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Apoptotic Activity of Glycoside Extract (Fraction I) from Leaves of *Convolvulus arvensis* on Rhabdomyosarcoma (RD) tumor Cell line is associated with its induction of DNA damage

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Abstract: Plants play an important role as a source of many anticancer drugs, thus screening of these plants in any region in our world may give a new valuable drug against cancer. The mechanism of anti-proliferative activity of new candidate plant extracts (Glycoside extract Fraction I from leaves of Convolvulus arvensis) as anticancer drug against RD tumor cell line after 72hrs treatment was indicated previously by the same investigators need to explanation. Glycoside was separated and fractionated by Thin Layer Chromatography (TLC) using silica gel. This study was designed to describe the activity of glycoside extract Fraction I(FI) from leaves of Convolvulus arvensis against the proliferation of RD cells and attempt to get an integrated view and better understanding of its modes of action . The result of present study showed that the Rate of flow (Rf) of glycoside fraction I was 0.8,whereas the melting point was 160 ^{0}C. This study demonstrated that the glycoside extract Fraction I from leaves of Convolvulus arvensis caused apoptosis. Since the light and fluorescent microscope observation showed morphological characteristic of apoptosis including cell volume shrinking, chromatin condensation and nuclear fragmentation after the treatment of RD tumor cell line with different concentrations (1250, 2500, 5000, and 10000, µg/ml) of glycoside at 72hrs. Beside that the apoptotic effect was confirmed by DNA fragmentation into low molecular weight fragments 180-200 pb as well as fragments with high molecular weight by using of agarose gel electrophoresis when treated with different concentrations.

Keywords: apoptosis, *DNA* fragmentation, *Convolvulus arvensis*, RD tumor cell line, glycoside extract Fraction I

Introduction

Programmed cell death or apoptosis is natural processes to eliminate the cells that have been produced redundantly, developed improperly, or have genetic damages. Generally, three alternative pathways are able to initiate apoptosis: one is referred to as the extrinsic pathway, which is mediated by death receptors on cell surface; the other is termed as intrinsic pathway, which is mediated by mitochondria (Igney and Krammer 2002). The third type is endoplasmic

reticulum(ER) stress signaling pathway, ER death signals include disturbance in calcium homeostasis or an accumulation of unfolded /misfielded proteins (Keller *et al.*, 2005).

In all pathways, caspases are activated to cleave the cellular substrate, including Poly(ADP- ribose) polymerase (PARP), and lead to cell morphological alterations, such as nuclear condensation, large-scale DNA fragmentation, the plasma membrane is budding, and finally the cell is fragmented into compact membrane-enclosed structures, called apoptosis body, all of which are features of apoptosis. (Saraste and Pulkki, 2000; Zhao *et al.*,2001).

The deregulation of apoptosis can impair the balance between cell proliferation and apoptosis, and then lead to a variety of disorders, including cancer. The evasion of apoptosis has been suggested as one of the six hallmarks of cancer (Hanahan and Weinberg, 2000). Either upregulation of oncogenes or inactivation of tumor suppressor genes is able to paralyze the cell death signaling, leading to increased cell survival (Thompson 1995; Danial and Korsmeyer
,2004). Some of the studies demonstrate the apoptosis induced by pure and crude extracts of plants. Montrirattigri *et al.,(*2008) found the aporphine extracted from *Stephania venosa* cause cell death via apoptosis. Treatment of pancreatic cell line (Panc-1) with *Petunia punctata* was shown to increase caspase-3 activity, indicating that the observed cytotoxicity was mediated via apoptosis (Sherine *et al.*, 2010). The chloroform extract of *Physalis minima* leads to DNA fragmentation, which is a biochemical hallmark of apoptosis (Ooi Kheng *et al*., 2011).

