

Full Length Research Paper

# Anticancerous potentials of *Achillea* species against selected cell lines

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**Cancer, heart failure and stroke are among the majority frequent causes of death worldwide. Cancer is the second most common reason of death following the cardiovascular diseases. In current study, two Iranian species of *Achillea* species as *Achillea millefolium* and *Achillea biebersteinii* have been collected from East Azerbaijan of Iran and assayed on six cancerous cell lines; AGS human Caucasian gastric adenocarcinoma, MCF7 human breast ductal carcinoma, SW742 human colorectal adenocarcinoma, SKLC6 human lung carcinoma, A375 human melanoma cancer cell and PLC/PRF/5 human liver hepatoma. Complementary assays were also carried out to verify selectivity of herbal extracts. Reduced hemolytic potential of herbal extracts plus antifungal test findings in comparison with their cytotoxicity effects on several cancerous cells, may present selectivity effects of them in inducing of cytotoxicity on human cancerous cells.**

**Key words:** *Achillea millefolium*, *Achillea biebersteinii*, anticancer, cancerous cell lines.

## INTRODUCTION

Cancer forces a serious burden on the public health system and has created a challenge to the medical science researchers. Though, the century-long drift of cancer mortality in the world was reversed in the middle of 19th century and now cancer remains the second leading cause of death. In addition, cancer presents a rationally complex set of difficulties because of multiple sites and causation, inefficiently understood biology, and numerous intervention approaches. Cancer, heart failure and stroke are among the most common causes of death worldwide. Cancer is the second most general reason of death following the cardiovascular diseases. Annually, further 10 million new case of cancer are diagnosed based on the World Health Organization (WHO) report (Vicker et al., 2009). By 2020, the world population is expected to have risen to 7.5 billion; of this number, around 15 million novel cancer cases will be diagnosed, and 12 million cancer patients will die. The critical

undesirable data demonstrate that cancer is described as a serious challenge in human healthcare and survival. While we have witnessed the expansion of lots of drugs against cancer, the death rates for the most prevalent cancers have not been decreased (Zhang et al., 2007). Wide ranges of anticancer drugs (over of 60%) employed in cancer chemotherapy have been derived from natural source such as vincristine, irinotecan and etoposide with herbal source and doxorubicin, bleomycin and dactinomycin extracted from marine source (Ghose et al., 2006).

Investigations in the traditional ethnobotany and ethnomedicine of West Azerbaijan of Iran presented that *Achillea millefolium* has been used to treat haemorrhoids, cancer, vertigos, anemia, anorexia, dyspepsia, gastralgia, haemorrhage, dysmenorrhoea; very efficacious in female complaints (Miraldi et al., 2001). Flowers are used for treatment of gastritis, tannins occurring in this species render it is used, for treatment of haemorrhage, dysmenorrhoea and diarrhoea; it is also used to treat haemorrhoids and as a tonic and cicatrizant. Similar investigations in British Columbia, Canada, have explained mastitis, wounds and sternal abscess effects of

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**Table 1.** Collection and extract preparation of 2 candidate herbal extracts.

voucher specimen number	Scientific name	Used parts	Family
77-23	<i>Achillea biebersteinii</i>	Green leaves, stem and flower	Compositae
77-39	<i>Achillea millefolium</i>	Green leaves, stem and flower	Compositae

*A. millefolium* (Lans et al., 2007). Also, in Chinese and American Indian (Chumash) medicine its abdominal discomfort and antibacterial effects is reported (James et al., 2010). In herbal market of Thessaloniki (N. Greece) the haemorrhoids, hypertension, diabetes, colitis, constipation, gallstones, gastritis, intestinal disorders, liver disorders, influenza, arthritis, rheumatism, greasiness, diuretic, dysmenorrhoea, menstruation disorders, antipyretic, appetizer, blood purification and stimulant effects have been indicated as *A. millefolium* properties (Hanlidou et al., 2004). The Siberian medicine pointed to antihaemorrhage and antiphlogistic effects of *A. millefolium* (Kokoska et al., 2002).

