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Cytotoxic Effects of Aqueous, Methanolic and Secondary Metabolites Extracts of Capparis Spinosa Fruit on Tumor Cell Lines In Vitro

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Abstract: The present study **aims** to prepare two types of extracts; (methanolic and aqueous) crude extracts and (polyphenol and rutin) secondary metabolite extracts of immature fruit of *Capparis spinosa* to evaluate the cytotoxic effects of all these prepared extracts on Human larynx carcinoma (Hep-2) and Human cervix adenocarcinoma (HeLa) tumor cell lines *in vitro*. The data of immature fruit extracts in present study was compared with that obtained by the same investigators in previous study for mature fruit extract to evaluate which one is more effective against the proliferation of tested tumor cell lines. The **results** of present study showed that the yield of extraction % of methanol and aqueous crude extracts; 16.1% and 15%, respectively, whereas that of polyphenol and rutin secondary metabolite extracts were 12.7% and 12.1%, respectively. The results revealed that the more effective extracts against the proliferation of Hep-2 tumor cell line was aqueous extract after 24 hrs treatment. After 48 hrs treatment each of methanol, aqueous, and rutin was more effective than polyphenol extract. Methanol and rutin extracts were more effective after 48 hrs than 24 hrs treatment. All types of immature fruit extracts had CC50 values on Hep-2 cell line > 10000 µg/ml for both periods of exposure. The result revealed that the effect of both methanol and rutin on proliferation of HeLa cell line after 24 hrs treatment was more than that of aqueous and polyphenol extracts. After 48 hrs treatment the activity of methanolic extract and aqueous against the proliferation of HeLa cell line more than that of polyphenol and rutin, the values of CC50 on HeLa cell line treated with methanol extract after 48 hrs was 9700 µg/ml. Aqueous extract was more effective after 48 hrs than 24 hrs. The present study shows that HeLa tumor cell line was more effective than Hep-2 tumor cell line. There were some notable cytotoxic activities of mature fruit extract of *C. spinosa* against the tumor cell lines than for that of immature fruit extract as compare with the previous study that done by the same investigators.

Key words: Cytotoxicity, crude extracts, capparis spinosa, fruit, tumor cell lines.

1. Introduction

Worldwide there are about 274,000 women deaths in 2002, because of cervical cancer [1]. In Iraq the cancer of the cervix is 2.1% [2]. Cancers of the mouth, pharynx, and larynx, together, are the seventh most commonly occurring types of cancer worldwide. Most of the cancers of the larynx begin in cells that line the inner walls of the larynx [3]. 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 [4].

The medical plants are used by people for medical purposes to build or maintain health, because the plants are an important source of molecules that may be useful as a drug [5]. Use of medicinal plants comes from ancient especially in the Africa, Asia and Latin America where the majority of the world's people live. Every day, a new study is published in the world journals to confirm pharmacological effects of medicinal plants that have been used traditionally. If one search for the key word of medicinal plants and pharmacology together in a general search engine like google, about 1,180,000 records would be found [6]. It is estimated that 30–40% of all pharmaceutical

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preparations that are used nowadays are derived from or based on plant metabolites [7]. Over 80,000 species of plants are in use throughout the world and the traditional and folk medicinal practice based on the use of plants and plant extracts is known as herbalism [8]. Capers belonging to the family Capparidaceae the genus *Capparis* comprise 250 species including shrubs, trees and woody climbers [9]. Probably it originated in the dry regions in west or central Asia [10]. Known and used for millennia, capers were mentioned by Iosco rides as being a marketable product of the ancient Greeks. Capers are also mentioned by the Roman scholar Pliny the Elder. More rarely, mature and semi-mature fruits are eaten as a cooked vegetable. Additionally, ash from burned caper roots has been used as a source of salt [5]. Capers contain considerable amounts of the antioxidant bioflavinoid, rutin [11, 12]. Peter [5] suggests that these antioxidants play a role in limiting cardiovascular disease and cancers. Caper seeds yield about 35% pale yellow oil containing palmitic, stearic, oleic and linoleic acids [13]. Two (6*S*)-hydroxy-3-oxo-alpha-ionol glucosides, together with corchoionoside C (6*S*, 9*S*)-roseoside) and a prenyl glucoside, were isolated from mature fruits of *C. spinosa* [14].

Capparis spinosa is said to be native to the Mediterranean basin, but its range stretches from the Atlantic coasts of the Canary Islands and Morocco to the Black Sea to the Crimea and Armenia, and eastward to the Caspian Sea and into Iran. *Capparis spinosa* is one such plant established to have highly diverse economic and medicinal value in different system of medicines. Seeds of *C.spinosa* contain glucocapparin, glucocleomin [15, 16]. palmitic, oleic acid and linoleic acid [17].

