



## **Antioxidant, Anti-proliferation and Cytotoxicity Activities of *Gossypium hirsutum* toward Standard HepG2, A549, MCF-7 and U87 Cancer Cell Lines Compared to Huvec, 3T3 Normal Cells**

**Fatemeh Sharifi<sup>1</sup>, Fariba Sharififar<sup>2\*</sup>, Iraj Sharifi<sup>3\*</sup>, Mostafa Pournamdari<sup>4</sup>,  
Touba Eslaminejad<sup>1</sup> and Mehrdad Khatami<sup>5,6</sup>**

<sup>1</sup>Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran.

<sup>2</sup>Herbal and Traditional Medicines Research Center, Department of Pharmacognosy, Kerman University of Medical Sciences, Kerman, Iran.

<sup>3</sup>Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran.

<sup>4</sup>Department of Medicinal Chemistry, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.

<sup>5</sup>School of Medicine, Bam University of Medical Sciences, Bam, Iran.

<sup>6</sup>Nanomedicine and Nanobiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

### **Authors' contributions**

*This work was carried out in collaboration between all authors. Authors F. Sharififar and IS designed the study. Author MK performed the statistical analysis. Author F. Sharifi wrote the protocol, and wrote the first draft of the manuscript. Author MP managed the analyses of the study. Author TE managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** Cancer has become a growingly medical and health care issue with no effective treatment modality. Chemotherapy besides existing drugs is associated with severe adverse side effects. The present study was aimed to explore the anti-proliferative/cytotoxic and antioxidant activity of *Gossypium hirsutum* boll crude extract on cancer cell lines compare to the normal cell lines.

**Study Design:** This study aims to explore the cytotoxic effect of *G. hirsutum* crude extract on human cancer cell-lines, compared with normal cells by using colorimetric cell viability assay and also the antioxidant activities of the crude extract were carried out by DPPH and FRAP assays.

**Methodology:** Fresh *G. hirsutum* bolls were gathered from the south-eastern of Iran. Soxhlet apparatus method was used for extracting the bolls. Complete medium was used to prepare six final concentrations (1-1000 µg/mL) for experiments. The cytotoxicity and antioxidant activities of the bolls extract were carried out by using colorimetric cell viability, DPPH and FRAP assays. All experiments were repeated in triplicate and analyzed by t-test. 50% cytotoxicity concentrations (CC50) value was calculated by Probit test.

**Results:** The extract performed a potent index of anti-proliferation/cytotoxicity effect on three cancer cell lines as demonstrated by the low IC<sub>50</sub> values (2.72, 4.02 and 4.22 µg/mL) on human breast cancer (MCF-7), human lung cancer (A549) and glioblastoma cells (U87), respectively compared to the normal cells. Results also confirmed the best antioxidant activities of the crude extract. The selectivity index (SI) as the measure of toxicity ranged in a normal and safe level.

**Conclusion:** It could be concluded that the *G. hirsutum* boll extract displayed potent anti-proliferation/cytotoxicity index against cancer cell lines and great antioxidant power. Thus, experiments are essential to analyze, isolate and characterize its compositions for future *in vivo* studies.

**Keywords:** *Gossypium hirsutum*; anticancer; medicinal plants; antioxidant activities.

## 1. INTRODUCTION

Over the past years, cancer has become a significant medical and health care issue [1,2]. The treatment of cancer involves one or all of the following procedures: surgery, radiation therapy, bone marrow transplantation, chemotherapy and photodynamic therapy (PDT) [3]. Although chemotherapy is quite an effective treatment, the side-effects are severe, especially when administered as systemic drugs to the patient. Earlier studies revealed that among women in the United States, breast cancer is the second major cause of cancer deaths [4,5]. There are chronic side effects associated with chemotherapy including nausea, bone marrow failure [6] and the development of multidrug resistance (MDR) [7,8]. Hence, it is of great importance to discover natural plant compounds that could serve as alternative for cancer treatment. Traditional remedies invariably involve crude plant extracts containing multiple chemical constituents, which vary in potency from highly active to very weak. Synergy and other interactions can take place between the constituents of a single extract as well as in a mixture of herbs [9,10]. The study on plants used in traditional medicine has currently received new impetus with introduction of a wide variety of small-scale bioassay methods and improved

methods for fractionation, isolation and characterization of compounds [11]. For example, individual compounds of plants have been isolated and used as lead molecules for the reputed activity [12].