Kotan and his research team detected 1,8-cineole, borneol, thymol, carvacrol, pathulenol, caryophyllene epoxide,  $\alpha$ -bisabolol oxide B,  $\beta$ -eudesmol-45.28, *epi*- $\alpha$ -bisabolol,  $\alpha$ -isabolol oxide A, isolongifolol oxide, (*Z,Z*)-farnesyl acetone, methyl palmitate, *n*-hexadecanoic acid, sphytol, methyl linoleate, *n*-eicosane, *n*-heneicosane, *n*-tricosane, *n*-etracosane and *n*-hexacosane have been detected in hexane extracts of the aerial parts of *A. millefolium* by GC (Gas chromatography), GC-MS (Gas chromatography-mass spectrometry) and IR (infrared) methods and reported their antibacterial effects against plant pathogenic bacteria (Kotan et al., 2010), anti-tumor activity of the derived flavonoid Casticin of *A. millefolium* by G2/M arresting (Haïdara et al., 2006). Also, the antihepatoma activity of *A. millefolium* on five human liver-cancer cell lines, that is, HepG2/C3A, SK-HEP-1, HA22T/VGH, Hep3B and PLC/PRF/5 have been investigated (Liang-Tzung et al., 2002) antioxidant, anti-inflammatory and antiproliferative properties of *A. millefolium* (Trouillas et al., 2003). In another research, it was found that *A. millefolium* was effective against mouse leukemia cells as anticancer agent (Goun et al., 2002).

*Achillea biebersteinii* distributed in North and West of Iran is a perennial herb, 30 to 60 cm high; villose, stems erect, simple or branched from the base; leaves up to 10 cm, oblong-lanceolate in outline, pinnatisect into numerous narrow segments, segments divided into minute linear-lanceolate mucronate lobes; the heads are radiate, in large dense compound corymbs; involucre 4 to 5 mm, oblong-ovoid (Karimi, 2002; Ghahreman, 1996; Goun et al., 2002). 1,8-cineole, *cis*-sabinenehydrate, linalool,  $\alpha$ -campholenal, camphor, borneol, *p*-cymen-8-ol,  $\alpha$ -terpineol, *cis*-carveol, piperitone, *b* ornyl acetate, thymol, carvacrol, spathulenol, caryophyllene epoxide, methyl linoleate, linoleic acid, *n*-eicosane, *n*-heneicosane and *n*-tricosane have been detected in hexane extracts of

the aerial parts of *A. biebersteinii* by GC (Gas chromatography), GC-MS (Gas chromatography-mass spectrometry) and IR (infrared) (Kotan et al., 2010). Sokmen et al. (1999; 2004) reported the *in vitro* antioxidant and antimicrobial activities of the essential oil and methanol extracts of *A. biebersteinii* Afan (Sökmen et al., 2004; Sokmen et al., 1999).

In current research, investigations were performed for studying the anticancer property of *A. biebersteinii* at the first time parallel to the anticancer activity of *A. millefolium* against six cancerous cell lines; AGS, MCF7, SW742, SKLC6, A375 and PLC/PRF/5. Complementary assays as hemolytic test and antifungal assay against *Candida albicans* (ATTC10231) were done to determine selectivity of herbal extracts.

## MATERIALS AND METHODS

### Collection of plant material

Source of information for plant material and its authentication is "A dictionary of Iran's vegetation plants" and "Flora of Iran", then the herbs were collected during trips from North-West of Iran, the voucher specimens for collected herbs were prepared and deposited to our herbarium in drug design lab (Table 1).

### Preparation of herbal extracts and drugs

Plant materials were dried at room temperature for 4 to 7 days. Dried herbal powder was extracted by ethanol (Merck, Germany) (80%) according to percolation method; the samples were steeped in solvent in the percolator for 24 h before each extraction and the process was repeated three times (Yrjönen, 2004; Sarker, 2006). The extracts were then collected and pooled. After evaporating, 10 mg of each herb was dissolved at 1 ml DMSO (Fluka, USA) and ethanol (50%) as two stocks, then extract dilution was done with RPMI (Gibco, England) 10% FBS (Gibco, England). Doxorubicin (50 mg/25 ml) was obtained from EBEWE Pharma Ges.m.b. Nfg. KG. A-4866 Unterach, AUSTRIA and was diluted with RPMI (10% FBS).