A dimeric 62-KDa lectin exhibiting a novel N-terminal amino acid sequence was purified from *C. spinosa* seeds [18]. Glucosinolates like sinigrin, glucoiberin and glucocleomin were isolated from the seeds and leaves of *C. spinosa* [19]. *Capparis spinosa* fruits contain alkaloids, glucosides, reducing sugar, fats, resins, ascorbic acid [13] and isothyiocyanate [11]. Alkaloids have been isolated and identified from *C. spinosa* fruits [20]. Triterpenoids like α-amyrin, sterols, β-carotene, saponins were found in the preliminary phytochemical screening [21].

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* [22]. Aqueous and methanolic root extract of *C.spinosa* has ability to reduce the tumor volume *in vivo* [22]. The total alkaloids of *C. spinosa* can inhibit the growth of human gastric adenoma cells SGC-7901 [23]. The lectin potently that isolated from seeds of *C.spinosa* inhibited the proliferation of both hepatoma HepG2 and MCF-7 cell lines [18].

Recently, the study of AL-Asady *et al*. [24] revealed that the secondary metabolite extract polyphenol from mature fruit extract of *C. spinosa* has inhibition activity against Hep-2 tumor cell line after 24 and 48 hrs treatment, and against HeLa tumor cell line after 48hrs treatment, the CC50% of Hep-2 cells was 6400 and 6800 µg/ml after 24 and 48 hrs, respectively. The CC50% of HeLa cells was 7100 µg/ml after 48 hrs.

According to the prevalence of *C. spinosa* in Iraq, and there are no reports on the cytotoxicity of immature fruit of this plant on tumor cell lines in Iraq. Hence, this work was conducted to evaluate the cytotoxic effects of Aqueous, Methanolic and Secondary Metabolites Extracts of *C. spinosa* immature fruit on Human larynx carcinoma (Hep-2) and Human cervix adenocarcinoma (HeLa) tumor cell lines *in vitro*.

2. Materials & Methods

2.1 Plant Collection

Capparis spinosa was collected from Duhok governorate/Iraq in September 2008. The whole plant was deposited to be identified, the identification done by Prof. Dr. Salem Shahbaaz plant taxonomist, Department of Forestry, College of Agriculture, University of Duhok, Duhok, Iraq. Then whole

immature fruits were dried at room temperature. According to Harborn [25]. the fruit was ground into powder by electrical grinder (mesh No. 0.5 mm), and the powdered parts were kept in plastic tubes in deep freeze -20°C until the time of use. Crude extracts(aqueous & methanol) from whole immature fruit of *C.spinosa* were prepared according to Harborn, [25]. Extraction of Secondary Metabolites Polyphenol from immature fruit as described by Yu and Dahlgren, [26]. Whereas rutin was extracted from immature fruit of *C. spinosa* according to Kim *et al.* [27]. 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 [28] and lead acetate solution according to Harborn [25] was used to test the presence of polyphenol (tannins). The presence of flavonoids in extracts was tested according to AL- Shahaat [29]. The identification of rutin according to Harborn [25]. 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 [25]. To test the presence of carbohydrate compounds Molish reagent was used [30]. The presence of glycosides was detected according to AL- Shahaat [29]. Saponins were identified according to Harborne [25].

2.2 Cell Line

Human Larynx 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 (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) (31):

$$C = N \times D \times 10^4$$

Where C is the number of viable cells per milliliter, N is the number of viable cells counted, and D is the dilution factor (D = 10). 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 (polyphenol & rutin) of immature 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, [31]. 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 [31, 32]. 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 (33).The cytotoxic concentration 50% (CC50%) for each extract was calculated from concentration-effect-curves after linear regression analysis [34].

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 [35].

3. Results

The Properties of C. spinosa Immature Fruit Extracts, Yield of Extraction, and Qualitative Chemical Analysis: The results of C. spinosa; Aqeous, Methanol crude extracts and secondary metabolites Polyphenol and rutin from Immature fruit 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.

Thin Layer Chromatography (TLC) of Rutin Extract Rutin extract from Immature fruit of *C.spinosa* was analyzed by TLC. The identification of rutin was done by comparison rate of flow (Rf) for each extract with Rf of standard rutin as well as the color of spot under U.V. light Table 3.

Cytotoxic Effect of Aqueous, Methanol crude Extracts and Secondary Metabolites (Polyphenol and Rutin) Extracts of Immature fruit of *C. spinosa* on Hep-2 Tumor Cell Line *in vitro:*

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

Compound	Rate of flow (Rf)	Color of spot under U.V. light
Rutin standard (a)	0.58	Yellow
Immature rutin extract (b)	0.48	Yellow

Table 1 The nature and color of dried product extracts and solutions of immature fruit 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 %
Im.f.	Crude extracts	Methanol	Viscous→ dark brown	Green	16.1
		Aqueous	Viscous→ dark brown	Brown	15
	Secondary metabolite extracts	Polyphenol	Viscous → dark brown	Greenish brown	12.7
		Rutin	Solid → brown	yellowish brown	12.1

Im.f = immature fruit.

Table 2 The results of qualitative chemical analysis for Aqueous, Methanol, and Polyphenol Extracts of Immature fruit of *C. spinosa*.

