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Research Article

GC-MS Analysis of Extract of *Rubia tinctorum* having Anticancer Properties

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ABSTRACT

Atomic Absorption Spectrometry determined the percentage of Cu, Zn, Cd, Mn, K, Fe, Co, Ti and N. Their amounts were: (0.002281, 0.004115, 0.000205, 0.007772, 0.833, 0.093952, 0.000332, 0.043 and 1.02) %. There was no Phosphorus. Total Crude protein was 5.8125 %. Thirty-five compounds identified in methanol extract of *R. tinctorum* using Gas Chromatography- Mass Spectrum analysis (GC-MS) in a single run. The major three components present were 9,12-Octadecadienoic acid (Z,Z)- (29.75 %); 9-Octadecenoic acid (Z)-, hexadecyl ester (26.1 %) and 1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)- (10.09 %). The results of cytotoxicity on cancer cell lines showed reduction in cell viability of Human Glioblastoma cell line (AMGM) treating with all concentrations of extract with IC50 reached to 76.44 mg/ml. Inhibition rate ranged between (28.54- 66.1) %. In Ahmed-Mohammed-Nahi-2003 (AMN3) cell line, the lower concentration (50 µg/ml-5 mg/ml), reduced cell viably while the higher concentrations (50-100) mg/ml induced it. IC50 is 21 mg/ml, the best Inhibition rate is 93.693% at 50 µg/ml. The extraction was less effect in cell viability of Human Larynx Epidermoid Carcinoma (HEp-2) cell Line, the maximum Inhibition rat was 22.12 % at 10 mg/ml. All other concentrations were not significant compared with control. Previous results of cytogenetic effects of plant extract on human lymphocytes showed non-significant changing in mitotic index, blast index, cell cycle progression and replication index but the extract induced chromosomal aberration and sister chromatid exchange. These inductions in chromosomal aberration were structural. They were: addition, deletion and ring chromosome. The deletions were the most common abnormalities. There were more than two chromosomal aberrations in the same cell.

Keyword: AMGM; AMN3; cell line; cytogenetic; Hep-2; GC-MS; *Rubia tinctorum*.

INTRODUCTIONS

Rubia tinctorum L. (Family: Rubiaceae), is one of Iraqi plants, its origin is native to southern Europe and Asia. Their common name is Madder. All parts of the plant contained an iridoid, asperuloside. Parts used is roots, they contain anthraquinone and their glycosides, including alizarin, purpurin, purpuroxanthin, pseudopurpurin, rubiadin, ruberythric acid and lucidin primeveroside, nordamnacanthal[1-4]. Other studies found other compound in roots and rhizomes such as: di- and tri-hydroxyanthraquinones, alizarin, purpurin and their derivatives, ruberythric acid (alizarin-primeveroside), pseudopurpurin and lucidin-primeveroside[5].

The action of roots used for menstrual and urinary disorders and liver diseases[1]. Madder is used in amenorrhea and dropsy, and when taken into the stomach imparts a red color to the milk and urine, and to the bones of animals, without sensibly affecting any other tissue[6].

R. tinctorum L. revealed antimicrobial activity against some Gram (+) and Gram (–) bacteria, yeasts, filamentous fungi and actinomycetes[7-8]. Aqueous extract of this plant has anti-diarrheal effect[9].

Some study refers that this plant is toxic, their understanding is not very precise because they mixed between natural extractions of plants and dye that extract from roots of plant using deferent solvents which has long tradition in dyeing processes of textiles and are toxic due to presence of toxic hydroxyanthraquinones[10], such as: alizarin[11], and rubiadin[12-13], this is different from natural aquatic or methanol crud extractions of plants which are weak toxicity, the results of Ino and others[14] suggest that dietary exposure of madder root (MR) has no acute or subacute toxic effects on mice. In addition, methanol extract of plant roots exerted here weak toxicity targeting liver, kidney, and possibly RBC and WBC in rats, some renal parameters being apparently affected from the 0.6% dose level[15].

Recently, the treatment with the aqueous extract exhibited a strong decrease in two different metastatic potency human melanoma cell lines in 10−6–10−5 M concentration range, which activity was demonstrated already after 48 h. This inhibitory effect was more pronounced in A2058 cell line (10^{-6}–10^{-5} M: 87.4–55.0 %) than in HT168-M1 cells (10^{-6}–10^{-5} M: 87.5–63.7 %) after 72 h. In normal fibroblast cells derived from normal lung tissue (MRC-5 cells) a similar, concentration dependent antiproliferative/cytotoxic effect (10^{-6}–10^{-5} M: 77.2–62.0 %) was observed for this aqueous extract[16].

