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The effect mechanism of *Ginnalin A* as a homeopathic agent on various cancer cell lines

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Abstract: Epidemiological and experimental studies have shown that natural products are beneficial for the protection against cancer. Maple syrup is a natural sweetener often consumed throughout the world. Maple syrup contains various phenolic compounds such as lignans, coumarin and *Ginnalin A* (GA). The aim of this study was to investigate the effects of GA shown to have cytotoxic and apoptotic effects in several human carcinoma cell lines. The effect of GA on cell viability was determined by a XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay as described in the manufacturer's instruction. Total RNA was isolated from cancer cells using TRIzol Reagent and reverse transcription was performed using Script™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Expressions of important genes in apoptosis including *MMP-2, MMP-9, TIMP-1, TIMP-2, CDH1,* and *CDH2,* were investigated in dose and control groups by qPCR (quantitative real time- polymerase chain reaction).

When compared with the control group, qPCR results illustrated that a significant increase in gene expression was observed in the expressions of *CDH1*, *TIMP-1* and *TIMP-2* by 3.52, 5.13 and 2.67 times respectively. Research has shown that *Ginnalin A* can demonstrate an anti-metastatic effect by regulating the expression of important genes in metastasis on cancer cell lines. Furthermore, in this study the activation of caspase-8 in apoptotic signaling pathways and the pro-apoptotic caspases required for extrinsic apoptotic signal transduction was defined.

Keywords: Ginnalin A; cancer cell lines; apoptosis.

1 Introduction

Cancer is a disease caused by an uncontrolled division of abnormal cells in the body. Cancer is classified according to the tissue type, the affected organ and the stage of the disease. Taking control of this disease and treating it is is extremely difficult and therefore alternatives to medical treatment have been developed [1,2] The use of complementary and alternative medicines has steadily increased over the last decade. Recently supportive and complementary therapy studies on classical homeopathy have created positive results in global health and well-being in cancer patients [3-5]

One factor affecting the success of the homeopathic principle, except for single drug therapy, is the minimum dose principle [6] Although it is important to understand whether homeopathy treatments have lasting undesirable effects, the minimum dose principle should be applied in order to avoid any side effects that may occur. Homeopathic medicines are derived from natural substances and therefore are likely more compatible with the immune system and the human body environment. Some, for example allopathic treatments do not suppress the body's natural system and are not addictive [7,8] but have been shown to to reduce cell proliferation and increase apoptosis of cancer cells. One of the most effective drugs in the treatment of cancer are those extracted from specific phenolic compounds and used as adjuvant active substances are *Ginnal A* or *Ginnalin A* (shown in Figure 1). Their effect on different cancer cell lines were included in the study.

In recent years, the scientific community hemeopath, which contains a wide range of secondary metabolites or flavonoids, have focused on the effectiveness of plant foods in living cells. Homeopathy in the treatment of various diseases, including cancer, is one of the commonly used complementary and alternative treatment area to reduce the side effects of radiotherapy and chemotherapy. Homeopathic remedies, used in the ultra low dose use diluted versions of various natural products. [9] The effectiveness of the homeopathic medicine has rarely

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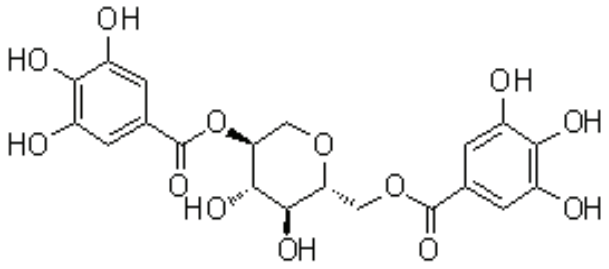

Figure 1: *Ginnalin A*.

been tested in in vitro models or animal models and their mechanisms of action have been in recent studies.

In the in vivo and in vitro studies, *Ginnalin A* and the *p38 MAPK* inhibitors were shown to be cytotoxic and apoptotic on various cancer cells [10]. The aim of this study is to search the inhibitory effect of *Ginnalin A* in the *p38-MAPK* signal pathways. Furthermore, my goal is to evaluate the effectiveness of the active phytomedicine ingredient of *Ginnalin A* in the cell signaling pathway that is as inhibitor in the treatment of cancer. Traditional and complementary medicine use *Ginnalin A* formulations to treat different cancer cases or cell lines. Basic research on homeopathic preparations of *Ginnalin A* are partially less and the use of cell-based models in drug screening is a reliable source of evidence.

