The First Molecular Insights to the Effect of Ashwagandha and Propolis on Cancer cells: Comparative Study

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***Abstract*—**Ashwagandah a naturally occurring plant are a commonly used herb in Ayurveda medicine that exhibit a broad spectrum of biological activities as they have an antioxidant, anti-inflammatory, immunomodulatory and anticancer effects. Propolis is one of the most important of naturally bioactive products having anti-bacterial, anti-viral, anti-fungal and anti-parasite activates . In this work, we try to compare their cytotoxic effect as well as the molecular mechanisms of the two natural compounds. The antitumor activity was evaluated against two cell lines, (MCF7 and HepG2). Ashwagandah and Porpolis caused a decrease in the viability of McF7 and HepG2 cells in a dose dependent manner. The result showed that porpolis is the most cytotoxic effect with the IC_{50} (0.70, 0.69) at low dose compared to Ashwagandah (0.90, 0.86) in HepG2 and MCF7 respectively. Cell cycle analysis result showed that Ashwagandah treated cells were high significant percent in G2\M phase arrest and low in S-phase on both cell lines, with a more percent in HepG2 than MCF7 cells. On the other hand, MCF7 cells treated by propolis showed low percent in $Go\backslash G_1$ and $G_2\backslash M$ phase arrest and high S-phase percent. While, HepG2 cells showed increase in $G_2\backslash M$ and S-phase and low percent in $Go\backslash G_1$ phase. The result showed high DNA degradation in both cell lines treated with ashwagandah and porpolis. Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. This is confirmed by the result of morphological observation which detected by inverted microscope.

***Keywords*—**cytotoxcity , propolis, ashwagandah, MCF7, HepG2.

Ashwagandha roots

Ashwagandha leave

Propolis granules

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I. Introduction

CHOICE of cancer treatment is influenced by several factors, including the specific characteristics of the tumor; patient`s overall condition; and whether the goal of treatment is to cure cancer, keep it from spreading, or to relieve the symptoms caused by cancer. Depending on these factors, patient can receive one or more of the following clinical traditional therapies such as surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy and or biological therapy, but these types of treatments are hampered limited success on treating cancer and have many side effects for patients.[1-5]

Surgery is often the primary treatment modality for cancer. Most people with cancer will have some type of surgery; it is the oldest form of effective cancer therapy. The size, type, and location of the primary tumor may determine operability and outcome. Surgery providing local treatment of the cancer, information gained during surgery is useful in predicting the likelihood of cancer recurrence and whether other treatment modalities will be necessary [3]

Radiation therapy kills some healthy cells that are in the path of the radiation or near the cancer being treated. The effect of radiation therapy on healthy tissue in the treatment field can produce changes in normal physiologic function that may ultimately diminish a patient's nutritional status by interfering with ingestion, digestion, or absorption of nutrients [1], these in addition to the other side effects [4].

Unlike surgery and radiation therapy, cancer chemotherapy is a systemic treatment (not a localized treatment) that affects the whole body [5]. Chemotherapy damages rapidly dividing cells, a hallmark trait of cancer cells, in the process, healthy cells that are also rapidly dividing, such as blood cells and the cells lining the mouth and GI tract are also damaged. All of these leads to many side effects such as fatigue, phlebitis, alopecia, nausea, vomiting, mucositis, anemia, and myelosuppression or neutropenia. associated with an increased risk of infection [6].

As chemotherapy and radiation therapy cannot distinguish between cancer cells and healthy cells; Consequently, healthy cells are commonly damaged in the process of treating the cancer, which results in many serious side effects; indicate that there is an imperative need of new type of cancer treatment such as alternative treatment [5,7,8]

Natural products have been used as traditional medicines in many parts of the world like Egypt, China, Greece, and India from ancient times. These products have been used as prophylactic agents in numerous of conditions or as adjuvant therapy for many diseases [9-16]. Many natural occurring compound exerts anti-carcinogenic effects on different types of cancer, where they have different mechanisms of action including cell growth suppression, modulation of cell differentiation and induction of apoptosis [17-21].

