-tumor activity of plant extract, and its purified compound on human leukemia cell lines (CCRF-CEM and K562), we evaluated the mode of action of this anti-cancer plant. In that respect, we investigated the effects of the plant extract, the purified component, and *Taxol* (an anti-neoplastic natural agent from the bark of the Pacific yew tree and with established mechanism of action) on the regulation of TNF-α receptors and TNF-α secretion by the isolated human monocytes in the absence or the presence of LPS. Based on the literature data, *Taxol* inhibits cell growth by two mechanism of cell cycle dependent and independent pathways. In cell cycle independent pathway, it rapidly down-regulates TNF-α receptors and stimulates TNF-α release by the isolated human monocytes in a similar way to LPS(Beatler et al., 1986; Ding *et al.*, 1990; Carswell *et al.,* 1975; Allen *et al.,* 1992; Nagahira et al., 2001). Based on our data presented in this report, the *Dendrostellera* extract and its purified component exerts similar effects on the isolated healthy human monocytes and the co-culture of human monocytes and two

leukemia cell lines with non additive and non synergistic properties between the plant extract and LPS.

Materials Methods

Human tumor necrosis factor alpha (TNF-?), E. Salmonella typhimurium LPS, RPMI 1640, Neomycin, Penicillin and other chemicals were purchased from Sigma Chemical Co.(Sigma-Aldrich Chime GmbH, Germany). Radioactive NaI^{125} was obtained from Atomic Energy Agency of Iran. Human leukopacks of normal donors were obtained from Blood Transfer Institute of Iran. Human leukemia cell lines (K562, CCKF-CEM) were obtained from Iranian Cell Bank.

Plant material

Aerial parts of *Dendrostellera lessertii* were collected from suburb of Central province at the end of spring. A voucher specimen was deposited in the herbarium of the faculty of Science, Tehran University. The plant material was dried, far from direct light. The dried leaves were separated from the stems and powdered. The powder was kept in a closed container in a cold room.

Extraction &active component

The powdered plant material(500 g) was extracted with ethanol-water (1:1, v/v) mixture for three times. The accumulated alcoholic extract was concentrated, under reduced pressure to a final volume of 500 ml. The concentrated extract was divided into 25 mL aliquots and kept at –20°C for further investigation. The accumulated solution was concentrated under the reduced pressure to a final volume of 1 ml. The residue was fractionated on a silica gel column (40×1.5 cm) using diethyl ether: chloroform mixture (8:2, 6:4 and finally 4:6, v/v) as the eluting solvents, into three fractions. The active component was purified from the second fraction using TLC techniques. The molecular weight of the purified compound was 662 mass unit, using FAB/MS. The purity of the isolated compound has been confirmed by TLC, HPLC, ^{1}HNMR and FAB/MS and the structure elucidation of the new compound has been successfully achieved and the results have been submitted for publication.

Iodination of Human TNF-?'

The radiolabeling of human TNF-α was achieved according to the published method (Greenwood et al.,1963*)*. Briefly, hTNF-α (500 μg/mL) was mixed with radioactive NaI (18.5î 10^6 Bq) and Chloramin T (1 mg/mL) and incubated 5 minutes at room temperature. Then 10 μL sodium metabisulphite solution (2 mg/mL) was added and after two minutes, 10 μL potassium iodide solution (5î 10^5 M in Tris-HCl buffer) was added. Separation of labeled hTNF-α from free Iodine was achieved by Sephadex G-25, which has been equilibrated with PBS (0.1 M, pH 6.8) containing 0.25% gelatin.

Monocytes Isolation

The modified method of Almedia and his coworkers (Almeida et al., 2000) was used for the gradient separation of the monocytes from the leukopakes. Briefly at first a Ficol-Hypaque gradient (density = 1.070 g/mL) and then a slight hyper-osmolar Percoll gradient (density = 1.064 g/mL) was used. Mixing one volume NaCl (1.5 M) with nine volumes of Percoll (density = 1.130 g/mL) was used for isosmotic Percoll preparation. The Percoll gradients was done by mixing isosmotic Percoll with PBS/citrate(13 mM). Both gradients were centrifuged at room temperature, 400 g for 35 minute [11, 12].