2 Experimental

2.1 Chemicals

Ginnalin A was commercially obtained from Sigma-Aldrich Chemical Company (USA). It was dissolved in the appropriate solvent and was applied to the cells at varying doses. RPMI-1640 medium, penicillin/streptomycin, phosphate buffered saline (PBS), fetal bovine serum (FBS) and XTT [2,3 bis (2 Methoxy 4 nitro 5 sulfophenyl) 2H tetrazolium 5 carboxanilide] kit were purchased from Biological Industries. TRIzol reagent was purchased from Invitrogen, USA. Transcriptor first strand cDNA synthesis kit was commercially supplied from Bio-Rad.

2.2 Cell Cultures

HCC ATCC (Hep-3G and Hep-3B; Manassass,VA, USA), and PC3 (ATCC® HB-8064™) were used as the human cancer cell lines in this study. Each cell line separately in EMEM with 10% fetal bovine serum and media containing 1% Penicillin-Streptomycin, was cultured at 37°C, 5% CO_2 environment passaged coated and was stored in a freezer at -80°C. After *Ginnalin A* dissolved in the appropriate commercially available solvent and was applied to the cells at varying doses.

2.3 Cell Culture Preparation

Cancer cells in EMEM medium containing 10% fetal bovine serum and 1% penicillin-streptomycin, 5% CO_2 with media were incubated at 37°C with which to cells using *Ginnalin A* was administered in different doses.

2.4 Determination of Cytotoxicity by XTT Method

The cytotoxic effect of *Ginnalin A* on cancer cells with XTT [2,3 Bis-(2-Metoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide] were determined by the cell viability method. Simply cultivated on 96 well plates after 24 hours incubation time individually cancer cells *Ginnalin A* 24, 48 and applied at various concentrations for 72 hours, respectively. Specified dose and time XTT solution was finally added to the cells after 4 hours and absorbance values read at 570 nm on microplate reader. Percentage cell survival was calculated for each well, measured at optical density value of the control optical.

Cell Viability % = (Measured optical density value) / (Control optical density values) x 100

The concentrations of GA inhibited 50% of cell viability (IC_{50}) was determined with CompuSyn Version 1.0 software.

2.5 Caspase-3 Activity Assay

Ginnalin A effect on caspase-3 activity was performed according to manufacturers' instructions. 6 well plates at pre-determined doses after 24 hours cultivation the treated cells and the time period *Ginnalin A*, and *non-Ginnalin A* was administered, respectively. Analysis were performed with kits and appropriate solutions in the content. Caspase 3 activity in all groups with microplate reader absorbance values reading at 400 nm the interpretation of the absorbance values was determined.

2.6 Total RNA Isolation and cDNA Synthesis

Ginnalin A and control for the evaluation of the expression levels of target genes results in the application and dose group. Total RNA was isolated from the cells using TRIzol method was performed. The quantity and quality of RNA obtained was determined using measurements in Nanodrop. As a result of measuring A260/A280 ratio of 2 ± 0.1 RNA samples which were used in further analysis. Total RNA samples were stored at -80°C is used. The synthesis of cDNA from the samples was performed using the manufacturer's instructions by cDNA synthesis kit.

2.7 Determination of the expression levels of genes that play important roles in cell cycle and apoptosis, necrosis by Real-Time PCR (QPCR) analysis

In the control and dose groups apoptosis, necrosis and the levels of expression at the mRNA of genes important in the cycle cell were determined using qPCR method. The anticancer effects of *Ginnalin A*, important genes in apoptosis as *CASP3, CASP8, CASP9, CYCS* ; metastasis significant *CDH1, TIMP1* and *TIMP2* genes and in cell cycle genes were assessed by determining the differences in gene expression levels of *P53*.

2.8 Statistical Analysis

Differences in gene expression levels between the control and dosing groups were determined using $2^{\Delta\Delta C}$ method using β-actin housekeeping gene. Other analyzes were performed with SPSS 21.0 (SPSS Inc., Chicago). In all analyzes, $P<0.05$ was considered statistically significant.

Ethical approval: There is the conducted research is not related to either human or animals use.