Compound group	Im.f Extracts					
	Aqueous		Methanol		Polyphenol	
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	+		+		+	

Im.f = immature fruit; +=The extract contain the designated phytochemicals;

- =The extract does not contain the designated phytochemicals.

The results demonstrate a highly significant difference ($p \leq 0.0001$) among all crude and secondary metabolites extracts and among concentrations after 24 hrs treatment. The differences among extracts and among concentrations were significant ($p \leq 0.01$) after 48hr treatment. The interaction between extracts and concentrations were not significant after 24 hrs and 48 hrs. Table 4 shows that the concentrations of Immature fruit crude extract and secondary metabolites extracts after 24 hrs started their effects from 312.5 µg/ml up to 10000 µg/ml as compare with control group (Fig. 1a). The more effective extracts on the proliferation of Hep-2 tumor cell line was Aqueous extracts after 24 hrs treatment (Fig. 1b), the value of O.D. was 0.264±0.004, other extracts (Methaanol, Polyphenol, and Rutin) seem to have the same inhibition activity, the O.D. were 0.281±0.004, 0.279±0.006 and 0.283±0.005, respectively as shown in (Figs. 1c and 1d).

Table 5 shows that the concentrations of Immature fruit crude extract and secondary metabolites extracts after 48 hrs were effective with 5000 and 10000 µg/ml only. The same table also shows that the Methanolic extracts was more effective than Polyphenol extract (Figs. 2a and 2b), whereas it exhibit the same inhibition activity of Aqueous and Rutin extract, the value of O.D were 0.230±0.006, 0.250±0.006, 0.246±0.005 and 0.241±0.004, respectively. Statistical analysis revealed significant effect ($p \leq 0.01$) of exposure time of Hep-2 tumor cell line to (Methanol and Rutin) extracts, whereas non significant of exposure time of cells to each of Aqueous and Polyphenol extract of Immature fruit (Table 6). Methanol and Rutin extracts were more effective after 48 hrs than 24 hrs, the value of O.D. were 0.230±0.006, 0.281±0.004 and 0.241±0.004, 283±0.005, respectively. Other extracts (Aq. and P.) exhibit the same effect in both time of treatment, the value of O.D. were 0.264±0.004, 0.246±0.005, 0.279±0.006, 0.250±0.006 (Table 7). All types of Immature fruit extracts had CC50 values on Hep-2 cell line (>10000 µg/ml) for both periods of exposure.

Table 4 Mean ± SE for the effect of different concentrations of (Aqueous, Methanol, Polyphenol and Rutin)Immature fruit extract of *C .spinosa* on the growth of Hep-2 tumor cell line after 24 hrs treatments *in vitro*: (Observations of O.D).

Extracts	0	Concentration µg/ml								
		78.125	156.25	312.5	625	1250	2500	5000	10000	Over all concentrations
Aqueous	0.254±0.017	0.261±0.034	0.245±0.006	0.248±0.006	0.246±0.01	0.238±0.007	0.234±0.007	0.232±0.005	0.233±0.005	0.246±0.005
Methanol	0.254±0.017	0.247±0.019	0.249±0.01	0.246±0.012	0.240±0.006	0.222±0.02	0.241±0.012	0.174±0.013	0.194±0.004	0.230±0.006
Polyphenol	0.254±0.017	0.242±0.028	0.266±0.016	0.269±0.018	0.265±0.005	0.260±0.015	0.242±0.016	0.248±0.01	0.209±0.015	0.250±0.006
Rutin	0.254±0.017	0.248±0.01	0.247±0.011	0.245±0.014	0.246±0.011	0.245±0.005	0.248±0.014	0.222±0.01	0.219±0.019	0.241±0.004
Over all extracts	0.254±0.007	0.254±0.01	0.252±0.005	0.252±0.006	0.250±0.005	0.242±0.007	0.242±0.006	0.219±0.009	0.214±0.007	

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.0182	0.0273	-

Table 5 Mean ± SE for the effect of different concentrations of (Aqueous, Methanol, Polyphenol and Rutin)Immature fruit extracts of *C .spinosa* on the growth of Hep-2 tumor cell line after 48 hrs treatments *in vitro* (Observations of O.D).

Extracts	0	Concentration µg/ml								
		78.125	156.25	312.5	625	1250	2500	5000	10000	Over all concentrations
Aqueous	0.307±0.016	0.271±0.005	0.266±0.004	0.263±0.007	0.255±0.009	0.268±0.003	0.251±0.008	0.249±0.006	0.245±0.003	0.264±0.004
Methanol	0.307±0.016	0.293±0.003	0.288±0.004	0.292±0.011	0.286±0.01	0.286±0.007	0.266±0.003	0.251±0.002	0.259±0.001	0.281±0.004
Polyphenol	0.307±0.016	0.295±0.005	0.294±0.004	0.290±0.003	0.281±0.044	0.280±0.003	0.278±0.005	0.266±0.006	0.244±0.008	0.279±0.006
Rutin	0.307±0.016	0.298±0.009	0.300±0.008	0.297±0.008	0.293±0.008	0.277±0.001	0.274±0.008	0.266±0.005	0.234±0.006	0.283±0.005
Over all extracts	0.307±0.007	0.289±0.004	0.287±0.005	0.286±0.005	0.274±0.011	0.278±0.003	0.268±0.004	0.258±0.003	0.246±0.004	

