INVITRO EVALUATION OF ANTI-CANCER ACTIVITY OF 5-FLOUROURACIL AND CURCUMIN IN SINGLE AND COMBINED FORMS AGAINST BREAST AND COLON CANCER CELL LINES

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Abstract

The present study was undertaken to assess the chemotherapeutic activity of 5-fluorouracil and the herbal curcumin extract in single and in combination doses against the human breast adenocarcinoma cell line MCF-7 and colon cancer cell line Caco-2. The IC₅₀ values of curcumin and 5-FU against both cell lines revealed that curcumin had a higher cytotoxicity against breast and colon cancer cells (IC₅₀ = 30.01 and 27.57µg/ml) than 5-FU (IC₅₀ = 60.4 and 57.457µg/ml). The cytotoxic activity of 5-FU may be due to inhibition of DNA synthesis and the cytotoxic activity of curcumin may be due to inducing apoptosis by inhibiting proliferation of cancer cells by cell cycle arrest. Some toxic effect of test materials were observed including morphological, apoptotic changes such as cell membrane blebbing, nuclear materials fragmentation, shrinkage and margination. Anticancer potential was confirmed based on pro-apoptotic regulation of the two genes Bax and p53up compared to the internal control GABDH gene. It was found that 5-FU showed a better gene up regulation than curcumin. Synergetic potential of curcumin to 5-FU was detected regarding gene expression and related pathological changes.

Introduction:-

Cancer is a group of more than two hundreds neoplastic diseases, all of which are caused by dysregulation of multiple cell signaling pathways (Vogelstein and Kinzler, 2004), characterized by uncontrolled growth and spread of abnormal cells. If the spread of abnormal cells is not controlled, it can result in death (American Cancer Society, 2014)

Cancer involves genetics and epigenetic changes associated with molecular alterations involving certain types of genes, such as proto-oncogenes and tumor suppressor genes, as a result of genetic predisposition (Omar et al., 2003). Various therapies have been used for treating cancer such as surgery, radiotherapy, chemotherapy, and hormone therapy (WHO, 2004). But despite these therapeutic options, cancer remains associated with high mortality. Natural and some synthetic compounds can prevent, suppress, or reverse the progression of cancer (McCormick, 2001). Several anticancer agents are naturally produced by a wide range of different organisms including microorganisms, plants, and animals. These natural products have an effect on cellular signaling and gene expression (Harvey,2010). Natural products have proven to be the most reliable single source of new and effective anticancer agents (Newman and Cragg, 2007)
Curcumin is a natural pigment derived from turmeric (Curcuma longa L.), which has been known for its numerous pharmacological properties for many years (Chattopadhyay et al., 2004; Goel et al., 2001; Ireson et al., 2001). Several studies have been shown curcumin has anti-inflammatory, anti-angiogenic and antioxidant (Guzman et al., 2013; Xu et al., 2013). This nontoxic natural compound has been reported to possess several biological activities that are therapeutically beneficial to cancer treatment. Curcumin is also well known for its anti-inflammatory activity (Amanda and Robert, 2008). There are many types of curcumin products were developed to make curcumin more bioavailable and soluble, theracurmin one of the most curcumin analogue characterized by easing bioavailability and solubility (Kanai et al., 2012)

An apoptosis is a process mediated by two pathways: a mitochondrial dependent (Intrinsic) pathway which is activated by cellular stress such as DNA damage leading to proapoptotic Bcl-2 gene activation causing mitochondrial membrane permeabilization and subsequent activation of the caspase, and cell death receptor (extrinsic) pathway which involves production of death-inducing signaling complex (DISC) and the activation of initiator caspases. Curcumin was found to overcome the apoptosis resistance of cancer cells through both pathways (Lavrik et al., 2005)