3 Results and Discussion

The result of the uncontrolled proliferation of cancer cells in the body is one of the most important health problems occurring in our day. Cancer is known as the biggest cause of death worldwide. Cancer is a multistep process that leads to pathological metabolic changes in living cells. This pathological process, threatens the immune system which ensures the survival of healthy cells and poses serious modifications in the cell. Cancer cells in the tumor development process gain many phenotypic features. These changes may cause the rapid and uncontrolled proliferation of tumor cells and can spread into surrounding tissue. Cancer cells invade the lymphatic system and blood vessels and spread to other parts of the body i.e.metastasize. Normal cells may be damaged or completely degraded at this stage [11]. Depending on the disease it may be diagnosed later and the observed slow development of resistance to chemotherapeutic and radio-therapeutic agents used in the clinical treatment [12]. Therefore, for disease prevention and treatment there is the need for the development of new therapeutic strategies [13,14]. *Ginnalin A* is one of the most important phenolic compounds and studies have shown that *Ginnalin A* inhibits the growth of colon cancer cell line [15]. In a study conducted with colon and breast cancer cells and Cyclin D1 and by a reduction in the level of synthesis phase (S) the *Ginnalin A* cell cycle G2/M phases are said to ending phase [16]. In case of activation of mitogenic signaling pathways MAPKs reaches the nucleus and they induce binding DNA of the transcription factors. All of these proteins in eukaryotic cells, the cell membrane has a very important role in conveying information to the nucleus. MAPKs; p38-MAP kinase, extracellular signal regulated kinase (ERK), and c-Jun NH2-terminal kinase (JNK) is divided into three groups; This signal transduction pathways of life play a role in the regulation of proliferation and apoptosis process shown as in Figure 2. P38 has a significant biological effect as the stimulating agent of cellular pathway on cell cultures [17]. P38 creating a response to environmental stress also activates with mitogenic warning, and the cell cycle progression, differentiation, apoptosis, is effective in cases such as inflammatory response. Apoptosis death or survival is an important part of the mechanism which regulates the process of cell and is controlled by various signaling pathways [18]. Programmed cell death is apoptosis but also of tissue homeostasis is a central regulator. Apoptosis is needed to maintain the healthy conditions and eliminate damaged or infected cells in multicellular organisms. The failure of apoptotic pathways contribute to cancer formation by a suitable environment for the accumulation of gene mutations and genetic instability [19]. Therefore the induction of apoptosis pathways in cancer cells have been the main focus of the therapeutic strategy [20].

Ginnalin A is another agent which enhances the effect of these inhibitors [21,22]. Cancer is difficult to treat. Thus other methods of treatment as well as for medical therapy have been developed. In this case it is extremely