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.014	0.0204	-

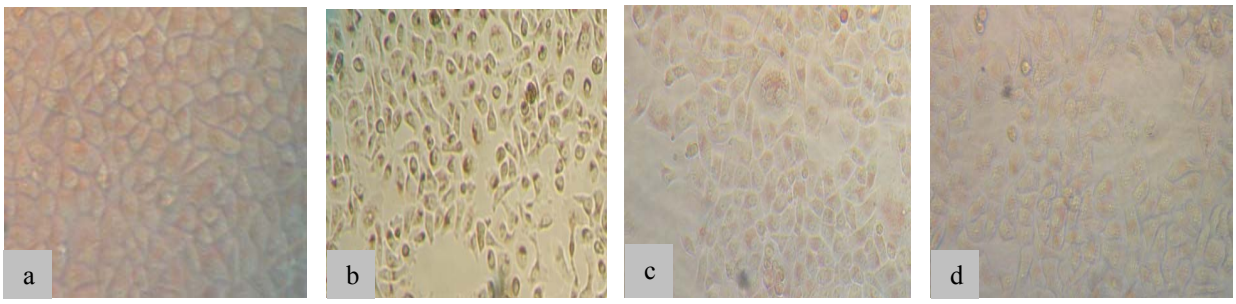

Fig. 1 Hep-2 tumor cell line (250X) after 24 hrs treatment: (a) Control confluent monolayer, (b) cells treated with 10000 µg/ml Aqueous immature fruit extracts, (c) cells treated with 10000 µg/ml Methanolic immature fruit extracts, (d) Cells treated with 10000 µg/ml Polyphenol immature fruit extracts.

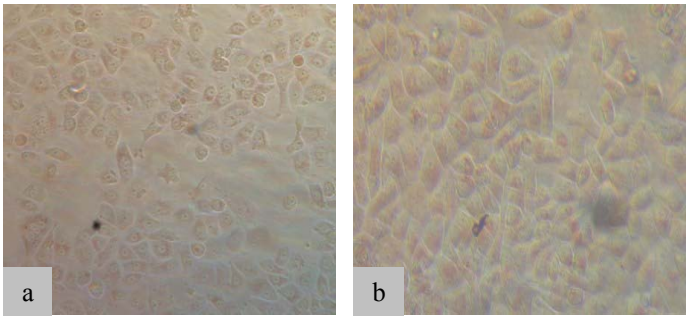

Fig. 2 Hep-2 tumor cell line (250X) after 48 hrs treated with: (a) 10000 µg/ml Methanolic immature fruit extracts, (b) 10000 µg/ml Polyphenol immature fruit extracts.

Table 6 Analysis of variance for the effect of exposure time to Immature fruit extracts of *C. spinosa* on the growth of Hep-2 tumor cell line *In vitro*.

S.O.V	F value				
	d.f	Aq.	Meth.	P.	R.
Time	1	5.37^{NS}	42.24^{*}	6.58^{NS}	32.09^{*}
Error	52				

S.O.V = source of variance, d.f = degree of freedom, NS = non significant * = ($P \leq 0.01$).

Table 7 Mean ±SE for the effect of exposure time to Immature fruit extracts of *C. spinosa* on the growth of Hep-2 tumor cells *in vitro* (Observations of O.D).

Extract	Time/hrs		L.S.D
	24	48	
Aqueous	0.264±0.004	0.246±0.005	-
Methanol	0.281±0.004	0.230±0.006	0.029
Polyphenol	0.279±0.006	0.250±0.006	-
Rutin	0.283±0.005	0.241±0.004	0.028

SE = standard error.

Cytotoxic Effect of Aqueous, Methanolic Crude Extracts and Secondary Metabolites (Polyphenol and Rutin) Immature fruit extracts. of *C.spinosa* on HeLa Tumor Cell Line *in vitro:*

The result of present study shows highly statistical differences among all extracts as well as concentrations ($p \leq 0.0001$) after 24 and 48 hrs of treatment. The interaction between concentrations and extracts were also highly significant ($p \leq 0.0001$) after 24 and 48 hrs. The effective extract on proliferation of HeLa cell line after 24 hr was methanol, which revealed more inhibition activity than Aqueous and Polyphenol extracts, Rutin extract exhibit similar effect with Methanolic extract. Rutin extract was more effective than Aqueous extract, while it had the same effect with polyphenol extract (Table 8). The same table shows that the concentrations of Immature fruit extracts have started their effects after 24 hrs from 78.13 µg/ml to the highest concentration 10000 µg/ml. Aqueous extract was effective only with concentration 10000 µg/ml as compare with control group (Figs. 3a and 3b). The concentrations that made of Methanol extracts effective were 2500 5000 and 10000 µg/ml, the higher concentration exhibits more inhibition activity (Fig. 3c), the value of O.D. were 0.231±0.005, 0.228±0.003