5-Flourouracil (5-FU) is one of the most chemotherapeutic compounds used for the treatment of a great variety of tumors (Ofverholm et al., 2010). 5-FU is a heterocyclic aromatic organic compound with a structure similar to that of the pyrimidine molecules of DNA and RNA. Due to its structure, 5-FU interferes with nucleoside metabolism and can be incorporated into RNA and DNA, leading to cytotoxicity and cell death (Noordhuis et al., 2004; Hulme et al., 2005; Ning et al., 2008). 5- Fluorouracil is widely used for the treatment of many types of cancers and it was routinely employed in the management of colorectal cancer (Wyatt and Wilson, 2009). It was reported that the synergism between 5-FU and curcumin is more effective in enhancing the apoptosis and reducing the proliferation of colorectal cancer (Atee et al., 2014)

Materials and methods:-
Reagents and chemicals:-
Curcumin and 5-flourouracil, Tissue culture media, serum, trypsin and antibiotics were purchased from (Sigma – Aldrich –USA)

Cell culture:-
Human breast adenocarcinoma cell line (MCF-7) and colon cancer cell line (Caco-2) were obtained from American Type Culture Collection (ATCC). Cells were grown as monolayer in Minimum essential medium with Earle’s salt (EMEM) supplemented with 10 % fetal bovine serum (FBS) at 37 °C in a humified atmosphere of 5 % CO₂.

Subculture of MCF-7 and Caco-2 cell Lines:-
Cell lines subculture was performed as described by (Alan 1998; Jennie1998) The cell monolayer was trypsinized by trypsin/EDTA solution, the excess amount of trypsin was decanted and the flask kept in incubator for at 37 °C. 1-5 minutes. The cells were examined using an inverted microscope to ensure that all cells are detached and rounded. The cells were resuspended in a small volume of fresh growth medium to inactivate the trypsin biodegradable activity. One hundred µl of cell suspension were taken to perform a cell count. The required number of cells was transferred to a new labeled flask containing pre-warmed medium

Treatment of MCF-7 and Caco-2 cell lines with 5-FU and curcumin:-
The monolayer cells were trypsinized and re-suspended in growth media (90 % media + 10 % F.B.S). Equal number of cells was seeded in 6 well tissue culture plate and the plates were incubated at 37 °C for 24 hrs. The spent medium was removed 24 hrs post culture and fresh medium containing IC₅₀ value of curcumin and 5-FU in sloley and in combination were added to each well.

Evaluation of IC₅₀ using sulforhodamine B assay:-
The cell survival was evaluated using SRB assay as described by (Skehan et al., 1990). The MCF-7 and Caco-2 cells were seeded overnight in a flat bottom 96-well tissue culture plate (Nunc, USA) and incubated at 37 °C in a humidified air atmosphere enriched with 5 % CO₂. The cells were treated with tenfold dilution of 5-FU and curcumin separately ranging between 0 - 100 µg/ml for 72 hrs. The cells were stained with 0.4 % SRB dissolved in 1 % acetic acid 70 µl/well for 10 minutes the optical density (OD) was measured spectrophotometrically at 490 nm
with an ELISA micro plate reader (Biotek – EL-x 800-USA) and the IC₅₀ values was calculated using (Sigma Plot software) as:

Percentage (%) of Cell survival = Experimental (OD₄₉₀)/ Control(OD₄₉₀)× 100

**Statistical analysis:-**
Statistical analysis was performed using the ANOVA test to determine significant differences between treatment groups, with p values < 0.05 indicating statistically significant differences and p value < 0.001 indicating highly statistically significant. A randomize complete block design was used for data analysis. The treatment means were compared by least significant difference (L.S.D.) test as given by (Snedecor and Cochran, 1994). Statistical analysis was carried out by a special statistical program, ASSISTAT (Silva et al., 2009)