The aim of the present study was to develop an efficient anticancer drug using aquatic roots' extract of *R. tinctorum*

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and if there are any toxicity in cytogenetic of human lymphocyte.

MATERIALS AND METHODS

Plants extraction

The roots of *Rubia tinctorum* plant obtained from Iraq Medical Herbarium/ Ministry of Health/ / Baghdad / Iraq. Roots dried at 38 C° and ground by a grinder. Fifty grams of powdered material extracted by 250 mL of 70% methanol using a Soxhlet extractor at 40 ℃ for 3 h.[17]. The extracts filtered using Whatman No.1, evaporated and sterilized by 0.22 µ micro filters.

Chemical analysis

Atomic Absorption Spectrometer

Atomic Absorption Spectrometer (Nove AA-350, analytik Jena) was used to determine the concentrations of some element in plants. The elements were: Cu, Zn, Cd, Mn, K, Fe, Co, Ti, P and N. The samples prepared according to Kodama in (1963)[18]. The level of Nitrogen and total proteins were recorded by Macro Kjeldahl method[19].

Gas Chromatography- Mass Spectrum analysis (GC-MS) analyses

GC-MS analyses were done using Shimadzu GC-2010 Plus coupled with Shimadzu GCMS-Q2010 Ultra. Capillary column (InertCap 1 MS, 0.25 mm, 30 m, 0.25 µm, Gl Sciences, Japan). Carrier gas was helium. Constant flow rate is 1 ml/min and auto injector is AOC-20i, Shimadzu. Injection volume is 5 µl. Column oven temperature program were in the following order: 100 ℃. Oven temperature program was: 100 ℃ for 3 min., 240 ℃ for 9 min., 280 ℃ for 5 min. and 300 ℃ for 2min. The rates were 15. For identification of components, direct comparison of the retention times and mass spectral data of components had done with those for NIST Standard Reference Database 1A: NIST/EPA/NIH Mass Spectral Library (NIST 08) and NIST Mass Spectral Search Program (Version 2.0f)[20].

Cytotoxicity on cancer cell lines

Cell culture

Three cancer cell lines obtained from cell bank unite/ Department of Experimental Therapy / Iraqi Center for Cancer and Medical Genetic Research (ICCMGR). The cell lines were: Human Glioblastoma cell line (AMGM) (passage 69), Ahmed-Mohammed-Nahi-2003 (AMN3), murine mammary adenocarcinoma cell line (passage 203) and Human Larynx Epidermoid Carcinoma (HEp-2) Cell Line (passage 275). They maintained using RPMI 1640 (USbiological - USA) supplemented with 15% calf bovine serum (Gibco, USA), 100 units per ml penicillin, and 100 µg/ml streptomycin as recommended by cell bank unite at ICCMGR. The cells seeded in Flat bottomed 96-well polystyrene and incubated at 37℃ under a humidified atmosphere containing 5 % CO2 for 24 hr. A population was 1.5*104 cells per well.

Cytotoxic activity

Cytotoxicity assay had done according to method of Department of Experimental Therapy / Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) with some modifications. The spent media of the confluence cells, (90-95) % within 24 h. incubation, was removed.

Table 1: The level (%) of some elements and total proteins in *R. tinctorum*.

Elem.	%
Cu	0.002281
Zn	0.004115
Cd	0.000205
Mn	0.007772
K	0.833
Fe	0.093952
Co	0.000332
Ti	0.043
P	NIL
N	1.02
Prot.	5.8125

Elem.: element, %: Percentage, Cu: Copper, Zn: Zinc, Cd: Cadmium, Mn: Manganese, K: Potassium, Fe: Iron, Co: Cobalt, Ti: Titanium, P: Phosphorus, N: Nitrogen, Prot.: Crude protein.