and 0.18±0.003, respectively. This effect was also pronounced in Polyphenol extract, whereas these concentrations have the same effect. Rutin extract shows similar effect with concentrations 1250, 2500, 5000 and 10000 µg/ml (Fig. 3d). The growth of HeLa cells was affected after 48 hrs with all concentrations started from 78.13 µg/ml up to 10000 µg/ml (Table 9). Methanolic extract was more effective than Polyphenol and Rutin extracts. It had similar effect with Aqueous extracts. Aqueous extracts were more effective than Polyphenol extracts, while it exhibit the similar effect with Rutin extract. Aqueous extract starting its inhibition activity from 2500 up to 10000 µg/ml and all concentrations revealed the same activity against the proliferation of HeLa cells (Fig. 4a), the value of O.D. were 0.188±0.004, 0.182±0.009 and 0.183±0.07, respectively. Methanolic extract was effected with concentrations 5000 and 10000 µg/ml the activity of extract increased with higher concentration (Fig. 4b), the value of O.D. were 0.153±0.014 and 0.107±0.004, respectively. Polyphenol extract appeared its effect with concentration 10000 µg/ml only (Fig. 4c), Rutin extract revealed its effect with concentration 5000 µg/ml which exhibit less activity than 10000 µg/ml with value of O.D. 0.184±0.011 and 0.137±0.004 (Fig. 4d).

The effect of exposure time was not significant on the growth of HeLa cell line when these cells are subjected to Immature fruit extracts (Table 10) except in Aqueous extract which showed significant effect. Table 11 shows that Aqueous extract was more effective after 48 hrs than 24 hrs, the values of O.D. were 0.194±0.003 and 0.272±0.003, respectively. The values of CC50 on HeLa cell line treated with Immature fruit extracts were more than 10000 µg/ml for each extracts in both times of exposure with exception that methanol extract after 48 hrs had CC50% 9700 µg/ml.

Table 8 Mean ± SE for the effect of different concentrations of (Aqueous, Methanol, Polyphenol, and Rutin) Immature fruit extracts of *C .spinosa* on the growth of HeLa-2 tumor cell line after 24 hrs treatments *in vitro*: (Observations of O.D).

Extracts	0	Concentration µg/ml								
		78.125	156.25	312.5	625	1250	2500	5000	10000	Over all concentrations
Aqueous	0.283±0.009	0.270±0.007	0.279±0.007	0.279±0.002	0.276±0.004	0.277±0.002	0.277±0.003	0.273±0.01	0.242±0.009	0.272±0.003
Methanol	0.283±0.009	0.257±0.003	0.256±0.004	0.256±0.006	0.255±0.005	0.255±0.006	0.231±0.005	0.228±0.003	0.180±0.003	0.244±0.006
Polyphenol	0.283±0.009	0.265±0.006	0.264±0.003	0.264±0.009	0.262±0.003	0.258±0.004	0.247±0.02	0.253±0.003	0.235±0.001	0.259±0.003
Rutin	0.283±0.009	0.269±0.005	0.256±0.024	0.265±0.007	0.266±0.003	0.245±0.006	0.242±0.004	0.240±0.006	0.236±0.013	0.252±0.004
Over all extracts	0.283±0.004	0.265±0.003	0.263±0.007	0.266±0.004	0.265±0.003	0.259±0.004	0.249±0.006	0.249±0.007	0.224±0.008	

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.0097	0.015	0.0292

Table 9 Mean ± SE for the effect of different concentrations of (Aqueous, Methanol, Polyphenol,and Rutin) Immature fruit extracts of *C .spinosa* on the growth of HeLa tumor cell line after 48 hrs treatments *in vitro*: (Observations of O.D).

Extracts	0	Concentration µg/ml								
		78.125	156.25	312.5	625	1250	2500	5000	10000	Over all concentrations
Aqueous	0.221±0.01	0.201±0.004	0.201±0.005	0.192±0.007	0.191±0.001	0.191±0.004	0.188±0.004	0.182±0.009	0.183±0.07	0.194±0.003
Methanol	0.221±0.01	0.202±0.003	0.204±0.003	0.203±0.004	0.198±0.007	0.198±0.011	0.200±0.006	0.153±0.014	0.107±0.004	0.187±0.007
Polyphenol	0.221±0.01	0.218±0.021	0.218±0.006	0.218±0.013	0.217±0.002	0.217±0.01	0.213±0.007	0.199±0.018	0.140±0.005	0.207±0.006
Rutin	0.221±0.01	0.210±0.006	0.210±0.007	0.209±0.013	0.209±0.005	0.205±0.007	0.208±0.002	0.184±0.011	0.137±0.004	0.1996±0.005
Over all extracts	0.221±0.01	0.208±0.005	0.208±0.003	0.206±0.005	0.204±0.004	0.203±0.005	0.203±0.004	0.180±0.008	0.142±0.008	

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.0107	0.016	0.032

SE = Standard Error.

