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Cytotoxicity Effect of 5-fluorouracil and bee products on the HTC-116 Human colon Cancer Cell Line *in vitro*

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Abstract: **Introduction**: Tumoris one of the most irresistible diseases all over the world, comprising large group of disorders distinguished by unmanaged cellular proliferation. Nowadays, there is an increased concern in the clinical application of natural products as an efficient, safe, and economic therapeutic alternative. Apitherapy or honey bee products therapy, was used to control different illness including cancer. **Aim:** The main target from this work was to study *in vitro* the potential cytotoxic impacts of honey bee products (RJ, H, PG, and mix) combined with or without 5-Fluorouracil (5-FU) against viability of colorectal cancer cells (HTC116). **Material and methods**: Human colon cancer cell line HCT116 were cultured in RPMI 1640 medium, enriched with 10% FBS and antibiotics. The cells were incubated under suitable environmental conditions (5% CO_2 and 95% air at 37°C and high humidity). Samples with cell viability of 95% and above were selected for use throughout this study. The cytotoxicity of 5-FU combination was tested against HTC-116 cells by sulforhodamine B (SRB) assay. **Results**: Treatment of in vitro HTC116 cultured cells with 5-FU alone resulted in elevation in IC50 value (6.94 uM, R-value 4.24%), in comparison with treatment with one or more of bee products (IC50 >100 uM/ml. In contrast, treatment with 5-Fu and supplementation with one or more of Honey products (RJ, H, PG & Mix), resulted in a significant decrease in IC50 value, which reached 3.39, 2.59, 1.9 and 2.04 uM, respectively. Therefore, during treatment with 5-FU, combine with one of honey products (RJ, H, PG) or their combination induced significant decline in cell viability as matched with the control (untreated) group, and the capability of 5-FUto affect the growth of HCT 116 cells in vitro was more enhanced only when the drug was supplemented with bee products. In conclusion, The combination of a single dose of one of Royall jelly, honey, pollen grains or their combinations with different concentrations of 5-FU confirmed significant suppressive action on HCT 116 viability in contrast to 5-FU alone at the same dosage. The obtained data proposed that bee products (RJ, H & PG) has a synergistic cytotoxic outcome with 5-FU in HCT 116 cell lines *in vitro*.
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Keywords: HCT-116, cell line, cytotoxicity, colorectal cancer, honey products

1. Introduction:

Worldwide, colorectal cancer (CRC) is the 2nd most common tumor in women and the 3rd in men, with a incidence of 9.2% and 10.0, respectively (Bray et al., 2013; Ferlay et al., 2015). In spite of the recent advancement in diagnostic tools and introduction of new techniques and therapies, CRC is one of the main reasons responsible for elevating the mortality rate among patients suffering from cancers, while the available therapies are not sufficient to manage CRC metastasis (Santandreu et al., 2011).

One of the most commonly used therapeutic drugs is 5-Fluorouracil (5-FU) which used as the first-line treatment for colorectal tumor. Longley et al., (2003) proposed that the mode of action of 5-FU as anticancer may be through disorderly synthesis of RNA & DNA throughout the miss-incorporation of fluoronucleotide into sequence and inhibiting the activity of thymydilate synthase (TS). The disadvantages of 5-FU treatment are attributed to the developed resistance to the drug, low availability within the cells due to its degradation in the liver by the enzyme dipyrimidine dehydrogenase in addition to higher toxicity associated with higher doses (Meregalli et al., 1998). To overcome the toxicity induced by high doses of 5-FU, new strategies are applied including many anti-cancer modulators in combination of 5-FU drug to enhancement its efficiency with less permissible toxicities. The advantageous observed benefits of using combined drug treatment encountered in increasing in the survival rate of patient and consequently the median survival time (Douillard et al., 2000; Giacchetti et al., 2000).

The application of more than one drug or multiple agents for treatment of tumor was proven as efficient in activity and reduced the toxicity (Mehta et al., 2010). The concern for application of natural compounds together with chemical drugs has become

a very promising approach as anti-cancer therapy. Plants are rich in phytochemicals, which are now being consumed for the purposes of chemoprevention (Rajamanickam and Aggarwal, 2008).