**RNA Extraction from Cell lines (MCF-7 and Caco-2):-**
RNA was extracted from 5-FU and curcumin in single and in combination treated and untreated MCF-7 and Caco-2 cells 72 post treatment according to manufacturer's protocol, where cells were collected by trypsinization, cold PBS washed twice and transferred to eppendorf tubes. RNA lysis buffer (175 µl) was added as to cell pellet and thoroughly mixed by inversion followed by addition of 350 µl of RNA dilution buffer, mixed by inverting 3-4 times and heated in a water bath at 70°C for 3 minutes. Cells were centrifuged at 14000 rpm for 10 minutes. The clear lysate was transferred to a clean tube, 200 µl of 95 % ethanol were added to the clear lysate and mixed well by pipetting. The mixtures were transferred to spin baskets assembly and centrifuged for 1 minute. The elute were decanted, 600 µl of RNA wash solution were added and centrifuged for 1 minute. The elute were decanted again and 50 µl of DNase incubation mix (40μl Yellow Core Buffer, 5μl 0.09M MnCl₂ and 5μl DNase I enzyme) were added to each spin basket and incubated at room temperature for 15 minutes. Reaction was stopped by adding 200 µl of DNase stop solution and centrifuged for 1 minute. Each spin basket was first washed with 600 µl of RNA wash solution, centrifuged for 1 min followed by second wash with 250 µl of RNA wash solution and centrifuged for 2 min to ensure removal of RNA impurities. Finally, RNA was eluted using 100 µl of nuclease free water. Extracted RNA was stored at -70 °C.

**Determination of RNA yield and quality:-**
Concentration and purity of the extracted RNA were assured according to (Wilfinger et al., 1997), where RNA was diluted with distilled water and the optical density was measured spectrophotometrically at 260 and 280 nm. RNA concentration and purity were calculated as follows:

Concentration of the extracted RNA (µg/ml) = A₂₆₀ × dilution factor X 40
RNA purity = A₂₆₀ / A₂₈₀
The expected absorbance range of pure extracted RNA should be within 1.7 to 2.1

**Reverse transcription:-**
For each sample, extracted RNA (1 µg), random hexamer primer (1 µl) and DEPC-treated water (to 12 µl) were mixed, centrifuged briefly and incubated at 65 °C for 5 minutes. Samples were placed on ice and the following components were added to each sample in the indicated order (Table 1).

<table>
<thead>
<tr>
<th>Table 1:- Reverse transcription reaction mixture.</th>
<th>Volume</th>
</tr>
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<tbody>
<tr>
<td>Component</td>
<td></td>
</tr>
<tr>
<td>Buffer 1x</td>
<td>5µl</td>
</tr>
<tr>
<td>DNTPS 1µl 200 µM</td>
<td>1µl</td>
</tr>
<tr>
<td>RNAs Inhibitor 10 unit</td>
<td>0.63 µl</td>
</tr>
<tr>
<td>Random Primer 0.6 µM</td>
<td>1µl</td>
</tr>
<tr>
<td>DEPC water</td>
<td>11.37 µl</td>
</tr>
<tr>
<td>RT Enzyme 2.5 units</td>
<td>1µl</td>
</tr>
<tr>
<td>RNA template 2 µg</td>
<td>5 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>25 µl</td>
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</table>
Verification of cDNA synthesis:-
GAPDH specific control primers (designed to be complementary to human GAPDH genes) were used to verify the synthesis of cDNA from the extracted RNA, where the following were mixed as follows:

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<tbody>
<tr>
<td>Dream Taq green master mix</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>cDNA template</td>
<td>2 µl</td>
</tr>
<tr>
<td>Forward GAPDH primer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Reverse GAPDH primer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>5.5 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

Preparation of Tri acetate EDTA (TAE) Buffer:-
TAE buffer is commonly prepared as a 50X stock solution according to (Brody and Kern, 2004). A 50X stock solution can be prepared by dissolving 242g Tris base in water, adding 57.1mL glacial acetic acid, and 100mL of 500mM EDTA (pH 8.0) solution, and bringing the final volume up to 1 liter. This stock solution can be diluted 50:1 with water to make a 1X working solution. This 1X solution will contain 40mM Tris, 20mM acetic acid, and 1mM EDTA.

