Journal of Medicinal Plants and By-products (2015) 2: 171-178

Original Article

Total Phenolic Content, Antioxidant Activity and *In vitro* Cytotoxicity of the Essential Oil of *Jurinea leptoloba* DC.

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Article History: Received: Received: 05 February 2015/Accepted in revised form: 13 September 2015

Abstract

The essential oil from the aerial parts of *Jurinea leptoloba* DC., collected from Shiraz (South of Iran), was investigated for total phenolic content (TPC), antioxidant and cytotoxic effects. TPC of *J. leptoloba* oil was determined to be 16.53 ± 5.69 µg gallic acid equivalent/mg sample (µg GAE/mg oil) using the Folin-Ciocalteau assay. The ferric reducing power of the oil was determined 0.117 ± 0.01 GAE (mg/g). *J. leptoloba* oil exhibited a dose-dependent scavenging of DPPH, with an IC_{50} value of 24.50 mg/ml. Antioxidant activity percentage of the essential oil of *J. leptoloba* determined by β-carotene bleaching test revealed less potential than both standards BHT and BHA (62.01 ± 2.45%, 2.5 mg/ml oil). Cytotoxicity was measured using a modified MTT assay. IC_{50} values for HeLa and lymphocyte cells were calculated to be 290.76 and 2900.97 µg/ml, respectively. The results suggest application of *J. leptoloba* essential oil as a moderate antioxidant and anticancer agent.

Key words: *Jurinea leptoloba*, Total phenolic content, Antioxidant activity, Cytotoxicity

Introduction

The large genus *Jurinea* from *Compositae* (tribe *Cynareae*, subtribe *Carduinea*) with about two hundred and fifty species is distributed in Europe and Asia [1]. Several species of *Jurinea* have been examined chemically and presence of sesquiterpenes, especially elemanolides and melampolides have been reported [2]. The extract of the aerial parts of *Jurinea leptoloba* DC., afforded in addition to several germacranolides albicolide, pectorolide, salonitenolide, jurinelloide and its derivative, four melampolides, two elemanolides, glucopyranoside and dihydrosyringenin [3]. The essential oil from the aerial parts of *J. leptoloba* was analyzed by GC and GC-MS. Thirteen components of the oil of *J. leptoloba* identified, representing 70.55% of the total components detected. Non-terpenoid hydrocarbons and phenolic compounds were the major components in the oil of *J. leptoloba*. Non-terpenoid components and hydrocarbons were the major component in this oil. The major constituents were identified as 6-n-butyl-2,3,4,5-tetrahydropyridine (15.6%) and Cyclohexene, 3,5,5-trimethyl (11.26%). 1,3-Menth-2-ene (1.09%) was identified as monoterpenoid component in this oil [4]. To the best of our knowledge, no study has been performed about the pharmacological and biological activities of the essential oil of *J. leptoloba* in the literature. Hence, the objectives of this research were to evaluate the total phenolic content, *In vitro* cytotoxic and antioxidant activities of the essential oil of *Jurinea leptoloba* DC collected from Shiraz.

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Material and Methods

General

The major equipment types used were a hydro-distillation (clevenger apparatus), Shimadzu UV-2501PC spectrophotometer and DNM-9602G ELISA reader (Perlong Group, Beijing, China). Microbial and cell culture media and laboratory reagents were from Merck, Germany. Other chemicals were of analytical grade.

Plant Materials

The aerial parts of *J. leptoloba* were collected in August 2011 at the flowering stage from 40 km south of Shiraz, Iran. Voucher number 324R, deposited at the herbarium of the Department of Botany, Shahid Beheshti University, Tehran, Iran.

Isolation of the essential oil

The aerial parts of *J. leptoloba* were dried at room temperature for several days. Air-dried aerial parts of *J. leptoloba* (100g) were separately subjected to hydrodistillation using a clevenger-type apparatus for three hours. After decanting and drying the oil over anhydrous sodium sulfate, the oil was recovered. Results showed that essential oil yield was 0.1% (w/w).

Total Phenolic Content Assay

Total phenolic content (TPC) of oil was determined using the Folin-Ciocalteau assay [5]. Samples (300 µl) were introduced into test tubes followed by 1.5 ml of a Folin-Ciocalteau's reagent (10 x dilutions) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were allowed to stand for 30 min before measuring absorbance at 765 nm. TPC was expressed as gallic acid equivalent (GAE) in mg per 100 g material.

Antioxidant Activity

Antioxidative properties of the essential oil of *J. leptoloba* was determined by three methods: The Ferric-Reducing Antioxidant Power (FRAP), Radical-scavenging capacity of the oil or bleaching of 2,20-diphenylpicrylhydrazyl (DPPH) and β-carotene-linoleic acid assay.