Ashwagndha (Withania somnifera), is a natural plant which used in wide range for stress, strain, fatigue, pain, skin diseases , rheumatoid arthritis, epilepsy and as anticancer agen [22-29]. Recent researches revealed that Ashwagndha water extract (ASHWEX) found to have selective cancer cell growth arrest, it`s considered as good example for natural and economic recourse for anti-cancer medicine [28,30,31].

Prospolis is one of the most important naturally bioactive products produced by bees (Apis mellifera), it have many biological properties including immune-modulatory, anti-inflammatory, anti-oxidant, anti-bacterial, anti-viral , anti-fungal and anti-parasite activates [32-36]. Recent ,research shown the cytotoxic action of propolis and its isolated compounds on various tumor cells[37-44].

Aim of the work: The purpose of this study is to highlight and comparing the potential cytotoxisity and molecular mechanism of the antitumor activity of Ashwagndha and propolis.

II. Materials And Methods

1. Plants Preparation

A- Egyptian Ashwagndha, roots were harvested from Rafah, El-Arish, North Sinai, Egypt in September 2008. Dry powder of Ashwagndha roots was prepared by suspending 10g of dry powder in 100 ml of distilled water and stirring it overnight at 45±5 ºC, followed by filtration under sterile conditions. The filtrate thus obtained was treated as 100% W.S. It was stored at –20 ºC in 1 ml aliquots until further use.

B- A water-soluble derivative of propolis (WSDP) was prepared by the method described elsewhere [45]. Briefly, propolis was extracted with 96% ethanol, which was filtered and evaporated to dryness in vacuum evaporator. The resultant resinous product was added to a stirred solution of 8% L-lysine (Sigma Chemie, Deisenhofen, Germany) and freeze-dried to yield WSDP, a yellow-brown powder. WSDP was stored under sterile conditions at 4°C. Before use WSDP was dissolved in distilled water.

Cell lines:

A human hepatocellular carcinoma cell line (HepG2) and Human breast cancer cell line (MCF7) preserved and passage in NCI, Cairo, Egypt laboratory. Cells were cultivated in RPMI-1640 culture medium containing 10% fetal bovine serum, and penicillin / streptomycin at 37 ºC in a 5% CO_2 incubator.

Cell viability test by Trypane blue. Cells were cultured in 24-well plates and incubated for 24hrs. Cells treated with gradual concentrations ranges ***(0.6 % to 1%)*** of each tested compound then cells incubated for 24hr and the viability was examined using trypane blue dye.

Microscopic Examination:

MCF7 and HepG2 cells were examined after treatment by ashwagndha, and propolis extracts and morphological changes were observed by inverted microscope. Cells were photographed using digital camera.

MTT assay:

MCF 7 and HepG2 cells were treated by ashwagandah and porpolis using the colorimetric methyl tetrazolium test (MTT) as described and modified by Tim Mosmann[46]. Percentage of relative viability and the half maximal inhibitory concentration IC50 was calculated by the prism program

Cell cycle analysis:

The cells were treated by IC_{50} dose of each tested material, at different intervals (6 and 12 h), then subjected to flow cytometry analysis after staining their DNA using Coulter Epics XLTM Flow Cytometer (Beckman).

DNA Fragmentation assay:

DNA was extracted from different treated cells and loaded in agarose gel then allowed to run.

Approximately 20 mg DNA was loaded in each well, visualized under UV light, and photographed.

III. Results

Morphologic appearance:

The result revealed that cells treatment with 1% conc. Ashwagndha root extract for 24 hrs markedly affected both type of cell lines (HepG2 and MCF7), (Fig 1A) (they showed condensed, shrank and aggregated shapes). However, at lower concentration cells appeared to be growth arrested without change in its morphology compared to control untreated cells. Meanwhile the HepG2 & MCF7 cells treated with propolis, at concentration of 0.8% showed apoptotic fragments (Fig. 1B).

