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Cytotoxic and Cytogenetics Effects of Aqueous, Methanolic and Secondary Metabolites Extracts of *Capparis spinosa* on Tumor Cell Lines *in vitro*

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Abstract

Capparis spinosa is one of the most aromatic plants growing in wild in the dry regions around the west or central Asia and the Mediterranean basin, and reports to contain a wide range of phytochemical constituents. The present study evaluates the cytotoxic effects of aqueous, methanolic crude extracts and secondary metabolites extracts polyphenolic, rutin, and alkaloids of mature fruit of *C. spinosa* on Human larynx carcinoma (Hep-2) and Human cervix adenocarcinoma (HeLa) tumor cell lines *in vitro*. The present study also includes the investigation of the effect of polyphenol mature fruit extracts on mitotic index (M.I.) of HeLa tumor cell line. The effect of (aqueous and methanol) crude extracts and secondary metabolites extracts (polyphenol, rutin, and alkaloids) of mature fruits of *C. spinosa* on Hep-2 and HeLa tumor cell lines have been showed highly significant difference ($P \leq 0.0001$) or ($P \leq 0.01$) among all types of extracts, and among all concentrations for each extract in two periods 24 and 48 hrs of the treatment. However the study reveals that the effective extracts against the proliferation of tested cell line were polyphenol extracts with concentration 10000 µg/ml in Hep-2 cells after 24 and 48 hrs. and with concentrations 10000 and 5000 µg/ml in HeLa cell line after 48 hrs. Polyphenolic extract showed a cytotoxicity concentration 50% (CC50%) 6400 and 6800 µg/ml on Hep-2 tumor cell line after 24 and 48 hrs. of treatment, respectively. The CC50% of HeLa cells was 7100µg/ml after 48 hrs. Other extracts; aqueous, methanolic crude extracts and secondary metabolites extracts (rutin and alkaloids) of mature fruit of *C. spinosa* caused less inhibition activity on the growth of Hep-2 and HeLa tumor cell lines. The CC50% for all these extracts were more than 10000 µg/ml. The result of present study shows that non significant difference of polyphenol mature fruit extracts effect on the type of tumor cell line either HeLa or Hep-2 cell lines. The cytogenetic study on HeLa cell line shows that polyphenol mature fruit extract has antimitotic index against tested cell line. Some of structural and numerical chromosomal aberrations were observed in both treated and untreated groups. Structural chromosomal aberrations include: ring chromosome (R. Ch.), dicentric chromosome (D. C.Ch.), chromatid gap and symmetrical interchange of chromosome as well as pulverization in treated group with higher concentrations 3550and 1775 µg/ml of extract. Numerical chromosomal aberrations include: octoploidy, euploidy and aneuploidy.

keywords: Cytotoxic, cytogenetics, extracts, Capparis spinosa, tumor cells.

1. Introduction

Cervical cancer is the second most common form of cancer among women worldwide about 274,000 deaths in 2002, and accounted for 15% of all female cancers (Fayed, 2008). In Iraq the cancer of the cervix is 2.1% (IARC, 2008). Laryngeal cancer is the most common non cutaneous malignancy. Cancers of the mouth, pharynx, and larynx, together, are the seventh most commonly occurring types of cancer worldwide. These cancers are three times more common in men than in women (AICR, 2007), especially those older than 60 years (Parkin *et al.*, 1990). Over 550000 cases were recorded in 2002, accounting for around 5 % of cancer cases overall (AICR, 2007).

Larynx carcinoma can develop in any part of the larynx. Most of the cancers of the larynx begin in cells that line the inner walls of the larynx (Parkin *et al.*, 1990). In the last three decades, cancer has been transformed from a fatal disease to one in which the majority of people diagnosed with cancer receive highly effective treatments that result in either cure or long-term survivorship (Angela *et al*., 2007).

Medicinal plants possess an important position in the drug discovery (Newman *et al*., 2000). According to the estimates of the WHO, more than 80% of people in developing countries depend on traditional medicine for their primary health needs (Sivalokanathan *et al*., 2005; Pandey and Madhuri, 2006). A major reason for the

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increase seems related to consumers' perceptions that these products are environmentally pure and without side effects (Montbriand, 1997 and 2000). Plant extracts have longer been a fertile source of cure for cancer (Mukherjee *et al*., 2001).

Several studies were carried out during the last years in Iraq to evaluate the cytotoxic activity of several local plants *in vitro* and *in vivo*. Al-Atby (2001) has found that the ethanolic extract of *Withaniasomnifera* had a growth inhibitory action against plasmacytoma cell line. In addition, the study of AL- Dabhawi (2005) revealed the great growth inhibition of aqueous and ethanolic extracts of *Artemisia herba alba* on Hep-2 and AMN-3 cell lines. Aqueous root extract of *Echium sericeum*posses inhibitory affect on the growth of Hep-2 and RD cell lines (Al-Habbib and Al-Asady, 2009). Seeds of *C.spinosa* contain glucocapparin, glucocleomin (Bhargara and Soni, 1980; Mathur, 1986) palmitic, oleic acid and linoleic acid (Attila and Ozcan, 1999). A dimeric 62-KDa lectin exhibiting a novel N-terminal amino acid sequence was purified from *C. spinosa* seeds (Lam *et al.*, 2009). Glucosinolates like sinigrin, glucoiberin and glucocleomin were isolated from the seeds and leaves of *C. spinosa* (Romeo *et al*., 2007). *Capparis spinosa* fruits contain alkaloids, glucosides, reducing sugar, fats, resins, ascorbic acid (Rastogi and Mehrotra, 1995) and isothyiocyanate (Mitchell, 1974). Alkaloids have been isolated and identified from *C. spinosa* fruits (Fu *et al*., 2008). Yang *et al*.,(2010) found that fruit of *C.spinosa* contain a significant amount of compounds with many health benefit, Three new alkaloids, (1) Capparisine A, (2) Capparisine B, (3) Capparisine C and (4) two known alkaloids, (4)2-(5-hydroxymethyl-2-formylpyrrol-1-yl) propionic acid lactone and (5)N-(3'-maleimidy1)-5-hydroxymethyl-2-pyrrole formaldehyde were isolated from the fruit of *C.spinosa* . Indole-3 acetonitrile glycosides and (6S)-Hydroxy-3-oxo-α-ionolglucosides were isolated from *C. spinosa* mature fruits in Turkey (Çalis*et al*., 1999; Çalis*et al*., 2002). Triterpenoids like α-amyrin, sterols, β-carotene, saponins were found in the preliminary phytochemical screening (Satyanarayana*et al*., 2008). Recently study of A. Al-Sqeer (2011) revealed that the total antioxidants in hot-water extracts of *C.spinoso* were 115.66µmol per 100mL.