Cells washed with PBS (0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl) and 0.2 ml of fresh free serum media (RPMI 1640) was added. Series of dilution of crude plant extract, dissolved in PBS which were diluted with free serum medium (RPMI 1640), were added to plates. There was a negative control (PBS) and six replicates for each tested. Plates incubated at 37℃ with 5% CO_2 for (24, 48,72) hr. of AMN3, AMGM and Hep-2 respectively. At the end of exposure time, cells washed twice with PBS. A fifty micro letter of Neutral Red solution (Sigma, U.S.A; 50 µg/ml dissolved in PBS), added to each well and incubated at 37℃ for two hr.

The wells washed three times with PBS. Fifty microliter of solution (PBS and absolute ethanol 1:1 V/V) added to each well. The optical density read by a Micro-ELISA. Percentage of cell viability[21] and inhibiting rate[22] calculated. In addition to Compusyn Computer software, (version 2011), which used for IC_{50} calculation.

Cytogenetic effects on human lymphocytes.

Blood samples (0.2 mL) obtained from nonsmokers, 24-25 years' age with no history of drugs, radiation therapy or viral infection. They planted in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma/ U.S.A) supplemented with: AB^+ Plasma, 10 µg/mL bromodeoxyuridine (Serva/ Germany) and 0.1 ml of 10 µg/ml of Phytohemagglutinin (Sigma/ U.S.A). Cells treated with (100, 10 and 1) mg/ml of crude plant extract. There were negative controls and positive control (adding Mytomycin C, Kogyo Company, at 50 ng/ml final concentration. There were triplicates for each treatment. All experiments were carried in the dark.

Lymphocytes were cultured for 72 h at 37 ℃. For CA and SCE analysis, 0.06 µg/mL colchicine (Sigma/ U.S.A, dissolved in distilled water) was added to cultures at the last two hours of exposure time. Cells were harvested by centrifugation (216×*g*, 10 min) and suspended in a hypotonic solution of 0.075 M KCI for 30 min at 37 ℃. Cells were centrifuged and fixed by cold methanol acetic acid (3:1) for 20 min. This step repeated three times. Solution dropped in clean slides and air drying. For

Table 2: Composition of methanolic extraction of *R. tinctorum*.

P	R.T	Area %	Comp.	C.F	M.W
1	5.23	0.45	1,2-Cyclopentanedione	$C_5H_6O_2$	98
2	8.44	0.08	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144
3	10.24	0.44	N-Methyl-4-(3-nitrobenzamido)phthalimide	$C_{16}H_{11}N_3O_5$	325
4	10.50	0.52	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150
5	10.82	0.59	Ethylene diacrylate	$C_8H_{10}O_4$	170
6	10.96	0.51	3-Allyl-6-methoxyphenol	$C_{10}H_{12}O_2$	164
7	11.27	0.46	6,7,8,9-Tetrahydro-5H-[1,2,4]triazolo[1,5-a]azepin-2-ylamine	$C_7H_{12}N_4$	152
8	11.81	10.09	1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)-	$C_6H_{14}O_3$	134
9	12.94	0.46	Pentadecanoic acid	$C_{15}H_{30}O_2$	242
10	13.01	1.23	3-Deoxy-d-mannoic lactone	$C_6H_{10}O_5$	162
11	13.29	1.35	3-Deoxy-d-mannonic acid	$C_6H_{12}O_6$	180
12	13.63	0.05	Carbonic acid, allyl nonyl ester	$C_{13}H_{24}O_3$	228
13	13.80	0.04	Dodecanoic acid	$C_{12}H_{24}O_2$	200
14	14.14	0.04	Thiocyanic acid, 4.alpha.-methyl-5.alpha.-cholestan-3.alpha.-yl ester	$C_{29}H_{49}NS$	443
15	14.33	0.10	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180
16	14.63	0.16	Tetradecanoic acid	$C_{14}H_{28}O_2$	228
17	16.23	0.53	Oleyl alcohol, trifluoroacetate	$C_{20}H_{35}F_3O_2$	364
18	16.42	6.05	l-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	652
19	16.58	0.49	4-Methoxy-2-nitroformanilide	$C_8H_8N_2O_4$	196
20	17.79	0.42	9,10-Anthracenedione, 1-hydroxy-	$C_{14}H_8O_3$	224
21	17.90	1.17	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H34O_2$	294
22	18.00	0.86	9-Octadecenoic acid, methyl ester, (E)-	$C19H36O_2$	296
23	18.40	29.75	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280
24	18.50	26.10	9-Octadecenoic acid (Z)-, hexadecyl ester	$C_{34}H_{66}O_2$	506
25	18.80	5.49	Octadecanoic acid	$C_{18}H_{36}O_2$	284
26	19.27	1.05	Methanone, [4-(methoxymethyl)phenyl]phenyl-	$C_{15}H_{14}O_2$	226
27	20.85	0.15	Glycidol stearate	$C_{21}H_{40}O_3$	340
28	23.74	0.59	Iron, dicarbonyl(.eta.5-2,4-cyclopentadien-1-yl)(2,3,3,4,4,5,5,6,6-nonafluoro-1- cyclohexen-1-yl)-	$C_{13}H_5F_9FeO_2$	420
29	24.54	2.59	6,9-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294
30	24.67	1.92	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-	$C_{57}H_{104}O_6$	884
31	25.78	0.67	Octadecanoic acid, 5,9,13,17-tetramethyl-, methyl ester, [5R-(5R*,9R*,13R*)]-	$C_{23}H_{46}O_2$	354
32	26.12	3.59	9,10-Anthracenedione, 1,8-dihydroxy-3-methyl-	$C_{15}H_{10}O_4$	254
33	26.32	0.62	(+)-9-Norartemisinin, 9-n-butyl-	$C_{18}H_{28}O_5$	324
34	26.40	0.75	3.beta.-Fluoro-5-hydroxy-7-keto-17.beta.-acetyloxy-androstane	$C_{21}H_{31}FO_4$	366
35	26.77	0.64	Methyl 17-methyl-octadecanoate	$C_{20}H_{40}O_2$	312
		100.			

