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Cytotoxicity studies of *Tamus communis* root and berries extracts on human HEp-2 and AMN-3 carcinoma cells

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Abstract

Plan
In the present study, the steroidal fraction was isolated from the ethanolic extract of T.communis root and berries (TCRE &TCBE, % yield: 0.035 and 0.032 respectively) and screened for their cytotoxicity against human epidermoid laryngocarcinoma (HEp-2) and Ahmed-Mohammad-Nahi-2003 mammary adenocarcinoma (AMN-3) cell lines.

Methodology
Tamus communis (F: Dioscoreaceae) is a plant, growing wildly in the northern part of Iraq, mainly contains the steroidal sapogenin, diosgenin, in its roots and berries. In the present study, the steroidal fraction was isolated from the ethanolic extract of T.communis root and berries (TCRE &TCBE, % yield: 0.035 and 0.032 respectively) and screened for their cytotoxicity against human epidermoid laryngocarcinoma (HEp-2) and Ahmed-Mohammad-Nahi-2003 mammary adenocarcinoma (AMN-3) cell lines. The presence of diosgenin was confirmed in the extract by HPLC methods with standard diosgenin sample.

Outcome
The plant extracts, TCRE and TCBE showed better cytotoxic effect (IC_{50} 342.56 µg/ml and 386.55µg/ml) on HEp-2 cell line than pure diosgenin (IC_{50} 409.29 µg/ml), while diosgenin was more toxic to AMN-3 cell lines (IC_{50} 360.04µg/ml) compared to the plant extracts (TCRE: IC50 400.28 and TCBE: 465.64µg/ml respectively).

***Key words**: AMN-3 cell lines, HEp-2 cell lines, cytotoxicity, TCRE, TCBE, diosgenin*

1. Introduction

Tamus communis(F: Dioscoreaceae) is a climbing plant[1,2] in woods and hedges with weak stems twining round anything within reach, and thus ascending or creeping on the trees and bushes. In Iraq, the plant is distributed[3] in many areas like Erbil and Sulaimanya and has common local names such as Al-Karam Al-barri, Black Bryony, Lady's Seal, Nueza Negra, Siyah Akasma, Mewa Marwana, and Dara Liner etc. The plant is considered poisonous due to its diosgenin (steroidal sapogenin) content.

In folklore medicine the plant is used as cathartic, diuretic, emetic, hemolytic, and rubefacient. A poultice prepared from the fresh root is used for removing discoloration caused by the bruises, scars, and black eyes, etc [1, 4].*T.communis* root saponins normally considered as a dangerous irritant poison and the poisoning symptoms include irritant purgation with burning of the mouth and skin blister[1, 4, 5]. A tincture made from the root and fruits are used for curing unbroken chilblains in Iraqi folklore medicine[5].

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Reports showed that diosgenin has antibacterial activity against *E.coli* and *P.aeruginosa* [6, 7]. In the light of the above folklore claims and reports, an attempt has been made to evaluate the cytotoxic activity of *Tamus communis* plant extract and pure isolated diosgenin on two cell lines (HEp-2 and AMN-3) in this study.

2. Materials and methods

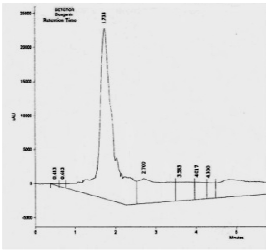
2.1. Plant material

The roots and berries of the plant *Tamus communis* (Dioscoreaceae) were collected during the months of September and October from Shaqlawa city, Erbil. The plant was authenticated by National Iraqi Herbarium, Botany Directorate at Abu-Ghraib. Voucher specimens (PCOG /321/CP-BU/2004) were deposited at the herbarium of the Pharmacognosy lab, College of Pharmacy, Baghdad University.

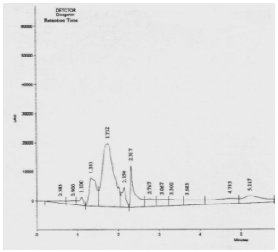
2.2. Extraction of T.communis saponins

A 50 gm quantity each of the dried powdered plant material (berries & roots) was separately soaked in water for 24 h, extracted with 80% ethanol (500ml) in a soxhlet extractor for 10 h, and then evaporated to dryness under vacuum. The residue was reflexed with 2N sulfuric acid / water containing 70% 2-propanol (500ml) at 100 ^{o}C for 4 h. The residue was neutralized with 5% ammonia and partitioned with equal volume of petroleum ether (60-80 °C) using a separating funnel .The petroleum ether layer was taken and evaporated to dryness under vacuum. The yield obtained was 0.035 % (from roots) and 0.032 % (from berries). The residues obtained from the berries and roots were designated as TCBE& TCRE respectively. Phytochemical screening [ref] was performed to confirm the presence of steroids in the extract.

