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European Journal of Experimental Biology, 2014, 4(2):164-171

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Cytotoxic and cytogenetic effects of aqueous and methanol crude extracts of *Nicotiana tabacum* on Rhabdomyosarcoma (RD) and L20B cell lines *in vitro*

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ABSTRACT

The present study was carried out to evaluate the cytotoxicity of aqueous and methanol crude extracts of locally Nicotiana tabacum leaves on two cell lines, Rhabdomyosarcoma (RD) tumor cell line, and Murine fibroblast (L20B) cell line in vitro. The cytogenetic effects of methanol crude extract was studied also on both cell lines by evaluation the mitotic index (M.I) after estimating cytotoxic concentration 50% (CC50%) value for extract. The results of cytotoxic effect of aqueous and methanol crude extracts of N. tabacum revealed that both extracts had a cytotoxic effect against both RD and L20B cell lines, in which a significant decrease in proliferation of RD tumor cell line was observed at 78.125 to 10000µg/ml of methanol crude extracts after 72hrs treatment. Aqueous extract showed cytotoxic effect on both RD and L20B cell lines at all concentrations also after 72hrs treatment. The CC50 values were 2100µg/ml for RD and 2150µg/ml for L20B cell lines after 72hrs exposure with methanol extract. The cytogenetic effects of methanolic crude extract of leaves of N. tabacum revealed a significant decrease in M.I in both cell lines.

Keywords: Cytotoxicity, *Nicotiana tabacum*, RD tumor cell line, L20B cell line, Cytogenetics, M. I.

INTRODUCTION

Rhabdomyosarcoma is the most common soft tissue sarcoma of childhood, with an annual incidence of four to seven per million children 15 years old. Approximately 250 new cases are diagnosed each year in the USA, and approximately 65% of cases are diagnosed in children less than six years old [**1]** .In Iraq the incidence of RD is about 3% of childhood cancer cases less than14 years of age in 2010 [**2]**. Al-Niaimi , (2006) [**3]** showed that the incidence of RD in north of Iraq is about 0.8% ,whereas in Mosul it is about 2.4% [**4]** and in Basrah RD constitutes about 5.94% of cancer [**5]**.

In last decades researchers used medicinal plants for studying antitumor pharmacological activity. Medicinal plants and natural products were used for cytotoxic study against tumor cell lines, because the plants represent promising sources of anticancer agents with lower side effect as compared with chemical drugs. *Nicotiana tabacum* is the largest genus of tobacco plant. *Nicotiana* consist of over sixty species in the Solanaceae family [**6]**. It is a double edged sword plant. Tobacco smoke causes many harmful disease to the body, while researches on *N. tabacum* leaves extract show that tobacco has many medical uses, as antibacterial, anti-inflammatory, cardiovascular effect and anticancer [**7**,**8**,**9]**. Tobacco leaves consist of many organic alkaloid compounds which found in all plant parts.

The organic compounds exist in tobacco are Nicotine, Nicotinine, Nicotelline, Nornicotine, Nicotyrine, resin, myosmine, albumin and glycoprotein. Tobacco leaves contain solanesol, a long chain terpenoids alcohol [**7]**,

polyphenols and carotinoids [**10]**, tocotrienols [**11]**. Rodu and Ou, (2000) [**12]**, demonstrated that tobacco products had a range of antioxidant activity from moderate to high, a significant linear relationship was observed between the overall antioxidant capacity and the total phenolic content. The high antioxidant explains why low cancer risk is associated with long term use of these products. Nacoulma *et al.*, (2012) [**13]** demonstrated that methanol and aqueous extracts of *N. tabacum* leaves contain phenolic and flavonoids which have antioxidant activity. Jiang *et al.*, (2001) [**14**] found that chlorogenic compound in tobacco leaves induces cytotoxic effect of cancer formation. Ramage *et al.*, (2006) [**15]** showed that *in vitro* treatment of A549 lung epithelial cells with nicotine, causes a significant increase in the number of apoptotic cells. In addition treatment with both taxol and nicotine significantly increase apoptotic cells among the cells that had detached from the culture plate, and showed that after 4hrs of incubation ,they caused reduction in mitochondrial cytochrome C and increase in cytosolic cytochrome C, which means increase in tumor cell line death. Elias *et al.*, (2010) [**8]** studied the cytotoxic activity of tobacco extracts on human oral squamous cell carcinoma cell lines, and demonstrated that tobacco contain compounds that are useful in inducing apoptosis in cancer cell. No study was found about the effect of aqueous and methanol crude extracts of local N. *tabacum* leaves in Iraq against the proliferation of RD tumor cell line and L20B cell line. Therefore the present study was designed to assess the cytotoxic effect of aqueous and methanol crude extracts of N. *tabacum* on RD and L20B cell lines and cytogenetic effect of methanol crude extract of *N. tabacum* on the M.I of RD and L20B cell lines.

