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Effects of *Capparis spinosa L* on Cell Proliferation, Apoptosis and Epigenetic Events in Swiss Albino Mice Transplanted with Ehrlich Ascites Carcinoma.

Faten Z Mohammed[1]*, Adel A Gurigis[2], Tamer M Roshdy[2], and Alaa Gamal[1].

[1]Chemistry Department, Faculty of science, Zagazig University, Zagazig, Egypt.
[2]Genetic Engineering and Biotechnology Institute, University of Sadat City, Egypt.

ABSTRACT

Cancer disease has been considered as a result of progressive genetic alterations and epigenetics reprogramming. Naturally occurring compounds are considered as gold standard for cancer treatment. *Capparis spinosa L* is a multipurpose plant that contains a number of chemically active metabolites, particularly flavonoids such as rutin and hesperidin. We aim to explore the invivo anti-tumor activity of *Capparis spinosa L* extract and most potent constituents rutin and hesperidin against Ehrlich ascites carcinoma (EAC) and the possible involvement of epigenetic modification. The antitumor effect was assessed by evaluating tumor volume, tumor cell count, survival time and increase in life span of EAC bearing mice and investigated the possible involvement of epigenetic modification by detection of the relative gene expression of DNA methyltransferase genes (DNMTs) and detection of gene expression of apoptosis related gene (caspase 3). Treatment with plant, hesperidin and rutin increased the life span of EAC bearing mice by reducing the viability of EAC cells and decreasing the tumor volume .The anticancer effects of *Capparis spinosa L* extract and its potent constituents rutin and hesperidin may be partly attributed to its ability to demethylate and reactivate methylation-silenced apoptotic genes through decreasing expression of DNMT1.

Keywords: *Capparis spinosa L*, Caspase 3 , DNA methyltransferase, Ehrlich ascites carcinoma (EAC), Hesperidin, Rutin .

Corresponding author

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INTRODUCTION

Cancer is the result of DNA aberrations causing deregulation of cell cycle, apoptosis and cell survival. Carcinogenesis, cannot be explained only by genetic alterations, but also involve epigenetic processes, such as DNA methylation , histone modifications and miRNA deregulation **[1]**. DNA methylation is an epigenetic mechanism that commonly used by cells to control gene expression. DNA methylation occurs on cytosine at position 5 in a CpG dinucleotide which is mediated by DNA methyltransferases (DNMTs). Consequently, the DNA methylation pattern modifies a certain protein expression profile which is associated with various human aging disorders such as Alzheimer's (AD), and carcinogenicity **[2].**

Epigenetic aberrations are common in the development and progression of cancer cells and are mediated by DNA methyltransferases (DNMTs), histone deacetylases (HDACs) and microRNA (miRNA). These modifications alter gene function and malignant cellular transformation. DNA methylation is a reversible reaction, and catalyzed by three major DNMTs. One is DNMT1, which preserves the methylation patterns throughout each cell division. The others are DNMT2 and DNMT3, which transfer a methyl group to previously unmethylated genomic regions. Hypermethylation of DNA is akey epigenetic mechanism for the silencing of many genes, including those for cell cycle regulations, receptors, DNA repair, and apoptosis **[3].**

Medicinal plants have been know as one the most effective and safe therapeutic agents for the treatment of human diseases **[4].** There are numerous medicinal plants which possess multiple health-promoting effects **[5-8].** In addition, it is well known that synthetic drugs can cause a wide range of serious adverse effects **[9].** Nowadays, medicinal plants are known as an important source of bioactive natural products such as phenols and flavonoids **[10-12].** Medicinal plants used as folk medicine have strong antitumour activity against the Ehrlich ascites carcinoma (EAC) cell line **[13]**.

Caper (*Capparis spinosa L*.) is a common member of the genus Capparis (Capparidaceae family)**.**Caper consider a multipurpose plant used for the treatment of many human ailments as it has pharmacological effects and is utilized in phytomedicine around the world **[14]**. Phytochemical analysis showed that different parts of *C. spinosa* are rich sources of bioactive constituents, including polyphenolic compounds, which are responsible for its health-promoting effects. C. spinosa possesses different pharmacological effects including antioxidant, antimicrobial, anticancer and hepatoprotective effects **[4].** Hesperidin is a major dietary flavanone. Hesperidin has been recognized as a potent anti-inflammatory, anti-carcinogenic and anti-oxidantagent according to the data obtained from numerous in vitro and in vivo studies **[15].**Rutin is a flavonoid of the flavonol-type that is widespread in the plant kingdom **[16].** Rutin has a wide range of pharmacological properties (e.g., antioxidative activity) that have been exploited in human medicine and nutrition. Conventionally, it is used as an antimicrobial, antifungal, and antiallergic agent. Rutin has multi-spectrum pharmacological benefits for the treatment of various chronic diseases,such as cancer, diabetes, hypertension, and hypercholesterolemia **[17].**

The aim of the present study was to investigate anti-tumor activity of *Capparis spinosa L* extract and its main constituents rutin and hesperidin against Ehrlich ascites carcinoma (EAC) .Also to evaluate their effect on the expression of DNMTs and caspase3.