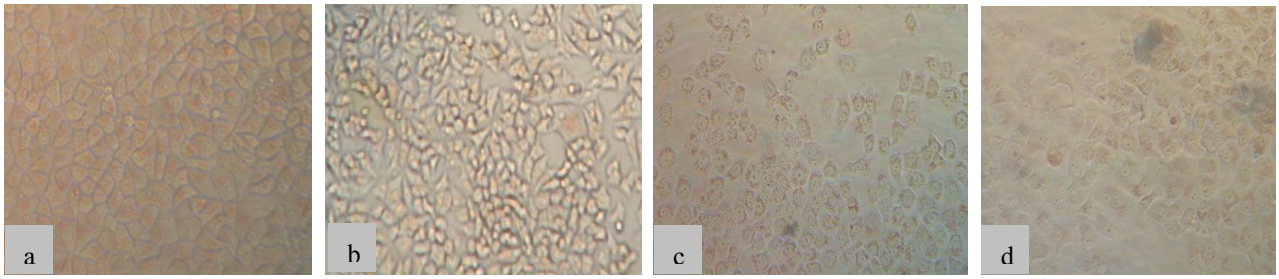

Fig. 3 HeLa tumor cell line (250X) after 24 hrs treatment: (a) Control confluent monolayer, (b) cells treated with 10000 µg/ml Aqueous Immature fruit extracts, (c) cells treated with 10000 µg/ml Methanol Immature fruit extracts, (d) cells treated with 10000 µg/ml Rutin Immature fruit extracts.

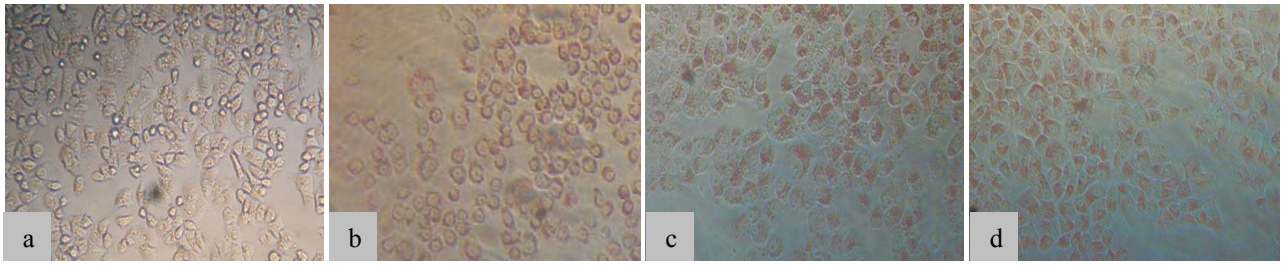

Fig. 4 HeLa tumor cell line (250X) after 48 hrs treated with: (a) 10000 µg/ml Aq. Im.f. Es, (b) 10000 µg/ml Methanol Immature fruit extracts, (c) 10000 µg/ml Polyphenol Immature fruit extracts, (d) 10000 µg/ml Rutin Immature fruit extracts.

Table 10 Analysis of variance for the effect of exposure time to Immature fruit extracts of *C. spinosa* on the growth of HeLa tumor cell line *in vitro*.

S.O.V	F value				
	d.f	Aq.	Meth.	P.	R.
Time	1	34.48^{**}	0.82^{NS}	2.82^{NS}	1.68^{NS}
Error	52				

S.O.V = source of variance, d.f = degree of freedom, NS = non significant ** = (P≤ 0.0001).

Table 11 Mean±SE for the effect of exposure time to Immature fruit extracts of *C. spinosa* on the growth of HeLa tumor cells *in vitro* (Observations of O.D).

Extract	Time/hrs		
	24	48	L.S.D
Aqueous	0.272±0.003	0.194±0.003	0.068
Methanol	0.244±0.006	0.187±0.007	-
Polyphenol	0.259±0.003	0.207±0.006	-
Rutin	0.253±0.004	0.199±0.005	-

SE = standard error.

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

Table 12 shows statistical differences (P ≤ 0.01) of Immature fruit extracts effect on cell lines types. The result revealed that HeLa tumor cell line was more effective than Hep-2 tumor cell line, the value of O.D. were 0.227±0.003 and 0.259±0.002, respectively (Table 13).

Comparison between the biological activity of immature and mature fruit extracts of *C. spinosa* against the proliferation of tumor cell line.

To compare between the cytotoxic effect of immature fruit extracts and mature fruit extracts of *C.spinosa* on both tested tumor cell lines ,the data of present study statistically analyzed with that of mature fruit (Tables 14–16) those obtained from previous study by the same investigators (AL-Asady *et al*., 2011).

Table12 Analysis of variance for the effect of Immature fruit extracts of *C. spinosa* on the types of cell lines.

S.O.V	d.f	SS	MS	F value
Cell line types	1	0.126	0.115	401.5^{*}
Error	286	0.084	0.0002	

S.O.V = source of variance, d.f = degree of freedom, SS = summation square, MS = mean square, *= (P ≤ 0.01).