However, it is also apparent that, in many cases, the mechanisms of action are due to a mixture of constituents, perhaps, each contributing a different biological effect to the overall activity. Thus, it is likely that not only will traditional medicines continue to provide new molecules for drug discovery, but they might also form the basis for a wider acceptance of crude extracts in standardized form, as another type of medicine in modern practice [13].

It has been estimated that over 60% of the anticancer drugs in current use are in some ways, derived from plants and other natural products, and are in the process of evaluation [14]. A successful anticancer drug should kill cancer cells without causing excessive damage to the normal dividing cells. This idea is difficult, or perhaps impossible to reach and that is why cancer patients frequently suffer unpleasant side-effects when undergoing treatments [9].

*Gossypium hirsutum* belongs to the Malvaceae family and is an annual flowering shrub, which is

cultured primarily for its herbal seed fiber. It has been reported that the seeds and roots of plants belonging to this genus are used in nasal polyps, uterine fibroids as abortifacient and in the treatment of other types of cancer [15]. *G. hirsutum* seeds contain Gossypol (a toxic dihydroxyphenol) which exhibited anticancer activity in new LL, WA and PS-150 tumor systems [16]. The capsules of the plant named "bolls" were used for treatment of cutaneous leishmaniasis (CL) in Iranian folk medicine [17].

For future studies, it is essential to select plant extracts with potential antineoplastic properties. This is achieved by using important preliminary data provided by cytotoxicity screening models [18]. Majority of the tissues require oxygen for their existence. Oxygen-derived species produce free radicals that lead to chain reactions and attach to macromolecules, most frequently, of proteins, DNA and lipids causing chronic degenerative diseases such as malignancies, diabetes, cardiovascular and neurologic disorders [19,20]. Antioxidant systems from endogenous and exogenous sources including dietary foods, vegetables, fruits, medicinal plants, vitamin supplements, minerals, polyphenols, flavonoids and various enzymes are principal sources which could prevent these oxidative damages to cellular components [21]. Medicinal plants consist of complex networks of overlapping antioxidants, which are able to either prevent this reactive radical species from being formed or remove them before they damage vital components of the body cellular structures. Thus, in addition to removing oxidants by-products, either antioxidant systems showed the ability to keep the tissues at an optimum level [22].

Therefore, the aim of the present work is to explore the cytotoxic effect of *G. hirsutum* crude extract on lung cancer (A549), hepatocellular carcinoma (HepG2), breast cancer (MCF-7) cells, and glioblastoma cells (U87), umbilical vein endothelial cells (HUVEC) and mouse embryonic fibroblast cell line (3T3). Colorimetric cell viability, DPPH and FRAP assays were used.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material and Chemicals

The bolls of *G. hirsutum* were obtained from Orzuiye Baft, Kerman province (southeastern of Iran) and kindly approved by Department of Botany, Bahonar University, Kerman, Iran. The herbarium of the Faculty of Pharmacy, Kerman

University of Medical Sciences provided a voucher specimen of the obtained plants as reference. Lung cancer (A549), hepatocellular carcinoma (HepG2), breast cancer (MCF-7) cells, and glioblastoma cells (U87), human umbilical vein endothelial cells (HUVECs) and mouse embryonic fibroblast cell-line (3T3) were obtained from Pasteur Institute of Iran.

WST-1, Butylated hydroxyanisole (BHA), 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP), fetal bovine serum (FBS), Dulbecco's Modified Eagle's medium (DMEM), 95% ethanol, dimethyl sulphoxide (DMSO), phosphate buffer saline (PBS), trypan blue dye solution, trypsin, EDTA solution and antibiotics were obtained from Sigma-Aldrich (St. Louis, MO, USA). Chloroform was prepared from Merck Company. All the other chemicals and solvents used were of the highest purity commercially available.