MATERIALS AND METHODS

Plant collection

Nicotiana tabacum leaves were obtained from Khosnawate region in Kurdistan-Iraq. The plant was identified in the Department of Biology, College of Science, Salahaddin University, Erbil, Iraq. Tobacco leaves were dried at room temperature (RT) and then grounded into powder by electrical grinder (mesh No. 0.5mm). The powder was kept in plastic bags in deep freezer (-20°C) until use [**16]**.

Preparation of Aqueous extract of *Nicotiana tabacum* leaves

Aqueous extract was prepared using the procedure described by [**16]**. Fifty grams of powdered dried leaves of tobacco plant were suspended in 200 ml of distill water (D.W) in conical flask and stirred using magnetic stirrer over night at 45˚C.The resultant filtrate extract was gently poured into pre-weighed glass Petri dishes and placed open in an electric oven at 37°C for two to three days till dryness. The yield of extraction percentage was calculated.

Preparation of methanol crude extract of *Nicotiana tabacum* leaves

In order to obtain the methanol extract, fifty grams of the powder from *Nicotiana tabacum* leaves were placed in a flask, with 300 ml of absolute methanol and tightly sealed. The mixture was left to stir on a magnetic stirrer for 3 days at RT. Extract was obtained using the procedure described by [**17]**. The yield of extraction percentage was calculated.

Biochemical tests of *Nicotiana tabacum leaves* extracts

One gm of each aqueous and methanol extract was dissolved into 100 ml phosphate buffer (PBS) (as solvent). The suspension was then filtered and sterilized using 0.45µm and 0.22 µm sterile Millipore filters and kept in deep freeze at -20˚C until use.

Different biochemical tests were used to detect the presence of some biological active compounds, Liebermann-Burchard test was used to test the presence of triterpenoids. Whereas the presence of glycosides was detected according to [**18]**.Wagner's Reagent and Hager's Reagent were carried out on plant extracts to test the presence of alkaloids. Ferric chloride solution and lead acetate solution according to [**16]** were used to test the presence of polyphenol (tannins). The presence of flavonoids in extracts was carried out using the procedure described by [**18]**. Saponins were identified according to [**16]**.

Cell lines

Rhabdomyosarcoma (RD) cell line was provided by Iraqi centre for cancer and medical genetic research (ICCMGR)/ Baghdad/Iraq. This human cell line was derived from a biopsy specimen obtained from a pelvic RD of a seven year old Caucasian girl [**19]**. Passages (217-226) of RD cell line were used throughout this study. Cells were grown in RPMI-1640 (Sigma, USA) with L-glutamine and Hepes , each 1000 ml of RPMI-1640 were supplemented with 100ml of fetal calf serum, benzyl penicillin solution 0.5 ml (1000000 IU), and 0.5 ml Streptomycin. Sodium bicarbonate 2 gm was added to give a final pH of 7.2. The final medium was then sterilized using 0.22 µ sterile Millipore filter paper and stored at 4 ^{o}C before use.

L20B cell line was kindly provided by (ICCMGR). L20B is a murine cell line derived from mouse L cells (fibroblasts) expressing the human polivirus receptor [**20]**. Passages number (10 to 15) of L20B were used in this study in Eagles MEM (Sigma, USA).One liter medium was supplemented with L-glutamine , 10% Hepes 10mM, 10% FBS, sodium bicarbonate (4.4%) 5-10 ml, benzyl penicillin solution 0.5 ml (1000000 IU), and streptomycin 0.5 ml.

The following formula was used according to [**21]** to calculate the viability of cell lines by using 1% Trypan blue Stain (Fine Chemical,Sweden Pharmac), $C = N \times D \times 10^4$, where C is the number of viable cells per milliliter, N is the number of viable cells counted, and D is the dilution factor (D= 10). A sample of cells counted by using an improved double Naubauer ruling counting chamber.

