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Anticancer effect of *Citrullus colocynthis* and *Capparis spinosa* against human Cervix and Hepatocellular cancer cell lines

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ABSTRACT

The current work aims to assess the anticancer effect of *Citrullus colocynthis*, *Capparis spinose* against human Cervix (Hela) and hepatocellular (Huh7) cancer cell lines and understand the mode of action at molecular level via gene expression and cell cycle analysis. The Methanolic extracts were prepared. Antioxidant activities and Phytochemical screening of every extract were estimated. To measure cytotoxicity, MTT test was employed, and Annexin/propidium iodide (PI) staining was used to measure apoptosis induction and change in gene expression level was detected by Real Time PCR (qPCR). Obtained findingsexplained anticancer potential of both *Citrullus colocynthis* and *Capparis spinosa* methanolic extracts against cervical and hepatocellular carcinoma. The anticancer effect of *Citrullus colocynthis* was higher than *Capparis spinosa* extract. This effect might be due to the role TPC and TFC through induction of apoptotic pathways influenced by upregulation of p3 and p21 genes. These results may help expand the importance of the *Citrullus colocynthis* and *Capparis spinosa*; and may be regarded in the detection of new anticancer compounds.

Key words: *Anticancer, Citrullus colocynthis, Capparis spinose, Human Cervix, Hepatocellular cancer.*

Introduction

One of the most important reasons of morbidity and death world side is cancer and about 1 in 6 deaths is due to cancer. In 2018 there were 9.6 million fatalities associated with cancer and almost 18.1 million additional cases identified. Over the next 20 years, the number of cancer cases is anticipated to reach 22 million per year (Hajjar *et al.,* 2017). Cancer pathophysiology is caused by cellular hyper proliferation, which is responsible for cell growth and invasion, cell differentiation, death, as well as metastasis and angiogenesis (Vorobiof *et al.,* 2007; Tavakoli *et al.*, 2012). However, conventional chemotherapy is still used in the majority of patients, particularly those that result in metastases. Resistance to drugs is a major obstacle in chemotherapy, thus new therapeutic drugs are urgently needed that are either more effective or work in tandem with existing ones. The application of herbs as a powerful supply for new treatments against cancer is becoming more prevalent (Denny, 2002). Plants contain a wide range of chemical substances, including anti-cancer, that have strong biological effects. Thus, the probability

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of herbal medicines in the development of new bioactive molecules to produce efficient pharmaceutical products is evident (Shoeb *et al.,* 2006).

Citrullus colocynthis (L.) Schrad, a useful cucurbit,is extensively dispersed throughout the world's deserts. *Citrullus colocynthis* is a species of *Citrullus colocynthis*. This fruit is well-known for its numerous therapeutic applications, as well as its pharmacological and nutritional potential (Hussain *et al.,* 2014).

Capparis spinosa L. (*C. spinose*), a winter-deciduous perennial, is utilized as an anti-inflammatory (Issac *et al.,* 2011), anti-oxidative (Siracusa *et al*., 2011), anti-diabetic (Huseini *et al.,* 2013), anti-hepatotoxic (Aghel *et al.,* 2007), anti-bacterial (Boga *et al.,* 2011), and anti-proliferative agent (Wu *et al.,* 2003).

Capparidaceae are a vast family of phanerogam gymnosperm dialypetalae plant species (Moghadamnia *et al.,* 2019). *Capparis spinosa (C. spinosa*) (CS) is from the Capparidaceae family. Phytochemical research has shown that the various sections of *C. spinosa* are rich polyphenol producers, and scientific work has consequently concentrated on the health-promoting properties of the active ingredients of such plant (Nabavi *et al.,* 2016). A substantial amount of scientific evidence indicating *C. spinosa* has anassortment of pharmacological actions (e.g. antioxidant, antibacterial, anticancer, as well as hepatoprotective activities exists (Aghel *et al.,* 2010; Tlili *et al.,* 2010; Gull *et al.,* 2015, Hikal *et al.,* 2017 and Al-Harbi *et al.,* 2019.

The current work was implemented to assess anticancer effect of *Citrullus colocynthis* and *Capparis spinosa* methanolic extract against human Cervix (Hela) and Liver (Huh7) cancer cell lines.

Materials and Methods

Plants materials and preparation of extracts

Plant materials were gathered from Al-Jouf, Kingdom of Saudi Arabia region and next dried and powdered. The powder was utilized to prepare methanolic extract using Soxhlet procedure. The solvent was taken out under vacuum conditions and extracts were then collected and stored in dark glass vials.