MATERIALS AND METHODS

Materials:

Plant material

Capparis spinosa L leaves were collected from Southern Egypt Sinai and a sample of plant was identified by cairo University herbarium, faculty of science, cairo University.

Chemicals

Rutin and Hesperidin were purchased from sigma Aldrich company , Trypan blue dye was purchased from El- Gomhouria Company, G-spin Total Kit DNA purification (Intron Biotechnology, Korea) ,PCR master mix (Invitorgen, USA), RNase and DNase free water (Invitrogen, USA),Hpa II (Bio Basic Inc., USA) , CutSmart Buffer

(Biolabs, USA),High purified agarose (Invitrogen, USA) , TriZol (Invitrogen, USA), RNase inhibitor (Ambion, UAS), High capacity cDNA synthesis kit (Qiagen, USA), SYBR Green master mix kit (Invitrogen, USA)

Experimental Animals

Adult female Swiss albino mice weighed (25-30g) purchased from Abo Rawash culture , Giza , Cairo, Egypt . Mice were housed in steel mesh cages (animal house, faculty of science , Zagazig University). The animals were maintained in controlled environment of temperature, humidity and light. The mice had free access to tap water and a commercial pellet.

Tumor cell line:

Ehrlich ascites carcinoma (EAC): EAC cells were initially supplied from the National Cancer Institute, Cairo, Egypt (only for the first transplantation), and maintained in female Swiss albino mice through serial intraperitoneal (I.P.) inoculation at 7 -10 days intervals in our laboratory in an ascites form. This process was repeated every 10 days for keeping the strain available throughout the present study.

Methods:

Plant extraction:

Ethanolic *Capparis spinosa L* extract was prepared according to the method **[18]**

HPLC analysis:

High performance liquid chromatography (HPLC) was used to identify the phenolic compounds present in *Capparis spinosa L* extract. The analysis of extracts were performed with HPLC (Hewlett Packard Series 1050, USA), the column (Hypersil BDS 5 um C18). Sampling injector by using quaternary HP pump (series 1100), solvent degasser, iso gradient separation was carried out with methanol and acetonitrile as a mobile phase at flow rate of 1 ml/min, temperature was maintained at 35°C. The ultraviolet UV detector set at wavelength 280 and 330 nm for phenolic and flavonoid compounds. Standards were obtained from Sigma Co. were dissolved in a mobile phase and injected into HPLC. Retention time and peak area were used to calculation of phenolic and flavonoid compounds concentration by the data analysis of HEWLLET packared software**[19].**

Toxicity study (LD_{50}):

Determination of the approximate acute median lethal dose (LD 50) was determined according to the method **[20].**

Experimental design:

In this experiment, the adult female Swiss albino mice were divided into 8 groups (15 mice in each group) as following:

Group (1): Negative Control: This group received sterile saline solution (0.9 NaCl) day after day for 9 days.
Group (2): Positive Control : This group received Ehrlich ascites carcinoma (EAC), (2.5×10^6 cells/ 0.3 ml/mouse) by (I.P) injection once in the first day.
Group (3): Preventive plant group : This group received plant extract (200 mg/kg b.w.)**[21]** one day before EAC injection (2.5×10^6 cells/ 0.3 ml/mouse) ,Then next days of EAC inoculation, Mice received plant extract (200 mg/kg b.w.) day after day for 9 days .
Group (4): Therapeutic plant group: This group received plant extract (200 mg/kg b.w.) **[21]** day after day for 9 days after the injection with Ehrlich ascites carcinoma (EAC), (2.5×10^6 cells/ 0.3mL/mouse) by (I.P) injection once in the first day..

Group (5): Preventive rutin group : This group received rutin (10 mg/kg b.w.)**[22]**one day before EAC injection (2.5×10^6 cells/ 0.3 ml/mouse) ,Then next days of EAC inoculation, Mice received rutin (10 mg/kg b.w.) day after day for 9 days.
Group (6): Therapeutic rutin group: This group received rutin(10 mg/kg b.w.)**[22]** day after day for 9 days after the injection with Ehrlich ascites carcinoma (EAC), (2.5×10^6 cells/ 0.3mL/mouse) by (I.P) injection once in the first day.
Group(7): Preventive hesperidin group : This group received hesperidin (50 mg/kg b.w.) **[23]** one day before EAC injection (2.5×10^6 cells/ 0.3 ml/mouse) ,Then next days of EAC inoculation, Mice received hesperidin(50 mg/kg b.w.) day after day for 9 days .
Group (8): Therapeutic hesperidin group: This group received hesperidin (50 mg/kg b.w.) **[23]** day after day for 9 days after the injection with Ehrlich ascites carcinoma (EAC), (2.5×10^6 cells/ 0.3mL/mouse) by (I.P) injection once in the first day.