P: peak; R.T: retention time; Comp.: compound; C.F: compound formula; M.W: molecular waight; M.S: Mass spectra.

chromosome aberrations CA, they stained with Giemsa according to[23].

For SCE, other slides stained with Giemsa according to the Fluorescence - plus giemsa (FPG), (Tawn and Holdswoth 1992)[24] which was modified by current study. Default method is described as fallowing: Hoechst stain was prepared at a final concentration 0.5 mg / ml by dissolving 5 mg of dye powder in 10 ml of water distilled, when there were need it, a 5µg / ml was prepared by PBS as a solvent, keeping the solution away from light under the temperature (-18) Cº. Slides Stained with Hoechst dye (5µg / ml) for 25 minutes, then washed with a solution of the BPS (BDH company) and left to dry, slides immersed in PBS solution in a suitable container and exposed to a source of ultraviolet radiation (UV light) a wavelength of (365) nm for (2.5-2 hours) at a distance of (10) cm, washed with PBS, and left to dry, stained with Giemsa prepared (1ml dye: 9ml Gurr's buffer) for (1.5 -1) minutes. This method has been modified as follows:

Hoechst stain prepared in the same way at a final concentration of 5 µg/ ml. Slides immersed in a Hoechst stain, which dilute (1 dye: 2 PBS) and exposed to ultraviolet radiation (UV light) at a wavelength (365) nm for (2.5-2 hours) at a distance of (10) cm distance, slides washed with distilled water and left to dry, the slide either: Staining directly by Giemsa (which prepared above) for (1.5-1) a minute.

or immersed into PBS solution (PH= 6.8) and exposed to direct sun light for 25 minutes, staining by Giemsa (which prepared above) for (1.5-1) hours, left them to dry and