Phytochemical screening tests

Total Phenolics and total flavonoids were quantified following standard procedures as described by (Khan *et al.,* 2011) as follow;

Determination of antioxidant power

Ferric inhibits antioxidant activity (FRAP) analysisa . A spectrophotometer set at 593 nm was used to measure the rise in absorbance. The antioxidant percentage was estimated employing the equation:

$$\text{Antioxidant (\%)} = [(\text{Abs}_{\text{Sample}} - \text{Abs}_{\text{Control}}) / \text{Abs}_{\text{Sample}}] \times 100.$$

Cell lines

To human cancer cell liens Cervix (Hela) and Liver (Huh7) were used. The cells were procured from the VACSERA Company, Egypt.

In vitro Cytotoxic Activity (MTT assay)

MTT reduction assay was used to determine the cellular cytotoxicity of the extracts investigated against Cervix (Hela) and Liver (Huh7) cells. The MTT is a colorimetric assay that uses in vivid cells, with mitochondrial succinate dehydrogenase enzymes, to transform 3-(4), 5-dimetial-2-yl)-2, 5-diphenyltetrazole bromide (MTT) to a spectrometrically measurable coloured, formazan product (Mosmann 1983). In duplicate, different concentrations of the tested extras were added to the 96-well platesincluding a confluent cell monolayer (10^6 cells per well). After 48 hours, we added 20 L of MTT solution [5 mg/mL in Phosphate buffered saline (PBS)]. After four hours of incubation under same circumstances, plates were treated with a 24:1 HCl/Isopropanol combination for thedissolution of the blue intracellular formazan product.As a negative control, DMSO was utilized. The absorbance (570_m) was used to decide viable cells. The IC50 was calculated by visually estimating the required concentration to impede viability by 50% after measurements were taken. A UV- Spectrophotometer was used to detect the absorbance (570_m), with blank wells (no sample containing cells serving). Using the following formula, the influence on the proliferation of utilised cells of the samples was declared as a percent of cell viability:

% cell viability = A_{570} of treated cells / A_{570} of control cells × 100%.

Measurement of apoptosis via Annexin V staining assay

The test used lines of 1×10^6 human cell cancer cells cultivated in six wave dishes. The HuH7 and Hela

cell liens were treated with *Capparis spinosa* and *Citrullus colocynthis* methanolic extract respectively when the IC50 concentration is present of each methanolic extracts for 24hrs as described by (Pumiputavon *et al.*, 2017). Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) co-sticking assay was employed to evaluate the quantity of apoptotic cells. Cells were, then, extracted and centrifuged for eight min at 1800_{rpm} at the end of the 24-hour incubation. This pellet was suspended in a 0, 5 µl Annexin V-FITC-binding buffer of 50 µl and incubated in dark at four °C for 30 min. Every tube was incubated with PI (50 g/ml) for 5 min in 200 µl buffer binding. In conclusion, the cytometry of the cells was evaluated (Pumiputavon *et al.*, 2017). The flow cytometry results of Annexin V and PI staining cell percentages may be divided in four groups.In both early and late apoptotic cells the cell populations resident in Annexin V+/PI - and Annexin V+/PI+ quadrants were identified. As living cell and necrotic cell, the annexin V"/PI+ and the annexin V"/PI+ were determined.

Cell cycle analysis

After treatment of each cell lien with IC50 concentration of plant extract 24 hrs as described by (Pumiputavon *et al.*, 2017), the washed cells were centrifuged for eight minutes at 1800 rpm. With 70 percent ethanol, the cells have been resuspended and fixed at four °C for 2 hours. The cells were rinsed and centrifuged after fixing with PBS for eight min at 1800 rpm. It was divided into 250 µl PBS, includingRNase A (20 µg/ml), PI (20 µg/ml), and Triton X-100 (0.1%) for another 30 minutes in the dark. Pellicular pellets had been broken down by vortex. Finally, flow cytometry analysis was conducted for the cells.

