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Assessment of the Anticancer and Antigenotoxic potential of *Costus speciosus* extract against HCT116 cell line

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ABSTRACT:*The aim of the present study was to analyze different effect of the costus speciosus extract against cancer cell line and to study its genotoxicity activity. The whole plant extract was compare to most effective and known compound diosgnen. For this purpose, antiproliferation and antigrowth activity was determined using cell viability assessment assays such as WST-1 assay and trypan blue dye exclusion staining assays against human colon tumor cell line (HCT116). Acridine orange dye and Erythrosine-B dye was used to understand the mechanism, whether the death of cells is due to necrosis or Apoptosis. Furthermore, Comet assay and Ames test were used for the detection of genetic toxicity of whole plant material. Our results showed that with concentration of 300 µg/ml of whole plant extract almost showed 100 % of cell suppression in HCT116 cell line. This data was also confirmed that the extract contained some compounds other than Diosgnen, which can suppress the growth of the cells. Therefore, we might concluded that, the whole plant of costus speciosus contains active compounds other than Diosgnen, which has the ability to kill cancer cells.*

Keywords: *costus speciosus, Diosgnen, Antiproliferation, Antigenotoxic*

1. Introduction

Human history used natural products as a traditional medicine [1]. Natural product sources contain compounds that are used for treatment of numerous diseases including cancer [2]. The third most common form of cancer is colon cancer, which is the fourth major cause of deaths that occurs due to cancer [3]. In most cases, the occurrence of aberrant crypt foci (ACF) results in colon cancer, an initial neoplastic injury that are bunches of mucosal cells having heavier coating of epithelia than the neighboring usual crypts which move into polyps that is followed by adenocarcinomas and adenomas [4]. Accumulation of numerous genetic changes in colonic epithelium is considered to be a cause these sequences of events [5]. Although colon cancer is not developed entirely by ACF, it is reported in a number of studies that the incidence of ACF progress into colon cancer [4].

In 90 % of cases, colon cancer is mainly associated with age; it arises in people of age 50 or above; both males and females have same possibility of colon cancer till the age of 50 while in males this possibility increases in later ages. It is reported in epidemiological studies that colon cancer indicates numerous hereditary cancer predisposition disorders that include inherited adenomatous polyposis, familial non-polyposis colorectal cancer, and past history of colorectal cancer and/or polyps and inflammatory bowel syndrome in individual or family [6]. Likewise, there are also some other

factors associated with higher risks of colon cancer that include obesity, absence of physical activity, drugs, smoking, high-fat diet, red meats and insufficient consumption of dietary fiber, vegetables and fruits [7-8,4].

Current studies reveal that in colon cancer, herbal extracts and phytochemicals that include extracts from ginger, grapes, turmeric, green tea soy and garlic, have antitumor effects [9-10]. *Costus speciosus* is member of the *Costaceae* family that belongs to the Malay Peninsula of Southeast Asia. Plant grows naturally in Sub-Himalayan region of India, in parts of Central India and in the Western Ghats of Maharashtra, Kerala, and Karnataka [11]. The tubers of Costus speciosus are rich in saponin like diosgenin, sapogenin, tigogenin, alkaloids and steroids [12, 13].

Many experiments on the pharmacological actions of *Costus speciosus* reported that extract possesses anti-inflammatory and antipyretic properties [14]. A number of beneficial properties for human health have been reported in plants that include inhibition of enzyme, vascular, cytotoxic antitumor activity, antiallergic, anti-fertility and hepatoprotective activity [15], diuretic activity [13], Antihyperglycemic Activity [16], anti-nociceptive [17]. In addition, studies showed that the C. *speciosus* leaf extract had anti-proliferative effect against cell lines of breast cancer (MCF-7 and MDA-MB-231) [18].

2. Material and Methods

2-1 Plant material extraction and preparation of Costus speciosus rhizome:

For the extraction 2 kg of rhizome dry powder was taken in individual aspirator bottle; 6 litres of hexane were used and was shaken occasionally for 72 hours. Then the extract was filtered. All extracts were decanted and combined and the process was done three times. Before drying, the extracts were filtered by Whattman filter paper no.2 on a Buchner funnel and vacuum distillation was used to remove the solvent in a rotary evaporator at 40 degree Celsius; the extracts were placed in pre-weighed flasks before drying.

2-2 Cell culture

The human colon tumor cell line HCT-116 was grown in McCoy's 5a Medium having 10 % fetal bovin serum (FBS), 100 units/ml penicillin + 100 µg/ml streptomycin antibiotics in tissue culture flasks at 37 degree Celsius in an incubator that contained 5% CO_2.

2-2-1 Cell viability assessment by trypan blue dye exclusion test.

In this test, *Costus speciosus* rhizome effects on the increase of the human colon cancer cell HCT116 tested using 0.4 % trypan blue exclusion test. In 24 well plates, cells were cultured at a density of $5\times10^4/cm^2$. After that, cells were treated with indicate concentrations of the plant rhizome extract (100, 140, 160, 180, 190, 200 and 300 µg/ cm^2) for 24 hour. Cells were then trypsinization, collected and mixed them with 1:1 trypan blue for 5 min. Cells were counted manually using hemocytometer under light microscope. At the end the percentage of live cells were calculated.

2-2-2 Cell viability assessment by tetrazolium salt WST-1 kit.