Table 13 Mean±SE for the effect of Immature fruit extracts of *C. spinosa* on the types of cell lines.

Cell lines	Hep-2	HeLa	L.S.D
Immature fruit Extract	0.259±0.002	0.227±0.003	0.023

SE = standard error

Statistical analysis shows highly significant difference between (Mature and Immature) fruit extracts of *C. spinosa*. Mature fruit extracts were more effective than immature fruit extracts, the value of O.D. were 0.213±0.002 and 0.234±0.001, respectively (Table 17).

Table 14 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 Hep-2 tumor cells *in vitro* (Observations of O.D).

Extract	Time/hrs		
	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 15 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.* (Observations of O.D).

Extract	Time/hrs		
	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 16 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 17 Mean ± SE for the effect of mature and immature fruit extracts of *C. spinosa* on tumor cell lines.

Cell lines	M.f. Es.	Im.f. Es.	L.S.D
Extract	0.213±0.002	0.234±0.001	0.02

4. Discussion

4.1 The C. Spinosa Immature Fruit Extracts

The reasons for the importance of *Capparis* genus has been a subject of interest from the phytochemicals that included particularly due to its glucosinolate content. Most of the species reported positive for triterpenoids (alpha-amyrin), sterols, beta-carotene, saponins and some tested positive for flavanoids, glycosides and alkaloids [21]. 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.* [37] in which they find that the proportion of crude product is variable and depending on the method of extraction as well as the part of the plant.

In the present study, some of chemical tests used to detection of alkaloids, tannins, flavonoids, glycosides, triterpenoids, carbohydrates as well as saponins qualitatively in the crude and secondary metabolites extracts that are prepared from the immature fruits of *C. spinosa*. The result shows that the chemical compounds are varied due to the solvent of extraction. The results of qualitative chemical tests differ according to the type of extract. The qualitative variation can be attributed to the fact that, the crude and secondary metabolites extracts of immature fruit contain different constituents that vary considerably in their relative concentrations. Study of Howard *et al.* [38] on the *Capsicum* species, revealed 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.

In addition to the previous compounds, rutin extract is qualified as a flavonoids secondary metabolites by comparison of its Rf value with that of standard. The yield of rutin extraction for Immature fruit was 12.1, while Ramezani *et al*. [39] 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.

Aqueous and methanolic fruit extracts was prepared in the present study, because each crude extract containing several different compounds and revealed high activity against the proliferation of tumor cells. The study of Gold *et al*. [40] indicate that unpurified whole plant extract containing several different compounds these can interact together where the effect of the whole plant is greater than its individual compound. Mayalen *et al.* [41] report that crude extracts of *bifurcata*, *Cystoseira tamariscifolia*, *Desmarestia ligulata*, *Dictyotadichotoma*, and *Halidrys siliquosa* exhibited strong cytotoxic activities against three different Human leukemic tumor cells lines; Daudi, Jurka , and K562.

Chung *et al*. [42] suggested that aqueous extracts from plants used in allopathic medicine are potential sources of antiviral and antitumor agents.Usually methanolic extract has cytotoxic properties, because most of the biological active compounds are extracted with methanol. Since methanol has high polarity, it could dissolve both the polar and non polar compounds in it [36]. Rajendra and Ramakrishnan [43] reported that methanolic extract of *Artocarpus heterophyllus* has cytotoxic effect against the proliferation of Hep-2 cells. Study of Al-Asady [22] indicated that methanolic root extract of *C. spinosa* posses inhibition activity against AMN3 tumor cell line *in vitro* and *in vivo*.

Recently the study of AL-Asady *et al.* [24] revealed that the secondary metabolite extract polyphenol from mature fruit extract of *C. spinosa* has inhibition activity against Hep-2 and HeLa tumor cell lines.

Extraction of polyphenols that performed may isolate large number of polyphenol compounds. Decker [44] mentioned that whole variety of phenolic compounds, are widely distributed in grains, fruits, vegetables and herbs. Gadgoli and Mishra [45] reported that *C.spinosa* contain hydroxy cinnamic acid like caffic acid, ferulic acid, cumaric acid. Pierpoint [46] reported that high concentration of flavonoids are present in the skin of fruits and have important and varied roles as secondary metabolites. Rutin was extracted from *C. spinosa* fruit as a flavonoid, it may have antioxidant, anti-inflammatery and anticarcinogenic activities [47, 48]. The presence of alkaloids in *C. spinosa* reported by Sadykov *et al*. [49], they estimated about 0.86% was found in seeds. Total alkaloids of *C. spinosa* inhibit the growth of human gastric adenoma cells SGC-7901 *in vitro* [23].