RNA isolation

Total RNA has been isolated from treated cells with *Citrullus colocynthis* and *Capparis spinosa* methanol extracts utilizing Trizol Reagent (Invitrogen, Carlsbad, CA, USA) in conformity with the procedure of the manufacturer. The contamination of DNA was dismissed and the RNA was purified via Dnaseuntil further use, RNA was kept at -80 °C.

cDNA synthesis and real-time qRT-PCR

In real-time PCR, the mRNA level of the P53 and P21 genes was quantified. RNeasy Mini Kit was used to isolate total RNA (Qiagene-USA).The isolated RNA was used for first-strand cDNA generation in RT-PCR immediately (cDNA Synthesis Kit). The RT-PCR was implemented using the appropriate primers for P53 and P21 respectively. The sequences of the primers used were as follows: p53 F 5′-CTG TCA TCT TCT GTC CCT TC-3′ p53 R 5′-TGG AAT CAA CCC ACA GCT GCA-3′. P21 F 5'-GTC ACT GTC TTG TAC CCT TGT G-3′p21 R 5'-CGG CGT TTG GAG TGG TAG AAA-3' and GAPDH F 5'-GCA AGT TCA ACG GCA CGA TCA AG-3', GAPDH R 52-CTA CTC AGC ACC AGC ATC ACC-3′. In the ABI Step One TMRealTime PCR System (Applied Biosystems), 40 cycles of denaturation were done at 95 °C during 30s, 60 °C for 30s and 80 °C for 20s. Gene amplification and expansion were carried out for 30s and 20s.

Statistical analysis

Data have been evaluated using one-way ANOV Ausing PLC Version 20 and significant medial differences when $P < 0.05$ were considered (LSD post hoc test).

Results and Discussion

Antioxidant activity and phytochemical screening

Two plants extract were tested for Phytochemicals and antioxidant activity. The measurements include total phenolic compounds, total flavonoid contents (TFC) and antioxidant strength (Table 1). The results indicated that *Citrullus colocynthis* had a greater TPC, TFC and antioxidant strength than *Capparis spinosa* extract.

The Cytotoxic Effect

The MTT assay was employed to estimate the cytotoxic effect of methanolic extract of *Citrullus colocynthis* (S12) and *Capparis spinosa* (S10) on both Hela and HuH7 cancerous cell line.

Citrullus colocynthis (S12) represented cytotoxic effect on both of Hela and HuH7 cancerous cell having IC_{50} values of 11.127 and 19.572 µg/mL respectively, whereas the *Capparis spinosa* (S10) extract demonstrated a modest cytotoxic impact with IC percent 50 on both cell lines 4238 and 3726 µg/ml with Hela and HuH7 respectively as appears in Figures 1-8.

Apoptosis assessment and cell cycle analysis

The co-staining Annexin V and PI to examine prob-

able cell death induction (apoptosis and/or necrosis) were used for the investigation of the activity of cancer of the examined methanol extracts on lines of cancer cells. The two untreated controls had

Table 1. Antioxidant activity and phytochemical screening of *Citrullus colocynthis* and *Capparis spinosa* methanolic extract

Sample	Total phenol extract (mg/g)	Flavonoids extract (mg/g)	Antioxidant extract (mg/g)(as ascorbic acid)
Capparis spinosa	12.3	10.4	7.9
Citrullus colocynthis	16	11	9.5

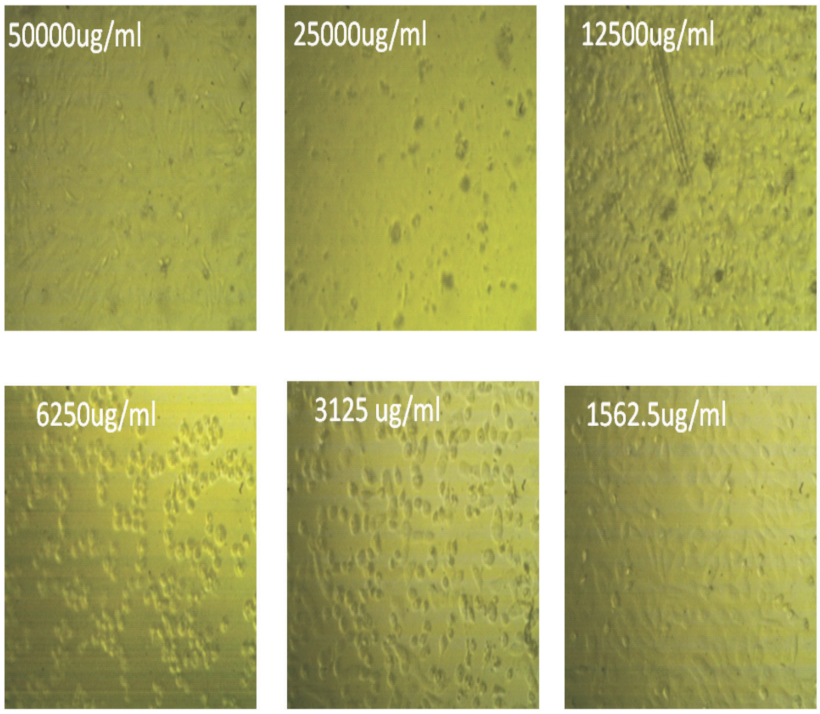

Fig. 1. Effect of *Capparis spinosa* (S10) on (Hela) cells at different concentration