Cytotoxicity study

The cytotoxicity protocol was according to [**22]**. About 200 µl of cells suspended (55000 cells/ml) in growth medium were seeded in to each well of a sterile 96-well micro-titration plate. The plates were incubated at 37°C, 5% CO2 incubator. When the cells are in exponential growth (approximately 70-80% confluent monolayer), the medium was removed and serial dilutions of each aqueous or methanol crude extract in maintenance medium (10000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125µg/ml) were added to the wells. Three replicates were used for each concentration of either extract. Three wells used for seeding cells in maintenance medium with PBS used as control. The plates were re-incubated at 37°C, 5% CO2 incubator for the selected exposure times (24 , 48 ,and 72hrs). Then the supernatants were removed from the wells, and 50 µl of 0.01% neutral red dye were added to each well, and re-incubated for 2 hrs; at the end of incubation, excess dye was removed by washing the wells twice with 150 µl PBS, then 125 µl of extraction dye solution was added [**22]**. The optical density (O.D) of each well was read using Enzyme Linked Immunosorbent Assay (ELISA) reader at a transmitting wavelength on 492 nm [**21]** .

The cytotoxicity percentage was calculated as (A-B)/A X100, where A is the mean O.D of untreated wells and B is the O.D of wells with plant extracts [**23]**. The cytotoxic concentration 50% (CC50%) for each extract was calculated from concentration-effect-curves after linear regression analysis [**24]**.

Cytogenetic Study on RD Tumor Cell Line and L20B cell line

Three replicates of RD tumor cell line were treated with 2100, 1050, and 525µg/ml of *Nicotiana tabacum* leaves methanolic crude extract. Also three replicates of L20B cells were treated with 2150, 1075, and 537.5µg/ml methanolic crude extract of *Nicotiana tabacum* leaves. Another three culture flasks were used as a control group treated with culture medium only. Then all flasks were incubated at 37°C and 5% CO_2 for 72hrs.

The chromosomes were prepared according to [**25]**. One thousand cells were examined under 40X objective lens using light microscope (Proway, China).

The M.I % was calculated according to [**26]** as follow:

M.I. % = (No. of dividing cells / No. of dividing cells + No. of non-dividing cells) X 100.

Statistical Analysis

Analysis of variance (ANOVA) and the least significant difference (LSD) were used for the statistical analysis of the results and P-values at levels ($P<0.01$) was considered to be statistically significant. These calculations were carried out according to program of SPSS, version 18 [**27]**.

RESULTS AND DISCUSSION

The result of present study shows that the highest percentage of yield extract was (%9.2) from methanol crude extract (Table-1). The same table shows the color of each type of extract. Whereas the chemical tests of aqueous and methanol extracts of N. *tabacum* are summarized in table (2).

Cytotoxic effects of methanol and aqueous crude extracts of *N. tabacum* leaves on RD and L20B cell lines *in vitro*

Both RD and L20B cell lines showed variable responses when treated with the methanol crude extract of *N. tabacum* leaves. Statistical analysis demonstrated that after 24hrs (Table-3) and 48hrs (Table- 4) of treatments, RD cell line was not affected when treated with all concentrations of methanol and aqueous crude extracts of *N. tabacum* leaves. Similarly, L20B cells those treated with crude aqueous extract of *N. tabacum* were not affected (Table -5 &6) .The activity of all these extracts against the proliferation of RD cells (Table-7) and L20B cells (Table-8) was increased after 72hrs of treatment at concentrations from 78.125 to 10000µg/ml. Our results in table (9) show the

effect of exposure time overall concentrations, this table reveals that the activity of aqueous extract against the proliferation of RD cells was more effective after 72hrs treatment, whereas methanol extract showed the same activity against RD cells after 48 and 72hrs treatment.