The Convolvulaceae family includes a large number of important plants which have the properties of treatment of many diseases (Al- Antaki,1952). C*onvolvulus* species are widely distributed all over the world some of them have medicinal activity (Abdel-Raheim *et al*.,2011). *Convolvulus arvensis* was evaluated as a potential new source of antioxidant activity (Borchardt *et al.*, 2008; Mohammed *et al.*, 2011).

Flavonoids, coumarins, sterols, saponins, resin, tanoids ,vitamin C , mineral substances, glycosides (kaempferol 3-mono-glycosides and Quercetin 3-mono or di-glycosides), tannins and stilbene derivatives have been isolated from the plants of this genus (Jenett-Siems *et al*., 1998; Dawidar *et al*., 2000; Menemen *et al*.,2002).Previous study by the same investigators was found that Glycoside extract Fraction I from locally *C. arvensis leaves* had cytotoxic effect against RD cell line especially at 10000 µg/ml after 72hrs treatment(AL-Asady *et al*.,2014)Therefore the present study was designed to detect the apoptotic potency of glycoside extract Fraction I from Leaves of *Convolvulus arvensis* in RD tumor cell line .

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Methods Plant Collection

Convolvulus arvensis plants were collected from Basrah university fields/Iraq at the beginning
of the flowering season (April-September). Plant samples were authenticated at the College of Agriculture, University of Basrah, Basrah, Iraq. Leaves of plant were dried at room temperature and grounded into powder by electrical grinder (mesh No. 0.5 mm) and kept in plastic tubes in deep freeze at -20°C until use**.**

Extraction and Fractionation of Glycosides from Leaves of *C. arvensis*

The separation and fractionation of Glycosides in this study according to Menemen *et al*.,(2002) . Fifty gram of dried ground of *C. arvensis* leaves were refluxed with 80% methanol for 8 hrs below 60^0C. After extraction, the extract was filtered and the pooled extract was evaporated to dryness under reduced pressure in a rotary evaporator. The dried extract was dissolved in 80% methanol and run by thin layer chromatography(TLC) using silica gel in BAW(n-butanol: acetic acid:water,5:1:4) as eluent. The glycoside spots were examined and their position and color reactions recorded. Rate of flow (Rf) value were recorded. To obtain a large amount of fractions, the solid residue (0.3gm.) was dissolved in (3 ml.) elution and then passed through a

chromatographic column (1.6x60cm.) consisting of 50 gm of silica gel (70-150 mesh) at flow rate of 0.7cm3./min. Elution was carried out in BAW (5:1:4), fractions of 5ml. were collected and their absorbance were measured by spectrophotometer (450 nm.).The fractions
with similar peaks were collected and dried at room temperature. Two peaks were recorded, Fraction I and II , were identified by infrared spectra (FT-IR) was recorded in potassium bromide(KBr) pellets using FT-IR 84005 Spectrophotometer at the range (500-4000) cm-1 .This was processed at the Department of Chemistry /College of Science. Fraction I was identify only by melting point by electro thermal melting point, because FII was oily in nature.

Rhabdomyosarcoma (RD)

Rhabdomyosarcoma (RD) provided by Iraqi Center for Cancer and Medical Genetic Research/Baghdad (ICCMGR). Passage No.45 was used and the cells were cultivated in Minimum Essential Medium (MEM) with L-Glutamine and hepes (Sigma, USA) was supplemented with 10% of fetal calf serum, and penicillin/streptomycin (Fresheny, 1994).

Morphological study

The RD tumor cell lines were seeded in MEM growth medium in three separated micro- titration plates (15 wells were used in each plate). When the cells formed 70-80% confluent monolayer , subsequentlycells were treated with different concentrations of the glycoside extract Fraction I of *C. arvensis* prepared with maintenance EME medium (1250, 2500, 5000, and 10000, µg/ml) three replicates for each concentration . Other three wells were used for seeding cells in medium with phosphate buffer (PBS) only as control .The cells in first plate were used for light microscope study, second plate for fluorescence microscopy study, while the third for Molecular analysis of cell death.