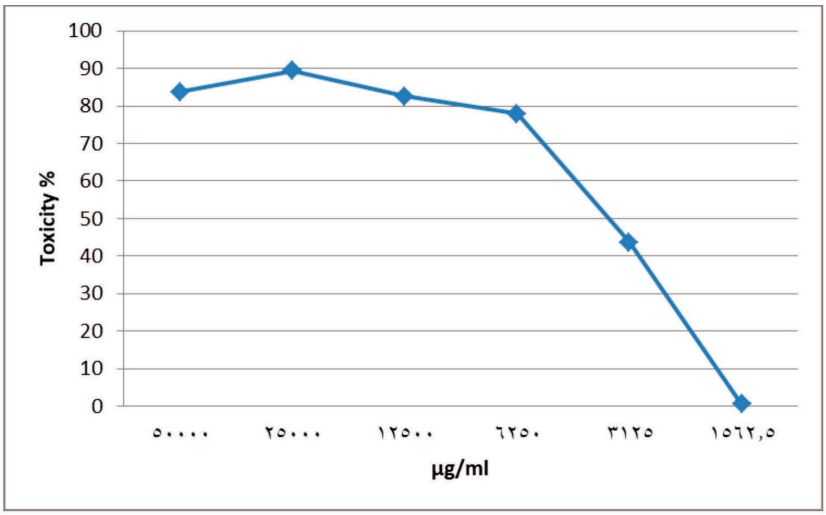

Fig. 2. Cytotoxic effect of *Capparis spinosa* (S10) on Cervix cancer (Hela) cells

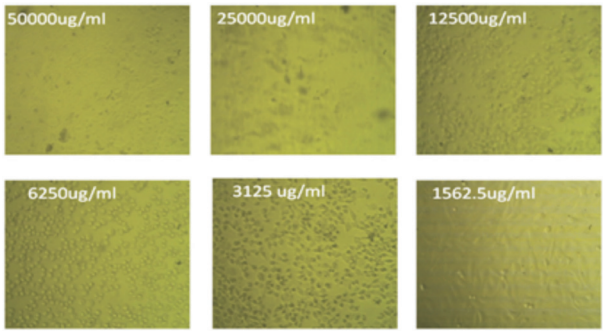

Fig. 3. Effect of *Capparis spinosa* (S10) on (Huh7) cells at different concentration

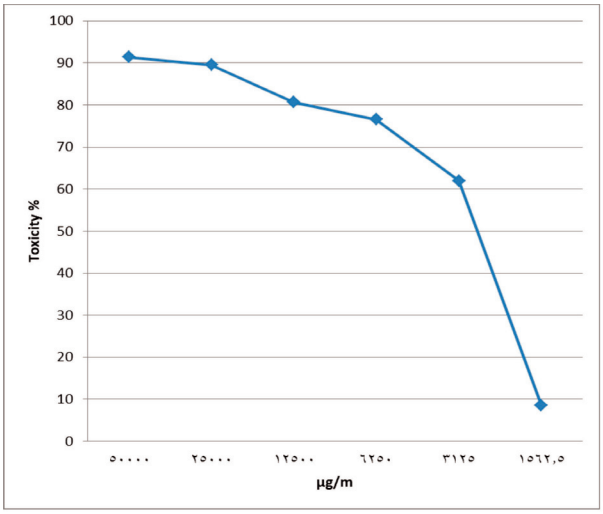

Fig. 4. Cytotoxic effect of *Capparis spinosa* (S10) on liver cancer (Huh7) cells

apoptotic cell percentages (the percent of the early and late apoptotic cells together). The early apoptotic events were higher than late events (Figure 9). The treatment of HuH7 cancer cell with *Capparis spinosa* (S10) extract induced 15.82% apoptosis in comparison with 1.79% in the control. While, the percentage of total apoptosis induced among Hela cells by *Citrullus colocynthis* (S12) was 27.39% in comparison with 2.08% in the control.

An analysisof cell cycle was then implemented to assess whether the death of apoptotic cell is related to the extracts' inhibitory influence on a cell cycle process. After treatment with each cell line with corresponding plant extract. The DNA content was measured in comparison with control as appears in (Table 2). The obtained outcomeselucidated that, the treatments arrested the cells growth at G2/M phase.