Table (1): Color and percentage of yield extracts of dried and solutions of N. *tabacum*

Plant	Extraction	Nature and Color of extracts	Solution color	(%) Yield
N. tabacum	Aqueous	Solid----Brown	Brown	6.5
	Methanolic	Viscous---Dark Brown	Brown	9.2

Table (2): Chemical compounds of *N. tabacum* leaves crude extracts

Plant	Extracts	Phytochemical Compounds						
		Flavonoids	Alkaloid		Glycosides	Triterpenoids	Tannins	Saponins
			D	W				
N. tabacum	Aqueous	–	+	+	+	+	+	+
	methanolic	+	+	+	+	–	+	+

D= Dragendorff test; W= Wagner's test; + = Positive reaction for designated phytochemicals; – = Negative reaction for designated phytochemicals

Table (3): Mean ± S.E for the effect of *N. tabacum* (Aqueous and methanolic) crude extracts on the proliferation of RD cell line after 24hrs treatment

Plant	Concentrations (µg/ml)	Extracts	
		Methanolic	Aqueous
N. tabacum	0	0.327±0.004	0.327±0.004
	78.125	0.338±0.002	0.314±0.002
	156.25	0.347±0.001	0.324±0.002
	312.5	0.336±0.002	0.334±0.002
	625	0.342±0.001	0.331±0.001
	1250	0.203±0.001	0.453±0.001
	2500	0.317±0.001	0.343±0.001
	5000	0.250±0.003	0.298±0.001
	10000	0.120±0.0001	0.226±0.002
Effectors	Extracts	Concentrations	Extracts & Concentrations
LSD(0.05)	0.14	0.27	0.17

S.E= Standard Error, LSD= Least significant difference 0.05

Table (4): Mean±S.E for the effect of *N. tabacum* (Aqueous and methanolic) crude extracts on the proliferation of RD cell line after 48hrs treatment

Plant	Concentrations (µg/ml)	Extracts	
		Methanolic	Aqueous
N. tabacum	0	0.226±0.002	0.226±0.002
	78.125	0.313±0.001	0.232±0.001
	156.25	0.206±0.001	0.232±0.001
	312.5	0.229±0.0001	0.306±0.001
	625	0.207±0.001	0.232±0.001
	1250	0.115±0.001	0.224±0.001
	2500	0.256±0.001	0.224±0.002
	5000	0.256±0.002	0.290±0.001
	10000	0.093±0.001	0.212±0.001
Effectors	Extracts	Concentrations	Extracts & Concentrations
LSD(0.05)	0.11	0.20	0.12

S.E= Standard Error, LSD= Least significant difference

Significant effects (P ≤0.001) were obtained also after treatment of L20B with methanol or aqueous crude extract of *N. tabacum* leaves regarding to the exposure time, so a highly effect was noticed on the cell growth after treatment with each extract after 72hrs (Table-10).The acceptable explanation to that can be traced to the difficulties in transport of molecules of crude extracts across the plasma membranes of RD and L20B cells. The result of the present study shows that the proliferation of L20B cells was decreased at both concentrations 5000 and 10000µg/ml after 24hrs (Table-5). After 48hrs treatment the effect of methanol crude extract showed its activity at 10000µg/ml only (Table- 6). All concentrations of methanol crude extract revealed their activity against the proliferation of L20B cell line after 72hrs treatment (Table- 8). These results indicated that most compounds present in aqueous and methanol crude extracts; triterpenoids, glycosides, alkaloids, polyphenol, flavonoids, and saponins, might be the causative agent to cell death. This death occurs due to different mechanisms of action (i.e. apoptosis). The activity of terpenoids in crude extracts of N. *tabacum* against the proliferation of tumor cell lines was studied by **[28];** they found that these compounds had ability to induce cellular death by apoptosis that already known in various tumor cells. Other studies have shown also the flavonoids compounds induce cell death by apoptosis [**29]**. The study of