At the end of the experiment the liver tissue from each group was collected and homogenized in liquid nitrogen. EAC cells were harvested from each mouse in centrifuge tube containing heparinized saline.

Viability and life span prolongation

The viability of EAC cells was determined by the *Trypan Blue* Exclusion Method **[24]**. Life span calculation was carried out according to the method **[25]**.

Real-time RT-PCR analysis

Quantitative real time polymerase chain reaction (q-RT-PCR) is a sensitive method for the quantification of mRNA expression levels using specific primers. Q-RT-PCR used to detect the expression levels of DNMT1, DNMT2, DNMT3, and Caspase3 genes. Total RNA was prepared using TRIzol reagent (Invitrogen, USA), according to the manufacturer's protocol. The total RNA was then used to performed cDNA using High capacity cDNA synthesis kit. The resulting cDNA was used as template for subsequent PCR amplification using DNA polymerase enzyme and primers specific for genes. The relative expression of DNMT1, DNMT2, DNMT3, and Caspase3 were detected using the QuantiTect SYBR Green PCR Kit (Qiagen) and oligonucleotides specific for each individual gene **(Table 1).** Levels of GAPDH were amplified using specific oligonucleotides, GAPDH-For-5'-TGGCATTGTGGAAGGGCTCA-3' and GAPDH-Rev-5'-TGGATGCAGGGATGATGTTCT-3' which was used for normalization. The results were analyzed using ΔΔ Ct equations **[26]**.

Table 1: Oligonucleotide sequences used for detection of indicated genes at RNA level

Description	Primer sequences 5'-3'
DNMT1-For	CCCATGCATAGGTTCACTTCCTTC
DNMT1-Rev	TGGCTTCGTCGTAACTCTCTACCT
DNMT2-For	CATACAATGCCCGTGTGAGTTCTTAAGG
DNMT2-Rev	CGTGTGTCTAAATGGCTTGAGTACAGT
DNMT3-For	TGCAATGACCTCTCCATTGTCAAC
DNMT3-Rev	GGTAGAACTCAAAGAAGAGGCGG
Cap-3-For	AAAATTTGGAACCAAAGATCATAC
Cap-3-Rev	TTTCTTCACGTGTAAGATCATTTTT

Gene amplification and restriction enzymes digestion:

Genomic DNA was extracted using G-spin Total Kit (Intron Biotechnology, Korea). Then the purified genomic DNA was used to amplify Caspase3 specific segment (250 nucleotides in length) using the specific primer sequences **(Table 1).** The following reagents have been prepared for each reaction; 15µl PCR master mix, 100 ng of genomic DNA, 10 pmol from each preimers (1 µl) and finally the total volume was adjusted to 25 µl with RNase and DNase free water. PCR was carried out in AmpGene DNA thermal cycler and the following PCR parameters were used, the initial denaturation step (95°C for 4 min), then 35 cycles (94°C for 40sec, 60°C for 45sec and 72°C for 60sec). One unit of Hpa II (Bio Basic Inc.) and 15 µl of CutSmart buffer were used to digest 1 µg of PCR product of all samples in total volume of 25 µl and one hour incubation at 37°C.

Finally, the digested product has been electrophoresed on 1% agarose gel for 30 min and monitored by gel documentation system**[27]** .

Statistical analysis

All statistical analyses were done by a statistical for social science package "SPSS" version 14.0 for Microsoft Windows, SPSS Inc. **[28]**. Numerical data were expressed as mean ± SE. The levels of markers were analyzed by ANOVA.

RESULTS

Total *Capparis spinosa L* extract yield

Capparis spinosa L leaves powder (100g) after undergoing extraction, yielded 8 g of ethanolic *Capparis spinosa L* extract (Thick green paste)

Identification of phenolic and flavonoid compounds for *capparis spinosa L* plant ethanolic extracts by using HPLC

Table 2: Fractions of phenolic compounds for *Capparis spinosa L* ethanolic extract by HPLC