Many investigations established that the natural compounds are capable for modulating the processes of carcinogenicity via reversing/antagonizing or blocking, with minimal cytotoxicity (Braun and Seymour, 2011; Lee et al., 2011). For instance supplementation with natural compounds in combination with synthetic drugs such as 5-FU may afford efficient treatment for colorectal tumor through augmenting carcinocidal action, in the same time diminishing dose-related toxicity and resistance.

The importance of nutraceutical compounds like carotenoids, flavonoids, terpenoids or anthocyanidins for tumor avoidance has been extensively studied, and there are many suggestions confirming that moderate intake of vegetables and fruits is liked with reduced possibility of CRC (Fernández et al., 2016) or helpful for the treatment of CRC. The mechanism of action of these nutraceutical compounds may be via modulating signaling pathways, regulate gene expression which play an important role in cell differentiation, apoptosis and cell cycle regulation (Pan et al., 2011). Moreover, many recent studies revealed that treatment of tumor by using different combinations is more valuable and efficient than the use of single drug (Singh et al., 2013). Therefore, the aim of the present study was to investigate the cytotoxicity of HCT116 cell line in vitro and the viability of cells following supplementation with bee products with or without 5-FU.

Material and Methods:

Chemicals and drugs.

5-fluorouracil and sulpharodamine-B (SRB) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Media (RPMI-1640), fetal bovine serum (FBS) and other cell culture materials were purchased from Gibco™, Thermo Fisher Scientific (Grand Island, NY, USA)

Bee product samples:

Bee products (royal jelly, honey, bee pollen) were obtained from company of wild honey (Riyadh, Saudi Arabia). Stock solution of honey was prepared by mixing the honey with RPMI-1640 medium and sterilized using 0.22um Millipore membrane filter fitted on syringe. The honey products used in the current work has been tested by an accredited laboratory and confirmed to be pure honey.

Cell culture.

Human colon cancer cell line HCT 116 (ATCC® CCL-247™) was obtained from King Fahd center for medical research (Jeddah, Saudi Arabia) and the cells were grown and cultured in RPMI 1640 (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum **(**GIBCO, USA), and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin**)** after sterilization by using 0.22 um filter membrane. They were incubated in a humidified incubator with 5% CO_2 and 95% air at 37°C. The HCT 116 (ATCC® CCL-247™) cells were subcultured every 2 to 3 days in a semi-confluent condition in which they were treated with a trypsin-like enzyme and phenol red (GIBCO, USA) for 5 minutes. The cells were then re-suspended in the medium with serum before being transferred into 2 or 3 new flasks. Samples with cell viability of 95% and above were selected for use throughout this study.

Cytotoxicity assay (SRB assay).

The assay was carried out in a 96-well plate, The cytotoxicity of 5-FU combination was tested against HTC-116 cells by sulforhodamine B (SRB) assay. Exponentially growing cells were collected using 0.25% Trypsin-EDTA and seeded in 96 flat-bottom microtiter plate (Nunclon, USA) at 1000–2000 cells/well. Cells were treated with serial concentration (0.01 to 1000 µM) of 5-FU and combination (Honey samples (400 mg/ml) in RPMI-1640 medium were aliquoted into the wells in triplicates and serially diluted, Untreated cells were used as a control). for 72 h and subsequently fixed with trichloroacetic acid (TCA) (10% w/v) for 1h at 4°C. After several washings with double distilled water, cells were stained with SRB solution 0.4% (w/v) for 10min in a dark place at room temperature and finally washed with 1% (v/v) acetic acid. After the plates became dry by overnight incubation, Tris-HCl (50mM, pH 7.4) was used to dissolve the SRB-stained cells and color intensity was measured at 540nm with ELISA microplate reader and calculated as percent viability of control cells (cells exposed to drug free media).