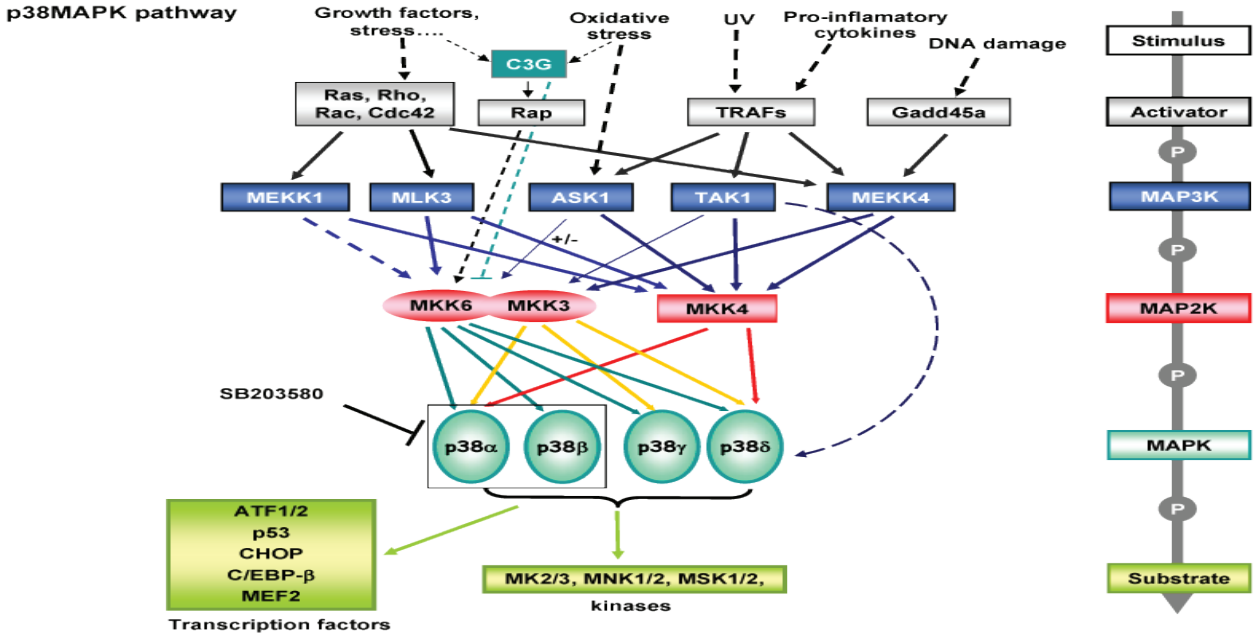

Figure 2: p38MAPK pathway [23].

important the controlled release of active substance into the cell and usage, dosage and duration of treatment in anticancer treatment using plant derived drugs. Thus it controls the microenvironment of the cancer cell are provided. Therefore, particularly in the early stages of metastasis formation prevention for the disease can be treated. For this purpose chemotherapeutic drugs/agents can be administered at the medical sense, the preparation of effective dose determination and combined preparations is particularly important. In this disease an urgent solution has been to improve the quality of life. In this respect an approach called homeopathy of the alternative methods has been negligible. Homeopathy is a holistic system of medical treatment which has been used by many practitioners to treat cancer patients. Homeopathic remedies are primarily sourced from plants, herbs, minerals, or animal products. Although there are still serious short-comings in clinical trials on cancer growth and treatment modalities, many researchers have studied the effects of homeopathy on the growth of cancer that uncontrolled cell proliferation in 2006, 2009 and 2016.

In one report, researchers found that homeopathic remedies inhibit the growth of prostate and liver cancer cells [21,22]. Polyphenols are bioactive compounds found in plant foods. Here I evaluated the anti-proliferative effects of *Ginnalin A* on liver (Hep3B and Hep3G) and prostate cancer cell lines (PC3). *Ginnalin A* were partially effective against the cancer cells than normal/healthy control cells. Among the polyphenols, *Ginnalin A* (70%, Hep3B and Hep3G; 80%, PC3) was effective at 132 µM concentrations. These results suggest that maple polyphenols may have potential cancer chemopreventive effects mediated through cell cycle arrest. *Ginnalin A* was administered as triple repeats to evaluate the cytotoxic effects on different cancer cells. 50, 75, 100, 150, 200, 250, 300, 350, 400, 500 µM of *Ginnalin A* XTT assay was performed following the dosing. The absorbance value of cells treated with the control group by comparing viability/cytotoxicity percentage was calculated. Through these tests the IC_{50} values of *Ginnalin A* at the end of 72 hours in cancer cells was found to be 132 µM. Briefly, as shown in Figure 3, 4a and 4b, *GA* inhibited the cell viability of prostate cancer cells in a time- and dose-dependent manner. However, after the application of GA, caspase-3 activity observed in the increase in rate is not statistically significant 1.23 ($p>0,05$). The changes in expression of genes that play an important role in apoptosis and metastasis relative to the control group after treatment with 1000 µM GA in human Hep3B, Hep3G and PC3 cancer cells were evaluated with qPCR analysis. These genes that analyzed for apoptosis were *CASP3*, *CASP8*, *CASP9*, and *CYCS* and *P53*. Whereas, *CDH1*, *CDH2*, *TIMP-1* and *TIMP-2* genes were analyzed for metastasis as shown in Figure 5.

New horizons in pathways associated with cancer treatment clinical trials with signal transduction

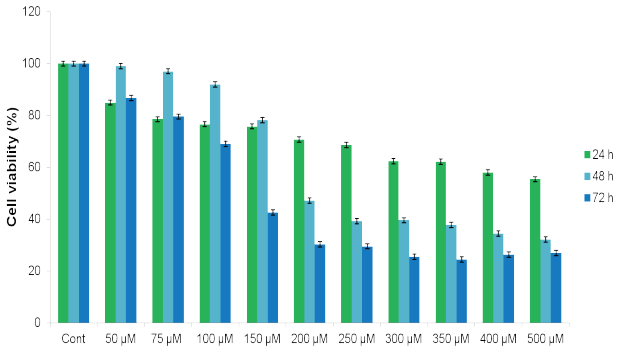

Figure 3: Cytotoxicity effect of *GA* on the viability of cancer cells. (Cont: control; untreated cells)