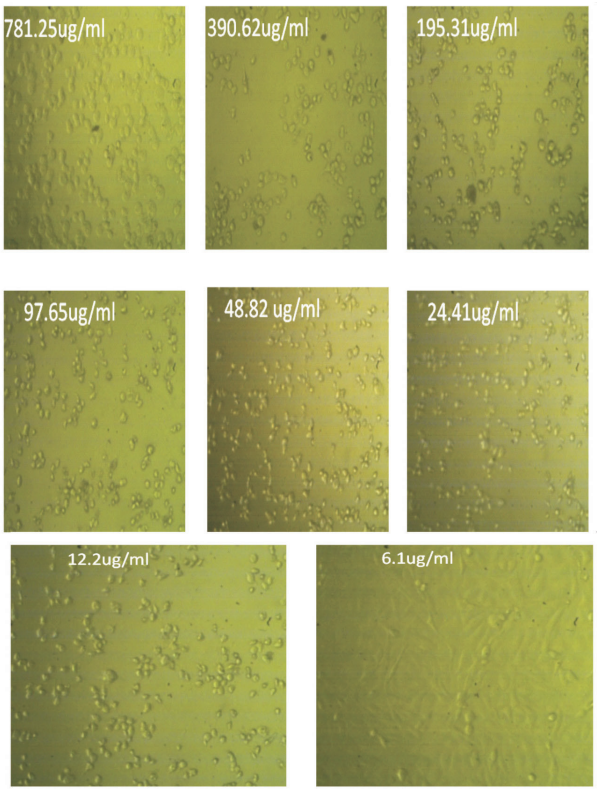

Fig. 5. Effect of *Citrullus colocynthis* (S12) on (Hela) cells at different concentrations

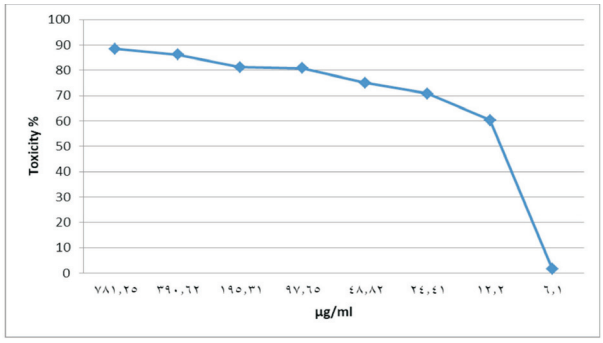

Fig. 6. Cytotoxic effect of *Citrullus colocynthis* (S12) on Cervix cancer(Hela) cells

Gene expression analysis

To examine the change of gene expression levels of P21 and P53 genes in HuH7 and Hela cancer cell line due to treatment with IC50 concentration of *Capparis spinosa* (S10) and *Citrullus colocynthis* (S 12), the change of mRNA level was analyzed quantitatively via Real Time PCR. The results illustrated in (Figure 10) reveals the over expression in p53 and p21 genes compared to their control due to treat-

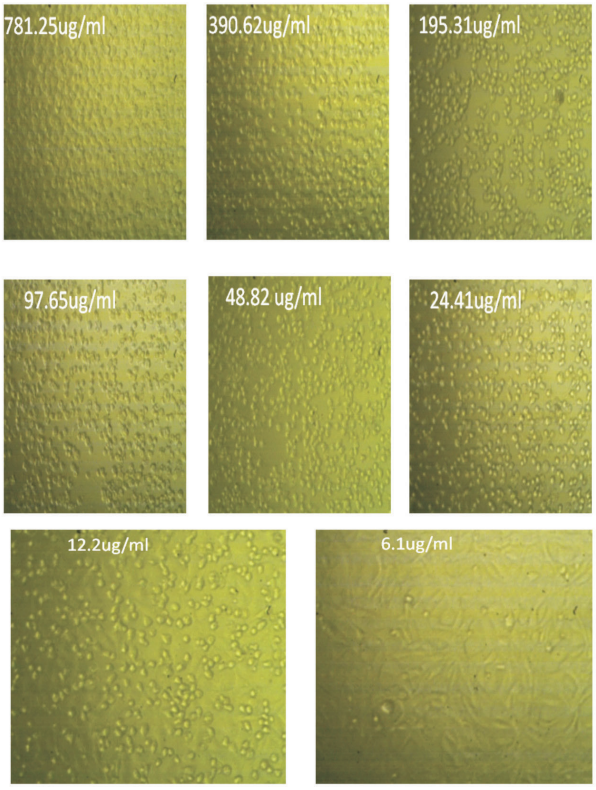

Fig. 7. Effect of *Citrullus colocynthis* (S12) on (Huh7) cells at different concentrations