Preparation of Agarose Gel:-
The agarose gel was prepared by dissolving 1.5 gm. of agarose in 100 TAE (1X), placed in microwave for 10 min and left to cool at 60 °C. Then 3µl of ethidium bromide was added and gel was casted in the electrophoresis marine cell. Sample was loaded post polymerization (Lerman and Frisch, 1982).

Polymerase Chain Reaction (PCR):-
Evaluating the expression of pro-apoptotic genes (p53 and Bax) was carried out using the newly synthesized cDNA as templates for PCR. Twenty five µl dream Taq green master mix, 4 µl cDNA, 2 µl forward primer (10 pmole /µl), 2 µl reverse primer (10 pmole / µl) and 17 µl nuclease free water were pre-denaturated at 94°C for 3 minutes. Amplification was performed (35 cycles), each cycle consisting of denaturation at 94 °C for 30 sec, annealing at 57 °C (p53), 58 °C (Bax) followed by extension at 72 °C for 45 sec. Reactions were terminated by heating at 72 °C for 5 minutes. Non-reverse transcribed RNAs were included to confirm the absence of genomic DNA. Negative control without adding template was also included to assess for reagent contamination. PCR product as well as DNA Ladder were loaded as 10 µl on 1.5 % agarose gel at 100 volts for 20-30 minutes, visualized using UV transillumiator and photographed after staining with Ethidium bromide(Huang et al., 2006).

Histopathology assay:-
Histopathology assay was carried out according to (Van, 2010). The 5-FU and curcumin treated MCF-7 and Caco-2 cells were harvested by trypsinization. The cells were centrifuged at 3000 rpm using cold centrifuge (Jouan-Ki22-France). A 50µl of cell pellet was dispensed to clean glass slides and spread in homogenous way to make a thin film. Slides were left to dry and fixed with methanol for 2 hrs at room temperature. None treated (control cells) were included. These films were stained by hematoxylin and eosin stain and investigated microscopically against morphological apoptotic features.

Results:-
Cytotoxicity assays:-
Data reordered revealed that the toxic effect of test drug was dose dependent; the cell viability decreased relatively to higher concentration of anticancer drugs (Table. 1) and (Fig. 1 – 4). Data recorded showed that there was a highly statistically significant difference (P<0.001). In the same time recorded IC50 revealed that curcumin showed a significant toxic effect than 5FU on both cell lines and there was a none significant difference of the IC50 values of 5FU as well as curcumin on both cancer cell lines (P>0.05)
Table 3: Evaluation of inhibitory concentration (IC$_{50}$) of 5FU and curcumin to breast (MCF-7) and Colon (Caco-2)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>IC$_{50}$ (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>MCF-7</td>
<td>5-FU</td>
<td>60.4</td>
</tr>
<tr>
<td></td>
<td>Curcumin</td>
<td>30.01</td>
</tr>
<tr>
<td>Caco-2</td>
<td>5-FU</td>
<td>57.4</td>
</tr>
<tr>
<td></td>
<td>Curcumin</td>
<td>27.57</td>
</tr>
</tbody>
</table>

PCR was performed to confirm the anticancer potentials of both test drugs based on evaluation of variability of expression rate of pro-apoptotic genes namely p53 and Baxin both breast (MCF-7) and colon (Caco-2). The treatments of 5-FU and curcumin showed up regulation of p53 and Bax genes (Fig. 5). Monitoring of gene expression revealed that both 5-FU and Curcumin showed up-regulation of pro-apoptotic genes in an insignificant was ($P>0.05$). Also, combination of curcumin showed a significant up-regulation of target genes than in case of being used singularly indicating that curcumin has a synergistic effect on up regulating the expression of pro-apoptotic genes. The normalization ratio of expressed gene was tabulated in (Table. 4). The normalization values of expressed genes in both cell lines was compared with control none treated cells. The recorded values assure the difference in expression rate and the none significant synergetic activity as well.
Table 4: Comparative evaluation of proapoptotic genes expression and normalization ration relative to control using RT-PCR