### 2.2 Preparation of the Plant Extracts

The bolls were allowed to dry in shade and powdered to provide a source of extract for further experiments. The method of extraction was selected on the basis of constituents of the plants and also the folk way of preparing these plants. Extracting solvent was chloroform and Soxhlet apparatus was used to extract the bolls of *G. hirsutum* for 4 h. The extract was filtered and concentrated under vacuum condition by a rotary evaporator. Finally, the extract was kept in oven at 40°C for 48 to 72 h by allowing to entirely dry and stored in dark glass container in refrigerator at 2-8°C for later usages [23]. The extract was diluted in DMEM medium to obtain final concentrations of 1, 5, 10, 50, 100, 500 and 1000 µg/ml for experiments.

### 2.3 Cell Lines and Culture Conditions

Four distinct tumor cell lines: human breast cancer (MCF-7) cells, human lung cancer (A549) cells, glioblastoma cells (U87) and human hepatocellular carcinoma (HepG2) were used. These cancer cell lines were grown in DMEM supplemented by 10% FBS and 100 µg/mL of penicillin/streptomycin at 37°C and 5% CO<sub>2</sub>. 3T3 fibroblasts and HUVECs as normal cells were used.

### 2.4 Cytotoxicity Assay

10<sup>4</sup> cells/well of each cell lines were seeded on the 96-well plate and kept for 24 h. Next day the medium was aspirated and 100 µl of each

concentration (1, 5, 10, 50, 100, 500 and 1000 µg/mL) were poured to each well. Then plates were incubated at 37°C and 5% CO<sub>2</sub> for a standard time of 24 h. 10 µL of WST-1 solution was added to each well, and incubated for 4 h. Then the absorbance was measured at 450 nm with the reference at 630 nm by ELISA reader (BioTeks Elx 800). Cell viability was expressed as 100% for control (untreated cells). All samples were performed in triplicates and the survival rate (%) was calculated as the following equation [24]:

$$\text{Eq. (A.1) Survival rate (\%)} = (\text{OD in treatment group} / \text{OD in control group}) \times 100$$

The inhibitory concentration required for 50% cytotoxicity (IC<sub>50</sub>) value was determined using the Probit test in SPSS and plotting the percentage of inhibition versus the concentration.

## 2.5 Antioxidant Activities

### 2.5.1 DPPH assay

Antioxidant assay was performed using the method developed by Benzie and Strain (1999). This method measured the ability of the plant extract to scavenge DPPH free radicals [25]. Briefly 0.1 mM of DPPH radical solution in methanol: water (8:2, v/v) was prepared and then 1 mL of this solution with 1 mL of sample solution at the concentrations of 0 to 400 µg/mL was mixed with sample solution (3 mL) in methanol: water (8:2, v/v). After 30 min, the absorbance was measured at 517 nm. Decrease in the DPPH solution absorbance showed an increase in the DPPH radical scavenging activity. This activity is given as % DPPH radical scavenging which is calculated using the following equation [26]:

$$\text{Eq. (A.2) \%DPPH radical scavenging} = (\text{control absorbance} - \text{sample absorbance}) / \text{control absorbance} \times 100$$

The DPPH solution without sample solution was used as control and butylated hydroxyanisole (BHA) was used as standard [27].

### 2.5.2 FRAP assay

The ability of antioxidant to reduce ferric iron was measured using ferric reducing antioxidant power (FRAP). It is based on the reduction of the ferric iron and 2, 3, 5-triphenyl-1, 3, 4-triaza-2-azoniacyclopenta-1, 4- diene chloride (TPTZ) complex to the ferrous form at low pH. To

measure the change in absorption at 593 nm, we used a diode-array spectrophotometer, which indicates the reduction power of the compound. In brief, 100 µL of diluted sample was mixed with three milliliter of prepared FRAP reagent; after 30 min incubation at 37°C, the absorbance was recorded at 593 nm. The absorbance of the sample was compared to a Fe<sub>2</sub>SO<sub>4</sub> standard curve and the FRAP values were expressed as Ferrous Equivalent (FE), the concentration of extract or chemical which gives the same absorbance as 1 mmol ferrous ion [28].