Light Microscope study

After exposure time the cells in first plate were fixed in bouni's fixative (75ml. of picric acid, 25 ml. of formalin and 5 ml. of glacial acetic acid) for 20 min. at 4°C then stained by haematoxilin and eosin (Lillie,1965). Then cells were examined under a light microscope using the 40 X objective lens.

Fluorescence Microscopy study

The cells in the second plate were treated with Trypsin/versene .The resultant cells suspension was centrifuged for 10min 1200 rpm at 10 ºC. The supernatant was removed and re-suspended cell pellet in 1x PBS and washed twice with PBS.
Twenty microlitter of Acridine orange 25 µg/ml was routinely added to a 20 µl cell suspension placed on a microscope slide. Both suspensions were mixed briefly on the slide with an automatic pipette tip then gently covered with a glass cover slip, with care taken to avoid
trapping any air bubbles. The resulting cells suspension was then viewed immediately under a fluorescence microscope using the 100X oil immersion objective lens (McCarthy,1993).

Molecular Analysis of Cell Death (Apoptosis)

Extraction of DNA from RD tumor cell line

Trypsin/ versene was used to remove RD cells from the wells of third plate, cells suspension was centrifuged for 10min 1200 rpm at 10 ºC. The supernatant was removed and re-suspended cell pellet in 1x PBS and washed twice with PBS.
Cell pellets were re-suspended in 50µl lysis buffer and incubated in 50ºC water bath for 1 hr. The DNA was extracted according to Wyllie (1980).The resulting DNA was visualized by 1% agarose gel electrophoresis and stained with ethidium bromide.

Results

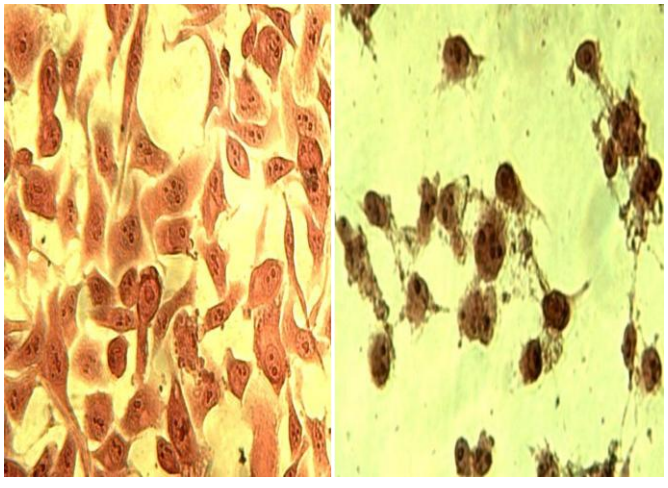
Cell Death Detection

Morphological changes characteristic of cell death by apoptosis were seen in RD treated cells. The morphological analysis was carried out by inverted and fluorescent microscope.
Figure (1-a) showed untreated monolayer

control group under phase contrast inverted microscope , cells characterized by irregular in shape, normal size with intact nucleus and nucleolus. In contrast Figure (1-b) revealed RD cells those treated with 10000 µg/ml of glycoside extract FI .These cells undergo apoptosis ,the chromatin has condensed and fragmented into several spherical particles and the cells number and volume were reduced with smooth surfaced in appearance .