Cell cultures and treatments with stimuli

A total of 10^6 cells/mL were incubated, in duplicate, in 24 well plates, containing the medium(RPMI 1640 containing 10% BCF, 100 IU penicillin, plus 100 μg/mL of streptomycin). After 20 hours various concentrations of the stimuli were added to each well to a final volume of 1 mL. The cultures were incubated for 24 hours at 37°C in a 5% CO_2 atmosphere (for the receptor assays, the cells were incubated under the influence of the stimuli for not more than two hours). The stimuli were:

plant extract (0, 10, 20, 40, 80, 160, and 320 μL of a 1:100 dilution of the original plant extract with a concentration corresponding to 0.5 g plant leaves powder per mL), LPS (100 ng/mL) and *Taxol* (0, 5, 10, 20 and 40 μM).

Determination of hTNF-?

The hTNF-α level in the supernatant of each culture monocyte sample was achieved by our developed biotin-streptavidin enzyme immunoassay system (Hedayati et al., 2001). Each sample supernatant (or the diluted sample 1:10) was added to a well of a 96 well plate(100 µL/well). The plate was incubated at room temperature for one hour on a plate shaker. After washing, 100 µL of biotinylated antibody was added to each well, incubated at room temperature for one hour on a plate shaker and rinsed with the wash buffer(PBS with 0.05% TWEEN 20). Then 100 µl of streptavidin -HRP conjugate was added to each well followed by 30 minutes of incubation at room temperature while shaking on the plate shaker.

Finally the plate was washed and the color was developed by adding the substrate buffer solution containing TMB and H_2O_2 (100 µL/well). The reaction was stopped after 30 minutes by the addition of 100 µl of 1.8 M sulfuric acid. The optical density of each well was recorded at 450 nm in an ELISA plate reader (Spectra, Tecan, Austria). The levels of the TNF-α in each sample was established using the corresponding calibration graph.

TNF -?Geceptor assay

The treated cells, along with the corresponding control samples, were incubated with ^{125} I-TNF-α solution (100µL of iodinated solution per well) for different time intervals. The cells were then washed with the tissue medium followed by centrifugation at 300 g for 5 minutes. The washing was repeated for three more times.

Results and Discussion

Tumor necrosis factor (TNF)-α, secreted mainly from the activated monocytes/macrophage in response to diverse extracellular stimuli, plays a major role in inflammatory diseases. LPS, a surface component of gram-negative bacteria, is one of the known stimulator of TNF-α production in monocytes/macrophage and exert its effect at the transcriptional and translational levels (Urban *et al.,* 1986 and Carswell *et al.,* 1975). It has been reported that macrophages respond to TNF-α

by means of specific receptors, TNF-R(Carswell *et al.,* 1975) recently, it has been repoted that Taxol, a plant-derived antitumor drug with the capability of inhibiting microtubule-depolymerization (Schiff and Horwitz, 1981) acts on macrophage mimicking two effects of LPS: to decrease TNF-α secretion. Based on this data, we planned to evaluate the effects of D. mucronata crude extract and one of its purified active components, with proven strong anti-tumor activity (Yazdanparast *et al.,* 2003) on TNF-α release and TNF-R in comparison to Taxol effects using co-culture of two different leukemia cell lines and human monocytes.

Primarily, it was necessary to evaluate the potency of the crude extract and its purified component. As it is shown in Fig 1a, 50% growth inhibitions were observed at 160 µL of the crude extract (each mili-liter of the extract corresponds to 0.5 g of the plant leave powder) and 2.6 µM of the purified component (2.6 µM). These data clearly show that the purified component is biologically active.However, in the presence of the human monocytes, and under all identical conditions, cell viability decreased almost by a factor of four. In other words, the concentration of the plant extract or the purified component required for a 50% reduction in cell number, decreased from 160 µL to 30µL (for the crude extract) and from 1.4 µM to 0.4 µM for the purified component (Figure 2).

