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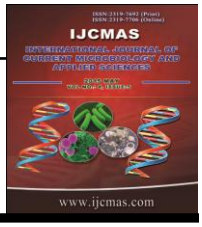

Original Research Article

Evaluation of the anticancer activities of pomegranate (*Punica granatum*) and harmal (*Rhazya stricta*) plants grown in Saudi arabia

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Rhazya stricta, *Punica granatum*, anticancer activates, Saudi Arabia

A B S T R A C T

Natural and some synthetic compounds can prevent, suppress, or reverse the progression of cancer. Natural products have proven to be the most effective in terms of their ability to alter the function of proteins relevant to cancer. Saudi Arabia has 2028 plant species out of which 300 have different medical applications. In the present study, the anticancer potentiality of pomegranate (*Punica granatum*) and harmal (*Rhazya stricta)* plants grown in Saudi Arabia were assessed. Different extracts of the two plants were utilized against Colon cancer (CACO) and Hepato-cellular carcinoma (HepGII) cell lines. All studied extracts showed a significant reduction in cell proliferation with dose dependant response. Moreover, all of the studied extracts showed different anti-proliferative profiles regarding extract type and concentrations. However, the Rhza H extract showed the highest cytotoxic effect among allextracts with HepG2 and Caco cells (IC50 25 µg/mℓ and 35 µg/mℓ), respectively.

Introduction

Natural products have proven to be the most effective in terms of their ability to alter the function of proteins relevant to cancer (Muhtasib, 2006). Plants have been an important source of medicine for thousands of years. Even today, the World Health Organization estimates that up to 80 % of people still rely mainly on traditional remedies such as herbs for their medicines (Tripathi and Tripathi, 2003; Demiray *et al*., 2009). Medicinal plants constitute one of the main sources of new pharmaceuticals and healthcare products.

Medicinal plants have been curing various disorders in human being from the time immemorial. Among the human diseases treated with medicinal plants is cancer (Koduru *et al*., 2007). Medicinal plants play important role in the development of potent therapeutic agents which developed to new drugs. Plant derived drugs came into use in the modern medicine through the uses of plant material as indigenous cure in folklore or traditional systems of medicine. From 1971 to 1990 new drugs such as ectoposide, E-guggulsterone, teniposide, nabilone,

plaunotol, Z-guggulsterone, lectinan, artemisinin and ginkgolides appeared all over the world (Samy and Gopalakrishnakone, 2007).

Saudi Arabia has 2028 plant species out of which 300 have different medical applications (Amin and Mousa, 2007). There have been many efforts to detect the anticancer potentialities of some Saudi indigenous plants species that are popular in Saudi traditional medicine. Yassen *et al*., (2012) published detailed pharmacological screening of 11 medicinal plants from Tabuk region at KSA. Cytotoxic effect of three plants was detected. Almehdar *et al* (2012) utilized different human cancerous cell lines to evaluate the anticancer activity of forty species of plants traditionally used in Saudi Arabia for the treatment of a variety of diseases. Interesting cytotoxic activity was observed for Hypoestes forskaolii, Withania somnifera, Solanum glabratum, Adenium obesum, Pistacia vera oleoresin, Caralluma quadrangula, Eulophia petersii, Phragmanthera austroarabica, and Asparagus officinalis. The anticancer prosperities of Achillea fragrantissima (Af) was reported by (Alenad *et al*., 2012) using human chronic myeloid leukemia (CML) (K562), T cell lymphoma (Jurkat) and hepatocellular carcinoma (HepG2) cell lines.

Harmal (*Rhazya stricta* Decne) and Pomegranate (*Punica granatum*) are two important plants that are used intensively in folkloric medicine to cure various diseases is Saudi Arabia. *R. stricta* is an important medicinal plant widely distributed in Saudi Arabia, South Asia and the Middle East. It is a member of the Apocynaceae family (Gilani *et al*., 2007; Baeshen *et al*., 2012). It possesses anti-oxidant, anti-carcinogenic and free radical scavenging properties (ElKady 2013). Four indole alkaloids were isolated from Rhazya stricta leaves and roots by Mukhopadhyay *et al* (1981). Three of these isolated are vallesiachotamine, sewarine and tetrahydrosecamine displayed cytotoxic activity. Pomegranate (*Punica granatum*) is one of the Kingdom's most in-demand fruits. Al-Baha and Taif are both well-known for farming this fruit, but Taif's pomegranate supply remains the most popular.