A 100µl of HCT116 cells were placed in 96 wells plate at a density of $5x10^4/cm^2$. After 24 hours of incubation, cells were handled with different amounts of *Costus speciosus* rhizome. All wells were added 10µl of fresh WST-1solution after the identified time. As a control for the microtiter plate, culture medium and WST-1 solution was combined in an unoccupied well. After 2 hours duration, using (BioTek Synergy HT, USA) micro plate reader, the concentrations of the treated and untreated samples were evaluated at 450 nm having reference wavelength 630nm for avoiding intervention of cell layer absorbance that stop passing of light through.

From absorbance reading from each well, ratio metric calculation was performed as

Survival rate (%) = A*sample **–A** blank **/ A** negative control **–A** blank

A* = absorbance

2-3 Single cell gel electrophoresis (Comet assay)

HCT116 cells were covered in 6 well cell culture plates at $1.3x10^4/cm^2$ density. After 24 hour of incubation, cells were handled with *Costus speciosus* at amount of (100, 150, 200 and 250.5 $\mu g/cm^{2)}$ and incubated for 24 hour. The media was removed from the tissue culture, and replaced with mincing solution (HBSS Ca^{++}, Mg^{++} free having 20 mM EDTA and 10 % DMSO, pH 7.0 - 7.5), then scraped the cells using a rubber policeman. Then the cells with medium was transferred and centrifuged at 400 xg for 5 minutes. Cells were washed once with ice cold 1X PBS (Ca^{++} and Mg^{++} free) and the final count of cells was 1-2 x 10^5 /cells/ml. A volume of re suspended cells, containing around 10,000 cells/slide was combined with 0.1 ml of 1% low melting point agarose (the volume containing cells was approximately 10% of the used LM agarose) and loaded on comet slides (Trevigen, USA), in duplicate after this, the gel was allowed to harden on a cold surface prior to further processing.

Following the embedding, each slide was placed in newly produced cold lysis solution (2.5M NaC1, 100 mM Na_2 EDTA, 10 mM Tris, pH 10, 1 % sodium sarcosinate, 1% Triton X-100 and 10 % DMSO was combined before using) at 4 degree Celsius for 30 minutes at the minimum. Each slide was then put in horizontal electrophoresis apparatus that was occupied with newly produced alkaline solution, pH ≥ 13 (300 mM NaOH, 1 mM EDTA) for a duration of 20 minutes for unwinding the DNA, after that electrophoreses was done at 0.9 Volt/cm for a duration of 20 minutes at room temperature. Three times, each slide was cleaned for 5 minutes with neutralizing solution (0.4 M Tris buffer pH 7.4) and then absorbed in absolute ethanol for 5 minutes for drying and DNA precipitation. After air drying, each slide was stained with 75µl ethidium bromide (20µg/ml) that was covered with glass cover slide. Slides were analyzed under fluorescence microscope and the extended dynamic range imaging (EDRI).

2-4 Ames test (Salmonella mutagenicity test)

Sterile aqueous extracts of *Costus speciosus* (8, 40, 200 and 1000 µg/plate) was tested by the *Salmonella typhimurium* mutagenicity test (Ames), with slight modification as described by [19].

3. Results and discussion

3-1 Cell viability and proliferation assessment

3-1-1 Cell viability assessment by trypan blue dye exclusion test

The effect of *Costus speciosus* rhizome on the viability of HCT116 cells was assessed by trypan blue dye exclusion. Cells were treated with increasing concentrations of *C. speciosus* rhizome (100, 140, 160, 180, 190, 200, and 300 µg/ cm^2) for 24, 48 and 72 hours. The result shows that the incubation with the extract efficiently inhibited cell viability in a concentration-dependent manner. **Figure (1)** shows the reduction in cell number and cell growth inhibition occurred when cells treated with 100, 140, 160, 180, 190, 200 and 300µg/ ml of the *C. speciosus* rhizome. In addition, the concentrations 160 and 300µg/ ml caused DNA fragmentation and formation of apoptotic bodies implying that cell death may be caused by apoptotic mechanism. The IC50 (concentration which inhibits 50% of cell growth) after 24 hours of incubation was 140 µg/ml and the concentration of 300µg/ml completely suppressed the proliferation **Figure (1)**. Clearly our data proves the anticancer activity of *Costus speciosus*, as has been documented previously. Leaves of *Costus speciosus* are also used in treating malignancies and other medical complications [20-21].

The cytotoxic effect of diosgenin on HCT116 cells was evaluated by trypan blue dye exclusion. Cells were treated with increasing concentrations of diosgenin (100, 140, 160, 180, 190, 200, and 300 µg/ cm^2) for 24, 48 and 72 hours. The result shows that the incubation with the extract efficiently inhibited cell viability in a concentration-dependent manner. **Figure (3)** shows the reduction in cell number and cell growth inhibition occurred when cells treated with 100, 140, 160, 180, 190, 200 and 300 µg/ ml of the diosgenin. In addition, the concentrations 160 and 300 µg/ ml caused DNA fragmentation and formation of apoptotic bodies implying that cell death may be caused by apoptotic mechanism. The