The total alkaloids of *C. spinosa* can inhibit the growth of human gastric adenoma cells SGC-7901(Yu *et al*., 2008). Aqueous and methanolic root extract of *C. spinosa* possess considerable inhibition of AMN3 cells ,whereas Hep-2 tumor cell line is sensitive to aqueous root extract as well as aqueous leave extract *in vitro*. Aqueous and methanolic root extract of *C.spinosa* has ability to reduce the tumor volume *in vivo* (Al-Asady, 2007). The lectin potently that isolated from seeds of *C.spinosa* inhibited the proliferation of both hepatoma HepG2 and MCF-7 cell lines. (Lam *et al.*, 2009).

According to the medical properties of *C. spinosa* fruits in the world those not detected in Iraq, the present study was designed to evaluate the cytotoxic effects of aqueous , methanolic crude extracts and secondary metabolites; polyphenol, Rutin, and Alkaloids of mature fruits of *C. spinosa* on Human larynx carcinoma (Hep-2) and Human cervix adenocarcinoma (HeLa) tumor cell lines *in vitro*.

2. Material and Methods

2.1. Plant Collection

Capparis spinosa was collected from Duhok governorate in September 2008. The whole plant was deposited to be identified, the identification done by Dr. Salem Shahbaaz, Department of Forestry, College of Agriculture, University of Duhok, Duhok, Iraq. Then whole mature fruits and seeds from mature fruits were dried at room temperature. According to (Harborne,1984), each part was ground into powder by electrical grinder (mesh No. 0.5mm), and the powdered parts were kept in plastic tubes in deep freeze -20°C until the time of use. Crude aqueous extract and crude methanolic extract, from whole mature fruit, alkaloid from seeds of mature fruit of *C.spinosa* were prepared according to (Harborne, 1984). Extraction of Secondary Metabolites Polyphenol from mature fruit as described by Yu and Dahlgren, (2000), whereas rutin was extracted from mature fruit of *C. spinosa* according to Kim *et al.* (2005).

Chemical test for plant extracts both Wagner's Reagent and Hager's Reagent was used to test the presence of alkaloids in extracts ,whereas Ferric Chloride Solution according to Gayon (1972) and lead acetate solution according to Harborne (1984) was used to test the presence of polyphenol (tannins). The presence of flavonoids in extracts was tested according to (AL-Shahaat, 1986). The identification of rutin according to Harborne (1984). Liebermann-Burchard test was used to test the presence of triterpenoids, whereas Peptides and Free Amino Group Test used to test the presence of peptides, primary or secondary amino groups (Harborne, 1984). To test the presence of carbohydrate compounds Molish reagent was used (Hawk *et al*., 1954).The presence of glycosides was detected according to AL- Shahaat (1986). Saponins were identified according to Harborne (1984).

2.2. Cell Line

Human Larynx Epidermoid Carcinoma (Hep-2) tumor cell line Passages 220-223 in RPMI-1640 medium (Sigma, USA) and Human Cervix Adenocarcinoma (HeLa) passage 240-243 tumor cell line in Eagles MEM (Sigma, USA) supplemented with L-glutamine, non-essential amino acids and 10% FBS. was kindly supplied by Tissue Culture Unit/Iraqi Center for Cancer and Medical Genetic Researches (ICCMGR)/Baghdad, Iraq.

To determine the viability of tumor cell lines, confluent monolayer were treated with trypsin-versene and cells were further dispensed by pipetting in growth medium then 0.2 ml of cells suspension was mixed with 0.2 ml of trypan blue solution and 1.6 ml phosphate buffer saline (PBS), and a sample of cells counted by using an Improved Double Naubauer Ruling Counting Chamber. Magnification powers of 100X and 400X were used to count the cells, viable cells do not stain, but dead cells stain blue.

The following formula was then used to calculate the number of cells per unit volume (cells/ml) (Freshney, 1994):

$$C = N \times D \times 104 \quad (1)$$

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).**

About 200 µl of cells suspended (55000 cells/ml) in growth medium was seeded in to each well of a sterile 96-well micro-titration plate. The plates were sealed with a self-adhesive film, lid placed on and incubated at 37°C. When the cells are in exponential growth (approximately 70-80% confluent monolayer), the medium was removed and serial dilutions of each aqueous, methanolic crude extracts and secondary metabolites extracts (polyphenolic , rutin ,and alkaloids) of mature fruit, separately in maintenance medium (10000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, and 0 µg/ml) were added to the wells. Three replicates were used for each concentration of either extract, and the plates were re-incubated at 37°C for the selected exposure times (24 or 48 hrs).

Cytotoxic effect of each extract on both tumor cell lines using neutral red dye assay according to Freshney (1994). The optical density (O .D) of each well after treatment was read using Enzyme Linked Immunosorbent Assay (ELISA) reader at a transmitting wavelength of 492 nm (Mahoney *et al*., 1989; Freshney, 1994). The percentage of cytotoxicity was calculated as (A-B)/A X100, where A was the mean O.D of untreated wells and B is the O.D of wells with plant extracts (Betancur-Galvis*et al*.,1999).The cytotoxic concentration 50% (CC50%) for each extract was calculated from concentration-effect-curves after linear regression analysis (Hayslett and Patrick, 1981).

Cytogenetic Study on HeLa Tumor Cell Line before and after Treatment with Polyphenol mature fruit extracts .Three replicates were used for each concentration (3550, 1775 and 887.5 µg /ml) dependent on CC50%. Another three culture flasks were used as a control group treated with maintenance media + phosphate buffer (PBS). All flasks were incubated at 37°C for 48 hrs., the chromosomes was prepared according to Modi (1987). One thousand cells were examined to calculate the M.I. In these cases the slides were examined fewer than 10 X magnifications, and observed chromosomes or chromatides aberrations under 100X magnification. The M.I % was determined as a ratio of the mitotic cells to the cells in interphase in 1000 calculated cells. M.I. % = (No. of dividing cells / No. of dividing cells + No. of non-dividing cells) X 100 (Babu *et al*., 2005, Kleinsmith, 2006).

2.3. 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 SAS system (SAS, 2000).

3. Results

3.1. The Properties of C. spinosa Fruit Extracts, Yield of Extraction, and Qualitative Chemical Analysis

The results of *C. spinosa* aqueous , methanolic crude extracts and secondary metabolites (polyphenol., rutin. and alkaloids) extracts from Mature fruits with respect to the nature and color of the obtained extract, color of each extract solution, yield of extraction %, and qualitative chemical analysis for each extract are summarized in tables (1 and 2).

3.2. Thin Layer Chromatography (TLC) of Rutin Extract

Rutin extract from mature fruit was analyzed by TLC. The result of the analysis shows spots of standard rutin and extracts (Figure 1), the identification of rutin was done by comparison rate of flow (Rf) for extract with Rf of standard rutin as well as the color of spot under U.V. light (Table 3).