### Cell lines

AGS (human Caucasian gastric adenocarcinoma) NCBI C131, MCF7 (human breast ductal carcinoma) NCBI C135, SW742 (human colorectal adenocarcinoma) NCBI C146, SKLC6 (human lung carcinoma) NCBI C566, A375 (human melanoma cancer cell) NCBI C136, PLC/PRF/5 (human liver hepatoma) NCBI C145 and HFFF (primary cell culture of human fetal foreskin fibroblast) NCBI C170 were obtained from National Cell Bank of Iran, Pasture Institute of Iran (Tehran). These cells were maintained in RPMI 1640 medium supplemented with 10% FCS in a humidified incubator (37°C and 5% CO<sub>2</sub>).

### Cytotoxicity experiments

Cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 2 mM glutamine (Sigma, USA), penicillin (Sigma, USA) (100 IU/ml) and streptomycin (Sigma, USA) (100, µg/ml) at 37°C in an incubator containing 5% CO<sub>2</sub>. Harvested cells with trypsin (Sigma, USA) (0.25%) were counted by Neubauer slide and then were seeded into 96-well plates (10<sup>4</sup> cell/well). The cells were incubated with 100 µl of different concentration of herbal extracts; in the first step 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml extract concentrations were prepared by serial dilution of DMSO extract stock and in another experiment, 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml extract concentrations were prepared by serial dilution of ethanol (50%) extract stock. Solvent control wells received 100 µl of DMSO (concentration ranging 1 to 0.12%) and ethanol (50%) (Concentration ranging 1 to 0.007%) (Serial dilution was performed by RPMI, 10% FBS). Doxorubicin (50 mg/25 ml) in 2, 1, 0.5, 0.25 and 0.125 µM concentrations were prepared and 100 µl of each concentration was added to wells as positive control. Twelve cell cultured wells without any treatments were used as negative control. Cells were incubated for 24 h. Each single dose and combination was in triplicate in each assay. For MTT assay well contents were taken out and 100 µl of MTT tetrazolium dye (Sigma, USA) (5 mg/ml in PBS) were added to each well and incubated in 37°C for 3 h. The insoluble formazan produced was dissolved in solution containing 100 µl DMSO and OD was read against blank reagent with multiwell scanning spectrophotometer (ELISA reader, Organon Tekninka, The Netherlands) at a wavelengths of 545 nm (Mosmann, 1983; Francis et al., 1986). The percentage of cytotoxicity was calculated according to following equations:

$$\% \text{Viability} = \left( \frac{\text{mean absorbance of treated cells}}{\text{mean absorbance of negative control}} \right) \times 100$$

$$\% \text{Cytotoxicity} = 100 - \% \text{Viability}$$

### Hemolytic test

Hemolytic test was done to determine biocompatibility of herbal extracts (Bock and Muller, 1994). The hemolytic test was performed in 96-well plates. Each well received 50 µl of 0.85% NaCl (Sigma, USA) solution containing 10 mM CaCl<sub>2</sub> (Sigma, USA). The first column was the negative control that contained only the 50 µl of saline solution and the first well of second column of plate received 50 µl of 0.2% triton (BioRad, USA) X-100 (in 0.85% saline) to obtain 100% hemolysis. At the end third column as solvent controls contained the vehicle (2% DMSO) and last sets of columns received 50 µl of herbal extracts that were diluted in half was added. The herbal extracts were tested at concentrations ranging from 3.25 to 200 µg/ml (DMSO extract stock was diluted by saline solution). Then, each well received 50 µl of a 2% suspension of sheep erythrocytes in 0.85% saline containing 10 mM CaCl<sub>2</sub>. Each single dose and combination was in triplicate in each assay. After incubation at room temperature for 30 min, and centrifugation (750 g, 3 min), the supernatant was removed and the liberated hemoglobin was measured spectroscopically as absorbance at 540 nm (Jumaa and Muller 1999; Cobo et al., 2002; Radwan, 2002; Jumaa et al., 2000; Aparicio et al., 2005; Yu et al, 2004).