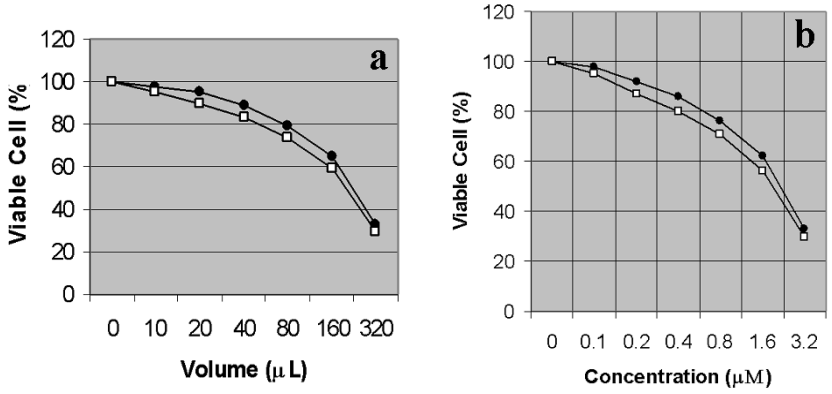

Figure 1 - The effects of *Dendrostellera* crude extract(a) and one of its purified component(b) on the survival rates of CCRF-CEM(?)# and K562(?)À cells.

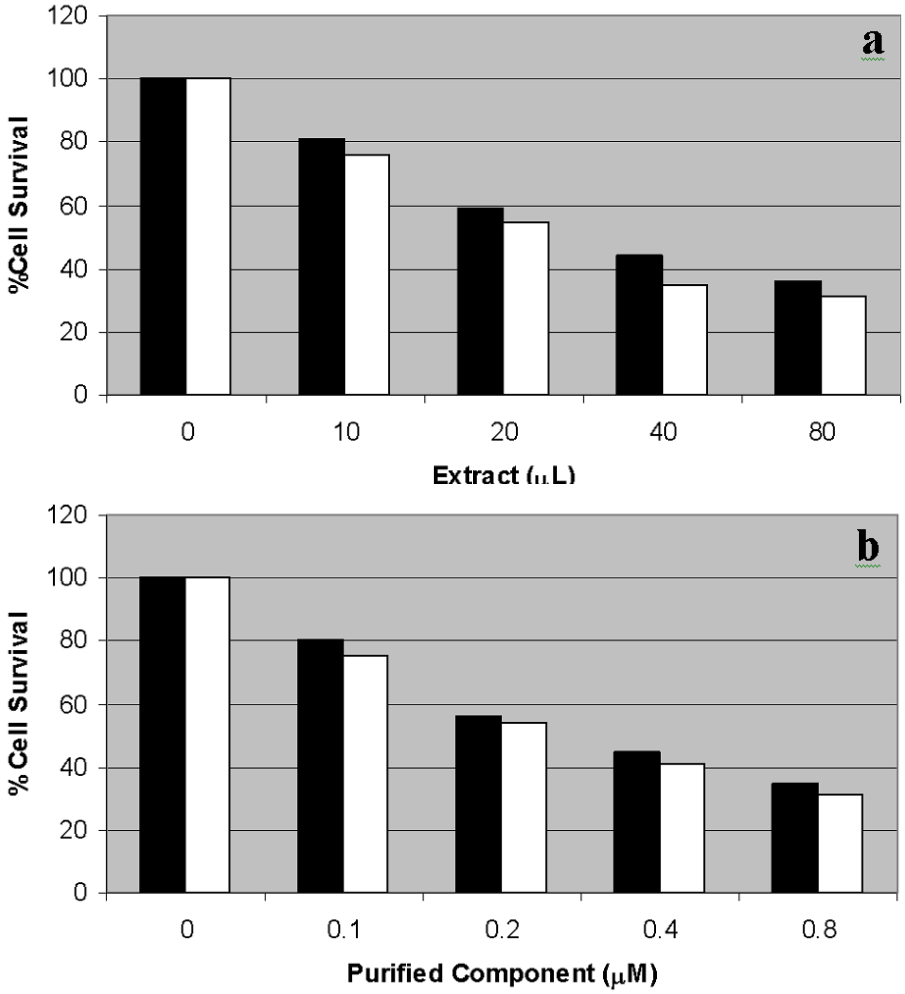

Figure 2 - The effects of *D.lessertii* crude extract(a) and the purified component(b) on the survival rates of CCRF-CEM (black) and K562 (white) cell lines in the presence of the isolated monocytes.