Yang *et al.*, (2005) [**30]** indicated that tobacco leaves contain chlorogenic acid and many other natural polyphenol compounds that showed antiproliferative activity. Zhou and Lin, (2006) [**7]** showed that solanesol, a long-chain terpenoid alcohol mainly existing in tobacco leaves, is the starting material for many high-value biochemicals, including vitamin K analogues and co-enzyme Q10 which is useful in the treatment of heart diseases, cancers and ulcers. Solanesol itself can be used as lipid antioxidant and antibiotics, and clinical trials are also developing the use of solanesol as an anti-cancer [**7]**. The result of present study showed that both cell lines were revealed sensitivity to the methanol crude extract of *N. tabacum* with the CC50 values 2100 µg/ml at 72hrs for RD cell lines, while for L20B cell line, the CC50 was 2150 µg/ml for 72hrs. Elias *et al.*, (2010) [**8]** studied the cytotoxic effect of tobacco crude extract (TCE) on human lineage of oral squamous cell carcinoma (OSCC-3), in which they found that exposure of human oral cancer cells to TCE induced cell death and decreased cell viability in a dose dependent manner, and after 48hrs the CC50 significant cell death was observed. The fragmentation of DNA and caspase-3 activation indicate that the type of cell death induced by TCE was apoptosis [**8]** .The results indicated that tobacco contains compounds that could be useful in inducing apoptosis in cancer cell. Another study carried out by [**31]** supported this finding they studied the effects of nicotine on lymphocyte proliferation and showed that nicotine activates the nuclear factor of activated T cells c2 (NFATc2), and reduces CDK4 expression leading to cytokine unresponsiveness and cell cycle arrest. The inhibitory effects of nicotine on lymphocyte proliferation may be due to inappropriate initiation of activation cascades that ultimately lead to an anergic state by increasing intracellular calcium (Ca^{2+}) levels. However other studies showed the reverse, in which the nicotine induced phosphorylation in association with the suppression of apoptosis, via the anti-apoptotic pathway in head and neck squamous cell carcinomas (HNSCC) [**32]**.

Table (5): Mean ±S.E for the effect of *N. tabacum* (Aqueous and methanolic) crude extracts on the proliferation of L20B cell line after 24hrs treatment

Plant	Concentrations (µg/ml)	Extracts	
		Methanolic	Aqueous
N. tabacum	0	0.201±0.001	0.201±0.001
	78.125	0.205±0.002	0.224±0.001
	156.25	0.182±0.033	0.213±0.002
	312.5	0.199±0.001	0.203±0.001
	625	0.204±0.003	0.204±0.001
	1250	0.207±0.001	0.203±0.001
	2500	0.205±0.002	0.193±0.002
	5000	0.093±0.001	0.191±0.0002
	10000	0.091±0.001	0.197±0.0001
Effectors	Extracts	Concentrations	Extracts & Concentrations
LSD(0.05)	0.10	0.10	0.06

S.E= Standard Error LSD= Least significant difference

Table (6): Mean±S.E for the effect of *N. tabacum* (Aqueous and methanolic) crude extracts on the proliferation of L20B cell line after 48hrs treatment

Plant	Concentrations (µg/ml)	Extracts	
		Methanolic	Aqueous
N. tabacum	0	0.248±0.0002	0.248±0.0002
	78.125	0.243±0.0004	0.244±0.001
	156.25	0.246±0.0006	0.245±0.001
	312.5	0.214±0.001	0.245±0.001
	625	0.214±0.001	0.211±0.001
	1250	0.175±0.001	0.233±0.001
	2500	0.246±0.001	0.233±0.002
	5000	0.216±0.003	0.237±0.001
	10000	0.018±0.001	0.114±0.013
Effectors	Extracts	Concentrations	Extracts & Concentrations
LSD(0.05)	0.15	0.14	0.07

S.E= Standard Error, LSD= Least significant difference

Cytogenetic effects of *N. tabacum* on RD and L20B cell lines *in vitro*

This result shows that all concentrations of *N. tabacum* methanol crude extract 2100, 1050, and 525µg/ml had a significant reduction effect on M.I% of RD tumor cell lines after 72hrs treatment, 12.6±0.66, 14.6±0.33, and 19.3±0.33, respectively as compare with control group 29.3±3.17 (Table- 11). L20B cell lines also showed significant decrease in M.I% after 72 hrs of treatment with 2150, 1075, and 537.5µg/ml of *N. tabacum* methanol extract, 15.6±1.2, 14.6±0.33 and 21.3±0.88, respectively as compared with the control, 25.6 ± 0.88 (Table- 11). This activity may be due to the presence of chlorogenic acid in tobacco leaves. Chlorogenic acid is a natural antioxidant and anticancer agent [**14]**. Analytical data also indicated that the concentration of chlorogenic acid and rutin is the highest (75%-95%) among the polyphenol compounds in tobacco leaves [**33]**. Calderon *et al.*, (2007)

[**34]** showed that the nicotine and cotinine exposure (that caused oxidative damage to DNA) may have implications in the decrease in cell replication (replication index) due to direct damage to DNA and/or a decrease in the DNA repair mechanisms. Alternatively, nicotine and cotinine may possibly induce apoptosis. Onoda *et al.*, (2001) [**35]** showed that nicotine adversely affects the cytotoxicity of DNA-damaging agents. Nicotine does not interfere with the repair of the damage but directly affects the signaling of the death pathway, reducing the signaling of the JNK1 pathway. The latter resulted in a decrease in efficacy of the anticancer treatment in tumors exposed to nicotine.