Peak No.	Identified compounds	Retention time (min)	Conc.(mg/100 g)
1	Galic acid	7.377	8.575
2	Pyrogallol	7.495	211.978
3	4-Amino-bebzoic	8.221	12.151
4	3-OH-Tyrosol	8.613	10.677
5	Protocatchuic	8.737	90.132
6	Catechein	8.883	96.842
7	Chlorogenic acid	9.486	132.638
8	Catechol	9.681	168.231
9	Epicatechein	9.912	79.869
10	Caffeine	10.141	46.262
11	P-OH-benzoic	10.286	20.758
12	Caffeic	10.565	65.282
13	Vanillic acid	10.680	108.247
14	P-Coumaric	12.031	41.215
15	Ferulic	12.316	169.089
16	Iso- ferulic	12.717	255.237
17	Reversetrol	13.113	29.723
18	e- vanillic	13.340	1232.783
19	Ellagic	13.412	294.416
20	Alpha- Coumaric	13.623	49.114
21	Benzoic	13.828	211.747
22	3,4,5-methoxy-cinnamic	14.254	38.158
23	Coumarin	14.341	38.981
24	Salicylic	14.483	355.066
25	Cinnamic	15.523	20.796

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Table 3: Fractions of flavonoid compounds for *capparis spinosa L* ethanolic extract by HPLC

Peak No.	Identified compounds	Retention time(min)	Conc.(mg/100 g)
1	Luteolin	12.481	374.93
2	Narengin	12.579	197.15
3	Rutin	12.684	392.69
4	Hisperidin	12.830	537.92
5	Rosmarinic	13.049	23.094
6	Quercetrin	13.602	110.3
7	Quercetin	15.017	60.5
8	Hisperetin	15.621	71.41
9	Kampferol	16.287	11.34
10	Apigenin	16.501	8.8086
11	7-OH-ydroxyflavone	17.363	1.1214

Toxicity study

As to determine the median lethel dose (LD_{50}) of *capparis spinosa* ethanolic extract , all doses was found to be safe up to 2000 mg extract/ kg mice, as non of the mice were dead, which suggests that extract may be a safe compounds.While, hesperidin was might safe up to 1000 mg / kg mice and rutin safe up to 200 mg / kg mice.

Tumor growth and survival parameters

Ascites fluid accumulation was observed in the peritoneal cavity of the experimental animals after the inoculation of Ehrlich Ascites Carcinoma cells. The tumor bearing animals showed a marked increase in tumor volume. On treatment with plant ,rutin and hesperidin there is a marked decrease in the tumor volume EAC by 74.8% and 79.07% ; by 47.09% and 51.7 % ,and by 55.4 % and 64.3% respectively in therapeutic and preventive group compared to positive control group ($p<0.001$) (table 4).

The increased viable cell count of ascites fluid of tumor bearing animals was found to be decreased on treatment with plant ,rutin and hesperidin by 73.2 % and 75.3% ; by 56.6 % and 57.7% , and by 63.2% and 67.3 % respectively in therapeutic and preventive group compared to positive control group ($p<0.001$) (table 5).

Table 4: EAC volume (ml) in different studied groups

Groups	Group(2) Positive Control	Group (3) Preventive *Capparis spinosa L* extract	Group(4) Therapeutic *Capparis spinosa L* extract	Group(5) Preventive rutin	Group(6) Therapeutic rutin	Group(7) Preventive hesperidin	Group (8) Therapeutic hesperidin
EAC volume(ml)	5.16± 0.16	1.08 ± 0.08	1.3 ± 0.09	2.49 ± 0.05	2.73 ± 0.06	1.84 ± 0.06	2.38 ± 0.09
%Change		79.07	74.8	51.7	47.09	64.3	55.4
P value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

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Table 5: EAC count (x10^{6}cells/ml) in different studied groups

Groups	Group(2) Positive Control	Group (3) Preventive *Capparis spinosa L* extract	Group(4) Therapeutic *Capparis spinosa L* extract	Group(5) Preventive rutin	Group(6) Therapeutic rutin	Group(7) Preventive hesperidin	Group (8) Therapeutic hesperidin
EAC count (x10^{6}cells/ml)	149.8 ± 2.80	37 ± 0.93	40.2 ± 0.96	63.3 ± 0.77	65 ± 0.66	48.9 ± 0.64	55.1 ± 0.54
%Change		75.3	73.2	57.7	56.6	67.3	63.2
P value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Effect of *capparis spinosa* ethanolic extract , hesperidin and rutin treatments on Life Span Prolongation

On treatment with *capparis spinosa* ethanolic extract , hesperidin and rutin increased lifespan was noticed in tumor bearing animals and the results were tabulated in (Table 6).