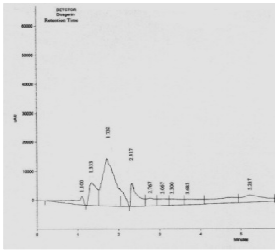
Qualitative HPLC analysis[23] was done using C18-column using the mobile phase, acetonitrile: water (25:75), with a flow rate of 1.5 ml/min. The measurements were carried out, using UV detector, at 205 nm. The retention time for the diosgenin (1.732) present in TCRE and TCBE were recorded and found to be identical to the standard diosgenin reference.



HPLC of Diosgenin



HPLC of TCRE



HPLC of TCBE

2.3. Anticancer activity studies

Cell line toxicity study of *Tamus communis* root and berry extracts (TCRE and TCBE) were carried out in Iraqi center for Cancer and Medical Genetic Research. The two cell lines: HEp-2 (Human epidermoid laryngocarcinoma) and AMN-3 (Ahmed-Mohammad-Nahi-2003 mammary adenocarcinoma) were selected for the studies. The investigation included the comparative study between the cytotoxic activity of the plant extracts, TCRE & TCBE and that of pure diosgenin isolated from *Tamus communis*.

The data obtained was statistically analyzed using student T-test. The IC_{50} value of plant extracts (TCRE &TCBE) and diosgenin were determined graphically from the dose response curve for the cell lines selected.

2.4. Culture of Cell lines [8]

Human epidermoid laryngocarcinoma cells from a 57-years old male and mammary adenocarcinoma cells from a female mouse were cultured in culture bottles (falcons) then stored at 20 °C.The cultured cells were washed with phosphate buffer saline (PBS). The trypsine-versine solution was added with gentle shake, then the final mixtures were poured to another culture bottles (sub-culturing) and incubated at 37 °C for 15 minutes. Counting of viable cells was carried out using trypan-blue dye (0.4%), dead cells take up the dye and appear blue under microscope while living cells exclude the dye and appear white.

Cytotoxicity studies of the plant extracts (TCRE, TCBE) and pure diosgenin were carried out using crystal violet cytotoxic assay. The plant extract and pure diosgenin were dissolved in dimethylsulfoxide, then four concentrations (62.5, 125, 250, 500 µg/ml) of each plant extract and pure diosgenin were prepared. 0.2 ml of cultured cells were transported to 96-well microplates, so each well contained 10^5 cells, followed by addition of 0.2 ml of prepared concentrations of plant extract and pure diosgenin, leaving some wells contained cultured cells but without any treatment by plant extract or diosgenin, used as negative control. The cells were incubated for 48 h at 37 °C, and then washed with PBS (sodium chloride :8 gm, potassium chloride : 0.2 gm, sodium phosphate : 0.15 gm, potassium phosphate : 0.5 gm and distilled water: 1000 ml) followed by addition of crystal violet solution (0.4%) and incubation for 20 minutes at 37 °C. The cells were washed with PBS and the absorbance at 492 nm was measured for each concentration which represents the cell viability in each cell line. The method repeated three times for each concentration of plant extract and isolated diosgenin.

Results (table1&2) proved that both of plant extract and isolated diosgenin have cytotoxic activity on both cell lines. Statistical analysis revealed significant differences between means of cell viability of each, HEp-2 and AMN-3 cultures, treated with plant extract at concentrations of 125, 250 and 500 µg /ml and those of negative control ($P<0.05$), while plant extract at concentrations of 62.5 µg/ml showed no significant effect on cell viability ($P>0.05$).

Table 1: Cytotoxicity studies of TCRE, TCBE, and Diosgenin on HEp-2 cell lines.