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Normalization %</th>
<th>Test materials</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Curcin</td>
<td>5-FU</td>
<td>Mix</td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53</td>
<td>198</td>
<td>238</td>
<td>248</td>
<td>132</td>
</tr>
<tr>
<td>N%</td>
<td>1.43</td>
<td>1.83</td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td>Caco-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53</td>
<td>233</td>
<td>254</td>
<td>289</td>
<td>189</td>
</tr>
<tr>
<td>N%</td>
<td>1.23</td>
<td>1.34</td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td>102</td>
<td>132</td>
<td>175</td>
<td>89</td>
</tr>
<tr>
<td>N%</td>
<td>1.14</td>
<td>1.48</td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td>Caco-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td>99</td>
<td>122</td>
<td>168</td>
<td>78</td>
</tr>
<tr>
<td>N%</td>
<td>1.26</td>
<td>1.56</td>
<td>2.15</td>
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</tr>
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</table>

Normalization % = Gene expression rate compared to its rate in cell control

**Histopathology assay:**
Histopathology assay are shown in (Fig. 6-13). There was a clear apoptotic morphological changes post treatment of MCF-7 cells with the IC$_{50}$ and there was plasma membrane blebbing, chromatin material condensed and marginated at nuclear membrane (Fig. 7). While in case of treating of Caco-2 cells with curcumin the morphological changes were a nuclear material shrinkage, forming of apoptotic bodies, and plasma membrane blebbing (Fig. 8). Also, the combination of 5-FU and curcumin treated MCF-7 cells showed morphological changes including DNA cleavage into oligonucleosomal fragments and formation of apoptotic bodies (Fig. 9). In case of treating Caco-2 cell line with 5-FU, plasma membrane blebbing, nuclear material shrinkage, and DNA cleavage into oligonucleosomal fragments were clear (Fig. 11). While, in case of using curcumin treated cells plasma membrane blebbing, nuclear material shrinkage, and DNA condensation and margination (Fig. 12) were clear. And combined form of 5-FU and curcumin, treated Caco-2, cells produced DNA cleavage into oligonucleosomal fragments, nuclear material shrinkage, and plasma membrane blebbing (Fig. 13).
Discussion:
Although the use of chemotherapeutics in cancer therapy remains the predominant option for clinical control; it provides inadequate effect in addition to its effect on the normal cells as well as on cancer cells; also one of the major problems of chemotherapy is the resistance developed after initial treatment (El Sharkawi et al., 2015).

The results of this study lead to the following main findings: (1) Curcumin is more effective than 5-FU towards breast and colon cancer cells, where the IC_{50} of curcumin towards breast and colon cancer cells was 30.0 and 27.5 µg/ml respectively, while the IC_{50} of 5-FU towards breast and colon cancer cells was 60.48 and 57.48 µg/ml respectively as demonstrated by the cell viability determined by SRB assay. The lower concentration of curcumin was as effective in reducing the viability of breast and colon cancer cells as the high value of 5-FU; (2) the expression of proapoptotic genes of MCF-7 and Caco-2 cells were significantly increased when 5-FU and curcumin are combined; (3) apoptotic morphological changes such as formation of apoptotic bodies, nuclear material shrinkage, DNA fragmentation into oligonucleosomal fragments, plasma membrane blebbing and nuclear material condensed and margination into cell membrane were observed under microscope.