MTT Assay result showed that treatment both type of cells (HepG2 and MCF7) with ashwagndha root extract inhibited tumor cell growth in a dose dependent manner, with IC_{50} value of 0.90 %, and 0.86%, respectively as shown in (Fig.2A&B). Also propolis water extracts inhibited the growth of HepG2 and MCF7 cells in a dose dependent manner, with IC_{50} values of 0.70% and 0.69% respectively. Cytotoxicity was measured and expressed as the survival fraction compared with untreated cells (table 1).

Cell cycle analysis:

The distribution of cells in different phases of the cell cycle is illustrated in Figure (3). The untreated cells showed the expected pattern for continuously growing cells, whereas

cells treated with tested materials showed a significant shift in cell cycle phases as follow;

HepG2 and MCF7 cells treated with ashwagndha extract showed a progressive accumulation in the G_2/M phase correlating with decrease in number of cells in the S-phase; recording 13.74 and 36.7 in MCF7 cells and 15.40 and 23.39 in HepG2 Cells, respectively. Compared to 9.37 and 44.26 in control untreated cells. MCF7cells treated with propolis showed a significant shift of the cell cycle phases with decrease in number in both G_0\ G_1 and G_2\ M phases recording 44.14 and 6.23 respectively compared to 80.46 and 10.82 for control. meanwhile HepG2 propolis treated cells showed a significant decrease of cell percent in G_o\G_1 phase recording 29.40 compared to 64.27 in control and increase cell percent in both G_2\M and S-phase recording 11.14 and 59.45 respectively this in comparable to 5.79 and 29.93 in control cells.

4- Cell death assessment by DNA fragmentation:

Treatment of HepG2 and MCF7cells with extract of different studied compound using concentration (0.5, 1.0, 5.0 and 10%) for **48**hrs induced significant DNA ladder formation, suggesting apoptotic cell death, as Internucleosomal DNA fragments were observed in treated cells (Photos 4).A& B.

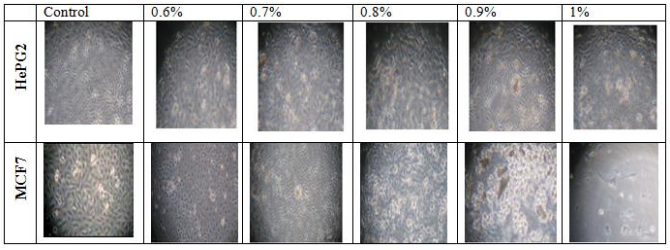

Fig.1 (A): Microscopic Picture For Ashwagndha- Treated Cells After 24 Hours

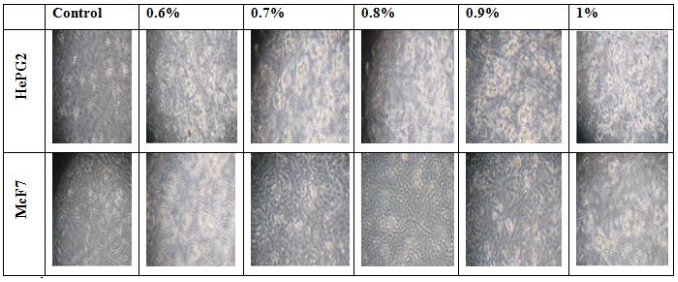

Fig.1 (B): Microscopic Picture Of Porpolis- Treated Cells After 24 Hours

TABLE I
IC50 FOR HEPG2& MCF7 CELLS TREATED WITH ASHWAGNDHA AND PROPOLIS

	IC50	
	HepG2	McF7
Ashwagndha	0.90%	0.86%
Propolis	0.70%	0.69%

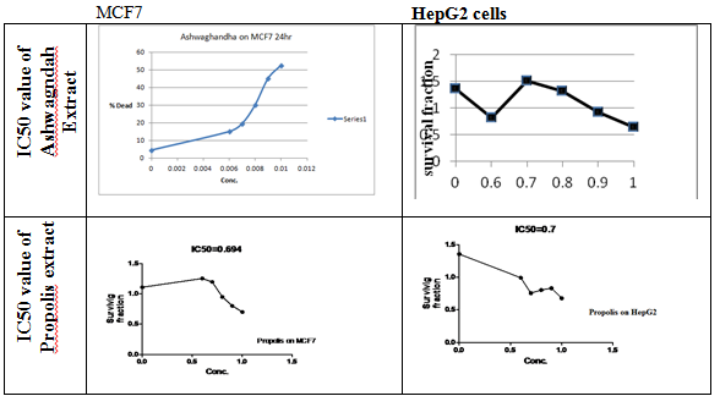

Fig. 2 Growth response curve for both type of tumor cell line treated with Ashwagndha and Propolis