3.3. Cytotoxic effect of aqueous, methanolic crude extracts and secondary metabolites (polyphenol, rutin. and alkaloids) extracts of mature fruits of C.spinosa on Hep-2 Tumor Cell Line in vitro

The effect of (aqueous and methanol) crude extracts and secondary metabolites extracts (polyphenol, rutin, and alkaloids) of mature fruits of *C. spinosa* on Hep-2 tumor cell line have been showed highly significant difference ($P\leq 0.0001$) among all types of extracts, and among all concentrations for each extract in two periods 24 and 48 hrs of the treatment, the interaction between extracts and concentrations was highly significant ($P\leq 0.0001$) after 24 and 48 hrs. Table (4) shows that the cytotoxic effect of mature fruit extracts varied with different types of extracts and concentrations levels. Interaction between concentrations and extracts revealed that the Polyphenol extract was more effective than other extracts and its activity against the growth of Hep-2 cells was started from 1250 µg/ml as compare to control group(Figure 2a) which shows complete confluent monolayer of cohesive malignant cells and the value of O.D. was 0.248±0.005. The concentration 10000 µg/ml have higher inhibition activity 0.061±0.003 than 5000 µg/ml 0.177±0.01 (Figure 2 b and c), and this concentration is more effective than lower concentrations those exhibited the same effect. Aqueous extract was effective only in concentration 10000 µg/ml (Figure 2d) and the value of O.D. was 0.231±0.003. Methanolic extracts became effective in higher concentrations 5000 and 10000 µg/ml. The concentration 10000 µg/ml which have O.D. value 0.171± 0.002 and exhibit more inhibition activity than 5000 µg/ml with value 0.204± 0.006 (Figure 2e). The inhibition activity of rutin extract started from the concentration 2500 µg/ml up to 10000 µg/ml, these concentrations have the same effect (Figure 2f), the values of O.D. were 0.236±0.01, 0.236±0.014 and 0.226±0.01, respectively. Statistical analysis shows that both concentrations of alkaloid extracts 5000 and 10000 µg/ml has the same inhibition activity on the growth of Hep-2 cell line (Figure 2g) and the value of O.D. were 0.239±0.005 and 0.210±0.007, respectively. After 48hrs. treatment aqueous extracts became effective with concentration 5000 µg /ml as well as 10000 µg /ml both have the same effect and the values of O.D were 0.224 ± 0.001 and 0.230 ± 0.0104 respectively (Figure 3a). The effective concentrations of methanolic extracts after 48 hrs were 5000 and 10000µg/ml, the values of O.D. were 0.183±0.004 and 0.138±0.17, respectively. The higher concentration 10000 µg/ml was more effective (Figure 3b).

The effect of polyphenol extracts started from 2500µg/ml up to 10000µg/ml. The effect was concentration-dependant manner, the values of O.D. were 0.213±0.007, 0.178±0.005 and 0.076±0.0006 respectively (Figure 3c). The higher concentrations of rutin extract 5000 and 10000µg/ml were effective and both of them exhibit the same effect (Figure 3d). Alkaloid extracts started in its effect from 2500 µg/ml up to 10000 µg/ml, the effect of these concentrations have the same inhibition activity against proliferation of Hep-2 cell line (Figure 3 e)(table-5). The exposure times had a highly significant effect (P<0.0001) on the growth of Hep-2 tumor cell line treated with aqueous, methanol, and alkaloid extracts whereas no significant effect on the growth of Hep-2 cell line was noticed when subjected to other extracts Polyphenol and rutin extracts. Table (6) demonstrated that aqueous, was effective at 48 hrs more than 24 h. Methanolic extract also was more effective at 48 hrs more than 24 hrs. Similarly Alkaloid extracts was more effective after 48 hrs. Polyphenolic extract reduce the viability of Hep-2 cells down to 50% and presented CC50 value of 6400 and 6850µg/ml after 24 and 48 hrs., respectively, while those of (aqueous methanol, rutin and alkaloid) mature fruit extracts were more than 10000 µg/ml.

3.4. Cytotoxic Effect of aqueous, methanolic Crude Extracts and Secondary Metabolites (polyphenol, rutin, and alkaloids) extracts of mature fruits of C. spinosa on HeLa Tumor Cell Line in vitro

The results showed statistical differences among all types of extracts (P≤0.01) after 24 hrs of treatment and highly significant difference (P≤0.0001) after 48 hrs. The results also showed that the effect of tested extracts on HeLa cell line proliferation was highly significant (P≤0.0001) among concentrations after 24 and 48 hrs. The interaction between concentrations and extracts was not significant after 24 hrs of treatment and was highly significant (P≤0.0001) after 48 hrs.

The result revealed that each extract (aqueous, methanol, and rutin) has the same activity against proliferation of HeLa cells after 24 hrs treatment (Table 7). The activity of extracts started from concentration 78.13 µg/ml up to 10000 µg/ml. The more inhibition activity of polyphenol extract after 48 hrs. of treatment concentrated in both 10000 and 5000 µg/ml, and both have the same effect(Figure 4 a and b). Methanolic extract revealed its effect with concentrations 10000 and 5000 µg/ml both of them have the same inhibition activity (Figure 4c and d), the same result was obtained by alkaloid extracts treated cells (Figure 4e). Rutin extract revealed its activity with concentration 10000 µg/ml only (Figure 4f) (table 8).

The effect of exposure time of extracts on the proliferation of HeLa tumor cell line was highly significant (P<0.0001) after treating with each (methanol, rutin, and alkaloid.) extract and was significant (P<0.01) when treated with each (aqueous and polyphenol) extract. Table (9) appeared that crude and secondary metabolites extracts were more effective in 48 hrs than 24 hrs. Polyphenolic extract presented CC50 value of 7100 µg/ml after 48 hrs on HeLa cell line, while those of (aqueous, Methanol, rutin and alkaloid) mature fruit extracts were more than 10000 µg/ml.

3.5. Effect of mature fruit extracts. of C. spinosa on the Type of Tumor Cell Lines

Statistical analysis shows highly significant differences (P ≤ 0.0001) of mature fruit extracts effect on the types of tumor cell lines. The result reveals that HeLa tumor cell line is more affected than Hep-2 tumor cell line, the value of O.D. are 0.183±0.0022 and 0.244±0.0024, respectively (Table 10). The result of present study revealed that non significant difference of polyphenol mature fruit extracts effect on the proliferation of HeLa and Hep-2 cell lines. The value of O.D. for Hep-2 and HeLa cell lines are 0.227±0.004 and 0.172±0.003, respectively.