Table 6: The effect of different treatments on Life Span Prolongation

Groups	Group(2) Positive Control	Group (3) Preventive *Capparis spinosa L* extract	Group(4) Therapeutic *Capparis spinosa L* extract	Group(5) Preventive rutin	Group(6) Therapeutic rutin	Group(7) Preventive hesperidin	Group (8) Therapeutic hesperidin
Survival days	9	20	19	18	16	15	16
life span T/C%	---	222.22	211.11	200	177.77	166.66	177.77
Increase in life span %	---	122.2	111.11	100	77.77	66.66	77.77

Effect of Capparis spinosa ,rutin and hesperidin on the transcript levels of DNMTs

The effect of capparis *spinosa* ,rutin and hesperidin on the DNMTs expression was determined by the quantitative analysis of mRNA for each of the three DNMTs(DNMT1 , DNMT2 , DNMT3). Our result indicated that the relative expression of DNMT1 significantly increased in EAC bearing mice while other DNMTs (DNMT2 and DNMT3) showed negligible differentiations. After treatment with *capparis spinosa* ,rutin and hesperidin, there was a marked decrease in the transcript levels of the DNMT1 (Figure 1).The most significant reduction was observed in preventive plant extract group on DNMT1 , as compared to other groups.

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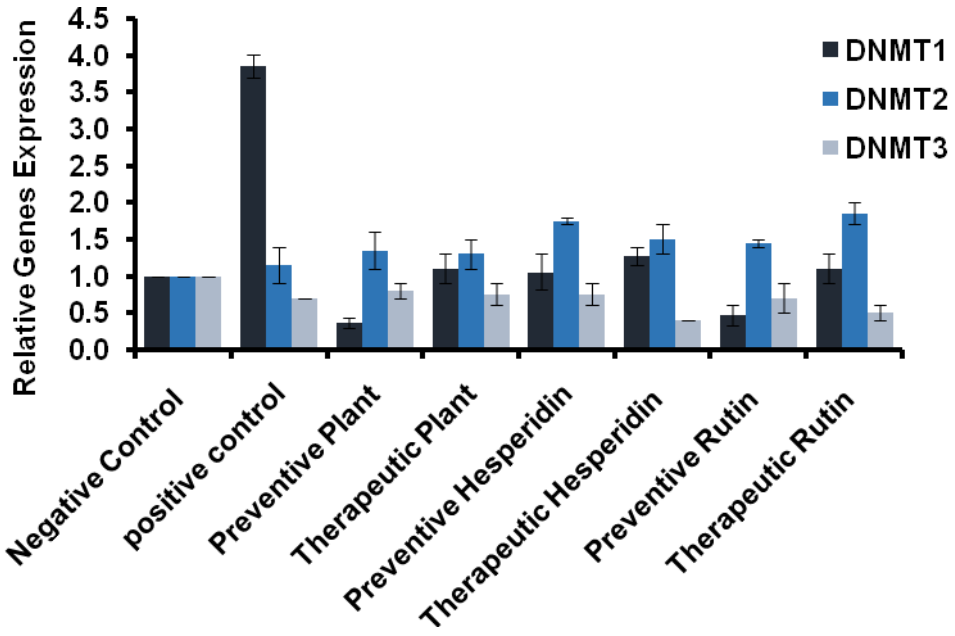

Figure 1: Relative expression of DNMTs in in experimental groups

Effect of *capparis spinosa* ,rutin and hesperidin on Relative expression of Caspase-3

Real time PCR was used to detect the relative expression of apoptosis genes Caspase3. Our results revealed a significant down regulated in EAC and upregulation of caspase3 gene expression following treatment with plant , rutin and hesperidin as compared to positive EAC group (Fig. 2). The most significant increase was observed in preventive plant extract group on caspase 3 , as compared to other groups.

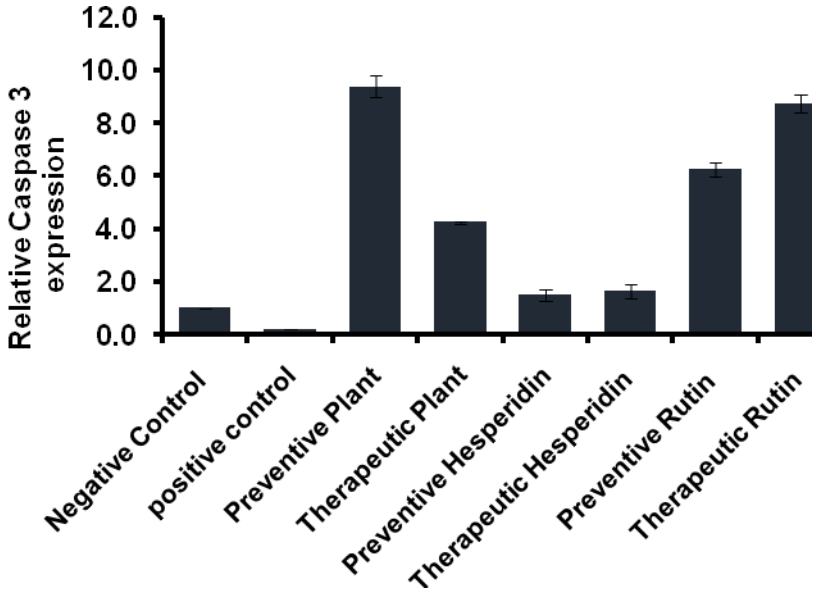

Figure 2: Relative expression of Caspase-3 in experimental groups

Treatment with Capparis spinosa L extract , rutin and hesperidin demethylates apoptosis Caspase3 genes and reactivates their expressions in EAC mice