Lower cell viability in the co-cultured systems is probably due to the effects of the plant extract and its purified component on monocyte in enhancing TNF-α secretion. In that respect,TNF-α concentrations in

culture media with different amounts of extract and purified component were determined. Determination of TNF-α in the cell culture media of the co-cultures revealed that under the effect of the plant extract (Figure 3a) or its purified active component (Figure 3b) the concentration of TNF-α increase in a dose dependent way. Based on these observations, it may be concluded that the higher sensitivity of K562 or CCRF-CEM cells to *D.lessertii*, in the co-culture samples, is most probably due to the secretion of TNF-α by the monocytes. The TNF-α secretion activity of the isolated human monocytes was separately determined under the effects of various concentrations of the pure compound. Figure 4a shows that this compound, similar to LPS, induced TNF-α secretion by the monocytes. Taxol exerted, in accordance with the literature (Allen *et al.,* 1992; Ding *et al.,* 1990), similar effects on TNF-α secretion by the monocytes (Figure 4b). It is interesting to note that the effects of the purified compound and LPS on TNF-α secretion by the monocytes are neither additive nor synergistic. Additionally, the effects of the plant extract and its purified component, along with taxol, on TNF-R were also evaluated in the same culture systems. Figure 5a indicates that purified component, as the crude extract (data not shown), is capable of reducing the number of receptors in a dose dependent manner. In less than two hours, as in the case of *Taxol* (Figure 5b) the number of TNF-α receptors decreased to a non-detectable level. Based on these data, it may be concluded that *D.lessertii*, is capable of down regulating TNF-α receptors in the cultured monocytes. In that respect, the plant extract as its purified active component act exactly as taxol (Ding AH, et al, 1990). Further investigations are in progress to find out whether correlation exist between the finding of this report and the in vivo anti tumor activity of *D.lessertii* reported previously (Yazdanparast *et al.*, 2003).

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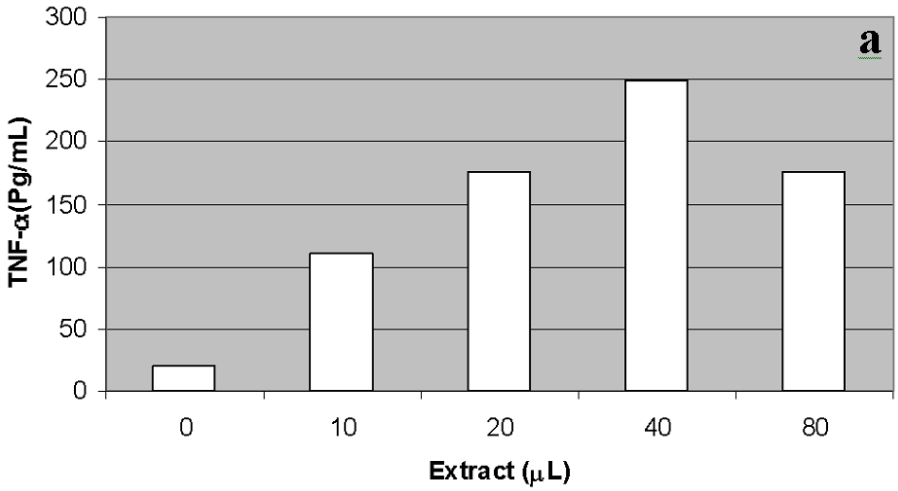