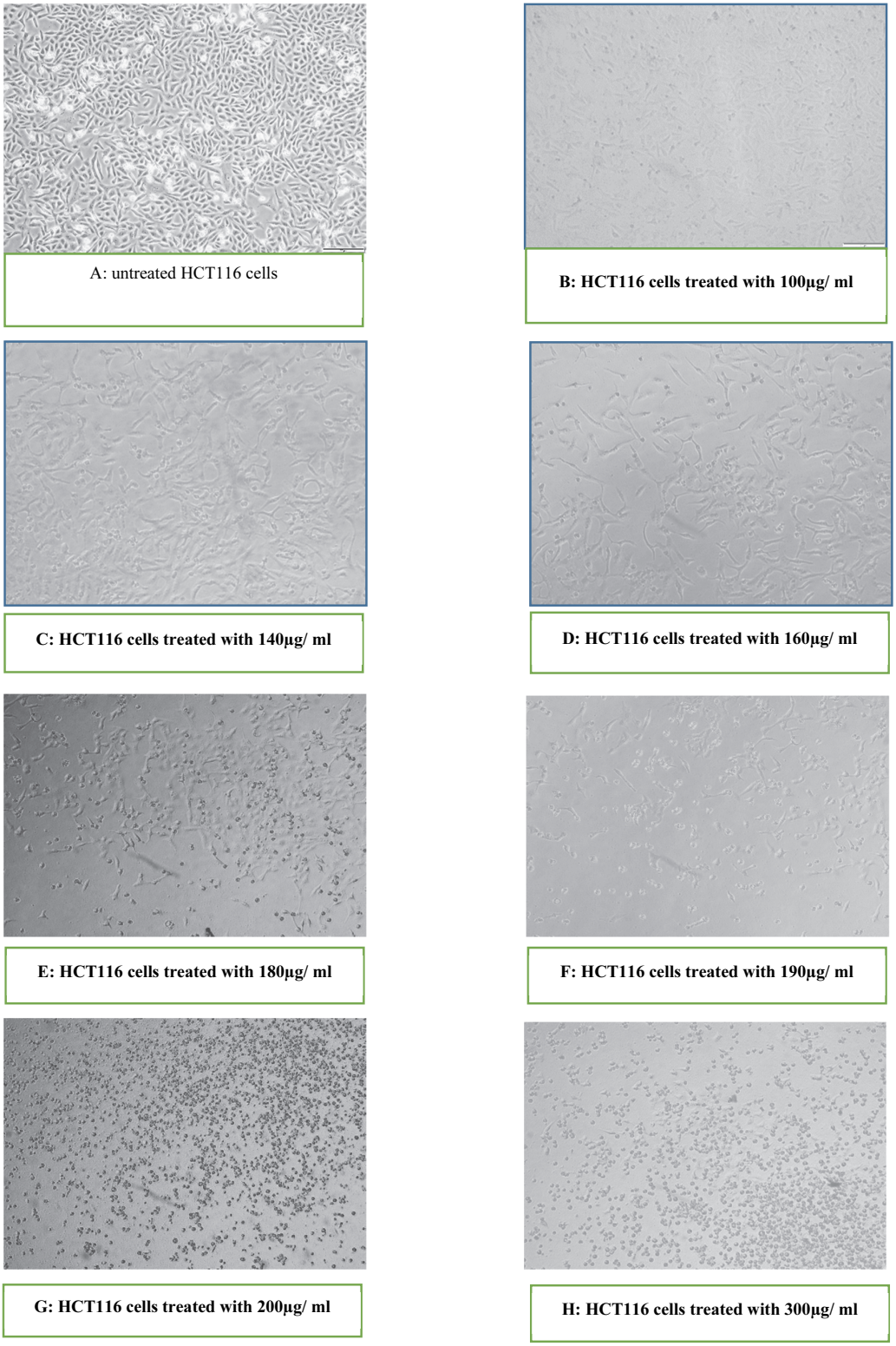


Figure 1 Photographic images showing the cytotoxicity of *C. speciosus* on HCT116 cells and DNA fragmentation.

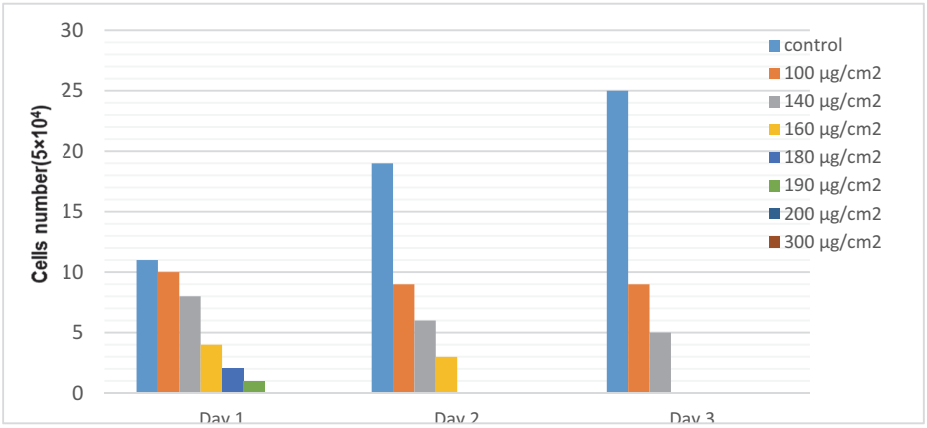


Figure 2 Cell viability of colon cancer cells HCT 116 treated with different concentrations of *C. speciosus as* determined by trypan blue.