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To confirm the possible epigenetic reprogramming of apoptosis Caspase3 gene, HpaII restriction enzyme has been used to digest the indicated segment of Caspase3 gene. HpaII has been shown to cleave the sequence CCGG. Importantly, the cleavage with this restriction enzyme is blocked when the substrate DNA is methylated by CpG methylase. Noteworthy, the sequence analysis of Caspase3 gene showed restriction sites that can be targeted by HpaII (Fig.3).Therefore, genomic DNA has been isolated and purified from tissue samples.The indicated segment of Caspase3 gene was amplified by using specific oligonucleotides and then was digested by using HpaII restriction enzyme. Interestingly, the digested EAC samples have been changed in size and number of indicated fragments in comparison with control samples. Moreover, the digested products of control samples showed two different fragments while EAC digested samples showed only one fragment (Fig. 4).Also the digested products of plant extract , rutin and hesperidin groups showed two different fragments again. These data indicated that the digestion activity of HapII is interrupted in positive EAC group due to methylation changes of cytosine nucleotides in its specific restriction sites and confirm the epigenetic reprogramming of Caspase3 gene in EAC. Collectively, our results indicated that the epigenetic reprogramming of gene expression through methylation of promoter region is involved in EAC and demethylation occur in treatment with plant extract , rutin and hesperidin.

(a)

Indicated Caspase-3 sequences with targeted CCGG methylated site.

GGAGAACACTGAAAACTCAGTGGATTCAAAATCCATTAAAAATTTGGAACCAAAGATCATACATGGAAGCGAATC
AATGGACTCTGGAATGTCCTGGGACACCGGTTATAAAATGGATTATCCTGAGATGGGTTTATGTATAATAATTAAT
AATAAGAATTTTCATAAAAGCACTGGAATGACATCTCGGTCTGGTACAGATGTCGATGCAGCAAACCTCAGGGAA
ACATTCAGAAACTTGAAATATGAAGTCAGGAATAAAAATGATCTTACACGTGAAGAAATTGTGGAATTGATGCGT
GATGTTTCTAAAGAAGATCACAGCAAAAGGAGCAGTTTTGTTTGTGTGCTTCTGAGCCATGGTGAAGAAGGAATA
ATTTTTGGAACAAATGGACCTGTTGACCTGAAAAAAATAACAAACTTTTTCAGAGGGGATCGTTGTAGAAGTCTAA
CTGGAAAACCCAAACTTTTCATTATTCAGGCCTGCCGTGGTACAGAACTGGACTGTGGCATTGAGACAGACAGTG
GTGTTGATGATGACATGGCGTGTCATAAAATACCAGTGGATGCCGACTTCTTGTATGCATACTCCACAGCACCTGG
TTATTATTCTTGGCGAAATTCAAAGGATGGCTCCTGGTTCATCCAGTCGCTTTGTGCCATGCTGAAACAGTATGCCG
ACAAGCTTGAATTTATGCACATTCTTACCCGGGTTAACCGAAAGGTGGCAACAGAATTTGAGTCCTTTTCCTTTGAC
GCTACTTTTCATGCAAAGAAACAGATTCCATGTATTGTTTCCATGCTCACAAAAGAACTCTATTTTTATCACTAA

(b)

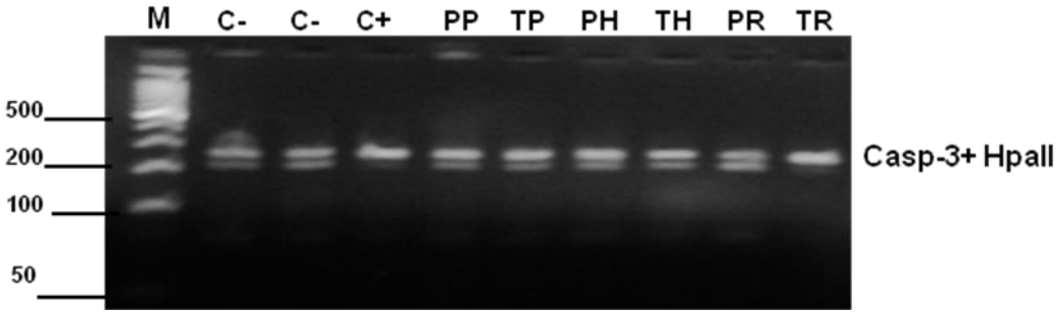

Figure 3: Possible methylation sites of apoptosis realted genes caspase 3

(a) : Indicated Caspase-3 gene sequences with targeted CCGG methylated site.