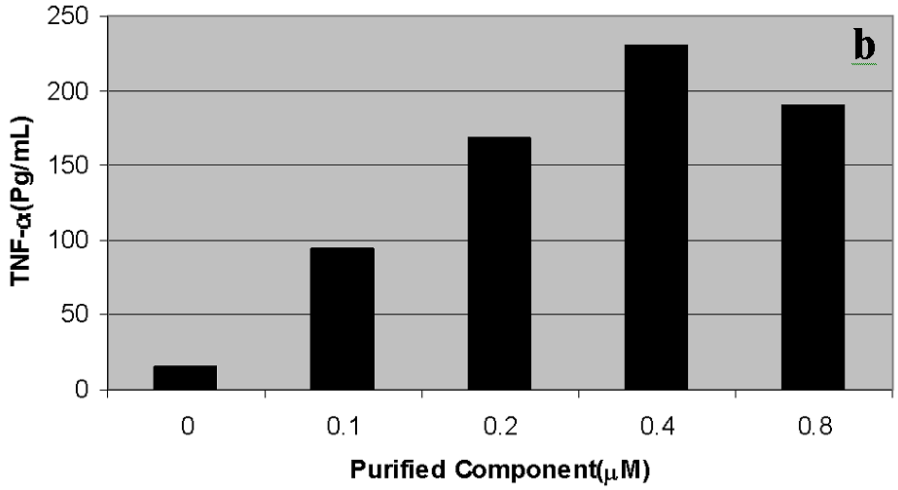
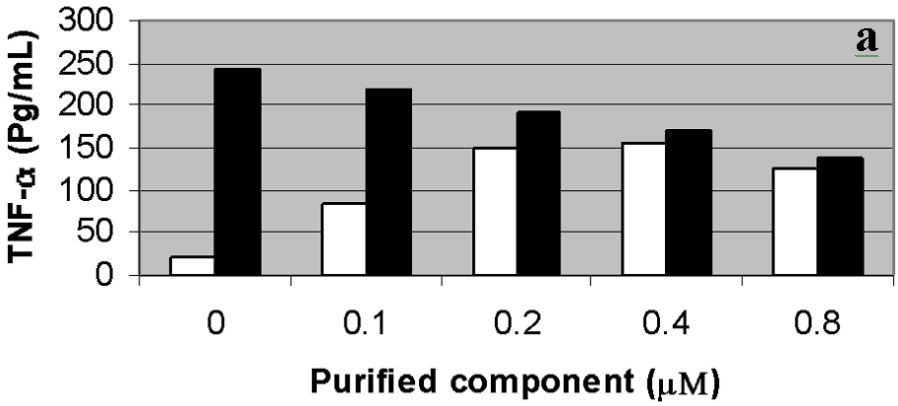


Figure 3 - The effects of *D.lessertii* crude extract(a) and the purified component (b) on TNF-?× secretion in the culture media of the co-culture of K562 cell lines with the isolated monocytes.