Ferric-reducing Antioxidant Power (FRAP) Assay of the oil

The FRAP assay was carried out according to the procedure employed by Lim *et al*., [6]. One milliliter of the extract dilution was added to 2.5 ml of 0.2 M potassium phosphate buffer (PH 6.6) and 2.5 ml 1% potassium ferricyanide. The mixture was incubated for 20 minutes at 50 ºC, after which 2.5 ml of 10% trichloroacetic acid was added. The mixture was then separated into aliquots of 2.5 ml and mixed with 2.5 ml of deionized water. Then, 0.5 ml of 0.1% (w/v) $FeCl_3$ were added to each tube and allowed to stand for 30 minutes. Absorbance for each tube was measured at 700 nm. The FRAP was expressed as gallic acid equivalents (GAE) in mg/g of samples used.

Bleaching of 2,20-di phenylpicrylhydrazyl (DPPH)

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-coloured methanol solution of 2,2-diphenylpicrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent. Fifty milliliters of 1:5 concentrations of the essential oil in methanol were added to 5 ml of a 0.004% methanol solution of DPPH. Trolox (1 mM) (Sigma-Aldrich), a stable antioxidant, was used as a synthetic reference. The essential oil from *Thymus x-porlock* was used as a natural reference. These results were compared to those of *Thymus x-porlock* essential oil, used as a reference ingredient [7,8]. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH in percent (I%) was calculated in following way [9]:

$I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100;$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Tests were carried out in triplicate.

β-Carotene-linoleic Acid Assay

Antioxidant activity of essential oil was determined using the β-carotene bleaching test [10]. Approximately 10 mg of β-carotene (type I synthetic, Sigma-Aldrich) was dissolved in 10 ml of chloroform. The carotene-chloroform solution, 0.2 ml, was pipetted into a boiling flask containing 20 mg linoleic acid (Sigma-Aldrich) and 200 mg Tween 40 (Sigma-Aldrich). Chloroform was removed using a rotary evaporator at 40 ºC for 5 min and, to the residue, 50 ml of distilled water were added, slowly with vigorous agitation, to form an emulsion. Five milliliters of the emulsion were added to a tube containing 0.2 ml of essential oil solution, prepared and the absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The

tubes were placed in a water bath at 50 ºC and the oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm over a 60 min period. Control samples contained 10 µl of water instead of essential oils. Butylated hydroxy anisole (BHA; Sigma-Aldrich), a stable antioxidant, was used as a synthetic reference. The antioxidant activity was expressed as inhibition percentage with reference to the control after 60 min of incubation, using the following equation:

$AA = 100(DR_C - DRS_S)/ DR_C$; where

AA = antioxidant activity,

DR_C = degradation rate of the control = [ln(a/b)/60],

DR_S = degradation rate in presence of the sample = [ln(a/b)/60],

a = absorbance at time 0, b = absorbance at 60 min.

Cytotoxicity Assay

The human cervical carcinoma HeLa cell line NCBI code No. 115 (ATCC number CCL-2) were obtained from Pasteur Institute, Tehran-Iran. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 1% (w/v) glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were cultured in a humidified atmosphere at 37 ºC in 5% CO_2. Cytotoxicity was measured using a modified MTT assay. This assay detects the reduction of MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide] by mitochondrial dehydrogenase, to blue formazan product, which reflects the normal functioning of mitochondrial and cell viability [11]. Briefly, the cells (5×10^4) were seeded in each well containing 100µl of the RPMI medium supplemented with 10% FBS in a 96-well plate. After 24 h of adhesion, a serial of doubling dilution of the essential oil was added to triplicate wells over the range of 1.0-0.005 µl/ml. The final concentration of ethanol in the culture medium was maintained at 0.5% (volume/volume) to avoid toxicity of the solvent [12]. After 2 days, 10 µl of MTT (5 mg/ml stock solution) was added and the plates were incubated for an additional 4 h. The medium was discarded and the formazan blue, which formed in the cells, was dissolved with 100 µl dimethyl sulphoxide (DMSO). The optical density was measured at 490 nm using a microplate ELISA reader. The cell survival curves were calculated from cells incubated in the presence of 0.5% ethanol. Cytotoxicity is expressed as the concentration of drug inhibiting cell growth by 50% (IC_{50}).

Results

Total Phenolic Content

The total phenol content of the essential oil of *J. leptoloba* was determined to be 16.53±5.69 µg gallic acid equivalent/mg sample (µg GAE/mg oil) ($y = 0.001x + 0.0708$; $R^2 = 0.996$) (Fig. 1).

Antioxidant Activity

The antioxidant capacities of the essential oil as assessed by different assay methods are summarized in Table 1.

The ferric-reducing antioxidant power (FRAP) was expressed as gallic acid equivalent or known Fe (II) concentration (Fig. 2). The ferric reducing power of the essential oil of *J. leptoloba* was determined 0.117 ± 0.01 gallic acid equivalent (mg/g) ($y = 16.263x - 0.0699$, R^2=0.9944).