(B): Agarose gel electrophoresis of caspase3 gene segment that digested with CpG restriction enzyme HpaII indicating marker in lane 1 the negative control in lanes 2,3 and positive EAC in lane 4 and preventive and therapeutic Capparis spinosa L extract , hesperidin and rutin groups in lanes 5,6,7,8,9,10 respectively .

DISCUSSION

Cancer is a major worldwide problem that characterized by uncontrolled growth of abnormal cells. Although the cancer research has led to a number of new and effective solutions, the medicines used as treatments have clear limitations and unfortunately cancer is projected as the primary cause of death in the future currently there is a huge scientific and commercial interest in the discovery of new anticancer drugs. Therefore the search for potent, safe and selective anticancer compounds is a crucial aspect of modern cancer research **[29]**.

Caper (*Capparis spinosa* L.) belongs to the Capparaceae family. It has various medical uses in medical fields. *C.spinosa* which was commonly used as a medicinal plant contained many biologically active chemical groups including phenolic, flavonoids, alkaloids, glycosides, tannins, triterpenoids steroids, carbohydrates, saponins and a wide range of minerals and trace elements. It exerted many pharmacological effects including antimicrobial, cytotoxic, anti-diabetic, anti-inflammatory, antioxidant effect **[30]**

Hesperidin (3,5,7-trihydroxy flavanone-7-rhamnoglucoside) is a pharmacologically active bioflavonoid found in citrus fruits, with good free radical scavenging and anti-lipidperoxidation properties in biological membranes **[31] .**Hesperidin also have antioxidant, antiallergic, antiplatelet, anti-inflammatory and antiviral activities as well as antitumor properties **[32].**

Rutin, also known as vitamin P or rutoside, has a number of pharmacological effects including antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective **[33]**.

Phenolic compounds are very important plant constituents because of their scavenging ability due to their hydroxyl groups**.** Therefore, total phenolic compounds of *C. spinosa* ethanolic extracts were identified by HPLC. Data are illustrated in Table (2) showed that twenty five phenolic compounds were identified in plant. It can be noticed that e- vanillic are the most abundant phenolic compound being 1232.783 mg / 100g dry weight in extract. Furthermore, Galic acid acids was found in small amounts being 8.575mg mg / 100g extract .Also reflected that eleven flavonoid compounds were identified in plant. It can be noticed that hesperidin and rutin are the most abundant flavonoid compounds in plant being 537.92 mg / 100g and 392.69 mg / 100g, respectively. While, 7-OH-ydroxyflavone was found in small amount, 1.1214 mg / 100g. Our results are agreement with study revealed that the high performance liquid chromatography of extracted polyphenols are proved the presence of (Gallic acid,Caffeic acid, Coumaric acid , Vanillic acid , Syringic acid, Ferulic acid, Chlorogenic acid, Rutin and Quercetin) in the *Capparis spinosa L.* leaves extract **[34]**.

Acute toxicity tests are generally the initial step in the assessment and evaluation of the toxic characteristics of a substance. The acute toxicity was estimated by intraperitoneal administration of the compounds *C. spinosa* extract , hesperidin and rutin to determine the median lethal dose (LD50). Our results revealed that, dose up to 2000 mg /kg was considered safe for plant agreement with study **[35]** suggested that the plant extract is relatively safe in mice .Our results revealed that, hesperidin was considered safe up to 1000 mg / kg mice and rutin safe up to 200 mg / kg mice .Our result in agreement with study **[36]** indicated the safety of hesperidin in herbal formulations and other study indicated toxicity of rutin **[22]**.

One of the most reliable criteria for assessing the anticancer potential of the drug is the prolongation of the life span of cancer animals . In the present study, a rapid increase in ascitis tumor volume was observed in EAC tumor-bearing mice and treatment with *C. spinosa* extract , hesperidin and rutin slow down the growth of tumor by decreased the tumor volume and count of EAC and increase life span in comparisons to the positive EAC group. It can therefore be inferred that *C. spinosa* extract , hesperidin and rutin increased the life span of EAC bearing mice may be due to prevention of the tumor progression.