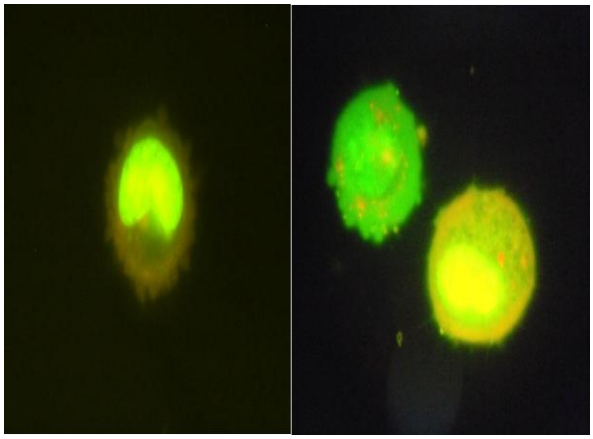
Acridine orange, an intercalating, fluorescent DNA binding dye, was used to identify apoptotic cells with the characteristic condensed nuclear DNA morphology. Differing DNA states within viable and apoptotic cells resulted according to interaction between acridine orange and DNA. Hence acridine orange gives out a diffuse yellow/green fluorescence when bound to DNA in viable cells and a bright green fluorescence when bound to condense fragmented DNA in apoptotic cells. RNA in both viable and apoptotic cells emits a red fluorescence.

Untreated control group of cells, figure (2-a,b) were shown the bright yellow/green color associated with viable cells with intact DNA in contrast cells treated with 10000 µg/ml of glycoside extract FI appear the chromatin condensed as a whole with nuclear chromatic stains bright green and was fragmented within the cell into many vesicles then marginates to the cell membrane the remainder of the cell will be devoid of chromatin will be almost transparent in appearance (Figure 2 c,d).

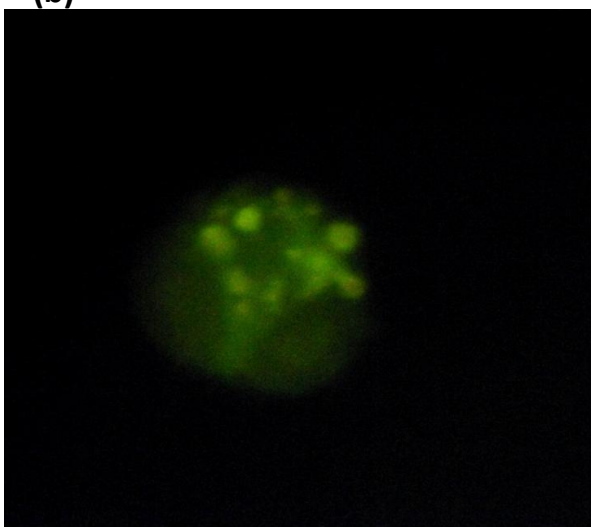


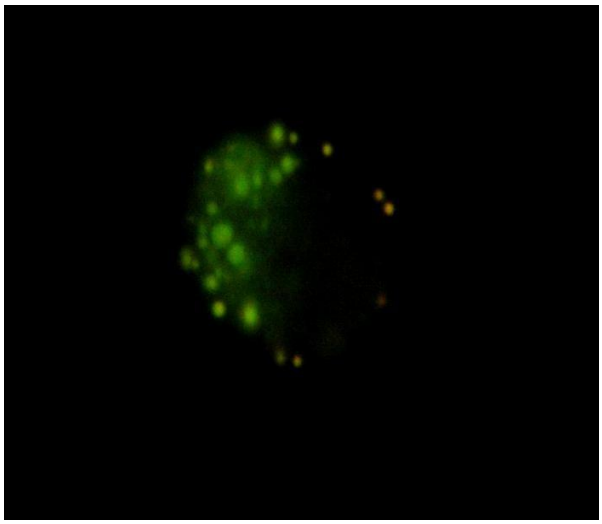
(a) (b)

Figure (1): Rhabdomyosarcoma cells in monolayer under inverted microscope (H & E, 40X) a-Untreated monolayer control group
b- Cells treated with 10000 µg/ml of glycoside extract FI undergo apoptosis



(a)
(b)