3.6. Cytogenetic Effect of polyphenol mature fruit extracts of C. spinosa HeLa on Tumor Cell Line in vitro

The results revealed highly statistical differences among treatments (P≤0.0001). The value of M.I in HeLa tumor cell line was decreased after treatment with concentrations 3550 and 1775 µg /ml of polyphenol mature fruit extracts only as compare with control groups 2.86±0.4, 3.4±0.26 and 6.76±0.24, respectively (table 11). Numbers of structural and numerical chromosomal aberrations were found in cells of control groups and in treated cells. The structural chromosomal aberrations in control group were: ring chromosome (R.Ch), chromosome break with fragment (Ch.B.W.F), gap chromosome (gap Ch.), dicentric chromosome (D.C.Ch.)(Figure 5a) and symmetrical interchange of chromosome (Figure 5b). The numerical chromosomal aberrations in control group were: euploidy (triploid 3n) (Figure 5c), octoploid (8n) (Figure 5d), aneuploidy (2n+2) (Figure 5 e). Pulverization of chromosomes was found in cells treated with high concentrations (3550 and 1775 µg\ml) of Polyphenol mature fruit extracts (Figure 6a, b). Other structural chromosomal aberrations in treated cells were chromatid break with fragment Cht.B.W.F., D.C.Ch and Cht. gap was found in cells treated with 1775 µg\ml Polyphenol mature fruit extracts (Figure 6c), ring chromosome, chromatid gap and D.C.Ch in cells treated with 887.5 µg\ml (Figure 6d). The numerical chromosomal aberrations were: triploid (3n) in cells treated with 887.5 µg\ml Es (Figure 6 e).

4. Discussions

4.1. The C. spinosa mature fruit extracts

The result of extraction in the present study reveals that the yield of extraction is varied according to the types of solvents those used in extraction method, and the method of extraction. This result agrees with that obtained by Henning *et al*. (2003) in which they find that the relation proportion between the amount of plant used for extraction and crude product. The result of present study revealed that the yield of extracts in mature fruit was high, may be according to the presence of high quantity of terpenoids and essential oils in mature fruits. This result is supported by Matthhaus and Ozcan (2005).

The result shows that the chemical compounds (alkaloids, tannins, flavonoids, glycosides, triterpenoids, carbohydrates, and saponins) and secondary metabolites extracts in the mature fruit under study were varied qualitatively due to the solvent of extraction. These qualitative variations can be attributed to the fact that the crude and secondary metabolites extracts from mature fruit contain different constituents that vary considerably in their relative concentrations. Study of Howard *et al*. (2000) on the *Capsicum* species, reveal that the concentration of chemical constituents such as carotenoids, flavonoids, phenolic acids and ascorbic acid increased as the *Capsicum annuum* , *C. frutescens* and *C. chinese* reached maturity.

Rutin extract is qualified as flavonoids secondary metabolites by comparison of its R_f value with that of standard. The yield of rutin extraction for mature fruit is 19.5, while Ramezani *et al*. (2008) purified rutin from different parts of *C. spinosa*, and they demonstrate that the yield of rutin extract from leaves, fruits and flowers are 18.22, 18.42 and 25.40% respectively, whereas the purity of rutin in leaves and flower extract is more than that extracted from fruits.

4.2. Cytotoxic Effect of Aqueous, Methanolic Crude Extracts and Secondary Metabolite (polyphenol, rutin, and alkaloid) extracts of mature fruit of C.spinosa on Hep-2 and HeLa Tumor Cell Lines in vitro

The cytotoxic effect of mature fruit extracts of *C. spinosa* on Hep-2 cell line varied with different types of extracts and concentrations levels. Polyphenol extract is the more effective extract in both periods of treatment (24 and 48 hrs). The activity of extracts concentrated in 10000 µg/ml against the growth of Hep-2 cells after 24 hrs and 48 hrs. The result revealed that HeLa cell line that treated with (aqueous and polyphenol) mature fruit extracts of *C. spinosa*, separately were more effective than other extracts (methanol, rutin, and alkaloid) after 24 hrs of treatment, whereas after 48 hrs, polyphenol extracts was the more effective with concentrations 10000 and 5000 µg/ml. The CC50 value for polyphenol extract on proliferation of Hep-2 was 6400 µg/ml after 24 hrs and was 6850 µg/ml after 48 hrs, whereas for HeLa cell line was 7100 µg/ml after 48 hrs. Other extracts (aqueous, methanol, rutin, and alkaloids) have CC50% more than 10000 µg/ml on proliferation of both Hep-2 and HeLa cell line. The highly inhibition activity of polyphenol mature fruit extracts against Hep-2 and HeLa cell lines can be due to its contents of active compounds such as caffeic, ellagic and ferulic acids, which have been reported to exhibit antioxidant and anticarcinogenic activities (Decker, 1995). Polyphenols were isolated from green tea, the powerful antioxidants were capable of scavenging H2O2and superoxide anions and thus preventing free radical damage to the body. This is a mechanism that has been associated with cancer (Khan *et al*., 1992). The results of present study agree with that obtained by Sa'eed, (2004) in which he found that the great growth inhibition of green and black tea extracts on Hep-2 tumor cell line. Plant extract rich in flavonoids exhibit antiproliferative effects on various cancer cell line (Adrienne *et al*., 2006). Apigenin is a widely distributed plant flavonoid that was reported as an antitumor agent, it inhibits the growth of human cervical carcinoma cells (Duthie and Crozier, 2000; Pei-Wen *et al*., 2005). Other extracts (aqueous, methanol, rutin. and alkaloid) reduce the proliferation of Hep-2 cells after 24 hrs. Simultaneously, whereas after 48 hrs methanol extracts shows its effect more than the other extracts (aqueous, rutin. and alkaloid). Methanolic extracts revealed this activity because most of the biological active compounds are extracted with methanol. Study of Betancur-Galvis *et al*. (1999) support the result that obtained in the present study. They found that seeds methanolic extract of *Annona* sp. has cytotoxic activity against Hep-2 tumour cells *in vitro*. Methanolic extracts was more effective than aqueous extracts because methnolic extracts contain the most potent antioxidant and phenolic compounds. Study of Khanavi*et al*. (2009) shows that methanolic extract from *Stachys* species contains the most potent antioxidant and phenolic compounds, whereas water extract afforded the lowest amount, therefore aqueous extracts lesser activity can be attributed to low quality of active compounds that included. Rutin extracts was also effective but the activity less than other extract, the less activity of rutin extracts may due to less purity of rutin that isolated from fruit (Ramezani*et al*., 2008). Alkaloid extracts have the same effect of methanolic extracts in HeLa cells after 48 hrs. treatment. Several studies reported that the plants have medical importance due to their alkaloids content, and these alkaloids have cytotoxic properties.