### Antifungal MIC test

MIC (minimum inhibitory concentration) assay was performed to determine inhibitory effect of candidate herbal extracts on fungal organism and calculate selectivity index (SI). After preparing of sub-culture from previous *C. albicans* (ATTC10231) cultures, stock fungal suspension in saline (10 ml of 0.85% NaCl (Sigma, USA)

solution) was supplied (measure the absorbance of sample by spectrophotometer in 530 nm to obtain transmittance between 73 and 75% (17). To make the culture medium, 50 g of Sabouraud Maltose Broth (SMB) (Difco, USA), powder was added to 1000 ml of deionized double distilled water and mix the suspension and autoclave (pH = 5.6 at temperature = 25°C). Fungal inoculum was prepared by adding of 10 µl stock fungal suspension to 10 ml culture medium. To prepare the plant extracts, Each ethanol extract (10 mg) was dissolved in 1 ml DMSO as a stock solution, then extract dilution was carried out with saline solution (NaCl 0.85% w/v). 1000, 500, 250, 125, 62.5, 31.25 and 15.62 µg/ml extract concentrations were used to determine MIC. Range of DMSO (solvent control) concentrations were from 10 to 0.078% (8 concentrations). Fluconazole (Zahravi, Iran) (10 mg/l) from 0.5 to 0.0019 µg/ml were prepared as a control drug (8 concentrations). The MIC assay was performed in 96-well plates. Each well received 100 µl of SMB. The first well of solvent control column contained 40 µl of DMSO and the first well of fluconazole group received 40 µl of the drug (10 µg/ml), also the first wells of plant extract columns received 40 µl of each sample. Then, mentioned wells of each column received 60 µl of SMB. Finally, each well received 100 µl of fungal inoculum. Each single dose and combination was in triplicate in each assay. After incubation for 24 h at 35°C, the MIC was defined as mean for the lowest concentration of the herbal extracts that completely inhibited the growth of the *C. albicans*. The results were expressed as the mean of triplicate readings (Cuenca-Estrella et al., 2002).

### Data analysis

After subtracting the solvent toxicity, the concentration giving 50% inhibition (IC<sub>50</sub>) was determined for the test samples by linear regression analysis of curves. GraphPad Instat – (DATASET1.ISD) version 3.00 was used to calculate IC<sub>50</sub>. Hemolytic test and cytotoxicity experiment results were expressed as mean ± SD. Mean difference among groups was calculated by paired *t*-test, one-way and repeated measures ANOVA (*p* < 0.05).

## RESULTS AND DISCUSSION

### Cytotoxicity experiment and anticancer property of selected herbs

Experiments with one normal fibroblast cell line HFFF and six cancerous cell lines; AGS, MCF7, SW742, SKLC6, A375 and PLC/PRF/5 exposed to 2 herbal extracts and cytotoxic drug were performed. Cell lines were treated with herbal extracts in two ways; first, 10 mg of evaporated herbal extracts dissolved in DMSO as stock (Table 2) and second, for confirming of findings resulted from first step, 10 mg of evaporated herbal extracts as second stock dissolved in ethanol (50%) (Table 3), then serial dilution were done by RPMI (10% FBS) in mentioned concentrations to perform MTT assay. Paired *t* test (*p* value < 0.05) was calculated between cytotoxic effects of herbal extracts and solvents to determine the effects of solvents that mixed with cytotoxic effects of herbal extracts (Tables 4 and 5). Findings obtained from paired *t* -test analysis showed that cytotoxicity effects of 2 on all cancerous cell lines were independent of cytotoxicity effect of DMSO as solvent;