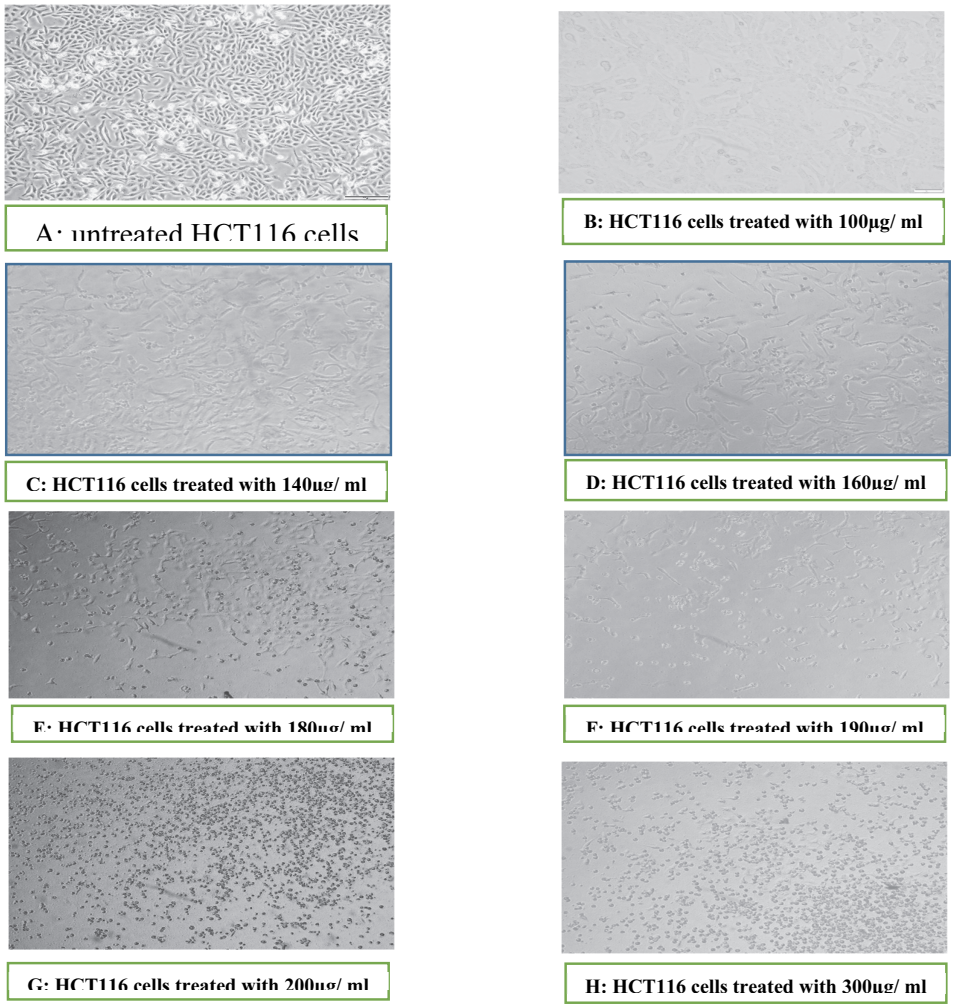


Figure 3 photographic image showing the cytotoxicity of diosgenin on HCT116 cell and DNA fragmentation**.**

IC50 value (concentration which inhibits 50 % of cell growth) after 24 hours of incubation was 160 µg/ml and the concentration of 300µg/ml completely suppressed the proliferation Figure (2, 3, 4). Diosgenin a steroid that is frequently used in pharmaceutical industry has anti-proliferative ability. It has been found that diosgenin induces apoptosis in cancer cells via caspases [22-23].

3-1-2 Cell viability assessment by WST-1 kit.

The anti-proliferative effect of *Costus speciosus* rhizome and effect of Diosgenin on HCT116 cells was examined by WST-1 kit, cells were treated with different concentrations of *Costus speciosus* rhizome (100, 150, 200, 250 µg/well) and the concentrations of Diosgenin (100, 150, 200 and 250 µg/well) for 24, 48 and 72 h. The result shows that the incubation with the rhizome efficiently inhibited cell viability and proliferation after 24 h in dose- dependent manner. The number of living cells is proportional to the quantity of formazan product (yellow color) formed by cellular mitochondrial dehydrogenases enzymes.

The concentration *Costus speciosus* rhizome of 100ug/well it caused inhibition in the survival rate by 69%, 75% and 86% after 24, 48 and 72 h of treatment respectively. At a concentration of 250µg/well, the treatment caused killing to almost 99.87% of the cells after 48h of treatment and the suppression occurred in a dose-dependent manner (**Figure 5**).

The concentration Diosgenin of 100ug/well it caused inhibition in the survival rate by 5.6%, 19.1% and 35.9% after 24, 48 and 72 h of treatment respectively. At a concentration of 250µg/well, the treatment caused high killing number 55.7% of the cells after 48h of treatment and the suppression occurred in a dose-dependent manner (Figure 6).

3-2 Genotoxicity of Costus speciosus rhizome extract on human colon cancer cells (HTC-116) as determined by comet assay:

A substantial difference was observed between the comet tail moments of cells treated with *Costus speciosus* for 30, 60 and 120 minutes and comet tail moments of the control group ($p>0.05$). After 30 minutes of treated with *Costus speciosus* 2 % and 160 mg/ml the corresponding mean comet tail moment were 3.52, 6.99 and 13.36 and cell with comet 45 %, 72 % and 84 % respectively, meanwhile the control cells tail moment was 0.2 and cells with comet were 2 %. After 60 minutes of treated with *Costus speciosus* 2 % and 200 mg/ml the corresponding mean comet tail moment were 6.56, 12.71 and 16.66 and cell with comet 49 %, 77 % and 86 % respectively. After 90 minutes of treated with *Costus speciosus* 2 % and 250 mg/ml the corresponding mean comet tail moment were 14.68 ,16.02 and 20.63 and cell with comet 51 %, 79 % and 92 % respectively as shown in (Figure 7).