The potential anticancer activity of *Rhazya stricta* against human breast cancer cells in vitro was confirmed by Baeshan and his coworkers (2012). They found that, the ethanol extract of *Rhazya stricta* potently inhibited cellular growth and colony formation of human breast cancer cell lines, MCF-7 and MDA-MB-231, in a dose- and time-dependent manner. Furthermore, it induced sequences of events marked by apoptosis, accompanied by a loss of cell viability, chromatin condensation, DNA fragmentation and proteolytic cleavage of poly (ADP-ribose) polymerase. In addition, the anticancer potentiality of *Rhazya stricta* aginest non-small lung cancer cell line A549 was explored by ElKady (2013) at different cellular and molecular levels. The suppuration of cell growth was observed which was correlated with apoptosis. However, no reports deals with the anticancer potentiality of *Rhazya stricta* against lung or colon cancer have been published. From the other hands, there are several lines of evidences for the anticancer activity of pomegranate. Several studies have been conducted to evaluate the anticancer activity of pomegranate against different types of cancer such as colon and breast cancer (Abdel Motaal and Shaker, 2011); breast cancer (Banerjee and Talcott, 2012); prostate cancer (Lansky et al., 2005); lung , breast and cervical cancer (Aqil *et al*., 2012). So far, no studies have been conducted to evaluate the anticancer properties of Saudi pomegranate cultivars.

Accordingly, the aim of the present work is to investigate, evaluate and compare the anticancer activity of Saudi *Rhazya stricta* and pomegranate cultivars against colon and hepatocellular carcinoma cell lines.

Materials and methods

Plant materials and preparation of plant extracts

Rhazya stricta plants were collected from naturally growing plants located along the roadsides of Jeddah-Makkah highway. The species was identified and authenticated to the genus and specie level by specialized botany taxonomist. The collected leaves were air-dried at room temperature for 2 weeks, then ground and stored at -20°C. To prepare the aqueous and ethanolic extract the ground herb was soaked in water (aqueous extract) or 70% ethanol (ethanol extract) for 24 h, then, the mixtures were filtered and passed sequentially through a 0.22 µm filter sterilization and kept in aliquots at 4°C according to Baeshen *et al*., (2012).

Fresh pomegranate fruits were collected from Al-Shafa farms at Taif governorate. Seeds and husks were peeled manually. The seeds were blended with 70% ethanol. The husks were left to dry at room temperature for 2 weeks and then blended with 70% ethanol. The mixtures were filtered and passed sequentially through a 0.22 µm filter sterilization and kept in aliquots at 4°C according to (Abdel Motaal and Shaker 2011).

Cell cultures

The Colon cancer (CACO) and Hepato-cellular carcinoma (HepGII) cell lines were kindly provided by the Holding Company for Biological Products & Vaccines, Egypt (VACSERA). Cells were cultured in RPMI 1640 medium (Gibico, USA) supplemented with 10% fetal bovine serum (Sijixin Inc., China) and 1% penicillin–streptomycin (Invitrogen, USA) at 37 ºC in a humidified atmosphere containing 5% CO2.

Determination of extract cytotoxicity

The methods reported by Van den Berghe *et al*., (1978) was applied. Growth medium was decanted from 96 micro titer plate after confluent sheet of Vero cell was formed. Each studied extract sample was applied in a series of 10 dilution (final concentration ranging from 10-100 ug/ml. in MEM medium without FCS. 0.2 ml of each dilution was tested in three different wells leaving two wells/ row as control, receiving only maintenance medium. Plate was incubated in incubator at 37oc and examined frequently for up to 3 days. Cells were checked for any physical signs of toxicity (e.g). Partial or complete loss of monolayer, rounding, shrinkage, or cell granulation). The maximum non-toxic concentration [MNTC] of each extract was determined and was used for further biological studies.

Trypan blue staining

After incubation 0.1 ml trypan blue was added and number of dead cells determined by using haemocytometer. The percent viability was calculated by using the following formula: *% viability = (live cell count/total cell count)*100*

Cell viability assay

Cell viability was assessed using the MTT assay (Chemicon, Temecula, CA) as a colorimetric method. The procedures were as follows: the medium was removed and replaced with 100 µl of fresh culture medium (RPMI 1620) containing indicated

concentrations of the tested extracts for the indicated time intervals table (1). Cells were treated with 20 µl of 5 mg/ml MTT. Four wells were used to represent each cell line. Each well of the 96-well microplate contained 5x103cells. A negative control was included by adding 20 µl of the MTT stock solution to 100 µl of cells-free RPMI 1620 medium. The microplate was incubated at 37°C for indicated time intervals in a humidified chamber.

The formed formazan crystals were solubilized with 150 µl/well dimethylsulfoxide for 10 minutes and then the absorbance values of the solution in each well were measured at 570 nm at the indicated intervals using microplate reader (BioTek Instruments, Winooski, VT).The % cell inhibition was determined using the following formula. *% cell Inhibition = 100- Abs (sample)/Abs (control) x100.*

Result and Discussion

During the present work the antitumor activity of two plants *Rhazya stricta* and pomegranate were assessed against two types of cancer. Two extracts of *R. stricta* (Rhazya A and Rhazya H) and the extracts of pomegranate seeds and husks were tested. The Hepato-cellular carcinoma (HepG2) and the colon cancer (Caco cells) cell lines and were used for the evaluation.