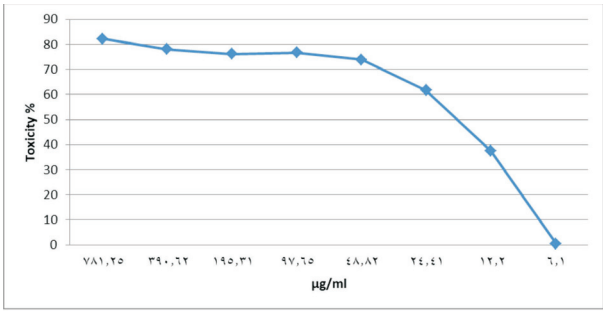

Fig. 8. Cytotoxic effect of *Citrullus colocynthis* (S12) on liver cancer (Huh7) cells

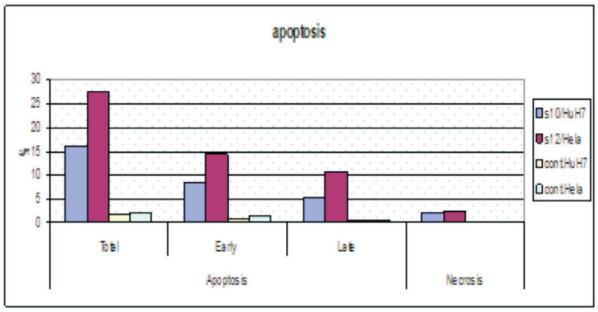

Fig. 9. Impact Effect methanolic extracts of *Capparis spinosa* (S10) and *Citrullus colocynthis* (S12) on the induction of apoptosis in the HuH7 and Hela cancer cells respectively

ments while, the change in the level of expression of p53 gene were higher than expression of p21 gene in all treated cells.

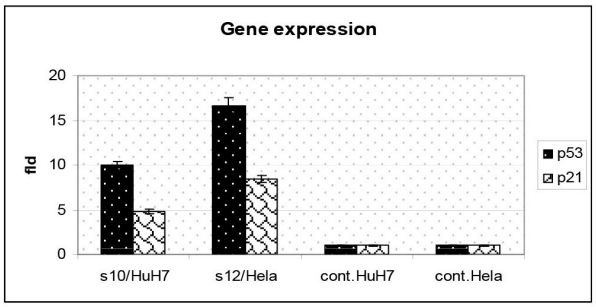

Fig. 10. Impact of *Capparis spinosa* (S10) and *Citrullus colocynthis* (S12) on the expression of different P53 and P21 genes. HuH7 and Hela cells were treated IC50 of the extract for 24 h. Expression of GAPDH was used to normalize the conditions.

Discussion

Given the present state of cancer-related fatalities, diagnostic and therapeutic options are on the increase (Chowdhury *et al.,* 2017). Overall cancer patients' survival and quality of life are, nevertheless, consistently improved for operational removal and

Table 2. Effect methanolic extracts of *Capparis spinosa* (S10) and *Citrullus colocynthis* (S12) on the cell cycle analysis of HuH7 and Hela cancer cells

Sample data		ResultsDNA content				
Ser.	**Code**	%G0-G1	%S	%G2/M	%Pre-G0	Comment
1	s10/HuH7	36.52	28.61	34.87	15.82	cell growth arrest @G2/M phase
2	s12/Hela	26.38	25.44	48.18	27.39	cell growth arrest @G2/M phase
3	cont.HuH7	55.29	35.49	9.22	1.79	cell growth arrest @G2/M phase
4	cont. Hela	47.92	37.18	14.9	2.08	cell growth arrest @G2/M phase

radiation, endocrine and other targeted therapy. Although the latest methods of treatment are able to treat the initial tumours, the main limits of the light currently are recurrence and metastasis. Chemical therapies also frequently have significant non-target effects, as well as systemic toxicity, likehepatic, nephrotoxicity and cardiovascular (King and Perry, 2001. Therefore, it is an important topic of study to identify new anticancer medications that prevent both tumour and metastasis while bypassing the recognised negative effects. The application of natural compounds, such as secondary metabolites, with effective tumour suppressant capability, seemed promising in recent years since, even at lesser dosages and because of their safe profile, they are proven to be effective (Kohen and Carter, 2005). So, the present investigation aimed to explore the properties of anti-cancer extracts from *Capparis spinosa* and *Citrullus colocynthis*. The obtained results showed that, the TPC, TFC and antioxidant power for *Citrullus colocynthis* methanolic extract was higher than *Capparis spinosa*. A possible justification of high antioxidant activity of *Citrullus colocynthis* methanolic extract would be due to high parentages of total phenolic contents and total flavonoid constituents in comparison with *Capparis spinosa* extract; alikeother reports Sahreen *et al.,* 2010).