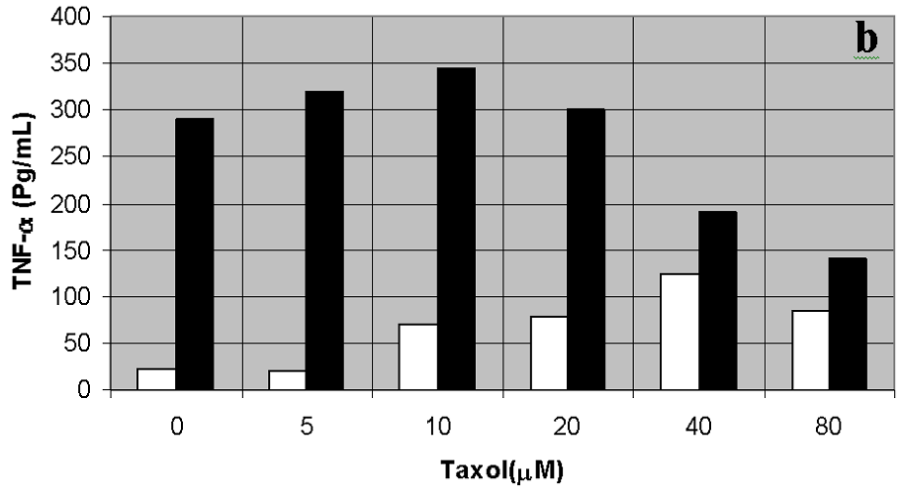
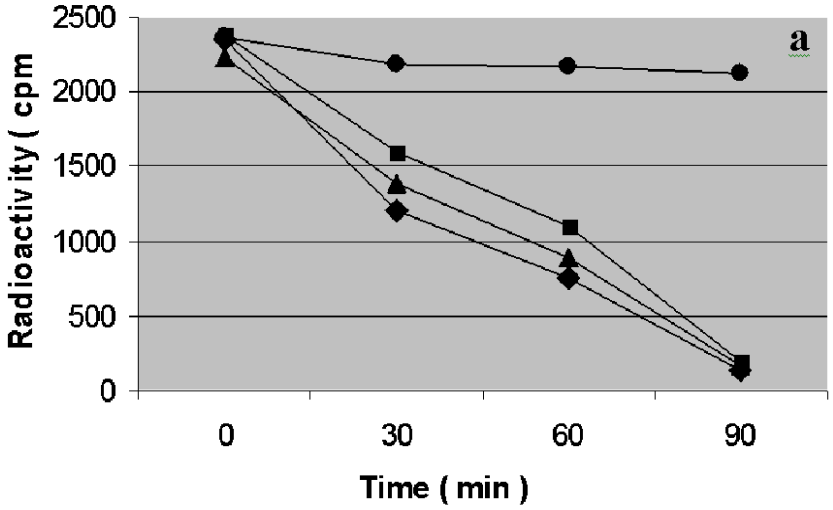


Figure 4 - The effects of purified active component of *D.lessertii* (a) or *Taxol* (b) On TNF-?b secretion by the isolated monocytes in the absence (white) or The presence (black) of 100 ng/mL of LPS.


a
Radioactivity (cpm
2500
2000
1500
1000
500
0
0
30
60
90
Time (min)

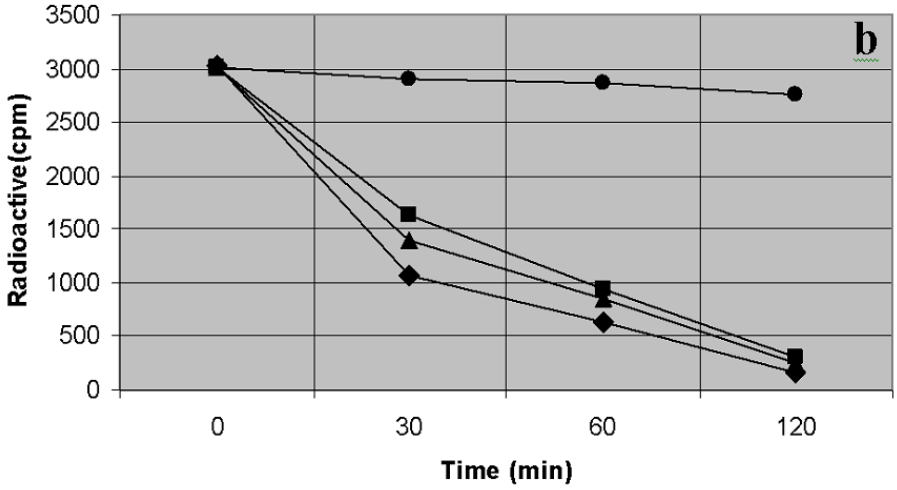

b
Radioactive(cpm)
3500
3000
2500
2000
1500
1000
500
0
0
30
60
120
Time (min)

Figure 5 - Time dependent down regulation of TNF-?7receptors in the isolated monocytes by (a) various concentration of the purified component of *D.lessertii* (0(?), 0.2(?), 0.4(?) and 0.8(?)?M) and (b) *Taxol* (0(?), 10(?), 20(?) and 40(?) ?M).

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