The study of Winter (2008) indicated that the low alkaloid lupin reduce the proliferation of mouse lymphoblast of P388 cell line even at 1mg/ml, and their cytotoxicity was assessed after 48 hrs. Vinca alkaloids, vinblastine and vincristine both act specifically to block mitosis of treated tumor cells (Richard *et al.,* 2001). Aparicio-Fernandez *et al*. (2006) demonstrated that methanolic crude extract of *Phaseolus vulgaris L.* have inhibitory effect on proliferation of HeLa cells. Other extracts (aqueous, methanol, and rutin) has the same activity against proliferation of HeLa cells after 48 hrs. Aqueous , methanol ,and alkaloid mature fruit extracts were more effective against the proliferation of Hep-2 cells at 48 hrs than after 24 hrs treatment , whereas the inhibition effect of Polyphenol and rutin mature fruit extracts against Hep-2 cells was similarly at both time of treatments 24 and 48 hrs. All types of mature fruit extracts against proliferation of HeLa cells were more effective after 48 hrs, except for polyphenol mature fruit extracts that revealed their inhibition activity against the proliferation of HeLa cell line after 48 hrs more than 24 hrs. This can be attributed to chemical constituents those found in polyphenol mature fruit extracts. These compounds may possess low ability to be absorbed by HeLa cells; therefore, they show their activity after 48 hrs of treatments. Marja (2004) stated that some plant extracts have low ability to adsorption by cell membrane such as glucosinolate and isothiocyanate, these can be found in polyphenol extract. In Hep-2 cells the similarity of polyphenol mature fruit extracts activity in both times, this reflect that Hep-2 cells have a specific properties in their membrane differs from HeLa cells that facilitate the movement of polyphenol mature fruit extracts compounds across it similarly in both time of treatment.

4.3. *The Effect of mature fruit extracts of C. spinosa* on the Type of Tumor Cell Lines

The *effects of mature fruit extracts on th*e types of tumor cell lines were detected. The results revealed that HeLa tumor cell line was more affected than Hep-2 tumor cell line. Lee *et al*. (2003) demonstrated that cell membrane receptors of tumor cells vary in their response to different drugs or crude extracts during chemotherapy treatments. Elisa et *al*. (2004) found that the cytotoxic activity of tannins and phenolic compounds extracted from *Cupheaaequipetala* was different in the three cancer cell lines; Hep-2, human colon cancer (HCT_15), and human prostate carcinoma (DV-145). The whole acetone-water extract does not show cytotoxic activity on (DV-145) cells.

The effect of polyphenol mature fruit extracts on the proliferation of HeLa and Hep-2 cells was also detected, both of them affected similarly with polyphenol mature fruit extracts. This may be due to the similarity in membrane properties in both Hep-2 and HeLa cell line as a target by phenolic compounds instead of mature fruit extracts.

This may be due to the presence of some chemical compounds in mature fruit extracts of *C. spinosa* with inhibitory properties against both Hep-2 and HeLa cell lines or may be due to the differences in membrane properties between Hep-2 and HeLa cells.

4.4. Cytogenetic Effect of polyphenol mature fruit extracts of C. spinosa on HeLa Tumor Cell Line in vitro

The present study shows that both tumor cell lines Hep-2 and HeLa were affected simultaneously after treatment with polyphenol mature fruit extracts. In this experiment, we have focused on determining the effect of polyphenol mature fruit extracts on M.I. of HeLa cell line. HeLa tumor cell line shows decrease in M.I. when treated with higher concentrations3550and 1775 µg /ml of polyphenol mature fruit extracts as compared with untreated cells. On the other hand, it was noticed that the low concentration 887.5 µg /ml has non significant effect. The acceptable explanation for ability of polyphenol mature fruit extracts to reducing the M.I. can be traced to its chemical constituents that have ability to effect on cell cycle progression. This result supported by Schoene *et al*. (2005) they have found the mixture of polyphenols from aqueous *Cinnamon* extract possessed anticancer properties by blocking cell cycle progression of leukemic cell lines at the G2/M phase. In addition to the G2/M arrest with mixture of polyphenols from aqueous Cinnamon, Schoene *et al*. (2005) also demonstrated that the extract reduced total phosphatase activity in the cell lines. Duthie and Crozier (2000) and Pei-Wen *et al*. (2005) suggested that apigenin is a strong candidate for development as an anti-cervical cancer agent. Apigenin's preventive effect is shown to be mediated through induction of p53 expression, which causes cell cycle arrest and apoptosis.

Chromosomal aberrations (numerical and structural) are found in HeLa cells those treated with polyphenol mature fruit extracts as well as in untreated cells. This result is in agreement with that obtained by Rocha-Guzman *et al.* (2009). Their result shows the ability of phenolic compounds to damage the DNA of HeLa cells and transformed human cells. Duesberg and Rasnick (2000) demonstrated the notion that aneuploidy is an autocatalytic process leading to the transition from a pre-neoplastic phenotype into a neoplastic one.

Table 1. The nature and color of dried product extracts and solutions of mature crude extracts of *C. spinosa*, and the yield of extraction %:

Part of plant	Type of Extract		Nature & color of Extract	Color of Solution	Yield of extraction %
Mature fruit	Crude extracts	Methanol	Solid→ greenish black	yellowish brown	15.8
		Aqueous	Solid→ brown	Brown	18.1
	Secondary metabolite extracts	Polyphenol	Viscous → greenish black	Brown	15.3
		Rutin	Solid → brown	Brown	19.5
		Alkaloid	Crystal → brown to black	Dark brown	11.7

Table 2. The results of qualitative chemical analysis for (aqueous, methnol, polyphenol and alkaloid) extracts of *C. spinosa* mature fruits.

	Mature crude Extracts			
Compound group	Aqueous	Methanol	Polyphenol	Alkaloid from (seeds)
Alkaloids				
a- Wagner′s reagent	+	+	+	+
b- Hagers reagent	+	++	+	++
Tannins				
a-lead acetate	+	+	+	+
b-Ferric chloride	+	+	+	+
Flavonoid test	+	+	++	-
Triterpenoid	-	++	++	+
Peptides& Free amino group	+	+	+	+
Carbohydrate	+	+	+	+
Glycosides				
a-before hydrolysis	++	-	+	-
b- after hydrolysis	-	-	-	-
Saponin	+	+	+	-

+ =The extract contain the designated phytochemicals.; - =The extract does not contain the designated phytochemicals.

Table 3. The results of TLC for rutin extract (R_f and color of spot under U.V. light) and comparison with standard rutin.

Compound	Rate of flow (R_f)	Color of spot under U.V. light
Rutin standard (a)	0.58	Yellow
Mature rutin extract (c)	0.48	Yellow

Table 4. Mean ± SE for the effect of different concentrations of (aqueous., methnol, polyphenol, rutin., and alkaloid) mature fruit extracts *spinosa* on the growth of Hep-2 tumor cell line after 24 hrs treatments *in vitro*: (Observations of O.D).

	Concentration µg/ml								
Extracts	0	78.13	156.25	312.5	625	1250	2500	5000	10000
Aqueous	0.289±0.014	0.268±0.011	0.269±0.006	0.269±0.008	0.266±0.004	0.265±0.005	0.268±0.002	0.258±0.006	0.231±0.003
Methanol	0.289±0.014	0.274±0.007	0.273±0.007	0.282±0.014	0.276±0.007	0.275±0.007	0.271±0.01	0.204±0.006	0.171±0.002
Polyphenol	0.289±0.014	0.280±0.007	0.282±0.006	0.284±0.005	0.270±0.008	0.248±0.005	0.235±0.01	0.177±0.012	0.061±0.003
Rutin	0.289±0.014	0.268±0.007	0.268±0.015	0.263±0.008	0.268±0.015	0.263±0.01	0.236±0.01	0.236±0.014	0.226±0.01
Alkaloid	0.289±0.014	0.272±0.003	0.270±0.006	0.270±0.002	0.271±0.007	0.265±0.005	0.259±0.004	0.239±0.005	0.210±0.007

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.011	0.015	0.033

SE=Standard Error.