Student t –test, ** p value < 0.001, * p value < 0.01

Groups	*Con. (µg /ml)*	*Cell viability (absorbance)*			*Mean S.D*	*IC50 µg/ml*
	500	0.199	0.294	0.220	0.237±0.312	
	250	0.269	414	0.363	0.34±0.154	409.29
Diosgenin	125	0.515	0.519	0.484	0.492±0.256	
	62.5	0.563	0.590	0.519	0.557±0.221	
	500	0.180	0.182	0.214	0.192±0.112	
	250	0.259	0.276	0.364	0.301±0.187	342.56*
TCRE	125	0.442	0.347	0.485	0.424±0.171	
	62.5	0.509	0.448	0.496	0.484±0.224	
	500	0.210	0.225	0.234	0.223±0.108	
	250	0.322	0.356	0.348	0.342±0.212	386.55
TCBE	125	0.485	0.474	0.490	0.483±0.129	
	62.5	0.524	0.487	0.495	0.502±0.112	
Negative control	-	0.598	0.537	0.574	0.569±0.312	

TCRE &TCBE: *T.communis* root and berries ethanolic extract

Table 2: Cytotoxicity studies of TCRE, TCBE, and Diosgenin on AMN -3 cell lines.

Student t –test, ** p value < 0.001, * p value < 0.01

Groups	*Con. (µg /ml)*	*Cell viability (absorbance)*			*Mean S.D*	*IC50 µg/ml*
Diosgenin	500	0.190	0.177	0.226	0.197±0.122	360.04*
	250	0.315	0.327	0.391	0.344±0.202	
	125	0.471	0.469	0.408	0.449±0.255	
	62.5	0.513	0.518	0.498	0.509±0.144	
TCRE	500	0.182	0.232	0.244	0.219±0.234	400.28
	250	0.411	0.343	0.441	0.398±0.102	
	125	0.438	0.456	0.501	0.465±0.212	
	62.5	0.507	0.481	0.492	0.493±0.188	
TCBE	500	0.250	0.245	0.255	0.25±0.233	465.64
	250	0.433	0.396	0.385	0.404±0.122	
	125	0.424	0.478	0.470	0.457±0.151	
	62.5	0.520	0.554	0.545	0.539±0.104	
Negative control	-	0.548	0.609	0.575	0.577±0.089	-

TCRE &TCBE: *T.communis* root and berries ethanolic extract

The results showed that cell survival in both plant extract and isolated diosgenin treated cultures was progressively decreased with increasing concentrations. The data showed that the plant extracts , TCRE and TCBE, had better cytotoxic effect (IC_{50} 342.56 µg/ml and 386.55µg/ml) on HEp-2 cell line than pure diosgenin (IC_{50} 409.29 µg/ml), while AMN-3 cell line was more sensitive to diosgenin (IC_{50} 360.04µg/ml) compared to plant extracts, TCRE and TCBE (IC_{50} 400.28 and 465.64µg/ml respectively).

3.Discussion

Diosgenin [9,10,11,12,13,14,15] inhibits cancer cell proliferation by induction of phosphorylated-p53 expression and localization in osteosarcoma, laryngocarcinoma, melanoma cells etc. It induces cell cycle arrest at S phase in human laryngocarcinoma, at G2/M phase in melanoma, at G1 phase in osteosarcoma by p53 activation, and G2/M arrest in human leukemia independently of p53, with lowering in cyclin B1 level [13, 14, 15]. Reports[16, 17, 18, 19] again indicated that diosgenin produces down-regulation of anti-apoptotic bcl-2 protein and up-regulation of pro-apoptotic bax protein in human leukemia, colon carcinoma, osteosarcoma, laryngocarcinoma and melanoma. It has been shown that diosgenin induces apoptosis in all above cancers via activation of cysteine proteases, known as caspase-9 and caspase-3 result in PARP cleavage and DNA fragmentation which are typical characteristics of apoptosis [20, 21, 22]. Diosgenin provokes a collapse of mitochondrial membrane potential with release of AIF from mitochondria into the cytosol and translocated to the nucleus leading to apoptosis in laryngocarcinoma & melanoma cells.

Even though diosgenin has potential cytotoxicity, the results indicated that the plant extracts TCRE and TCBE have enhanced toxic effect upon HEp-2 cell lines compared to diosgenin. Further research is warranted to quantify the amount of diosgenin in this plant and also to elucidate the exact mechanism of *T.communis* extract for its cytotoxic effect.

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