The anticancer activity of *Capparis spinosa L*. extract is probably due to presence of flavonoid and phenolic compounds in extract.Furthermore, flavonoids compounds have been shown to possess antimutagenic effect and anticancer properties **[37]** . N-butanol extract of *Capparis spinosa L.* had anti-tumor activity and significantly inhibited S180 sarcoma in terms of tumor weight **[38].** Another study proved that leaves and floral buds of *C. spinosa* were assayed for anticarcinogenic potential on HT-29 human colorectal adenocarcinoma cells **[39].**

The anticancer potential of hesperidin is demonstrated by inhibiting cancer cell growth and proliferation via apoptosis induction **[40].** Rutin has been extensively studied for anticancer/antineoplastic effects. Rutin is also known to inhibit cancer cell growth by cell cycle arrest and/or apoptosis, along with inhibition of proliferation, angiogenesis, and/or metastasis in colorectal cell lines **[41] .** Other study suggested that human leukemia HL-60 cells were implanted in a murine model, and rutin caused a significant reduction in tumor size justifying antileukemic potential**[42].**

Epigenetic regulation has been recognize to play a crucial role in etiology of cancer. DNA methylation is an epigenetic event that affects cell function by altering gene expression and refers to the covalent addition of a methyl group, catalyzed by DNA methyltransferase (DNMTs). Epigenetic mechanisms, responsible for regulation of gene transcription, have been shown to be deregulated in many cancers thereby altering the expression levels of many genes in tumor cells **[43,44]**.The expression of DNMT1 is increased in cancer and induces DNA methylation of various tumor suppressor genes. Up-regulation of DNMT2 was reported to activate viability and fertility. Furthermore, DNMT2-modulated cellular signaling pathways have been demonstrated in health and human disease conditions **[2].** Here, our result indicated that DNMT1 is highly expressed in EAC bearing mice in association with methylation of the promoter region of caspase-3 gene leading to low expression level of caspase-3 gene in EAC. We aimed to investigated whether the treatment with *C. spinosa* extract , hesperidin and rutin result in a reversal of epigenetic change. Subsequently, our findings here demonstrated that treatment with *C. spinosa* extract and rutin cause demethylation and significant increase expression level of caspase-3 gene.

Our results showed low expression level of caspase-3 accompanied with increasing expression level of DNMT1 in EAC bearing mice. These findings indicate the possible involvement of epigenetic reprogramming of caspase-3 via activation of DNMT1. Additionally, here we provide an easy and direct methods to confirm the methyaltion activity of specific DNA molecules by using HpaII restriction enzyme that targeted the CpG position and interrupted upon methyl alternation of cytosine nucleotides. Our finding indicated promoter hypermethylation for caspase-3 in EAC group and hypomethylation in treatment with plant , hesperidin and rutin .Our result agreement with study indicated that DNMT1, 3A, and 3B coordinate mRNA expression in normal tissues and overexpression in tumors. The expression levels of these DNMTs are reportedly elevated in cancers of the colon, prostate, breast , liver , and in leukemia**[45]**. Conversely, reduction of DNMT1 levels appears to have protective effects. Another study indicated the effects of dietary polyphenols such as EGCG on DNMTs appear to have their direct inhibition by interaction with the catalytic site of the DNMT1 molecule, and may also influence methylation status indirectly through metabolic effects associated with energy metabolism **[46]**. These bioactive components are able to modulate epigenetic events, and their epigenetic targets are known to be associated with breast cancer prevention and therapy **[47]**.

Apoptosis is a process of programmed cell death in which biochemical events lead to characteristic cell changes and death. Excessive apoptosis causes atrophy, while an insufficient apoptosis results in uncontrolled cell proliferation leading to cancer. Caspase-3 is also required for some typical hallmarks of apoptosis **[48]** . Our results showed treatment with plant , rutin , hesperidin cause increase expression level of caspase-3 gene compare to positive EAC control. The antitumor activity and antiproliferative activity of plant indicated in other study through apoptosis-induced effects on HepG2 cells **[49]** . Also, plant may have induced SGC-7901 cell apoptosis via the mitochondrial apoptosis pathway **[50]** . Rutin is also known to inhibit cancer cell growth by cell cycle arrest and/or apoptosis, along with inhibition of proliferation, angiogenesis, and/or metastasis in colorectal cell lines**[41].**The anticancer potential of hesperidin is demonstrated by inhibiting cancer cell growth and proliferation via apoptosis induction **[40]**. Hesperidin induces human colon cancer cell apoptosis as determined by increase of caspase-3 expression and activity **[51].**

In summary, Our data demonstrated that treatment with *C. spinosa* extract , hesperidin and rutin inhibited the growth of tumor by decreased the tumor volume and count of EAC and increase life span also by induction of apoptosis through activation of caspase 3.Also, we demonstrated that treatment with *C. spinosa* extract , hesperidin and rutin induce DNA demethylation and can restore or reactivate the expression of the DNA hypermethylated silenced gene in cancer by down regulation of DNMT1. The findings of the current study indicated that *C. spinosa* extract , hesperidin and rutin, may provide cancer preventive activity through the modification of the epigenetic process of the gene silencing.

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