Table 5. Mean ± SE for the effect of different concentrations of (aqueous., methnol, polyphenol, rutin., and alkaloid) mature fruit extracts of *C. spinosa* on the growth of Hep-2 tumor cell line after 48 hrs treatments *in vitro*: (Observations of O.D).

	Concentration µg/ml								
Extracts	0	78.13	156.25	312.5	625	1250	2500	5000	10000
Aqueous	0.258±0.004	0.245±0.004	0.244±0.003	0.247±0.002	0.243±0.006	0.239±0.0006	0.234±0.004	0.224±0.001	0.230±0.01
Methanol	0.258±0.004	0.248±0.011	0.248±0.02	0.240±0.012	0.246±0.007	0.244±0.007	0.241±0.007	0.183±0.004	0.138±0.017
Polyphenol	0.258±0.004	0.245±0.007	0.251±0.014	0.247±0.009	0.246±0.004	0.245±0.004	0.213±0.007	0.178±0.005	0.076±0.001
Rutin	0.258±0.004	0.257±0.005	0.258±0.013	0.258±0.007	0.260±0.01	0.248±0.004	0.246±0.007	0.210±0.008	0.186±0.006
Alkaloid	0.258±0.004	0.248±0.009	0.245±0.002	0.241±0.004	0.242±0.003	0.232±0.008	0.229±0.007	0.221±0.004	0.218±0.004

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.0091	0.0121	0.0272

SE=Standard Error.

Table 6. Mean ±SE for the effect of exposure time to mature fruit extracts of *C. spinosa* on the growth of Hep-2 tumor cells *in vitro* (Observations of O.D)

	Time/hrs		
Extract	24	48	L.S.D
Aqueous	0.265±0.003	0.240±0.002	0.01
Methanol	0.257±0.008	0.227±0.008	0.012
Polyphenol	0.236±0.014	0.218±0.011	-
Rutin	0.257±0.005	0.242±0.006	-
Alkaloid	0.260±0.005	0.237±0.003	0.008

SE=standard error.

Table 7. Mean ± SE for the effect of different concentrations of (aqueous., methnol, polyphenol, rutin., and alkaloid) mature fruit extracts of *C .spinosa* on the growth of HeLa-2 tumor cell line after 24 hrs treatments *in vitro*: (Observations of O.D).

	Concentration µg/ml								
Extracts	0	78.13	156.25	312.5	625	1250	2500	5000	10000
Aqueous	0.241±0.021	0.227±0.004	0.223±0.010	0.187±0.008	0.190±0.023	0.178±0.021	0.172±0.017	0.170±0.030	0.151±0.013
Methanol	0.241±0.021	0.233±0.008	0.234±0.009	0.231±0.009	0.227±0.001	0.212±0.016	0.217±0.005	0.173±0.003	0.157±0.014
Polyphenol	0.241±0.021	0.235±0.001	0.234±0.021	0.213±0.019	0.215±0.006	0.169±0.031	0.174±0.008	0.126±0.013	0.112±0.004
Rutin	0.241±0.021	0.216±0.005	0.216±0.008	0.214±0.007	0.210±0.018	0.211±0.018	0.210±0.009	0.185±0.012	0.153±0.013
Alkaloid	0.241±0.021	0.221±0.009	0.218±0.001	0.211±0.019	0.209±0.014	0.198±0.010	0.199±0.009	0.173±0.005	0.169±0.026

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.0192	0.026	_

SE=Standard Error.

Table 8. Mean ± SE for the effect of different concentrations of (aqueous., methnol, polyphenol, rutin., and alkaloid) mature fruit extracts of *C .spinosa* on the growth of HeLa-2 tumor cell line after 48 hrs treatments *in vitro*: (Observations of O.D).

	Concentration µg/ml								
Extracts	0	78.13	156.25	312.5	625	1250	2500	5000	10000
Aqueous	0.190±0.007	0.176±0.002	0.173±0.006	0.169±0.006	0.168±0.006	0.162±0.004	0.162±0.008	0.161±0.0003	0.161±0.005
Methanol	0.190±0.007	0.180±0.006	0.180±0.006	0.180±0.003	0.183±0.002	0.176±0.005	0.173±0.009	0.110±0.007	0.115±0.009
Polyphenol	0.190±0.007	0.177±0.007	0.171±0.0008	0.175±0.008	0.177±0.002	0.173±0.003	0.174±0.006	0.077±0.002	0.076±0.002
Rutin	0.190±0.007	0.176±0.007	0.176±0.009	0.177±0.013	0.178±0.008	0.171±0.005	0.172±0.004	0.170±0.004	0.144±0.009
Alkaloid	0.190±0.007	0.176±0.009	0.176±0.006	0.165±0.004	0.158±0.006	0.158±0.004	0.158±0.007	0.145±0.008	0.132±0.008

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.008	0.01	0.0422

SE=Standard Error.

Table 9. Mean ±SE for the effect of exposure time to (aqueous., methnol, polyphenol ,rutin., and alkaloids) mature fruit extracts of *C. spinosa* on the growth of HeLa tumor cell line *in vitro*.

	Time/hrs		
Extract	24	48	L.S.D
Aqueous	0.191±0.003	0.169±0.003	0.034
Methanol	0.214±0.006	0.165±0.007	0.012
Polyphenol	0.191±0.003	0.154±0.006	0.032
Rutin	0.206±0.004	0.172±0.005	0.014
Alkaloid	0.204±0.00	0.162±0.00	0.015

SE=standard error.

Table 10. Mean ± SE for the effect of mature fruit extracts of *C. spinosa* on the types of cell lines:

Cell lines	Hep-2	HeLa	L.S.D
Mature fruit Extract	0.244±0.0024	0.183±0.0022	0.0213

SE=standard error

Table 11. Mean ±SE for M.I of HeLa tumor cells after 48 hrs. treatment with polyphenol mature fruit extract of *C. spinosain vitro*:

	Concentration µg/ml	M.I%
Control	0	6.76±0.24
Treatment with Polyphenol mature fruit extracts	3550	2.8±0.4
	1775	3.4±0.26
	887.5	5.2±0.23
L.S.D		1.397

SE=standard error

Figure 1. TLC of rutin extract after treatment with ammonium hydroxide (NH_4OH) solution. a-Standard rutin b- Mature fruit