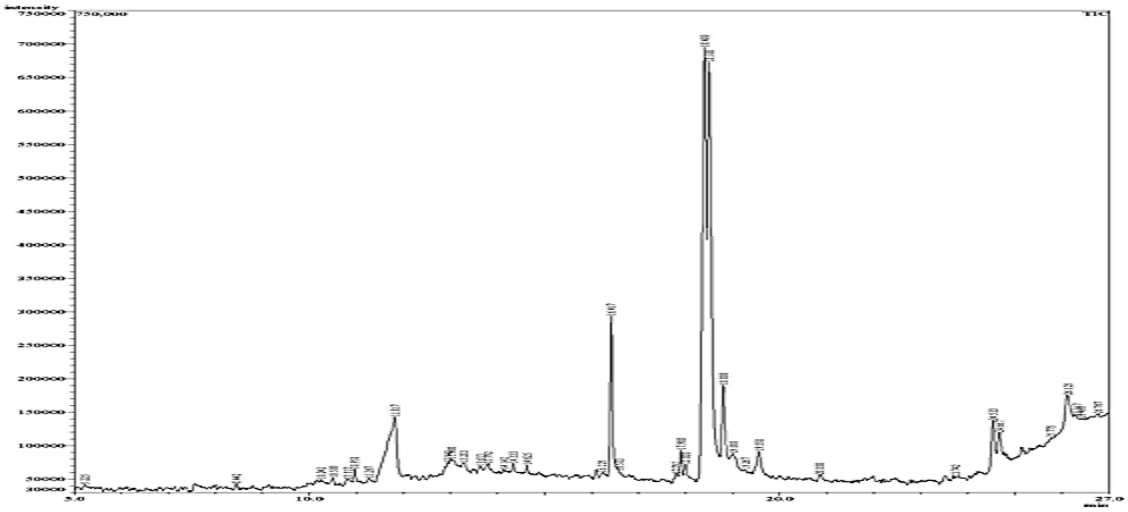


Figure 1: Chromatogram of methanolic extraction of *R. tinctorum*.

Table 3: percentage of mitotic index, blast index, chromosomal aberrations, sister chromatid exchange, cell cycle progression and replication index in human lymphocyte cultures exposed to *R. tinctorum* crude extracts.

Con. (mg/ml)	M.I	B.I	C.A/cell	SCE/cell	CCP			R.I
					M1	M2	M3	
100	2.6	37. 5	0.6 *	5 *	22.35	49.05	28.6	2.0625
10	2.562	39.915	0.52 *	4	21.35	50.05	28.6	2.074
1	2.552	49.2	0.38 *	4.584 *	24.975	39.275	35.75	2.11
Ct-	3.1687	40.577	0.16	4.167	46.65	40.02	13.33	1.668
Ct+	1.01 *	10.9*	3.1 *	12.24*	60.5	33.25	6.25	1.4

Data shows Means; Con.: concentration (mg/ml); M.I: mitotic index, B.I: blast index, C.A: chromosomal aberrations, SCE: sister chromatid exchange which appear by first method of modifying staining, CCP: cell cycle progression, R.I: replication index, Ct-: control negative (without treatment), Ct+: control positive (Mitomycin c with 50 ng/ml final concentrations, *: ($P < 0.05$).

washed with a Sorensen solution (Na2HPO4 with 9.08 g of KH2PO4 in a liter of distilled water).

Slides were examined under light microscope. Chromosomal aberrations were scored from 50 metaphases. It was classified according to the International System for Human Cytogenetic Nomenclature (ISCN). The number of SCE's was scored from 50 cells under second metaphases. Mitotic index and blast index were determined by scoring 1000 cells from each donor. One hundred cells were scored for the determination of the cell cycle progression and replication index (RI)[25-27].

Analysis of variance (ANOVA) and the least significant difference (LSD) were used for the statistical analysis of the results using SPSS, version 10, P-values at levels ($P \leq 0.05$).

RESULT AND DISCUSSION

Chemical analysis

Atomic Absorption Spectrometer

Atomic Absorption Spectrometry determined the percentage of Cu, Zn, Cd, Mn, K, Fe, Co, Ti and N. Their amounts showed in Table (1). There were no phosphorus. The level of total proteins was 5.8125.

GC-MS analysis

Thirty-five compounds identified in methanol extract of *R. tinctorum* using GC-MS analysis. The major three components present were 9,12-Octadecadienoic acid (Z,Z)- (29.75 %); 9-Octadecenoic acid (Z)-, hexadecyl ester (26.1 %) and 1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)- (10.09 %). Mass spectra from full scan analysis of components were showed in (Table 2). Figure (1) showed chromatogram of methanolic extraction of *R. tinctorum*.