## 2.6 Statistical Analysis

Student's t-test was used to analyze intergroup differences. Experiments were carried out in triplicate and repeated at least twice, and data are represented as the mean ± SD. A p-value less than 0.05 were considered statistically significant. The IC<sub>50</sub> value (50% inhibitory concentrations) was calculated using Probit test in SPSS.

## 3. RESULTS

### 3.1 Cytotoxicity Assay

#### 3.1.1 Cytotoxicity effect on cancer cell lines

The cytotoxic effect of *G. hirsutum* extract on four human cancer cell lines (A549, HepG2, MCF-7 and U87) was determined by WST-1 assay and compared to untreated cells as control group (Fig. 1).

The extract showed a potent index of antimultiplication/cytotoxicity effect on all four cancer cell lines as demonstrated by the low IC<sub>50</sub> values (2.72, 4.02 and 4.22 µg/mL on MCF-7, A549 and U87, respectively) (Table 1), whilst the cytotoxicity effect on HepG2 was considered as moderate activity (67.31 µg/mL).

**Table 1. The cytotoxic effect of *G. hirsutum* extract on human cancer cell lines (A549, HepG2, MCF-7 and U87)**

Cancer cell lines	IC <sub>50</sub> ± SD (µg/mL)
Human breast cancer (MCF-7)	2.718 ± 0.09
Human lung cancer (A549)	4.017 ± 0.05
Glioblastoma cells (U87)	4.22 ± 0.04
Human hepato cellular carcinoma (HepG2)	67.31 ± 0.4

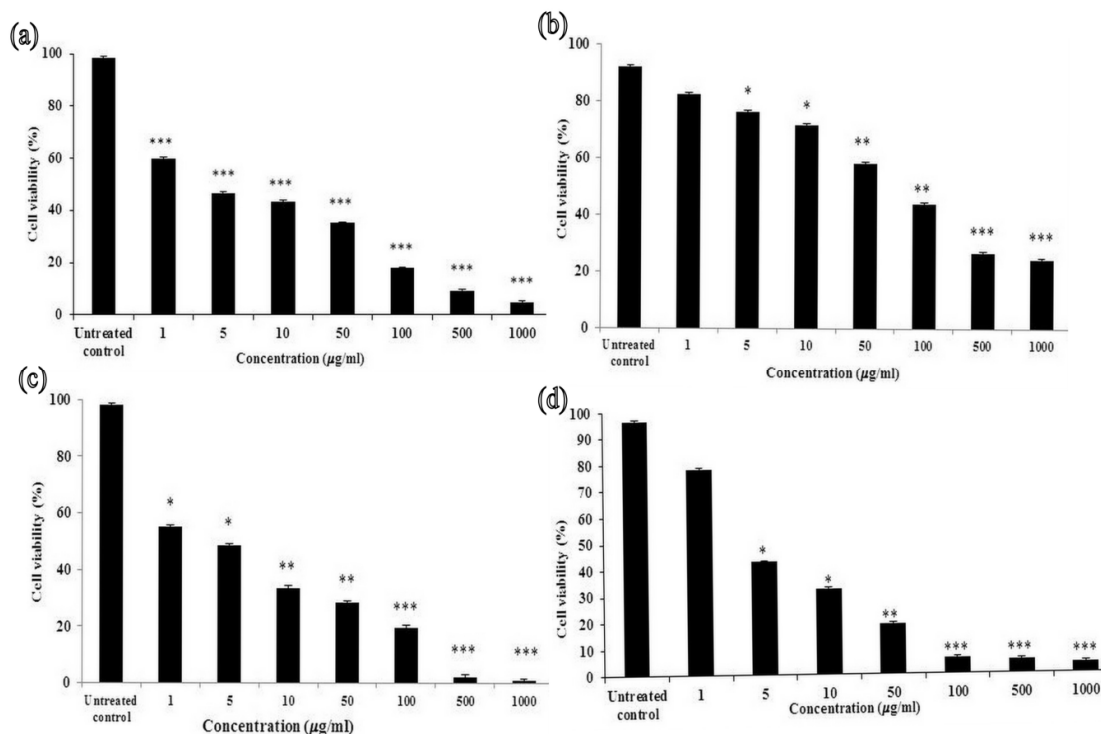
: Concentration of *G. hirsutum* extract caused 50% mortality