**Table 2.** Cytotoxic property of 2 herbs dissolved in DMSO.

	IC <sub>50</sub> <sup>*</sup>						
	AGS	MCF7	SW742	SKLC6	A537	PLC/PRF/5	HFFF
<i>Achillea biebersteinii</i>	32.773	47.468	41.103	57.024	36.178	41.806	35.238
<i>Achillea millefolium</i>	22.051	64.058	40.279	24.106	49.438	66.000	34.431
Doxorubicin	0.616	0.308	0.843	1.633	0.718	0.676	0.808

<sup>\*</sup>, Drug concentration with inhibit 50% growth of cell; IC<sub>50</sub> of herbal extracts (µg/ml) and IC<sub>50</sub> of doxorubicin (µM).

**Table 3.** Cytotoxic property of 2 herbs dissolved in ethanol (50%).

	IC <sub>50</sub> <sup>*</sup>	
	AGS	HFFF
<i>Achillea biebersteinii</i>	30.062	183.650
<i>Achillea millefolium</i>	29.872	111.065
Doxorubicin	0.694	0.883

<sup>a</sup> Drug concentration with inhibit 50% growth of cell; IC<sub>50</sub> of herbal extracts (µg/ml) and IC<sub>50</sub> of doxorubicin (µM).

**Table 4.** Calculation of *P* value between cytotoxic effects of herbal extracts and solvent (DMSO).

	<i>P</i> value (p<0.05) <sup>*</sup>					
	AGS	MCF7	SW742	SKLC6	A537	PLC/PRF/5
<i>Achillea biebersteinii</i>	0.0005	0.0007	0.0001	0.0094	< 0.0001	0.0068
<i>Achillea millefolium</i>	0.0003	0.0049	0.0075	0.0005	0.0013	0.0225
<i>Arnebia echioides</i>	0.0032	0.0124	0.0003	0.0381	< 0.0001	< 0.0001

<sup>\*</sup>, *P* value was calculated by GrophPad Instat - (DATASET1.ISD) version 3.00 on paired t test manner.

**Table 5.** Calculation of *p* value between cytotoxic effects of herbal extracts and solvent (ethanol (50%)).

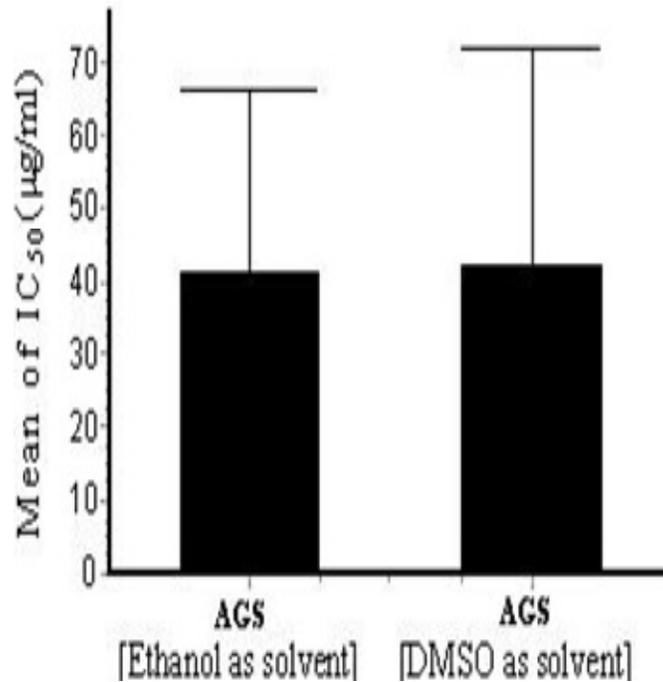
	<i>P</i> value (p < 0.05) <sup>*</sup>	
	AGS	HFFF
<i>Achillea biebersteinii</i>	< 0.0001	0.0214
<i>Achillea millefolium</i>	< 0.0001	0.0292
<i>Arnebia echioides</i>	< 0.0001	0.0003

<sup>\*</sup>, *P* value was calculated by GrophPad Instat - (DATASET1.ISD) version 3.00 on paired t test manner.