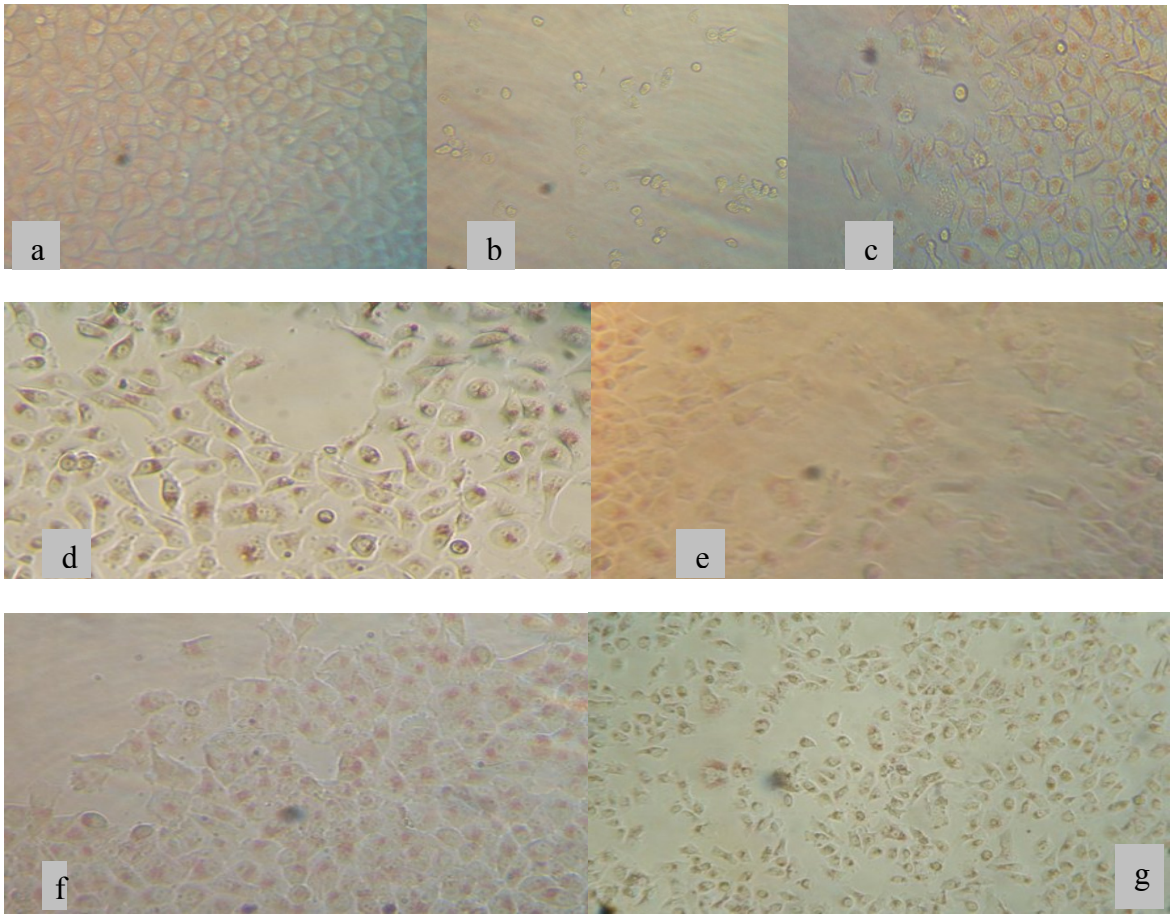

Figure 2. Hep-2 tumor cell line after 24 hrs (250X) treatment. (a) Control confluent monolayer (b) cells treated with 10000µg/ml Polyphenol mature fruit extracts (c) cells treated with 5000 µg/ml Polyphenol mature fruit extracts.(d) cells treated with 10000µg/ml aqueous mature fruit extracts (e) cells treated with 10000 µg/ml methanolic mature fruit extracts. (f) cells treated with 10000µg/ml rutin mature fruit extracts,(g) cells treated with 10000µg/ml alkaloids mature fruit extracts.

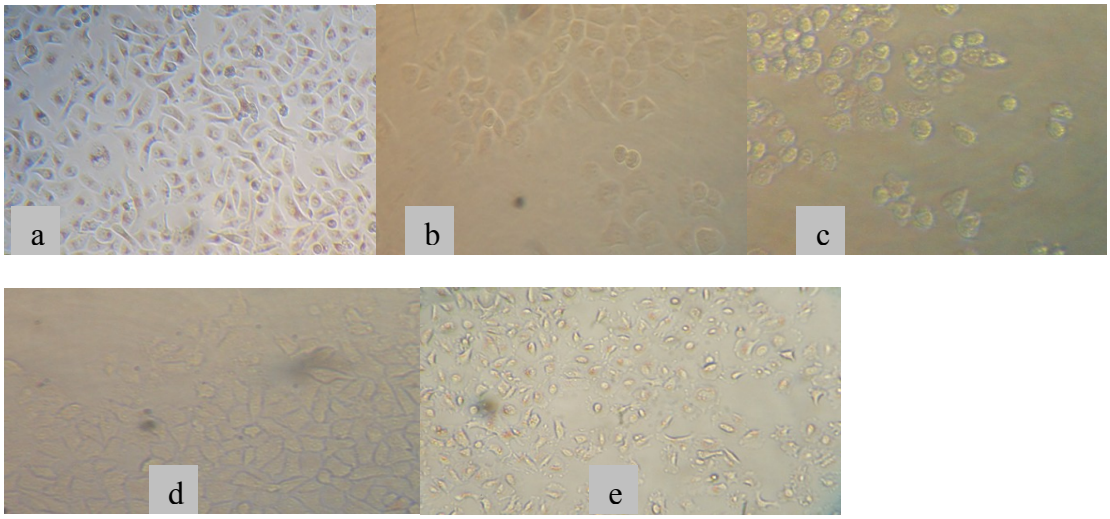

Figure 3. Hep-2 tumor cells (250X) after 48 hrs. treated with (a) 10000µg/ml aqueous mature fruit extracts (b) 10000 µg/ml methanolic mature fruit extracts (c) 10000µg/ml polyphenol mature fruit extracts (d) 5000µg/ml polyphenol mature fruit extracts (e) 10000 µg/ml rutin mature fruit extracts.