The three cancer cell lines (MCF-7, A549 and U87) demonstrated a similar pattern of affectivity (Figs 1.a, c and d), in contrast a lower activity was found for HepG2 (Fig 1.b). Comparison of various concentrations of the *G. hirsutum* boll extract showed significant inhibition of the proliferation rate of normal cell lines (Fig. 2 a, b), but in higher concentrations when compared to the cancer cell lines.

### 3.1.2 Cytotoxicity effect on normal cell lines

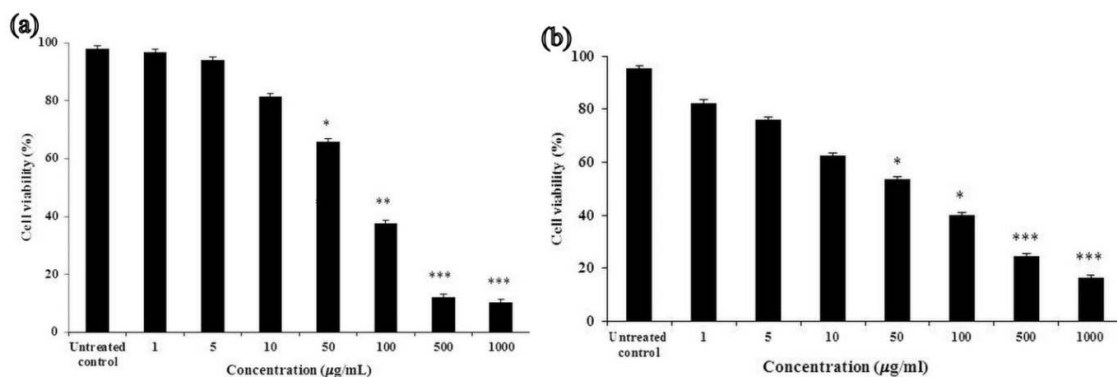
The cytotoxicity effect of *G. hirsutum* extracts on two normal cell lines (3t3 and HUVECs) was determined using the colorimetric assay (WST-1 based) and is presented in Fig. 2.

The *in vitro* cytotoxic activities ( $CC_{50}$ ) normal cell lines are shown in Table 2.



**Fig. 1. The cytotoxicity effect of *G. hirsutum* extract on four cancer cell lines (a) A549, (b) HepG2, (c) MCF-7 and (d) (U87)**

\*= *p* value < 0.01, \*\*= *p* value < 0.01 and \*\*\*= *p* value < 0.001



**Fig. 2. Cytotoxicity effect of *G. hirsutum* extract on two normal cell lines; (a) BALB/c 3T3 fibroblasts and (b) (HUVECs)**

\*= *p* value < 0.01, \*\*= *p* value < 0.01, \*\*\*= *p* value < 0.001

**Table 2. The cytotoxic effect of *G. hirsutum* extract on human normal cell lines (3t3 and HUVECs)**

Normal cell lines	CC <sub>50</sub> ± SD (µg/ml)
3T3 fibroblasts	70.16 ± 0.5
Human umbilical vein endothelial cells (HUVECs)	42.21 ± 0.4

: Concentration of *G. hirsutum* extract caused 50% mortality

### 3.2 Selectivity Index (SI)

The selectivity index of the cancer lines was calculated by following equation [29], whose data are shown in the Table 3.