The secondary plant metabolites that are omnipresent for plant and herbal goods are Phenolics or polyphenols. Most Phenolics have demonstrated substantial antioxidants concentrations (Razali *et al.,* 2008). Overall plant antioxidant activities are mainly supported by phenolic compounds because of their redox characteristics. In general, phenolic substances have antioxidant activity by neutralising free radical lipids and inhibiting the formation of free radicals when hydroperoxides decompose. (Javanmardi *et al.,* 2003; Li *et al.,* 2009).

Antioxidant activity in several plants correlates positively with phenolic concentration. (Hakimoglu *et al.,* 2007; Ko *et al.,* 2018). Several investigations have found that in scavenging free radicals, phenolic chemicals play a crucial function, and that phenol-rich plant samples have a high scavenging potential (Sharififar *et al.,* 2007; Moo-Huchin *et al.,* 2015; Alcântara *et al.,* 2019).

Flavonoids, having a benzo-pyrone structure, are the most prevalent and extensively dispersed category of plant phenolic chemicals (Abu Bakar *et al.,* 2009).

Flavonoids have the capacity to both suppress the formation of reactive oxygen species (ROS) and lower the level of ROS once they are created (Agati and Tattini, 2010).

Plants high in flavonoids may act as adequate sources of antioxidants, assisting in the augmentation of an organism's overall capacity of antioxidant and preservationversus lipid peroxidation (Sharififar *et al.,* 2009).

Natural phenolic substances are less effective antioxidants than flavonoids. Furthermore, plant products' antioxidant activity is linked to their total flavonoid content (Pietta, 2000).

Regarding to the cytotoxic effect, both examined extracts induces the effect of cytotoxic versus two utilized lines of cancer cells in dose dependent matter. This outcome is in consistence with previous work by (Sheukh *et al.,* 2017; Moghadamnia *et al.,* 2017) concerning the cytotoxic effect of *Capparis spinosa* extract on the line of hepatocellular carcinoma cells (hepG2) and breast cancer MCF7) and with results reported by (Rezai *et al.,* 2017) concerning the effect of cytotoxic of *Citrullus colocynthis* on breast cancer (MCF7) cell line. According to the National Cancer Institute's (NCI) cytotoxicity standards, a crude extract is considered inert, moderately active, or active when the IC_{50} values are less than 20 g/ml, between 20 and 100 g/ml, or greater than 100 g/ml, respectively (Ramos-Silva *et al.,* 2017), thus methanolic extract of *Citrullus colocynthis* was more toxic than *Capparis spinosa* with both utilized cell lines. This would be due to the increase of TPC, TFC as well as antioxidant activity of *Citrullus colocynthis* than *Capparis spinosa*. This suggestion is inconsistence with different reports Yarizade *et al.,* 2017; Bakari *et al.,* 2018).

It is generally known that cancer cells have the ability to proliferate indefinitely, which is primarily connected to the dysregulation of cell cycle progression and the stimulation of invasion (Lu *et al.,* 2014). Treatment of Hela and HuH7 cancerous cells with IC50 of *Citrullus colocynthis* and *Capparis spinosa* extract respectively deduced cell cycle arrest at G2/M associated with the induction of preG1 apoptosis, which was verified by the importantincrement in the populations of apoptotic cell. But the effect of *Citrullus colocynthis* on Hela cells was higher than the effect of *Capparis spinosa* on the HuH7 cells. This would be due effect of TPC and TFC in each extract (Kulisic-Bilusic *et al.,* 2012; Hajjar *et al.,* 2107).