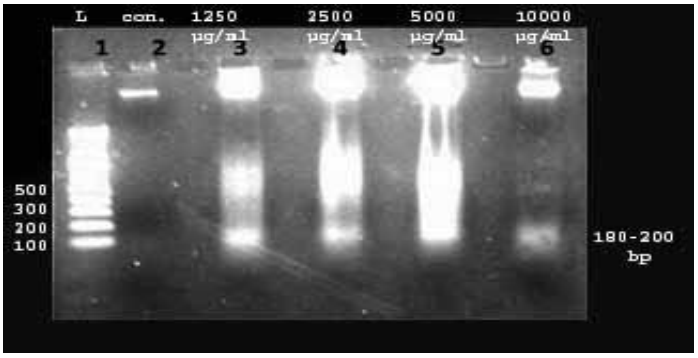


(d)

Figure (2):
Rhabdomyosarcoma cells stained with acridine orange under the fluorescent microscope (800X)
a,b -Untreated control group.
c, d -Apoptotic cells those treated with10000 µg/ml of glycoside extract FI, shows chromatin condensation in cell undergo apoptosis.

Molecular analysis of DNA of dead RD tumor cells

A further characteristic of many apoptotic cells is the activation of a calcium dependent endonuclease resulting in fragmentation of the DNA into 180-200 bp. Figure (3) showed an electrophoresis gel of low molecular weight DNA from RD cells after the treatment with glycoside extract FI of different concentrations from 1250,2500,5000 and 10000µg/ml (lanes 3- 6), the fragmentation into high molecular weight fragments being visible in all concentrations , whereas the fragmentation into multiple 180-200 bp was less obvious except in higher concentration 10000 µg/ml ,while untreated control group was shown genomic DNA without any fragmentation (lane 2).



Figure(3):
1% agarose gel electrophoresis analysis for DNA of RD cells treated with glycoside extract FI. The gel was run at 60 V, 10mAuntil bromophenol blue was near the end of the gel. Gel was removed and visualized bands under UV light 300nm.
Lane 1: **Ladder 100-2000 bp**.
Lane 2 :Untreated cells (control)
Lane 3: Cells treated with 1250 µg/ml of glycoside Lane
4: Cells treated with 2500 µg/ml of glycoside Lane
5: Cells treated with 5000 µg/ml of glycoside
Lane 6: Cells treated with 10000 µg/ml of glycoside with multiple **180-200 bp.**

Discussion

Induction of Cell Death

The morphological examination of the cells was carried out using both light and fluorescent microscope and evidence for the characteristic endonuclease activation resulting in fragmentation of the DNA was shown by agarose gel electrophoresis.
Morphological analysis of *C. arvensis* glycoside FI extract-treated cells demonstrated an increase in apoptotic RD cells compared to the untreated cells , suggesting that apoptosis might play a role in the growth inhibitory effects of *C. arvensis* -treated cells. The most remarkable changes could be observed in treated cells including cell number reduced, shrinkage, extensive detachment of cells from the culture surface and condensation of chromatin into several spherical particles revealed hallmarks of apoptosis under the light microscope.
Fluorescence microscopy of a typical viable cell staining with acridine orange demonstrating the bright yellow/green color associated with intact

DNA acridine bright diffuse staining DNA and highly irregular in shape, whereas apoptotic cells become smooth surfaced , distinctive condensed and fragmented of DNA which give off a brighter green fluorescence vesicles. Oberhammer *et al*.,(1983) and David *et al*.,(1993) concluded that the changes in nuclear

morphology at the first stage coincide with the activation of a nuclease enzyme that cleaves chromatin first into300 ,700 and 50-kbp fragments and ultimately into multiples of 180-200 bp. at the late stage (Wyllie,1980).

The DNA fragmentation into high molecular weight fragments observed in the present study when RD cells treated with glycoside extract F I, at 2500,5000,and 10000µg/ml,whereas the low molecular weight fragments observed at 1250µg/ml then became evident at higher concentrations. Jongsomboonkusol , (2005) reported that when cells undergo apoptosis, DNA will be digested into oligonucleosomal fragments about 180-200bp.