**Eq. (A.3)**  $SI = (CC_{50} \text{ normal cells} / CC_{50} \text{ cancer cells})$

### 3.3 Antioxidant Activity

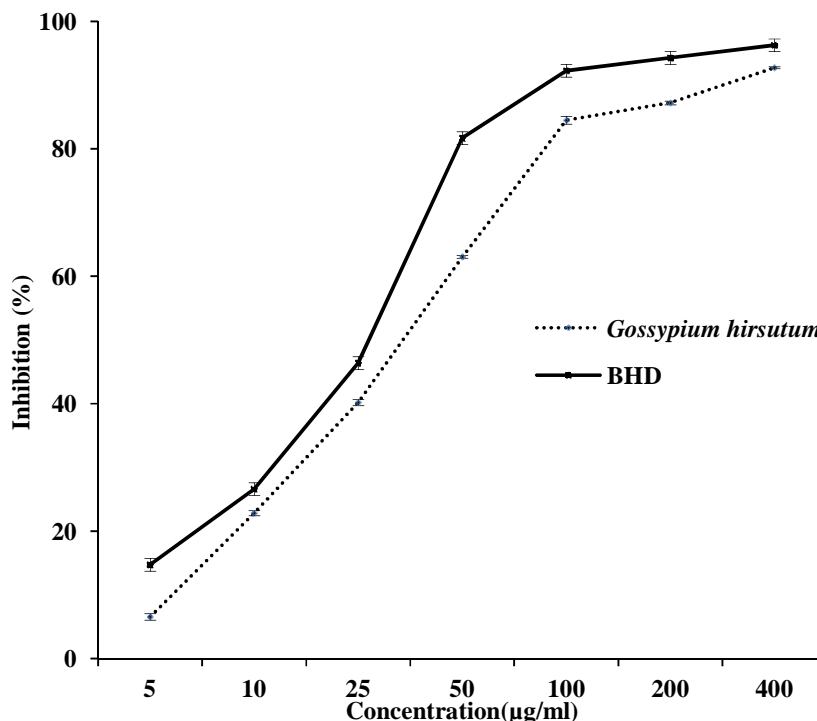
#### 3.3.1 DPPH assay

DPPH radical scavenging activity was assessed using crude extract of concentrations 0 to 400 µg/mL and showed statistically similar activity when compared to butylated hydroxyanisole (BHA) as standard (Fig. 3).

The IC<sub>50</sub> value for DPPH radical scavenging activity crude extract was 33.21 ± 0.05 µg/mL, which was not statistically significant as compared to BHA (21.44 ± 0.4 µg/mL) (Table 4).

**Table 3. The selectivity index of *G. hirsutum* extract**

Normal cell lines	Cancer cells SI			
	Human breast cancer (MCF-7)	Human lung cancer (A549)	Glioblastoma cells (U87)	Human hepato cellular carcinoma (HepG2)
3T3	25.89	17.466	16.62	1.04
HUVECs	15.52	10.52	10.049	-

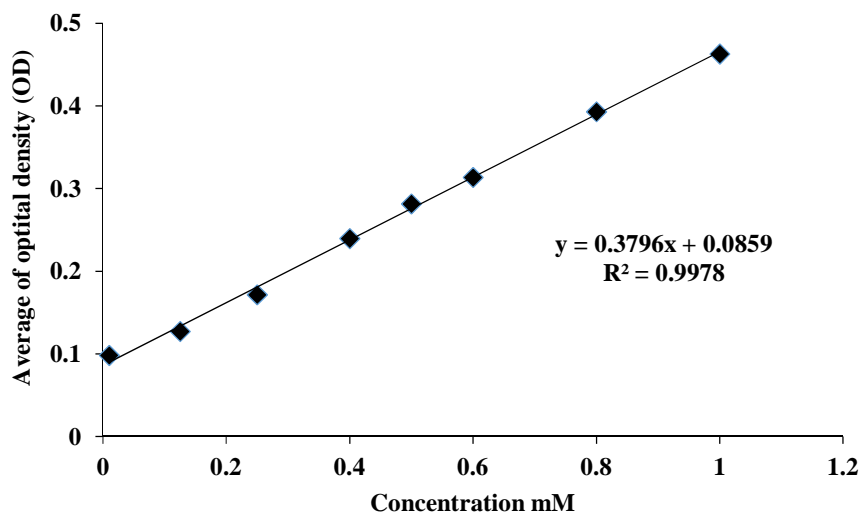


**Fig. 3. Scavenging effects of *G. hirsutum* crude extract on DPPH free radicals compared to BHA as a standard reference**