3-3 Genotoxicity of Diosgenin on human colon cancer cells (HCT-116) as determined by comet assay:

A substantial amount of difference was observed between the comet tail moments of cells treated with Diosgenin for 30, 60 and 120 minutes and comet tail moments of the control group ($p>0.05$). After 30 minutes of treated with Diosgenin 2 % and 160 mg/ml the corresponding mean comet tail moment were 0.3 ,0.41 and 0.7 and cell with comet 4 %, 7 % and 9 % respectively, meanwhile the control cells tail moment was 0.2 and cells with comet were 2 %. After 60 minutes of treated with Diosgenin 2 % and 200 mg/ml the corresponding mean comet tail moment were 0.6, 1.53 and 1.89 and 2.8 and cell with comet 12 %, 16 %, 17 % and 51 % respectively. After 120 minutes of treated with Diosgenin 2 % and 250 mg/ml the corresponding mean comet tail moment were 1.7, 1.72 and 1.93 and cell with comet 20 %, 24 % and 48 % respectively as shown in Figure 8.

3-4 Anti-genotoxicity of Costus speciosus rhizome extract on human colon cancer cells (HTC-116) as determined by comet assay:

Again a stark difference was between comet tail moments of cells treated with MNNG 30 µmol for (positive control) and comet tail moments of the (negative control) ($p<0.001$). Also, there was a substantial difference between the comet tail moments of cells pretreated with MNNG 30 µmol for 30 minutes then with *Costus speciosus* for 60 and 120 minutes and negative control ($p<0.001$) as shown in Figure 9.

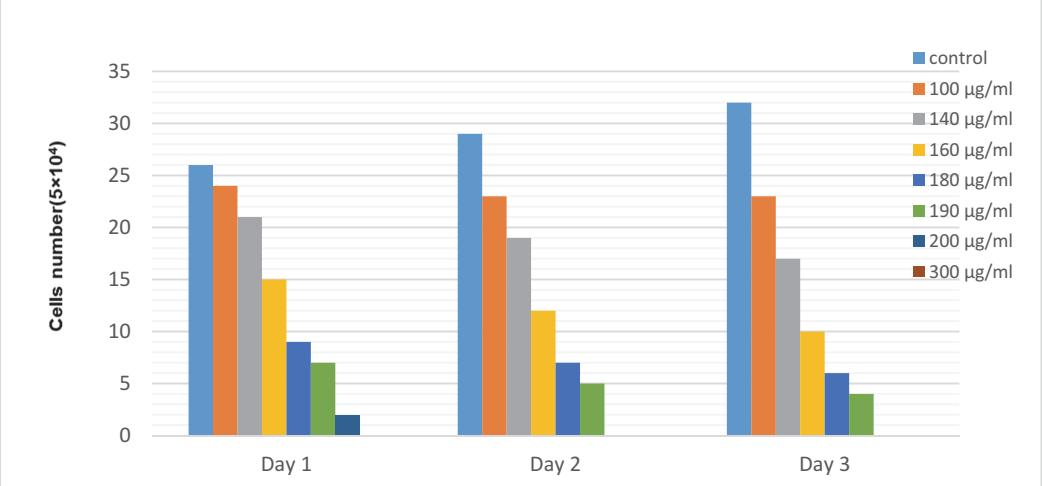
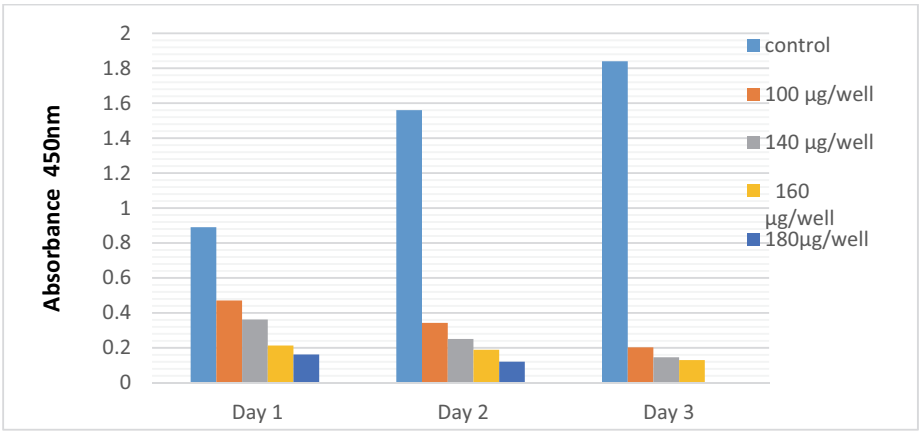


Figure 4 Cell viability of colon cancer cells HCT 116 treated with different concentrations of diosgenin as determined by trypan blue.