Determination of the Inhibitory concentration required for 50% cytotoxicity (IC50) value

The Inhibitory concentration required for 50% cytotoxicity (IC50) value was analyzed for each extract and results are summarized in tables (1&2). The values of IC50 were 30, 25, 45, and 40 µg/ml for Rhazya A, Rhazya H, Pomegranate seeds and Pomegranate husks respectively.

Table.1 IC50 concentration of each crude extract for HepG2 cells

Extract name	IC50 concentration µg/ml
Rhazya (A)	30
Rhazya (H)	25
Pomegranate seeds	45
Pomegranate husks	40

Table.2 IC50 concentration of each crude extract for Caco cells

Extract name	IC50 concentration µg/ml
Rhazya (A)	40
Rhazya (H)	35
Pomegranate seeds	45
Pomegranate husks	40

Determination of the maximum non-toxic concentration [MNTC] of the plant extracts

The maximum non-toxic concentration [MNTC] of each extract was determined and illustrated in table 3. It was 10-2 for both Rhazya H and Pomegranate seeds. The maximum non-toxic concentration [MNTC] of Rhazya A was 10-4 and 10-3 for Pomegranate Husks.

Determination of Metabolic activity

Metabolic activity can be evaluated by measuring the activity of a mitochondrial enzyme succinate dehydrogenase using MTT test. MTT is designed to be used for the quantification of both cell proliferation and cell viability in cell population using 96-well plate format. This test is widely used in the in vitro evaluation of the biosafety of plant extracts.

Table.3 The maximum non-toxic concentration [MNTC] of each extract

Extract	Selected dilution	Extract	Selected dilution
Rhazya A	10-4	Pomegranate seeds	10-2
Rhazya H	10-2	Pomegranate Husks	10-3

Table.4 MTT assay result of different Rhazya extracts against Hepato-cellular carcinoma (HepG2) cells

Extract	**Concentration µg/ml**	**O.D**	**% of cell survival**	**% of Anti tumor**
Rhazya (A)	100	0.00	0.00	100
	90	0.00	0.00	100
	80	0.003	1.2	98.8
	70	0.010	3.9	96.1
	60	0.048	18	82
	50	0.101	39	61
	40	0.125	49	51
	30	0.134	52	48
	20	0.190	74	26
	10	0.245	95	5
	0(cell control)	0.257	100	0.00
Rhazya (H)	100	0.001	0.4	99.6
	90	0.002	0.7	99.3
	80	0.009	3.0	97
	70	0.018	6.0	94
	60	0.027	10	90
	50	0.036	13	87
	40	0.055	20	80
	30	0.098	35	65
	20	0.174	63	37
	10	0.221	80	20
	0(cell control)	0.277	100	0.00

Table.5 MTT assay result of different Pomegranate extracts against Hepato-cellular carcinoma (HepG2) cells

Extract	Concentration µg/ml	O.D	% of cell survival	% of Anti tumor
Pomegranate seeds	100	0.010	4.2	95.8
	90	0.011	4.6	95.4
	80	0.012	5	95
	70	0.014	6	94
	60	0.036	15	85
	50	0.096	41	59
	40	0.134	57	43
	30	0.210	98	2
	20	0.202	86	14
	10	0.225	96	4
	0 (cell control)	0.235	100	0
Pomegranate husks	100	0.005	1.7	98.3
	90	00.9	3.2	96.8
	80	0.016	5.6	94.4
	70	0.039	14	86
	60	0.062	22	78
	50	0.101	36	64
	40	0.152	54	46
	30	0.189	67	33
	20	0.213	76	24
	10	0.254	90	100
	0 (cell control)	0.281	100	0

In the present study, we applied the MTT test to evaluate the biosafety of cytotoxic effect of different *Rhza strecta* and pomegranate extracts HepG2 and Caco cell lines. Therefore, cancer and normal cells were exposed to increasing concentrations (10-100 µg /ml of culture medium) of the tested extracts. The MTT assays data are presented respectively in Tables (4-7).

All studied extracts produced a reduction in cell proliferation. The obtained results show dose dependant response. The extracts showed different antiproliferative profiles regarding extract type and concentrations. The Rhza H extract was the highest cytotoxic extracts with HepG2 and Caco cells (IC50 25 µg/mℓ and 35 µg/mℓ) respectively.