The induction of apoptosis has been widely described as one of the active ways for stopping cancer

cell proliferation (Pistritto *et al.,* 2016). Apoptosis is the most important target and goal for research on cancer since cells eliminated through this mechanism of death of cell do not cause an inflammatory response, which can cause a variety of negative side effects (Rahman *et al.,* 2017). When compared to the control, the necrotic and apoptotic cell populations raisedconsiderably in both Hela and Huh7 cells after incubation with the studied extracts.When compared to the control, the apoptotic and necrotic cell populations raised significantly in both Hela and Huh7 cells after incubation of the studied extracts. Furthermore, apoptosis was much higher than necrosis in all treatments with all types of used cancer cells. Due to the treatment with plant extracts, early apoptotic events were higher than late apoptotic events in two lines of cancer cells.*Citrullus colocynthis*, on the other hand, had a greater apoptotic effect on Hela cells than *Capparis spinosa* did on HuH7 cells. According to several accounts, these symptoms could be caused by TPC and TFC.

P35 the gene of tumour suppression inhibits cell development and commits cells to apoptosis (Delbridge *et al.,* 2012). Meanwhile, in response to DNA damage, the p53 gene controls the expression of downstream effectors like p21, is a strong inhibitor of cell cycle kinases (Wang *et al.,* 2015). P53 is involved in a number of biological activities. It regulates the cell cycle, senescence, and apoptosis (Ferna´ndez-Majad *et al.,* 2016).

As shown in Figure (10), treatment with *Citrullus colocynthis* and *Capparis spinosa* methanolic extracts raised the amount of p53 expression. Additionally, the amount of p21, a cuclin-dependent kinase 2 inhibitor induced transcriptionally by p53, was enhanced. These findings confirm p53's crucial role in servex and liver cancer cell killing mediated by *Citrullus colocynthis* and *Capparis spinosa*, and are consistent with earlier research using plant extracts such as terrain, capsaicin, and flavonoid (Kim *et al.,* 2009; Porameesanaporn *et al.,* 2013; Zhao *et al.,* 2019, these data could due to the TFC and TPC in the plant extracts as described in previous studies. Polyphenols may have anticancer properties via a variety of signalling pathways, counting death receptor (extrinsic) system, the perforinzyme apoptotic pathway and the mitochondrial (intrinsic) pathway (Curti *et al.,* 2017). The p53 signalling route has played an essential role in the regulation of cell cycle, metabolism, ageing and developing, replicating and suppressing the tumour, etc. (Hu *et al.,* 2007; Kruiswijk *et al.,* 2015; Tanikawa *et al.,* 2017). polyphenols are employed to create anticancer action through apoptosis in many malignancies by p53 signalling pathway (Gupta *et al.,* 2012; Lee *et al.,* 2014; Periasamy and Alshatwi, 2013). Flavonoids are becoming more important as anti-cancer drugs and demonstrated significant possibility as an anti-cancer cytotoxic agent for cancer cell death (Abotaleb *et al.,* 2019). MDA-MB-453 cells of breast cancer have caused elevation of p53 expression and DNA fragmentation and phosphorylation, causing cell proliferation and therefore apoptosis (Li *et al.,* 2009). Caspase-3, and -7, p53, Bad, Bax, and Bcl-xL decreases have been reported for Kaempferol production of intrinsic apoptosis in cell lines OVCAR-3 and A2780/CP70 A2780Wt via mitochondrial pathways (Luo *et al.,* 2011). The cell death induced by apigenin was associated to the rise in p53, Bax/Bcl-2 expression, caspase 9 and 8 activation and PARP-1 cleavage (Masuelli *et al.,* 2017). In HepG2, Bax, caspas-3, -8, and -9 and DR5 and reduced Bcl-2 levels Chrysine alone, or in combination with cisplatin in cancerous cells produced intrinsic or extrinsic apoptotic pathways (Li *et al.,* 2015; Zhang *et al.,* 2016).

The results revealed *Citrullus colocynthis* and *Capparis spinosa* anti-cancer capability. These findings correspond with earlier studies such as Abdulridha *et al.,* (2019) who reported that the anticancer us effects of *C. colocynthis* on Colorectal cancer. The anticancer effect of *Capparis spinosa* on cervical and hepatocellualr carcinoma cells was examined by (Moghadamnia *et al.,* 2019). In HT29 human colorectal adenocarcination cells, the essential oil, which is hydro distilled from the flower of *C. spinoa* and the water infusion prepared with it, were tested against anticarcinogens. In addition, our results are able to shed more light on the molecular mechanism in which these extracts caused this anti-cancer effect by studying cell cycle analysis, apoptotic pathway and gene expression.

Conclusion

Our results reveal the anticancer affect of *Citrullus colocynthis* and *Capparis spinosa* methanolic extracts against cervical and hepatocellular carcinoma. This effect might be due to the role TPC and TFC through induction of apoptotic pathways influenced by upregulation of p3 and p21 genes.

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