4.2 Cytotoxic Effect of Aqeous Methanolic Crude Extracts and Secondary Metabolites (Polyphenol and Rutin) Extracts of Immature fruit of C. spinosa on Hep-2 and HeLa Tumor Cell Lines in vitro

The results of Immature fruit extracts on the proliferation of Hep-2 cell line revealed that the more effective extracts on the proliferation of Hep-2 tumor cell line after 24 hrs over all concentration was Aqueous extract. Other extracts (Methanol, Polyphenol and Rutin) seem to have the same inhibition activity. After 48 hrs, Methanolic extract, posses more inhibition activity than Polyphenol extracts. Methanolic extract exhibit the same effect with Aqeous and Rutin extracts. Methanolic and rutin extracts are more effective against the proliferation of Hep-2 cells after 48 hrs treatment, whereas Aqeous and Polyphenol extracts were effective at both time 24 and 48 hrs similarly.

The results of Immature fruit extracts on proliferation of HeLa cell line show the more effective extract after 24 hrs is methanol with concentration 10000 µg/ml, which reveal more inhibition activity than Aqueous, Polyphenol, and Rutin extracts. Rutin extracts is more effective than Aqueous extract, while it has the same effect with polyphenol extract. After 48 hrs Methanolic extract is more effective than Polyphenol and Rutin extract, whereas it possesses similar effect with Aqueous extract. Aqueous extract is more effective than Polyphenol extract, while it exhibit the similar effect with Rutin extract. Methanolic extract that prepare in the present study contains several

phytochemicals including alkaloids, tannins, flavonoids, triterpenoids, saponins, therefore such extracts is found to have this highly activity against the proliferation of both tested cell lines. This result supported by Ahn *et al*. [50], they mentioned that methanolic extract have anticancer activity, and antioxidant activity [51]. Arpornsuwan and Punjanon, [52] reported that the methanolic extract of *Morinda citrifolia* fruit is much more effective on breast cancer cells and neuroblastoma cells. The highest phenolic content is found in methanol extract of mulberry fruit. Total phenolic content of plant extracts is highly correlated with the radical scavenging activity. The antiproliferative effect of mulberry parts on human cell lines is different and connected to the concentrations of the extract [53].

Statistical analysis shows that each type of Methanol, Polyphenol, and Rutin has the same effect against the growth of HeLa cell line at both time of treatment, except in Aqueous extract that is more effective after 48 hrs. The CC50 values on HeLa cell line are more than 10000 µg/ml for all extracts in both periods of exposure with exception that methanol extract after 48 hrs have CC50% 9700 µg/ml, this demonstrate that Immature fruit extracts are not highly effective against both cell lines, the extracts became effective against both cell lines slowly, may be that Immature fruit of *C. spinosa* contains a trace amount of biological active compounds or may contain highly concentration of tannins which alone do not appear to have sufficient effect. It means that the extracts of *C. spinosa* have specifity in its activity according to its contents. Crozier *et al.* [54] demonstrated that many unripe fruits have very high tannin content, which is typically concentrated in the outer cell layers.

Tannin levels and/or the associated astringency decline as the fruits mature and the seeds ripen. Herbalists claim that unpurified whole plant extracts containing several different compounds can interact together where the effect of the whole herb is greater than its individual components [40]. The results of Immature fruit extracts in the present study do not agree with that obtained from Yu *et al.* [55]. They demonstrate that immature fruit of *P. salicina* L. can be regarded as a safe and promising new dietary source for decreasing the risk of developing breast cancer, because the extract of immature fruit from *P. salicina* contained higher levels of total phenolics than mature fruit.

4.3 The Effect of Immature Fruit Extracts of C. spinosa on the Type of Tumor Cell Line

The result revealed that HeLa tumor cell line is more effective than Hep-2 tumor cell line. The activities of plant extracts against the proliferation of tumor cells differ due to the properties of their chemical compounds [56] as well as cell membrane receptors of tumor cells [57]. Morrissey [58] demonstrated that a relatively small number of processes have been shown to be the targets of plant metabolites and these include electron transport chains, mitochondrial function and membrane integrity. It is now emerging, however, that other specific enzymes and processes may also be the targets of particular metabolites. There is a general hope that modern genomic approaches will identify new targets and modes of action of plant metabolites. Molecules, especially triterpenoids that trigger apoptosis or autophagy in tumor cells are of particular interest in this regard. There is a limited evidence for dietary components to influence late stages of cancer. Resveratrol, quercetin, curcumin, and genistein can inhibit one or more matrix metalloproteinase (MMPs). Vitamin C can inhibit MMP production by a number of human cancer cell lines and prevent invasion of these lines *in vitro* [59].

The difference between Mature and Immature fruit extracts of *C. spinosa* is also detected. Mature fruit extracts are more effective than immature fruit extracts, may be according to the presence of high quantity of terpenoids and essential oils in mature fruits than in immature fruit. This result supported by Matthhaus and Ozcan [60]. Pentacyclic triterpene from *Betula* spp [61]

and from *Zizyphus* spp. [62, 63] displayed selective cytotoxicity against human melanoma cell lines [64]. Study of Howard *et al*. [38] on the *Capsicum* species, indicated that the concentration of chemical constituents such as carotenoids, flavonoids, phenolic acids and ascorbic acid increased as the *Capsicum* spp. plant reached maturity.

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