(A)



(B)

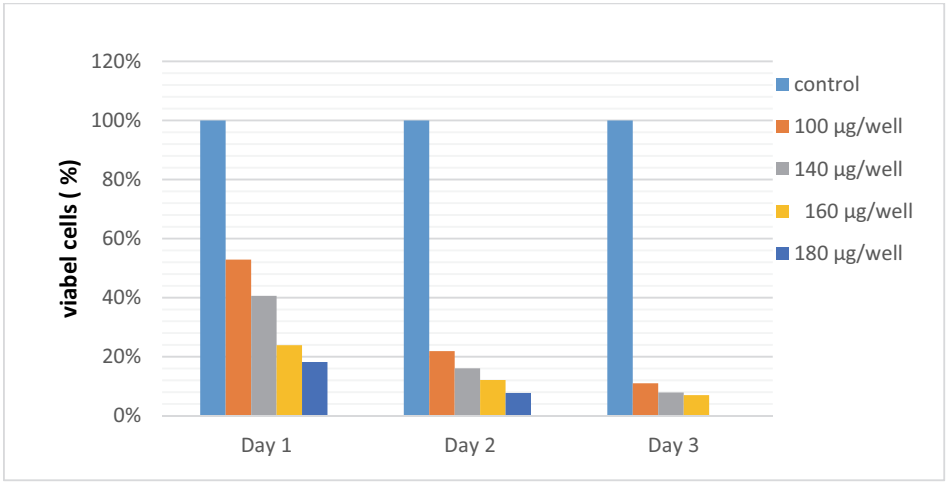
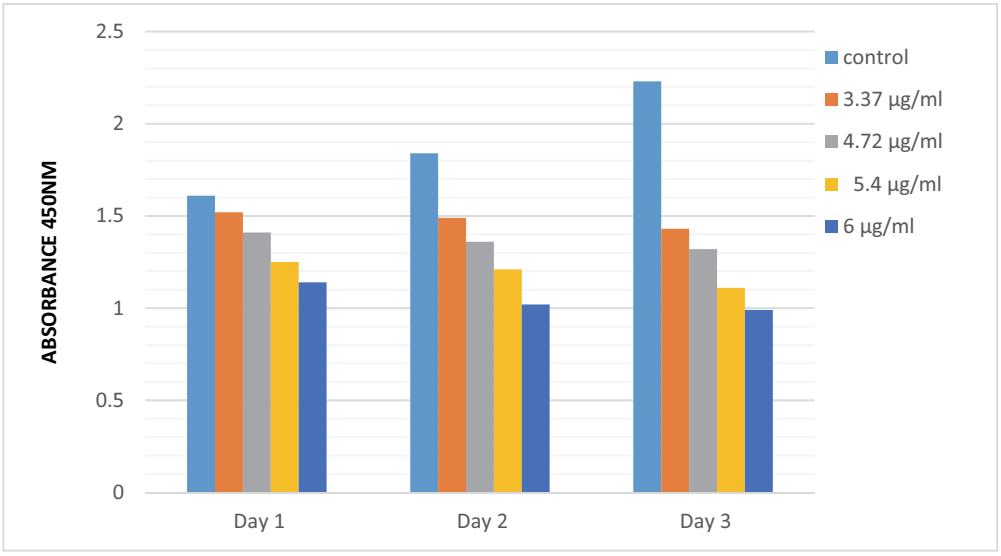


Figure 5 (A&B): Effects of *Costus speciosus* rhizome on HCT116 cells viability assessed by the tetrazolium salt WST-1 cell-proliferation assay kit. The results are the mean ± SD of triplicate independent experiments. Results shows that *Costus speciosus* rhizome inhibits viability and proliferation of HCT116 cell in a concentration-dependent manner.

(A)



(B)

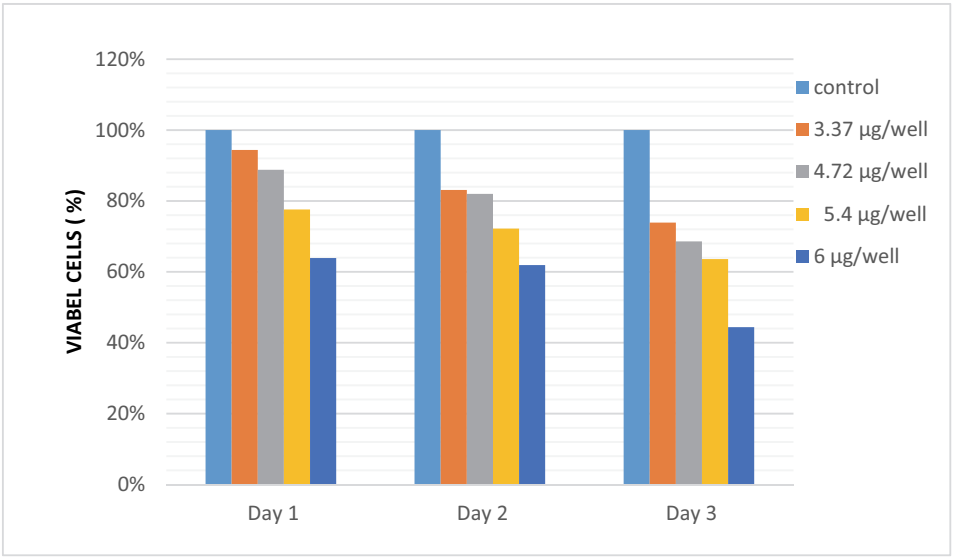


Figure 6 (A&B): Effects of Diosgenin on HCT116 cells viability assessed by the tetrazolium salt WST-1 cell-proliferation assay kit. The results are the mean ± SD of triplicate independent experiments. Results shows that Diosgenin inhibits viability and proliferation of HCT116 cell in a concentration-dependent manner.