These observations are in accordance with those obtained in other studies, where it was found that curcumin can suppress the proliferation of a wide variety of tumor cells, including breast carcinoma, colon carcinoma, renal cell carcinoma, hepatocellular carcinoma, T cell leukemia, B cell lymphoma, acute myelogenous leukemia, basal cell carcinoma, melanoma and prostate carcinoma (Somasundaram et al., 2002). Also, curcumin can increase the
cytotoxic effect of 5-FU to more than double, it significantly enhanced the efficacy of 5-FU chemotherapy without inducing any side effects. Curcumin is a pharmacologically safe compound and could clearly down-regulate NF-κB, both directly and also by thymidylate synthase, thereby circumventing 5-FU resistance (Cheng et al., 2001). In addition, curcumin can possess dual activities such as activation of p53 and inhibition of NF-κB thereby inducing apoptosis, curcumin induces p53-dependent apoptosis in basal cell carcinoma (Jee et al., 1998). It was reported that curcumin can increase p53 and Bax protein levels and was associated with marked oxidative stress and apoptosis in bile duct cancer (Suhim et al., 2010). The synergism between curcumin and 5-FU can induce apoptosis and p53 expression in cervical cancer cells. Curcumin induced apoptosis in cervical cancer cells, the apoptotic morphological features such as nuclear fragmentation and internucleosomal DNA fragmentation. Curcumin increased the apoptosis induced by 5-FU. Curcumin can increase the drug sensitivity of cervical cancer cells by up regulation of p53 and this is considered a novel therapeutic approach in cervical cancer (Ahn et al., 2010). Curcumin can induce apoptosis in numerous animal and human cell lines such as leukemia, melanoma, breast, lung, prostate, colon, renal hepatocellular and ovarian carcinomas. Curcumin also induces typical characteristics of apoptosis like cell shrinkage, chromatin condensation, and DNA fragmentation in immortalized mouse embryo fibroblast NIH 3T3 cells in a concentration- and time-dependent manner (Hanif et al., 1997)

The formation of Bax-Bax homodimers act as apoptotic inducers, while the formation of Bcl-2-Bax heterodimers can induce survival signal of the cell, moreover both Bcl-2 and Bax are transcriptional targets of p53 protein which induce cell cycle arrest or apoptosis in response to DNA damage, where the performance of these proteins is critical step for controlling life and death of a cell (Basu and Haldar, 1998). The antiapoptotic Bcl-2 members suppress apoptosis by blocking the release of cytochrome c; while proapoptotic Bcl-2 members induce apoptosis (Reed, 1996). The proapoptotic genes especially Bax is the most characteristic death promoting of Bcl-2 undergoes post translational modifications that include dephosphorylation and cleavage, resulting in their activation and translocation to the mitochondria leading to apoptosis (Scorrano and Korsmeyer 2003). The treatment of PC-3 and DU 145 prostate cancer cell lines with MS17 curcumin analogue showed various apoptotic morphological alterations such as cell shrinkage, formation of apoptotic bodies and membrane blebbing. Also it was found that there was significant decreased cell viability as the treatment doses increase in both cell lines (Saraste and Pulkki, 2000)

It was found that curcumin can effectively chemo sensitize breast cancer cells to 5-FU, thereby reducing the toxicity and resistance of 5-FU. It was reported that 10 μM 5-FU and 10 μM curcumin can induce a synergistic cytotoxic effect in different breast cancer cells. Curcumin was found to sensitize the breast cancer cells to 5-FU through TS-dependent down regulation of nuclear factor-κB (NF-κB) (Vino et al., 2013). In addition to previous findings the present study reported the consequences of apoptosis as DNA fragmentation in treated MCF-7 and Caco-2 cells (Figures 6 – 13).

Conclusion:-

In summary, the 5-FU and curcumin can inhibit tumor growth in MCF-7 and Caco-2 cells. Curcumin targets numerous pathways, highlighting its ability to inhibit carcinogenesis at multiple levels and thus, potentially circumventing the development of resistance. 5-FU is one of the most anticancer drugs, used for the treatment of a great variety of tumors due to inhibiting of DNA synthesis. The combination of 5-FU and curcumin can synergistically induce the up regulation of proapoptotic p53 and Bax genes and produce typical apoptotic morphological changes. The combination of 5-FU and curcumin may be useful to overcome 5-FU resistance in cancer treatment.

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