TABLES II
FOR CELL CYCLE ANALYSIS USING IC50

Cell cycle phases for MCF7 treated with Ashwagndah

Conc.	%G_0-G1	%G2-M	%S-Phase	%G2/G1
0	46.37	9.37	44.26	1.94
0.9	49.53	13.74	36.74	1.95

Cell cycle phases for MCF7 treated with propolis

Conc.	%G_0-G	%G2-M	%S-Phas	%G2/G1
0	80.46	10.82	8.72	1.90
0.7	44.14	6.23	49.62	1.94

Cell cycle phases for HepG2treated with Ashwagndha

Conc.	%G0-G1	%G2-M	%S Phase	%G2/G1
0	64.2	5.79	29.93	1.95
0.9	61.22	15.40	23.39	1.92

Cell cycle phases for HepG2 treated with propolis

Conc.	%G0-G1	%G2-M	%S-Phase	%G2/G1
0	64.27	5.79	29.93	1.95
0.7	35.74	5.28	58.98	2

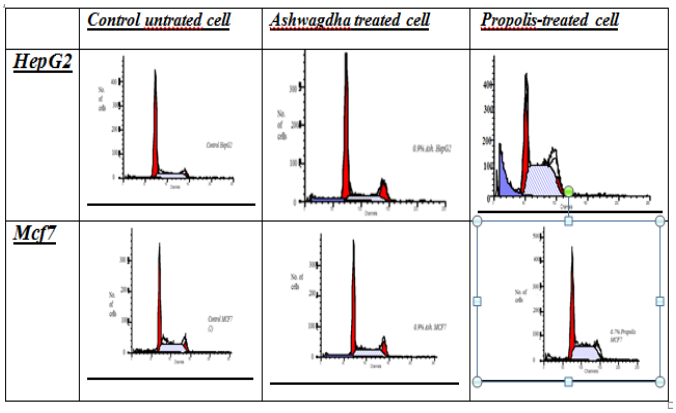

Fig.3: Cell Cycle Analysis For Propolis And Ashwagndha On Booth Cell Line

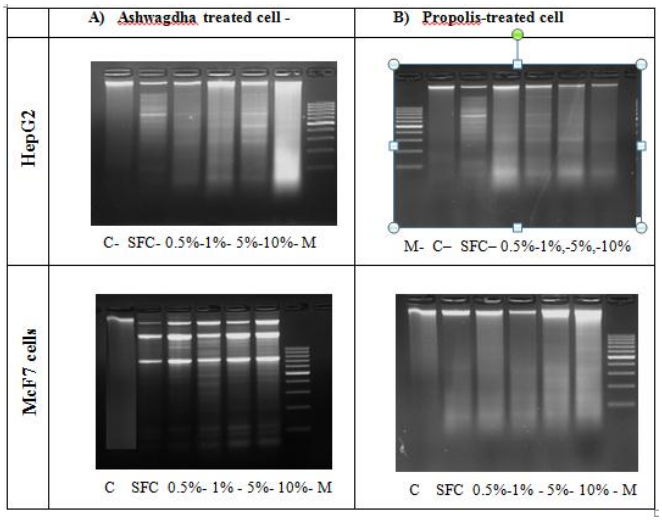

Fig.4: (*Effect Of Ashwaganda Root Extract On DNA Of MCF7 Cells* Colomn A.*& Effect Of Propolis On DNA Of MCF7 Cells Colomn B*
C:Control, SFC: Serum Free Control, 0.5, 1, 5, 10 % DNA Ladder, & M: DNA Marker