Data and statistically analysis. The dose response curves of drugs under investigation were analyzed using Emax model in the following formula:

$$\%\ Cell\ Viability = (100 - R) \times \left(1 - \frac{[D]^m}{K_d^m + [D]^m}\right)$$

Where "R" is the residual un affected fraction (the resistance fraction); "[D]" is the drug concentration used; "Kd" is the drug concentration that produces 50% reduction of the maximum inhibition rate and m is a Hill-type coefficient. "IC50" is defined as the drug concentration required to reduce absorbance to 50% of the control (i.e., Kd=IC50 when R=0 and E_{max}=100−R). Combination index (CI) was calculated from the formula:

$$CI = \frac{IC_{50}\ of\ drug\ (x)_{combination}}{IC_{50}\ of\ drug\ (x)_{alone}} + \frac{IC_{50}\ of\ drug\ (y)_{combination}}{IC_{50}\ of\ drug\ (y)_{alone}}$$

The nature of drug interaction is defined as synergism if CI1.2; and additive if CI ranges from 0.8–1.2.

Statistical analysis.

The numerical parameters were expressed as means ± standard error means (SEM). using Prism® for Windows, ver. 5.00 (GraphPad Software Inc., La Jolla, CA, USA). Analysis of variance (ANOVA) with LSD post hoc test was used for testing the significance using SPSS® for windows, version 17.0.0. $p < 0.05$ was taken as a cut off value for significance.

3. Results

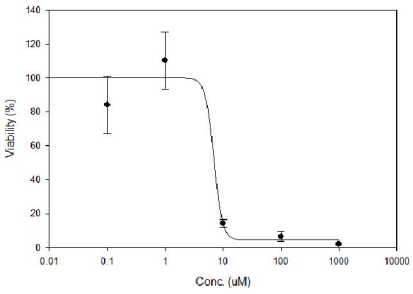
Table (1), showing The chemomodulatory effect of (Royal jelly, honey, bee pollen and combination of them) on the cytotoxicity of 5-FU in HTC-116 (**A**, **B, C, D, E**, **F, G, H, R**) colon cancer cell lines. Following an overnight incubation, HCT 116 colon cancer cells were then treated with either (A)5-FU (control positive group), (B) Royall Jelly, (C) Honey, (D) Pollen grains bee, (E) combinations of bee products and (F-R) treated with 5-FU with single treatment of RJ, H or PG or their combinations, respectively. SRB-assay was performed after 72hrs and the percentage of viable cells was measured in triplicate (presented as mean±SD, n=3). Cells were exposed to serial dilution of 5-FU, bee products or their combination for 72h. Cell viability was determined using SRB-assay and data are expressed as mean ± SD (n = 3). Statistical analysis was performed using Repeated-Measure ANOVA, followed by Tukey's Post-Hoc test. A p value of <0.05 was considered statistically significant. ***p<0.001 vs. control.

Table 1. Combination analysis for the cytotoxicity of 5-FU and bee products against HTC-116 colon cancer cell lines. Data is presented as IC50; n = 3.

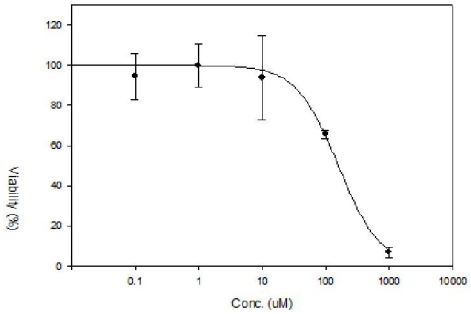
Exposure Time 72h	**HCT-116**	
	IC_{50} (µM)	**R-Value (%)**
5-FU	6.94	4.27
H (Honey)	>100	0
RJ (Royal jelly)	>100	0
PG (Pollen grains)	>100	0
(H+RJ+PG)	37.75	0
5-FU+H	2.59	0
5-FU+RJ	3.39	3.47
5-FU+PG	1.90	2.41
5_FU+(H+RJ+PG)	2.04	2.77s

Effects of Royal jelly, Honey and pollen grains bee with /or without 5-FU on HCT 116 cells viability *in vitro*:

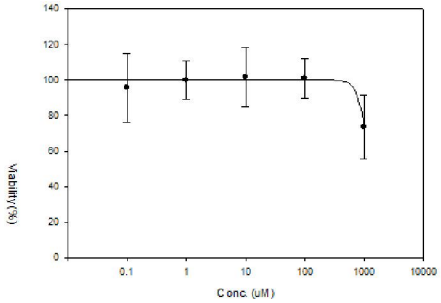
Figure 1B-1D showed effective growth inhibitory impacts of Royall jelly (B), Honey (C) and Pollen grains bee (D) honey on HCT 116 cells in a dose dependent manner. Single treatment with Royall jelly exhibited a gradual decrease in cell viability as the concentration of honey was increased reaching an IC50 of 200 um/mL, while in Honey IC50 reached 1000 uM and in Pollen grains IC50 reached 400uM, whereas in combinations of bee products (RJ+H+PG) IC50 reached 130uM. In contrast, supplementation with Bee products (RJ, H, PG) or their combinations with 5FU, showed much steeper decline with regards to its anti-proliferative ability against HCT 116 cells reaching an IC50 of 2.59,3.39, 1.9 and 2.04 UM/mL, respectively, in comparison with 5-FU alone (6.94 uM). This indicates that any of the bee products alone or combined are much more potent in inhibiting the growth of HCT 116 cells in comparison with control samples.



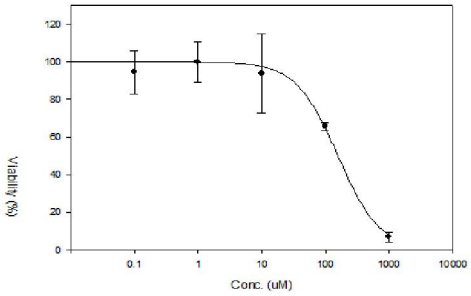
A): Viability of HCT116 cells after incubation for 72hr in different concentrations of 5-flurouraci I (5-FU +ve sample).



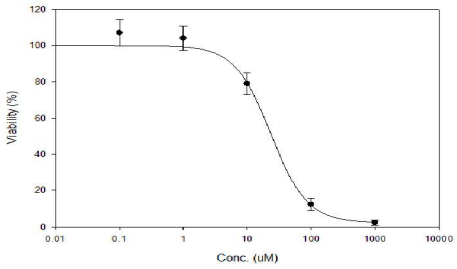
B) Viability of HCT116 cells after incubation for 72hr in different concentrations of Royal jelly.



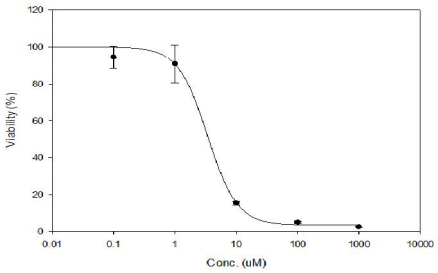
C)Viability of HCT116 cells after incubation for 72hr in different concentrations of Honey.



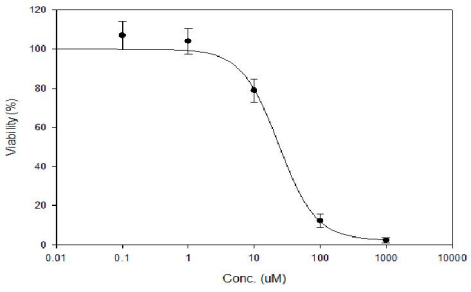
D)Viability of HCT116 cells after incubation for 72hr in different concentrations of pollen grain bee (PG).



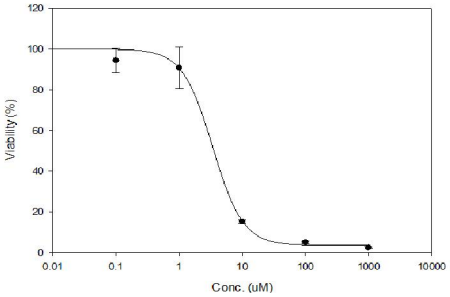
E) Viability of HCT116 cells after incubation for 72hr in different concentrations of combinations of Royall jelly, Honey and pollen grains bee.



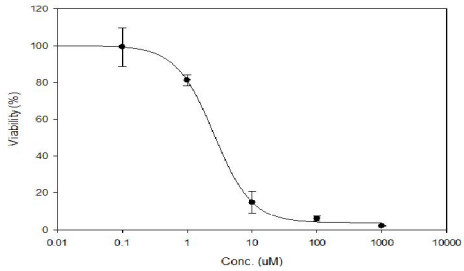
F) Viability of HCT116 cells after incubation for 72hr in different concentrations of 5-FU and Royall jelly.