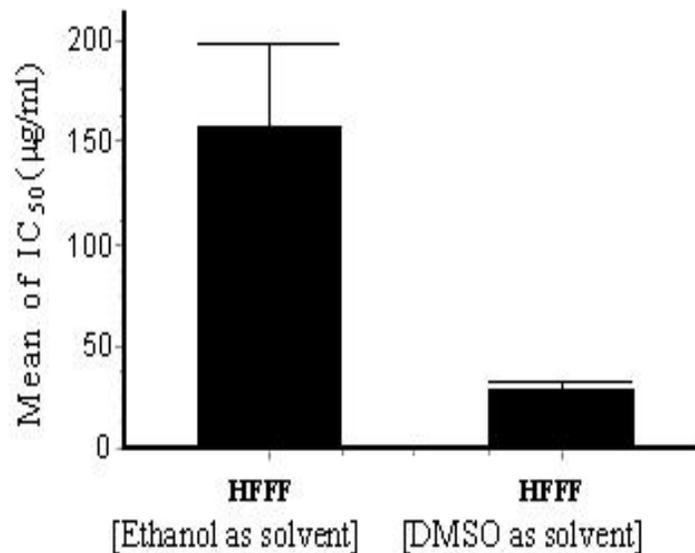
though cytotoxicity effects of 2 herbal extracts on HFFF (normal fibroblast cell line) were dependent to cytotoxicity effect of DMSO.

Our experiments showed cytotoxic effects of herbal extracts dissolved in two different solvent, DMSO and ethanol (50%), on AGS and HFFF were comparable for the first cell line but different for the fibroblast cells (Figure 2 and 3). Findings obtained from paired *t*-test analysis showed that cytotoxicity effects of 2 herbal extracts on six cancerous cell lines were independent of cytotoxicity effect of DMSO as solvent, thought

cytotoxicity effects of 2 herbal extracts on HFFF (normal fibroblast cell line) were dependent to cytotoxicity effect of DMSO. At the second step, herbal extracts were dissolved in ethanol 50% as stock solution. The results comparing cytotoxic effects of herbal extracts dissolved in two different solvent (DMSO and ethanol (50%)) on AGS and HFFF, illustrated that by replacing DMSO with ethanol 50% (presumably possessing less toxic property) cytotoxicity effects of herbal extracts on cancerous cell line AGS did not change significantly (*p* value is 0.1714, considered not significant), but cytotoxicity effects of



**Figure 2.** Comparison of cytotoxicity effects of 2 herbal extracts dissolved in DMSO with ethanol (50%) on AGS cancerous cell line ( $p$  value is 0.1714, considered not significant), results were expressed as mean  $\pm$  SD.



**Figure 3.** Comparison of cytotoxicity effects of 2 herbal extracts dissolved in DMSO with ethanol (50%) on normal fibroblast cell line HFFF ( $p$  value is  $<0.0001$ , considered extremely significant) results were expressed as mean  $\pm$  SD.

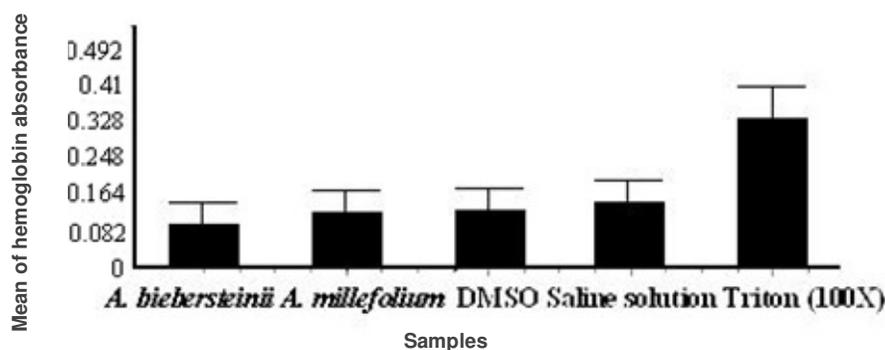
herbal extracts on normal cell line HFFF, decreased and the change was considered significant ( $p$  value is  $<0.0001$ , considered extremely significant). Final findings

resulted from two steps of cytotoxicity experiment may point at selectivity effects of mentioned herbal extracts in inducing cytotoxicity on cancerous cell lines.