Although the condensation of chromatin-DNA ladder has become one of the hallmarks of apoptotic death but the absence or incomplete of a detectable DNA ladder in present study may be due to asynchronous apoptosis or relatively little fragmentation to low molecular weight DNA. Cohen *et al.*, (1992) and Collins *et al*., (1992) showed that some cell lines will morphologically go through apoptosis, but DNA fragmentation may be absent or incomplete, possibly due to limited cleavage of the DNA into high molecular weight fragments. McCarthy , (1993) mentioned that the method by which fragmentation produced by cytotoxic drugs in Burkat lymphoma cells (Raji-BL) were detected, but did not solve the problems of detecting fragmentation in either Akata or Cheptages-BL cell line. This implies that not all cells which die by apoptosis necessarily have to fragment their DNA to the extent documented, or may be the destruction of the DNA becomes unreadable by RNA polymerase is hypothetically the most important outcome of endonuclease activation and may explain why not all cells fully fragment their DNA, but do cut the DNA into higher molecular fragments, which were not visible on agarose gels . Zaker *et al*.,(1993) demonstrated the absence of an easily detectable nucleosomal ladder could arise in several ways: lack of a thymocyte-type nuclease; loss of DNA fragments during isolation; or existence of a rate-limiting endonuclease, so that DNA fragments do not accumulate in sufficient amount to be identified .

Yamashita *et al*.,(2000) demonstrated that some types of flavonoids that found in the form of glycosides inhibits topoisomerase I and II, thus inhibiting cell replication and DNA repair and promoting apoptosis.

Topoisomerase II and topoisomerase I were required during DNA replication and transcription to provide swivel points for the DNA, Topoisomerase II was also required for the segregation of chromatids prior to mitosis (Yang *et al.*, 1987). The subsequent DNA fragmentation has been shown to be the result of apoptosis-fragmentation of the DNA being produced by the activated endonuclease. It has been proposed that the block of replication forks by topoisomeraseII inhibition or cleavable complex formation triggers apoptosis (Jaxel *et al.*, 1988).Sukardiman *et al.*, (2000) found that the flavonoid inhibited DNA Topoisomerase I activity resulting in cleavage of DNA.

Glycosides perhaps increased the induction of apoptosis by increased of P53 protein levels

,Shi *et al.*, (2007) observed a significant increase of p53 protein level in three glycoside-treated cancer cell lines without increase of p53 mRNA level, indicating the possible effect of glycoside on p53 posttranscriptional regulation.

The present study has been supported by many studies , Aslamuzzaman *et al*.,(2003) was reported in addition to the appearance of these spherical and irregularly shaped cells, prostate cancer cells exhibited apoptotic characteristics, namely chromatin condensation, nuclear fragmentation, and cell shrinkage when treated with the higher concentrations of fraction extract from the *Musaceas* . Light and electron microscopic examinations of the treated cells with

Daphne mucronata extract indicates that the majority of different human cancer cells lines (K562, HL-60, and MOLT-4 leukemia cell lines) and WEHI-164, a mouse BALB/C fibrosarcoma cell line were undergo apoptosis ,chromatin condensation, apoptotic bodies formation, and loss of microvilli are markedly visible (Yazdanparast and Sadeghi,2003) . A medicinal herb extracted from the *Hemsleya amabilis* plant inhibits tumor cell growth, colony formation, and induces apoptosis in various tumor cell lines, due to the saponins, glycoside produced by plant(Wu *et al*.,2002) . Horinaka *et al*., (2005) found, that the flavonoids not only induced the mitochondrial pathway of apoptosis but also caspase 8/10 activation and death receptor (DR-5) expression in human malignant cells.

Conclusions

Glycoside extract Fraction I from leaves of *Convolvulus arvensis* plant caused RD tumor cell death by apoptosis. The apoptotic effect was confirmed by DNA fragmentation into low molecular weight fragments 180-200 pb as well as fragments with high molecular weight.

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