Table (7): Mean±S.E for the effect of *N. tabacum* (Aqueous and methanolic) crude extracts on the proliferation of RD cell line after 72hrs treatment

Plant	Concentrations (µg/ml)	Extracts	
		Methanolic	Aqueous
N. tabacum	0	0.245±0.002	0.245±0.002
	78.125	0.225±0.001	0.233±0.001
	156.25	0.224±0.001	0.204±0.001
	312.5	0.218±0.0003	0.223±0.001
	625	0.193±0.001	0.224±0.0005
	1250	0.219±0.002	0.220±0.004
	2500	0.173±0.001	0.201±0.0001
	5000	0.244±0.001	0.164±0.008
	10000	0.181±0.001	0.230±0.001
Effectors	Extracts	Concentrations	Extracts & Concentrations
LSD(0.05)	0.03	0.01	0.07

S.E= Standard Error, LSD= Least significant difference

Table (9): Mean ±SE for the effect of exposure time to aqueous and methanolic crude extracts of *N. tabacum* on the growth of RD tumor cells *in vitro*. (Observations of O.D)

Extract	Time/hrs			L.S.D
	24	48	72	
Aqueous	0.328±0.012	0.244±0.006	0.213±0.004	0.02
Methanol	0.282±0.016	0.209±0.014	0.210±0.004	0.03

S.E= Standard Error, SE=standard error.

Table (8): Mean±S.E for the effect of *N. tabacum* (Aqueous and methanolic) crude extracts on the proliferation of L20B cell line after 72hrs treatment

		Extract	
Plant	concentration(µg/ml)	Methanolic	Aqueous
N. tabacum	0	0.253±0.002	0.253±0.002
	78.125	0.237±0.002	0.212±0.001
	156.25	0.245±0.001	0.224±0.002
	312.5	0.236±0.002	0.234±0.002
	625	0.242±0.001	0.231±0.001
	1250	0.203±0.001	0.213±0.001
	2500	0.163±0.002	0.213±0.001
	5000	0.224±0.001	0.228±0.0002
	10000	0.140±0.001	0.126±0.002
Effectors	Extracts	Concentrations	Extracts & Concentrations
LSD(0.05)	0.09	0.01	0.04

S.E= Standard Error, LSD= Least significant difference

Table (10): Mean ±SE for the effect of exposure time to aqueous and methanolic crude extracts of *N. tabacum* on the growth of L20B cells *in vitro*, (Observations of O.D)

Extract	Time/hrs			L.S.D
	24	48	72	
Aqueous	0.19±0.008	0.22±0.008	0.21±0.006	0.02
Methanol	0.173±0.01	0.195±0.015	0.211±0.007	0.03

SE=standard error.

Table (11): Mean±SE for mitotic index of RD and L20B cells after treatment with *N. tabacum* methanolic extract after 72hrs treatment

	Methanolic extract of *N. tabacum* µg/ml	**%MI**
LSD	0	29.3±3.17
	2100	12.6±0.66
	1050	14.6±0.33
	525	19.3±0.33
	5.35	
	Methanolic extract of *N. tabacum* µg/ml	**%MI**
LSD	0	25.6±0.88
	2150	15.6±1.2
	1075	14.6±0.33
	537.5	21.3±0.88
	2.87	

SE=standard error

CONCLUSION

Methanol crude extract had more inhibitory effect in different concentrations and different time of exposure on RD and L20B cell lines. Both cell lines were more sensitive to methanol crude extract than aqueous extract. Methanol extracts of *N. tabacum* have antimitotic activity on RD and L20B cells *in vitro*.

Acknowledgements
The authors wish to thank the University of Salahaddin College of Education for support this work.We also express our deep thanks to the head and the staff of Iraqi Center for cancer and medical genetic research/Baghdad/Iraq for their help.

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