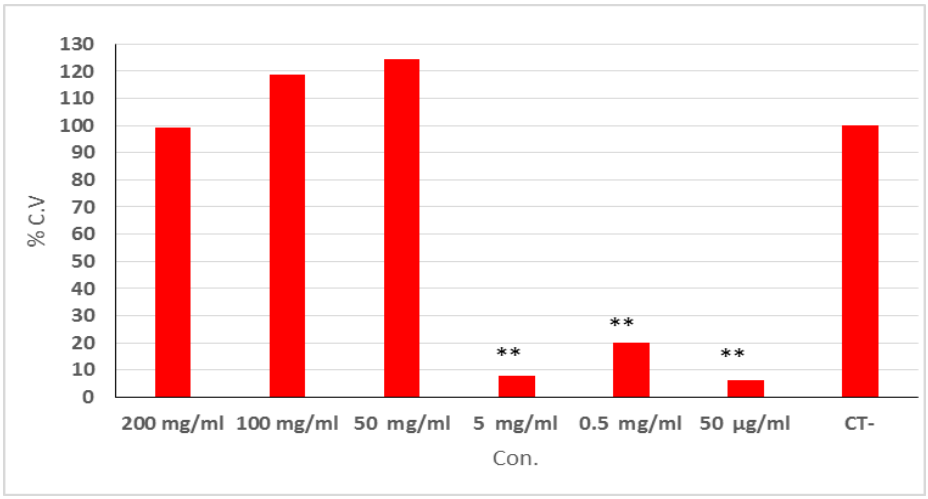
Cytotoxicity on cancer cell lines

In AMN3 cell line, the lower concentration (50 µg/ml-5 mg/ml), reduced cell viably at level ($P \leq 0.01$), while the higher concentrations (50-100) mg/ml induced it. IC50 is 21 mg/ml; the best Inhibition rate is 93.693% at 50 µg/ml. (Figure 2 A). The results of Figure (2) showed reduction, ($P \leq 0.05$), in cell viability of AMGM cell lines treating with all concentrations of extract with IC50 reached to 76.44 mg/ml. Inhibition rate ranged between (28.54- 66.1) %, (Figure 2 B).

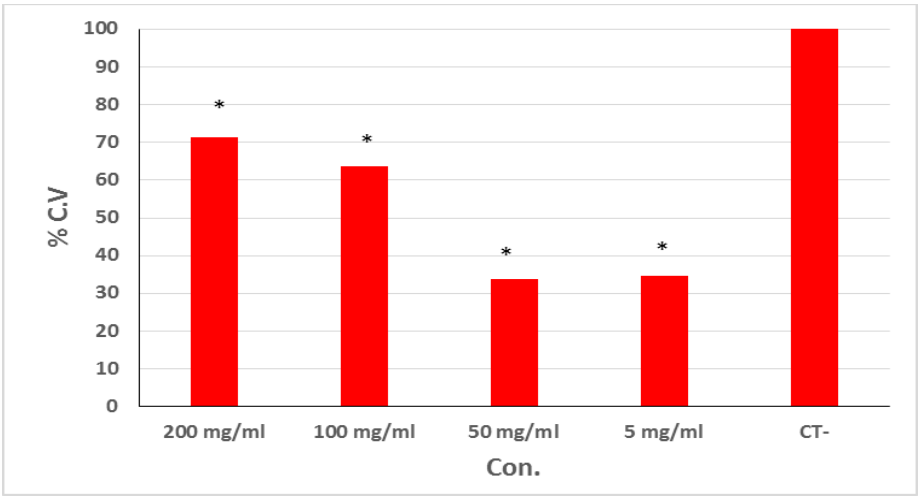
The extraction was less effect in cell viability of Hep-2 cell line, the maximum Inhibition rat was 22.12 % at 10 mg/ml, ($P \leq 0.05$). All other concentrations were not significant compared with control. There was no IC50 in this cell line, (Figure 2 C).

Cytotoxicity on human lymphocytes.

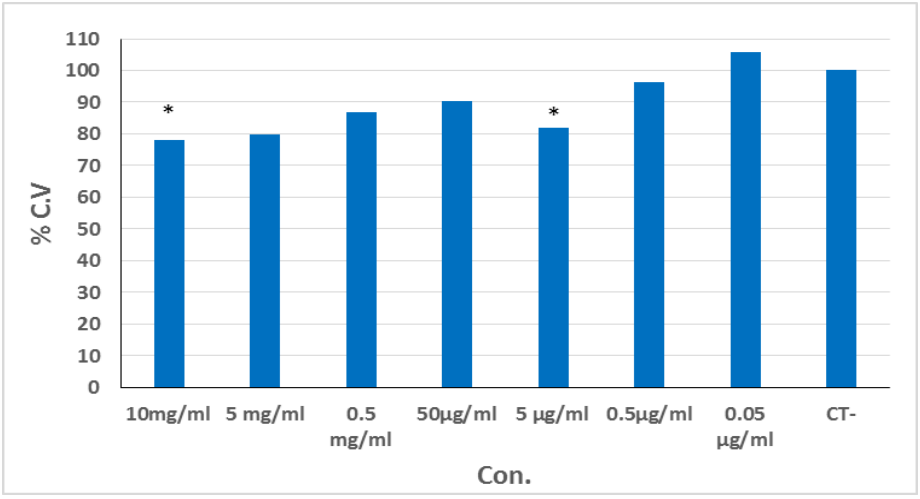
Two methods of staining were succeeding to appear chromosomes banding. The changing in mitotic index, blast index, cell cycle progression and replication index were not significant. But the extract induced chromosomal aberration and sister chromatid exchange. These