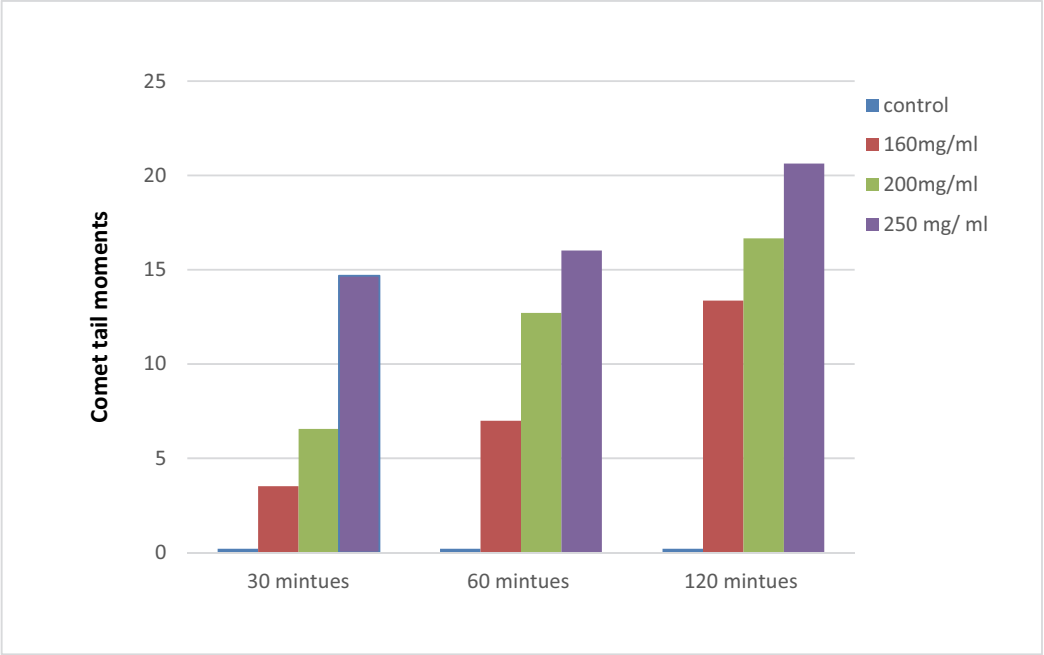


Figure 7 Genotoxicity of Costus speciosus rhizome extract on human colon cancer cell (HCT-116) as determined by comet assay.

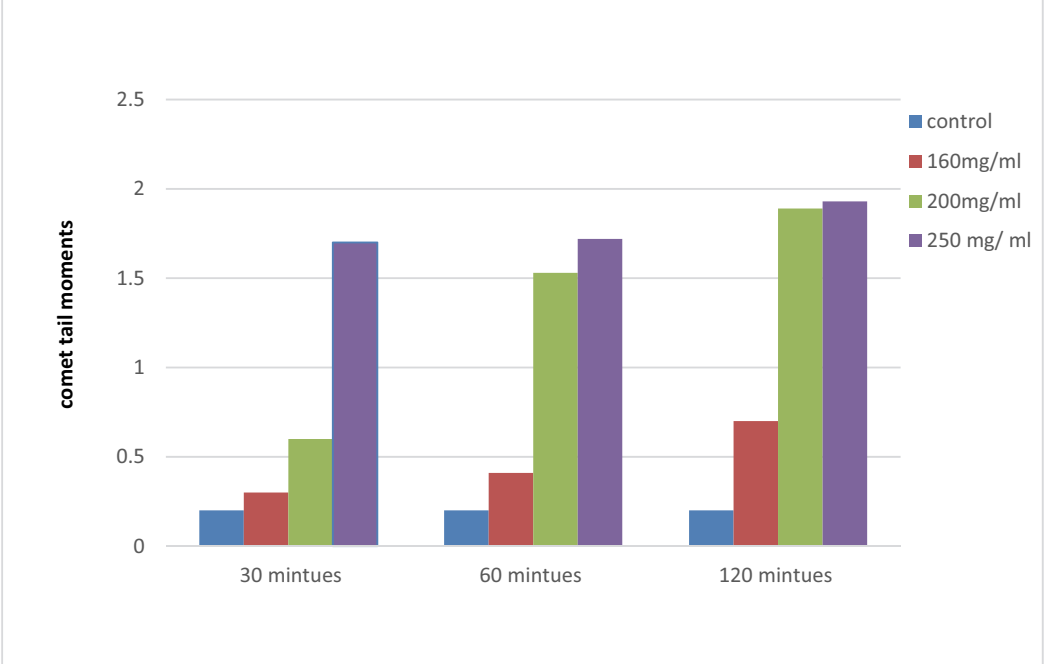


Figure 8 Genotoxicity of Diosgenin on human colon cancer cell (HCT-116) as determined by comet assay.