**Table 6.** MIC and selectivity Index of the candidate herbal extract against *Candida albicans* (ATTC10231) at 37°C for 24 h.

Samples	MIC	SI*					
DMSO (solvencontrol)	5% V/V						
Fluconazole (drug control)	No observed MIC( $\mu\text{g/ml}$ )	AGS	MCF7	SW742	SKLC6	A537	PLC/PRF/5
<i>Achillea biebersteinii</i>	250	7.628231	5.26670599	6.082281	4.384119	6.910277	5.980003
<i>Achillea millefolium</i>	500	22.67471	7.80542633	12.41342	20.74172	10.11368	7.575758

\*, SI calculated by dividing of MIC on IC<sub>50</sub> resulted from herbal extract (dissolved in DMSO) on cancerous cell lines.



**Figure 1.** The mean hemoglobin absorbance resulted from hemolytic induction. The mean hemoglobin absorbance related to 2 herbal extracts similar to salty solution (negative control) were considered extremely significant ( $p$  value < 0.0001) in comparison with Triton X-100 as positive control to obtain 100% hemolysis, results were expressed as mean  $\pm$  SD.

### Antifungal MIC assay

Antifungal MIC (minimum inhibitory concentration) assay was performed to determine effect of candidate herbal extracts on fungal growth and calculate selectivity index (SI) calculated by dividing of MIC ( $\mu\text{g/ml}$ ) on IC<sub>50</sub> ( $\mu\text{g/ml}$ ) (Table 6):

$$SI^a = \left( \frac{MIC^b \text{ of herbal extract}}{IC_{50}^c \text{ of herbal extract}} \right)$$

a, Selectivity Index

b, Minimum Inhibitory Concentration

c, Drug concentration exert 50% inhibition

The antifungal MIC test results indicated that *A. biebersteinii* had antifungal activity against *C. albicans* (ATTC 10231) in concentration of 250  $\mu\text{g/ml}$  independent of solvent toxicity for the first time. The antifungal MIC test showed that *A. biebersteinii* had antifungal activity against *C. albicans* (ATTC10231) in concentration of 250  $\mu\text{g/ml}$  independent of solvent toxicity for the first time. Indeed, in current research investigation of cytotoxicity effects related to candidate herbal extracts on human cancerous cell lines have been defined as the main goal. Antifungal MIC test just performed as complementary test

to compare cytotoxicity effects of herbal candidate extracts on *C. albicans* as primitive eukaryotic cells, normal advanced eukaryotic cells and cancerous cells. The SI values showed selectivity of cytotoxicity effect of herbal extracts on cancerous cells in comparison with primitive eukaryotic cells and introduced herbal extracts with upper SIs will be better candidates for further research.

### Hemolytic test to determining herbal extract biocompatibility

Hemolytic test was done for 2 herbal extracts to measure their property in induction of hemolysis. The liberated hemoglobin was measured spectroscopically as test result, mean of hemoglobin absorbance related to candidate herbal extracts similar to saline solution (negative control) were considered extremely significant ( $p$  value is < 0.0001, considered extremely significant) in comparison with Triton 100  $\times$  as positive control to obtain 100% hemolysis (Figure 1).

Finally, it is concluded from hemolytic test showed that candidate herbal extracts did not induce hemolysis similar to negative control, which is further evidence for

lack of general toxicity of the selected herbal extracts. Hemolytic assay as a screening test can indicate the potential cytotoxicity of natural and synthetic compound on RBC cells. In current study, poor hemolytic potential of candidate herbal extracts plus antifungal test findings in comparison with their cytotoxicity effects on several cancerous cells, may present selectivity effects of them in inducing of cytotoxicity on human cancerous cells.

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