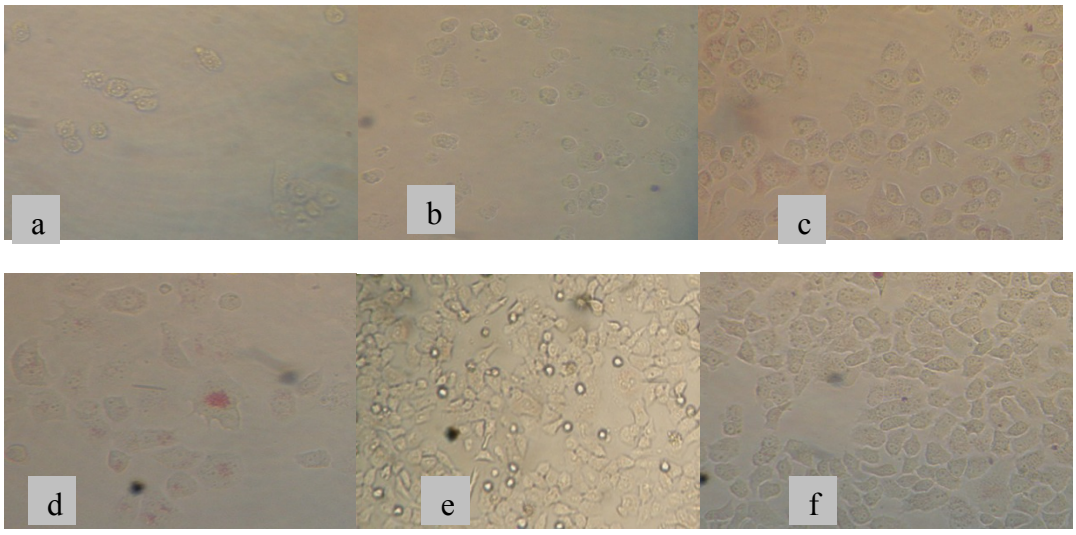

Figure 4. HeLa tumor cells(250X) after 48 hrs treated with (a) 10000µg/ml polyphenol mature fruit extracts (b) 5000µg/ml polyphenol mature fruit extracts (c)10000µg/ml methanol mature fruit extracts (d) 5000µg/ml methanol mature fruit extracts (e) 10000µg/ml alkaloids mature fruit extracts (f) 10000µg/ml rutin mature fruit extracts.

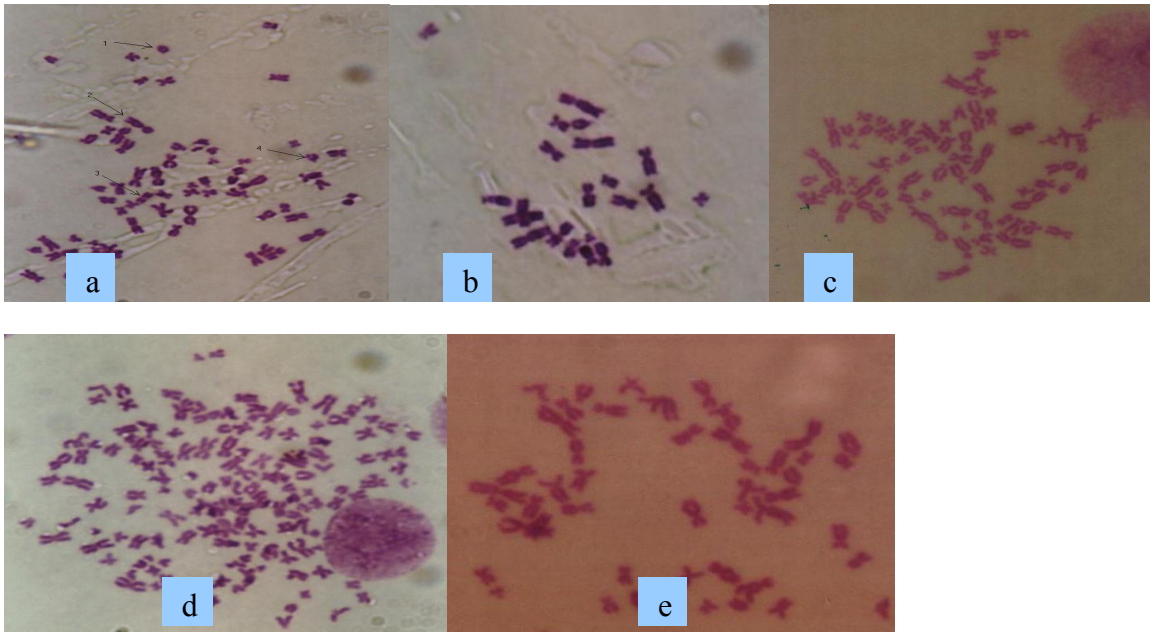

Figure 5. Chromosomes of HeLa tumor cell line untreated group(1000X, Giemsa stain) (a and b) structural chromosomal aberrations (a) 1-R. Ch.,2- D.C.Ch 3-Ch. B.W. F., and 4- gapCh. (b) symmetrical interchange of chromosome (c, d, and e) numerical chromosomal aberration (c) euploidy (triploid 3n) (d) octoploid, (e) aneuploidy(2n+2).

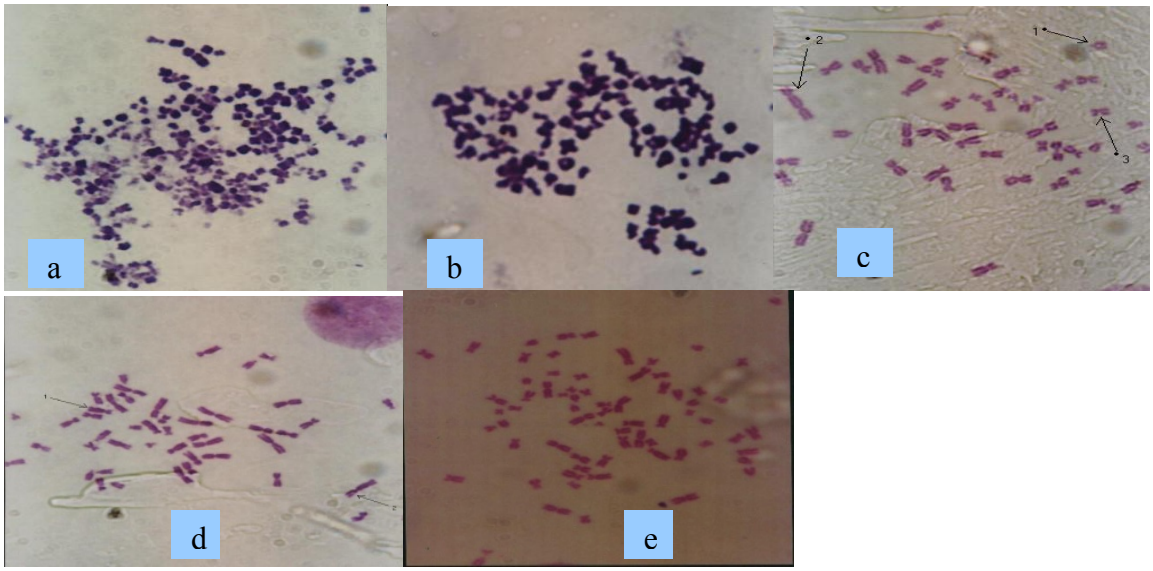

Figure 6. Chromosomes of HeLa tumor cell line(1000X, Giemsa stain) treated with polyphenol. mature fruit extracts (a and b) pulverization of chromosomes treated with 3550 and 1775µg/ml., respectively. (c, d and e) structural chromosomal aberration (c) 1-R. Ch., 2- D.C.Ch. 3-Chromtid gap in HeLa cells treated with 1775 µg/ml (d) 1-D. C.Ch 2- chromatide gap in HeLa cells treated with 887.5µg/ml (e)euploidy (3n) in HeLa cells treated with 887.5µg/ml

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