A



B



C

Figure 2: Cytotoxicity activity of *R. tinctorum* crude extract on (A): AMN3 cell line, 24 hr., IC50= 21 mg/ml, (B): AMGM cell line, 48 hr., IC50= 76.44 mg/ml, (C): Hep-2 cell line, 72 hr. *: ($P < 0.05$), **: ($P < 0.01$).

inductions in chromosomal aberration were structural. They were: addition, deletion and ring chromosome. The deletions were the most common abnormalities. There were more than two chromosomal aberrations in the same cell, (Table 3). These changing were less effect in treatment of Mitomycin C which induced CA and SCE with reduction in MI, BI and R.I compared with *R. tinctorum* crude extracts and negative control.

Madder is used in medicine, as its components are reported to exhibit various pharmacological and biological activities, including anticancer. Current results found that main components of madder roots extract are: 9,12-

Octadecadienoic acid (Z,Z)- (29.75 %); 9-Octadecenoic acid (Z)-, hexadecyl ester (26.1 %) and 1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)- (10.09 %), their common names are Linoleic; Oleic acid, hexadecyl ester and Etriol respectively. So, the anticancer activity of crud extract, which found in this study, may due to these compounds. Linoleic has good anticancer activity against mouse myeloma XS 63-5 cells[28], colon cancer cell- mediated by the CDK inhibitor[29], and Fights and prevention of cancer[30]. Different linoleic acid isomers inhibited the growth of mammary, colon, colorectal, gastric prostate, and hepatoma cell lines, mediated induction the expression of apoptotic genes[31].

The anticancer activity of madder may be due to oleic acid components rather than to Linoleic. Lot of studies approved the inhibition in cell proliferation induced by oleic acid in different cancer cell lines. Oleic acid could suppress the over-expression of HER_2 (erbB-2), a well-characterized oncogene, invasive progression and metastasis in different human cancers. Oleic acid could play a role in intracellular calcium signalling pathways linked to the proliferation event. Concerning to cell death, oleic acid has been shown to stimulate apoptosis in carcinoma cells. The mechanisms of it could be due to an increase in intracellular ROS production or caspase 3 activities[32]. In addition, Oleic acid is responsible for the prevention of breast cancer, precipitated a controversy[33].

Many anti-cancer plants have similar components, in current results there are: Tetradecanoic acid and hexadecyl ester in small quantities, they may be responsible for anticancer of *R. tinctorum*. Tetradecanoic acid was found in *Microcosmus exasperates* which has good anticancer activity against Dalton's Lymphoma Ascites (DLA) cells[34]. hexadecyl ester found in anticancer plant *Andrographis paniculata* leaf extract[35].

CONCLUSIONS

There were some elements in crud extract: Cu, Zn, Cd, Mn, K, Fe, Co, Ti and N but Phosphorus was absent. Thirty-five compounds identified extract using (GC-MS). The major three components present were 9,12-Octadecadienoic acid (Z,Z)- ; 9-Octadecenoic acid (Z)-, hexadecyl ester and 1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)-. The results of cytotoxicity of crud extract on cancer cell showed reduction in cell viability of (AMGM) cell line treating with all concentrations of extract. In (AMN3) cell line, the lower concentrations, reduced cell viably while the higher concentrations (50-100) mg/ml induced it. The extraction was less effect in cell viability of (HEp-2) cell Line. The toxicity human lymphocytes showed important changing in mitotic index, blast index, cell cycle progression and replication index but the extract induced chromosomal aberration and sister chromatid exchange. More study about toxicity of major three components that present in this plant will be benefit.

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