Table.6 MTT assay result of different Rhazya extracts against colon cancer (Caco cells)

Extract	Concentration µg/ml	O.D	% of cell survival	% of Anti tumor
Rhazya (A)	100	0.001	0.3	99.7
	90	0.002	0.7	99.3
	80	0.021	7.0	93
	70	0.061	20	80
	60	0.091	31	69
	50	0.118	39	61
	40	0.174	58	42
	30	0.194	65	35
	20	0.201	67	33
	10	0.291	98	2
	0 (cell control)	0.298	100	0
Rhazya (H)	100	0.00	0.00	100
	90	0.00	0.00	100
	80	0.016	5.7	94.3
	70	0.028	9.9	90.1
	60	0.073	26	74
	50	0.112	40	60
	40	0.124	44	56
	30	0.169	60	40
	20	0.271	96	4
	10	0.280	99	1
	0 (cell control)	0.281	100	0

Table.7 MTT assay result of different Pomegranate extracts against colon cancer (Caco cells)

Extract	Concentration µg/ml	O.D	% of cell survival	% of Anti tumor
Pomegranate seeds	100	0.003	1.0	99
	90	0.007	2.4	97.6
	80	0.013	4.4	95.6
	70	0.022	8	92
	60	0.083	29	71
	50	0.115	40	60
	40	0.193	66	34
	30	0.201	69	31
	20	0.249	86	14
	10	0.268	92	8
	0 (cell control)	0.291	100	0
Pomegranate husks	100	0.009	3	97
	90	0.010	3.5	96.5
	80	0.041	14	86
	70	0.069	24	76
	60	0.085	30	70
	50	0.127	45	55
	40	0.153	54	46
	30	0.197	70	30
	20	0.231	82	18
	10	0.271	96	4
	0 (cell control)	0.283	100	0

Nowadays it is well known that natural products not only offer protection against oxidative reaction but also suppress proliferation of cancer cells in culture as well as in vivo (Siriwardhana *et al*., 2003; Kuete *et al*., 2009; Choi *et al*., 2012). There are line of evidence indicating that Medicinal plants constitute a common alternative for cancer prevention and treatment in many countries around the world (Soobrattee *et al*., 2006; Mehta *et al*., 2010). The first antitumor drugs from plants with an application in cancer chemotherapy were developed five decades ago. A great achievement in this respect is the elaboration of drugs such as: vinblastine and vincristine (*Catharanthus roseus*), paclitaxel (*Taxus brevifolia*), silvestrol (*Aglaia foveolata*), eliptinium (*Bleekeria vitensis*) (Cragg and Newman, 2005), chrysin (*Passiflora incarnate*), artemisinin (*Artemisia annua*) (Newman and Cragg, 2007). Approximately, 60% of the anticancer drugs currently used have been isolated from natural products from the plants. At this time, more than 3000 plants worldwide have been reported to possess anticancer properties.

In the present study, the cytotoxicity of Rhazya stricta and pomegranate for the cancer cells were study and evaluated. All studied extracts produced a reduction in cell proliferation. The obtained results show dose dependant response. The extracts showed different antiproliferative profiles regarding extract type and concentrations. The Rhza H extract was the highest cytotoxic extracts with HepG2 and Caco

cells (IC50 25 µg/mℓ and 35 µg/mℓ) respectively. The obtained results were in agreement with different studies that were conducted to evaluate the cytptoxic effect of different pomegranate extracts against different cancerous cell lines (Abdel Motaal and Shaker 2011; Aqil *et al*., 2012; Yazici *et al*., 2012; Banerjee *et al*., 2012). On the other hand, *Rhazya stricta* showed antitumor effect among the two studied cancerous cell lines. These results in consistence with several studies were carried out to assess the anticancer effect of different Rhazya stricta extracts (Ali *et al*., 2000; Gilani *et al*., 2007; Baeshen *et al*., 2012; Elkady 2013).

Several reports described that the anticancer activity of the medicinal plants is may be due to the presence of antioxidants (viz., vitamins, carotene, enzymes, minerals, polysaccharides, polyphenols, flavonoids, lignins, xanthones, etc.). In addition, it was reported that these plants may promote host resistance against infection by re-stabilizing body equilibrium and conditioning the body tissues (G Pandey and S Madhuri). In fact, Harmal and pomegranate were shown to contain several of these antioxidants. Thus, the various combinations of the active components of these plants after isolation and identification can be made and have to be further assessed for their synergistic effects. Preparation of standardized dose and dosage regimen may play a critical role in the remedy of cancer. The rate with which cancer is progressing, it seems to have an urgent and effective effort for making good health of humans as well as animals. There is a broad scope to derive the potent anticancer agents from medicinal plants, which need thorough research. For the best of our knowledge, this is the first study that proves the anticancer activities of *Rhazya stricta* and pomegranate cultivated in Saudi Arabia against colon and hepatocellular carcinoma cell lines.

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