Data are shown as mean ± SD of triplicate experiments

**Table 4. The IC<sub>50</sub> values for DPPH radical scavenging activity (%) of *G. hirsutum* crude extract compared to BHA**

Compounds	IC <sub>50</sub> <sup>a</sup> ± SD (µg/ml)	p value
<i>G. hirsutum</i> extract	33.21 ± 0.05	
Butylated hydroxyanisole (BHA)	21.44 ± 0.4	Not significant

**Fig. 4. Linearity of FRAP (dose response line)**

### **3.3.2 Ferric reducing-antioxidant power (FRAP) assay**

The antioxidant linearity of FRAP (dose response line) for standard solution was shown in Fig. 4. The FRAP value of the crude extract was 600 µg/mL. The antioxidant activities were expressed as the concentrations of antioxidant having a ferric reducing ability equivalent to 1 mM of Fe<sub>2</sub>SO<sub>4</sub>.

## **4. DISCUSSION**

The development of chemotherapy in the 1950s and 1960s resulted in the availability of curative therapeutic approaches for patients with hematologic malignancies of several types of advanced solid tumors [30]. The present anticancer agents are extremely cytotoxic and exhibit serious adverse effects on various tissues of human at clinical level. Therefore, these limitations for use of synthetic anticancer compounds would be an essential need to screen and develop new medicinal plants extracts as alternatives which will be less toxic to normal cells, having greater therapeutic index, with different mechanism of action and shorter treatment cycles.

According to what earned in the present study “the *G. hirsutum* crude extract clearly displayed a cytotoxic effect on cancer cells lines of various types, more potent activity than the normal cell lines”. This product has been used as a male oral contraceptive [31] and antineoplastic activity particularly against melanoma and bladder carcinoma [32]. Reports indicated that gossypol is more cytotoxic in some tumor cell lines [33].

The exact biochemical and/or molecular mechanisms involved in the progress of such biological activity of antiproliferative/cytotoxic effect are not clearly explained. In light of the pivotal events involved in cancer cell death, serious efforts are being made to translate this knowledge into the rationale design and development of novel therapeutic approaches to improve the efficacy of chemotherapeutic agents [34].

Antioxidant activity was expressed as the concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM Fe<sub>2</sub>SO<sub>4</sub>. The present study demonstrated that *G. hirsutum* bolls possess potent antioxidant activity as shown by *in vitro* experiments using DPPH

and FRAPS assays. This characteristic indicates that the fresh crude extract of *G. hirsutum* bolls, as a rich source of antioxidants, have potentials to prevent chronic degenerative diseases induced as a results of oxidative stress [35].

The results of the current study demonstrated that the crude extract exhibited a cytotoxic effect on cancer cell lines, and had a rather moderate effect on normal cells. Furthermore, to get some insights into the role played by oxidation in cytotoxicity, the activity of the crude extract on the growth of these cells in the presence of radical scavengers was investigated. Cell sensitivity to the *G. hirsutum* extract in cancer cells was significantly higher when compared to normal tissue cell lines, with exception of the HepG2 which exhibited resistance (IC<sub>50</sub> value 67.31 µg/ml).

## 5. CONCLUSION

The findings of this study showed that the *G. hirsutum* crude extract consisted of potentially anticancer agent because of its strong antioxidant activity. Further investigations are needed to isolate, analyze and identify its compositions for evaluations of biological activity in animal model and clinical settings.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## ACKNOWLEDGEMENTS

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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