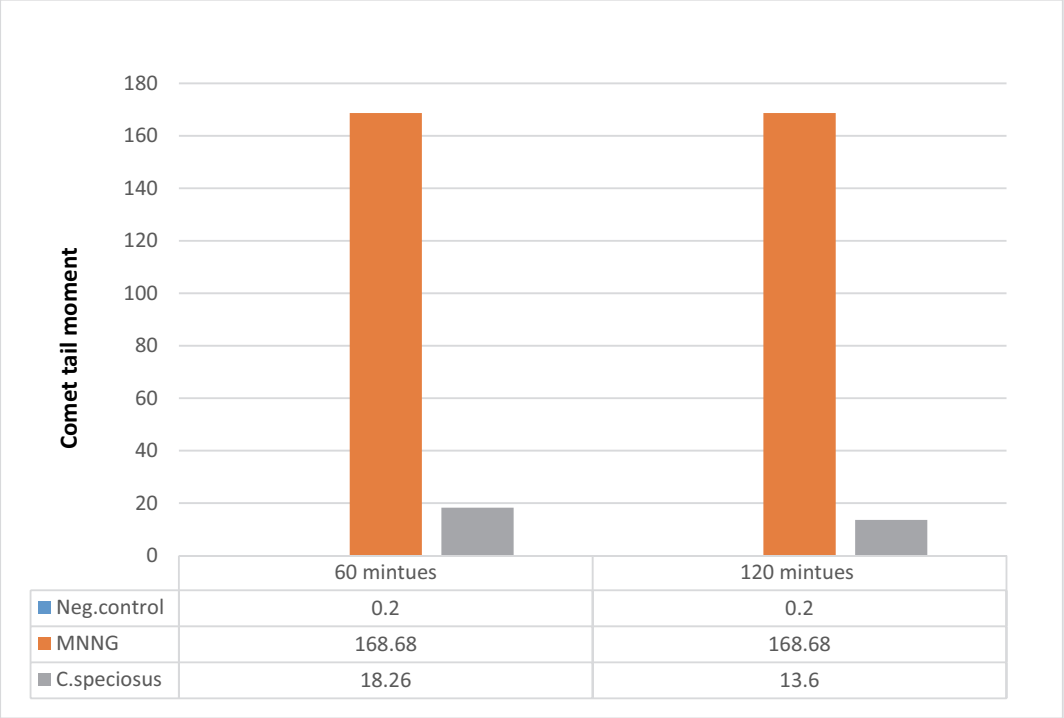


Figure 9 Antigenotoxicity of c.speciosus rhizome extract as determined by comet assay. HCT-116 were preteated with MNNG 30 µmol for 30 minutes then with Costus speciosus for 60 and 120 minutes.

After 30 minutes of treat with MNNG 30 µmol corresponding mean comet tail moment was 168.68 and cell with comet 97 % mean while the negative control cell mean tail

moment was 0.02 and cell with comet was 2 %. After 30 minutes for treated with MNNG 30 µmol , then with *Costus speciosus* 10 mg /ml for 60 and 120 minutes corresponding mean comet tail moment were 36 and 18.44 cell with comet were 87 % and 75 % treatment with *Costus speciosus* caused inhibition.

4. Conclusion

Our data highlights the anti-proliferative ability of diosgenin and whole rhizome extract of *Costus speciosus*. Diosgenin known to alter cell cycle and induce apoptosis is well documented [24]. Here we tried to compare the apoptotic ability of diosgenin and rhizome extract from *Costus speciosus*. From the data it can concluded that *Costus speciosusx* rhizome extract was more effective than diosgenin. This suggests the presence of other compounds in the rhizome with anti-proliferative and anti-cancer activity.

5. Recommendation

Before it as known that this plant contain diosgenin, which as an anticancer compound. But our results showed that this plant contains other compound that has the ability to show activity against cancer cell line. Further study is need to

identify that compound from rhizome extract of *Costus speciosus*.

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تقييم قدرة نبات القسط المضادة للنمو السرطانى والتسمم الجيني باستخدام خلايا القولون السرطاني HCT116

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المستخلص.أهداف الدراسة الحالية هي التحقق من الأنشطة المضادة للنمو والمضادة للأكسدة لنبات القسط الهندي *C.speciosus* باستخدام خلايا سرطان القولون البشري (HCT116) ومقارنة تأثير استخدام كامل النبات مع تأثير احد مكوناته الفعالة وهي مادة diosgnen والتي تشكل 3.37% من القسط الهندي. تم تحديد الأنشطة المضادة للنمو وسمية الخلايا باستخدام صبغة التريبان الزرقاء واختبار تكاثر الخلايا WST-1 . وتم التحقق من الالية الكاملة المحتملة وراء موت الخلايا سواء كان نخر أو موت خلايا مبرمج من خلال اختبار صبغة الاكريدين البرتقالية وصبغة الايثيديوم بروماید وتجربة هجرة الخلايا الكهربائية وحيد الخلية (المذنب) تم التأكد ايضاً من خلال تجربة السالمونيلا للتطفر (اختبار الايمز) وجميع تلك الاختبارات تستخدم للكشف عن السمية الجينية للمواد وكذلك فيما إذا كانت تلك المواد مضادة للسمية الجينية المستحدثة. ولم تثبت وجود أي تأثير مطفر لنبات القسط الهندي كما اثبتت النتائج ان مستخلص جذور القسط الهندي لديه القدرة الأعلى على منع تكاثر خلايا سرطان القولون بمقارنة بإحدى مكوناته الفعالة Diosgnen وهذا يتضمن احتمال لوجود أنشطة مكافحة للنشاط مضادات الاكسدة في خلايا السرطان لكامل نبات القسط الهندي.

كلمات مفتاحية : نبات القسط, diosgnen، الأنشطة المضادة للنمو ، المضادة للأكسدة