G) Viability of HCT116 cells after incubation for 72hr in different concentrations of 5-FU and Honey.



H) Viability of HCT116 cells after incubation for 72hr in different concentrations of 5-FU and pollen grains bee.



R) Viability of HCT116 cells after incubation for 72hr in different concentrations of 5-FU and combinations of Royall jelly, Honey and pollen grains bee.

N.B.: Each point in the diagrams represents the mean of the results of three independent experiments. Error bars represent means ± S.E.M. of the three independent experiments.

Figure 1(A-R). The chemomodulatory effect of (honey, Royal jelly, bee pollen and combination of them) on the cytotoxicity of 5-FU in HTC-116 (**A**, **B, C, D, E**, **F, G, H, R**) colon cancer cell lines. As illustrated in Figure 1(F-R), single and combination treatment of 5-FU with either Royall Jelly, or Honey or Pollen grains or combinations of bee products exhibited significant decline in cell viability as matched with the control (untreated) group. 5-FU's ability to affect the growth of HCT 116 cells was further enhanced only when the drug was supplementation with bee products. The combination of a single dose of either Royal jelly, honey, pollen grains or their combinations with different concentrations of 5-FU demonstrated significant suppressive effect on HCT 116 viability in contrast to 5-FU alone at the same dosage.

4. Discussion

Nowadays, in spite of the recent techniques and advancement in diagnostic tools and therapies for controlling of CRC, yet is still one of the main etiology of elevating the morbidity and mortality rates among patients suffering from cancers, while the available therapies are not sufficient to manage CRC metastasis (Santandreu et al.,2011). One of the most commonly used therapeutic drugs is 5-Fluorouracil (5-FU) which used as the first-line for colorectal tumor treatment (Longley et al., 2003).

Many literature established that the combination of more than one treatment is better than single drug, particularly mixes from chemical and natural products. In the current study, the effects of different bee products (with and without 5-FU) on HCT-116 colorectal cancer cell lines have been evaluated *in vitro*. The results revealed that supplementation with Royal jell plus 5-FU, inhibited significantly the growth of HCT116 cells, where IC50 was 3.39uM vs. RJ alone (IC50 >100uM) or 5-FU alone (6.94uM/ml). Also, combination of honey with 5-FU, suppressed significantly the growth of HCT116 cell lines, where IC50 was averaged 2.59 uM/ml, compared with honey alone (>100uM/ml), or different concentrations of 5-FU alone (6.94uM/ml). With the same manner combinations 5-FU with either PG (bee pollen) or mix of (RJ, H & PG) suppressed significantly the cellular growth of HCT116 cell lines *in vitro*, where IC50 were reached 1.9 and 2.04 UM/ml, respectively in comparison with 5-FU (6.94uM/ml), or PG alone (>100uM/ml) or mix of bee products (37.75uM/ml). Many authors dealing with influence of Gelam honey (one of bee products) as a chemoprotective agents due to its contents of phenolic compounds which are rich with anti-oxidants, in addition to their potent action against carcinogenic cells (Jaganathan and M. Mandal, 2009, Hussein et al., 2011). Hakim, et al., 2014, established that combination of 5-FU with honey product (Gelam honey) was effective in inhibiting the HCT-116 growth cells. In the current work, The CD50 value of 5-FU against HCT116 cell lines was 6.94uM/ml. The assessment of cytotoxicity revealed that the cytotoxic dose of RJ, H, PG and their combinations were >100uM/ml, but this values were declined to 3.39, 2.59, 1.9 and 2.04 uM/ml, respectively, when mixed with 5-FU. The combination of 5-FU with bee products (one or more) augmented the cytotoxicity of 5-FU against HCT116 cell lines in vitro. This finding is supported by the finding of Lee et al., 2015, who found that addition of Gelam honey to 5-FU was effective in treatment of colorectal cancer, via suppression of Wnt/β catenin, mTOR signaling pathways and initiation of apoptosis pathway.

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