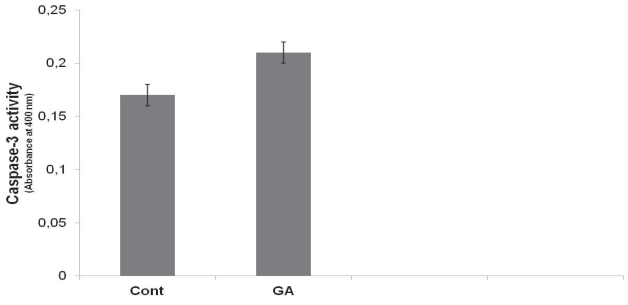

Figure 4a: The cells were untreated (control) or treated with GA. Data were presented as the mean ± standard deviation of three replicate experiments (Cont: control; untreated cells or Cont: control; dose 0). After the application of GA, caspase-3 activity observed in the increase in rate is not statistically significant 1.23 (p>0,05).

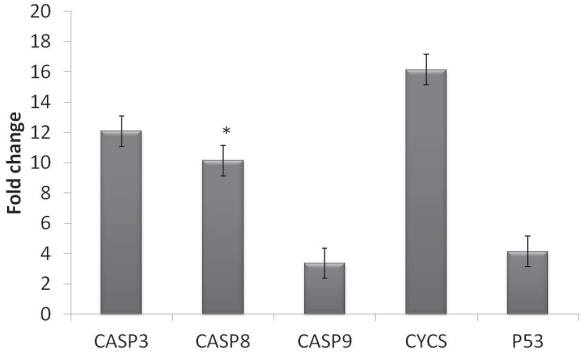

Figure 4b: Apoptotic effect of *GA* on cancer cells. In this study was defined the activation of caspase-8 in apoptotic signaling pathways and the pro-apoptotic caspases required for extrinsic apoptotic signal transduction.

molecules are expected in the future, for example specific inhibitory substances for the interaction of the signal transduction pathways and specific genes have also been used in carcinogenesis. The common aim of this approach

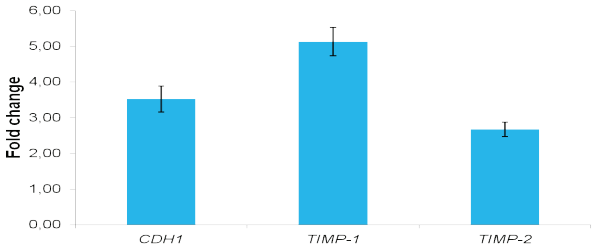

Figure 5: Effect of *GA* on expression of important genes in metastasis

is to achieve the most effective cancer treatment. In conclusion, the homeopathic agent appeared to be effective in reducing the severity of metastasis after you begin treatment with an agent. It is thought that GA partially demonstrates anti-metastatic effect by regulating expression of important genes in metastasis on cancer cell lines. The homeopathic agent appeared to be effective in reducing the spread of cancerous cells after treatment begins. When compared with the control group, qPCR results illustrated that a significant increase was observed in the expressions of *CDH1*, *TIMP-1* and *TIMP-2* genes as 3.52, 5.13 and 2.67 folds respectively. However, further molecular and functional analyses are required to clarify its effect on carcinogenesis. *Ginnalin A* alone exhibits fewer adverse effects than traditional chemotherapy cell proliferation and invasion.

4 Conclusions

The homeopathic agent appeared to be effective in reducing the spread of cancerous cells. It is thought that GA partially demonstrates anti-metastatic effect by regulating expression of important genes in metastasis on cancer cell lines. *Ginnalin A*, in the study of cancer cell culture lines, has not been effective in blocking cancer cell formation on the p38-MAPK signaling pathway. *Ginnalin A* and endogenous inhibitor agents would likely have fewer adverse effects than traditional chemotherapy cell proliferation and invasion. Further work and development is requried in the future but the concept is described in the literature and supported by the original experimental study. The current hypothesis to conduct more comprehensive research is planned on the effectiveness of complementary medicine in the treatment of cancer cases related to creating my work team in the coming days.

Highlights

GA showed cytotoxic effect in Hep3G, Hep3B and prostate cancer cells.

GA induced apoptosis and caspase-3 activity in human different cancer cells.

GA partially inhibited the cell invasion and the cell growth in human different cancer cells.

GA has the high potentials of natural products for human health.

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Conflict of interest: Authors state no conflict of interest.

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