IV Discussion

Clinical traditional therapies such as surgery, chemotherapy, radiation therapy have several side effects and that requires search for new safer cancer treatment [3,4,5]. Herbal medicine is one of the most effective treatments with low side effects [28,42] as it has anti-tumor effect, the current research showed and evaluated this effect. Ashwagndha is a well-known herbal medicine which used in many parts of the world, where it has anti-inflammatory, anti-oxidant and anti-tumor as well as neural protective properties. Ashwagndha antitumor mechanisms are multifactorial , it exhibits both anti-oxidant and pro-oxidant activities, also it inhibit nuclear factor kappa-B (NF-κB) a target genes involved in inflammation, angiogenesis, cell cycle, metastasis, apoptosis and multidrug resistance. Mathur and Singh showed that root extract of Ashwagndha down regulate the expression of p53 cdc2 which is cell cycle regulatory protein[47,48]. Propolis is a honeybee product with many biological properties, where it has anti-oxidant, anti-inflammatory, anti-parasite and anti-cancer properties [36,49], Propolis has been justly called Nature's premier preventive. The immune system is supported and strengthened by the ingestion of propolis. Modern scientific studies indicate that those who take propolis regularly escape winter colds and sore throats and seem to develop a natural immunity to common viruses, including the various strains of flu. There are many published studies suggesting that Ashwagndha and propolis exert a diverse of bioactivity effects, including anti proliferation of cancer cell lines [28, 31, 43, 44, 48]. However, so far very few studies have addressed comparison between mechanism of action in different medicinal herbal products. The current research evaluated the two products and compare between their effect on HepG2, and McF7 cell lines.

Our results showed that ashwagndha has strong cytotoxic effect on MCF7 cells with IC_{50} about 0.8%, while perpolis IC_{50} was 0.7% . The cell cycle analysis of MCF7, our study revealed that W.S produce a progressive accumulation in G2/M phase of the cell cycle and decreased in number of cells in S phase, recording 13.74 and 36.7 while propolis the results revealed that, there was clear decrease of cell number on G_0/G_1 and G_2/M phase recording 44.14 and 6.23 respectively compared to 80.46 and 10.82 for control non-treated cells while the percent of cells significantly increased S-phase. This result was agreed with Pretorius[50] who reported that viability was decreased when using high concentration of W.S .also, agreed with previous study thatpostulated that propolis is able to induce anti-tumor activity and death in cancer cells [51].

The results summarized in (table 3) revealed that Ashwagndha , inhibite the growth of HepG2 cells compared to control , also, propolis has cytotoxic effect on HepG2 cells and cause inhibition of cell growth . The cytotoxic effect of Ashwagndha on HepG2 cell lines with graded concentrations (0.6-1%), showed significant inhibition with IC50 0.9 % while, propolis results showed significant inhibition with IC_{50} 0.69. The cell cycle analysis revealed that Ashwagndha extract showed a progressive accumulation in the G_1/M phase correlating with decreased number of cells in the S phase when compared in control untreated cells, while propolis result revealed significant decreases in number of cell entered in G0/G1 phase. As it evidenced by the results the Ashwagndha exert their effect through cells cycle arrest and of apoptotic pathways. While propolis act as apoptotic inducer rather than cell cycle arrest.

Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. It occurs in response to various apoptotic stimuli in a wide variety of cell types. DNA fragmentation is a secondary consequence, rather than an integral cause, of apoptosis. The result revealed that high concentration of WS extract produced DNA fragmentation in treated cells when compared with untreated control cells on both cell lines. Also, porpolis, exert, a high significant effect on DNA degradation as showed by results on HepG2 and McF7cells., This is confirmed the results of morphological observation which detected by inverted microscope and assessed by DNA fragmentation test as a leader DNA formation. This result with agreement with [44, 52, 53] who reported that propolis exerted his antitumor activity on HepG2 cells via decreased cell proliferation and induction of HepG2 cellular apoptosis as well as cell cycle arrest.

In conclusion: our results revealed that both Ashwagndha and propolis cause a decrease in cell viability on a dose dependent manner, prevent cell proliferation and cell cycle progression, induction of apoptosis through DNA fragmentation effect. These results, suggest that both of Ashwagndha and propolis can be a promising anti-cancer therapeutic agents.

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