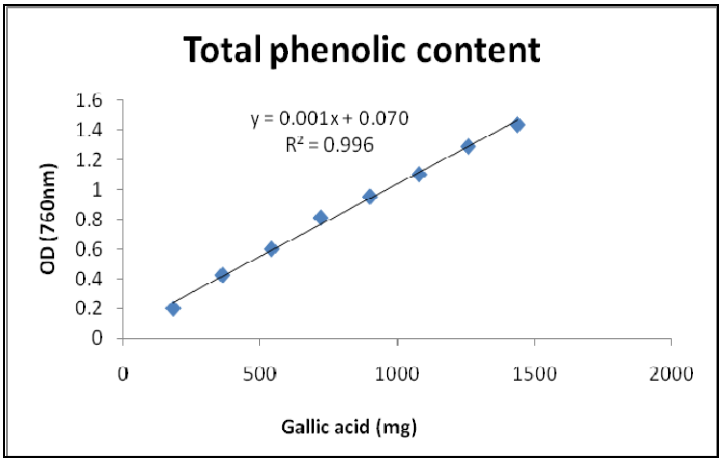

Fig. 1 Folin-Ciocalteau Gallic acid standard curve

Table 1 Antioxidant activity of the essential oil of *Jurinea leptoloba* DC.

Bleaching of 2,20-diphenylpicrylhydrazyl (DPPH)					
J. leptoloba oil (mg)	20	22	24	26	IC_{50} (mg/ml)
DPPH radical scavenging	36.93 ± 2.08	43.37 ±1.04	46.60 ±1.97	55.43±0.59	24.50
	Standard	1mM	BHT	38.26±0.54	
		1mM	BHA	49.14±0.75	
		1mM	Trolox	34.17±0.53	

β-Carotene - linoleic acid assay of the *J. leptoloba* oil			
	Sample	Concentration	% Antioxidant activity
	J. leptoloba oil	2.5 (mg/ml)	62.01 ± 2.45
Standard	BHT	1mM	86.21 ± 2.24
	BHA	1mM	80.88 ± 2.36

Comparison of antioxidant activity and total phenolic content of *Jurinea leptoloba* DC.oil				
DPPH IC_{50} (mg/ml)	DPPH radical scavenging (%)	FRAP (mg/g GAE)	β-Carotene-linoleic acid assay (%)	TPC (µg/mg GAE)
	Oil (mg)		Oil (mg/ml)	
24.50	55.43 ± 0.59	0.117 ± 0.01	62.01 ± 2.45	16.53 ± 5.69
	26 mg		2.5 (mg/ml)	

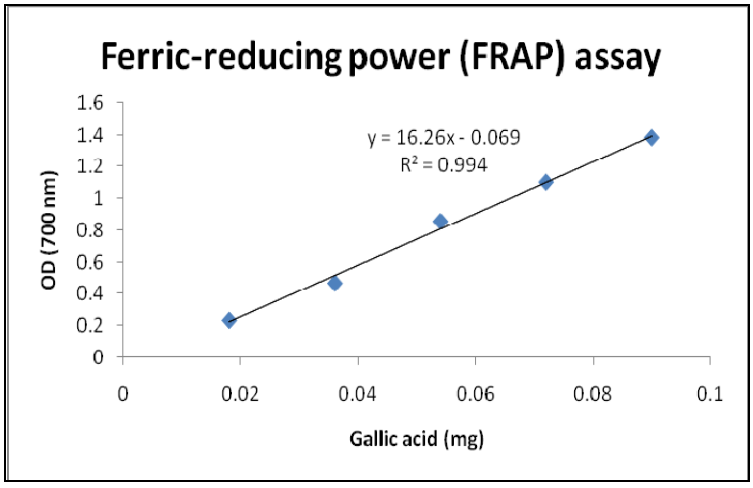

Fig. 2 Gallic acid standard curve for ferric reducing antioxidant power assay

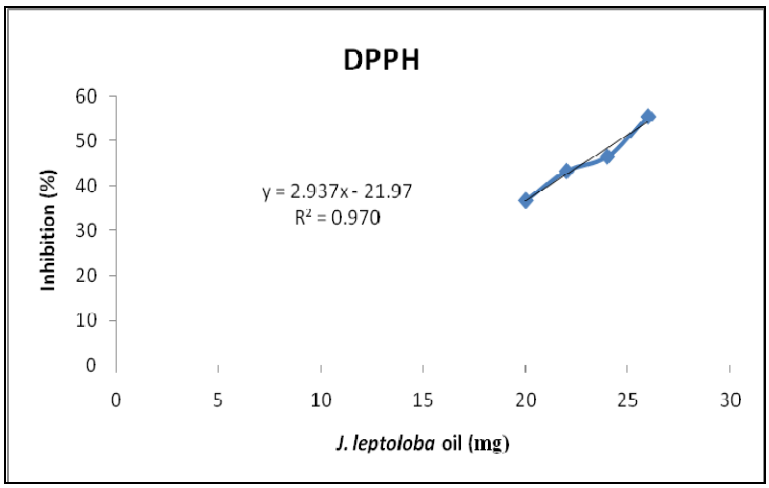

Fig. 3 Inhibition of DPPH activity

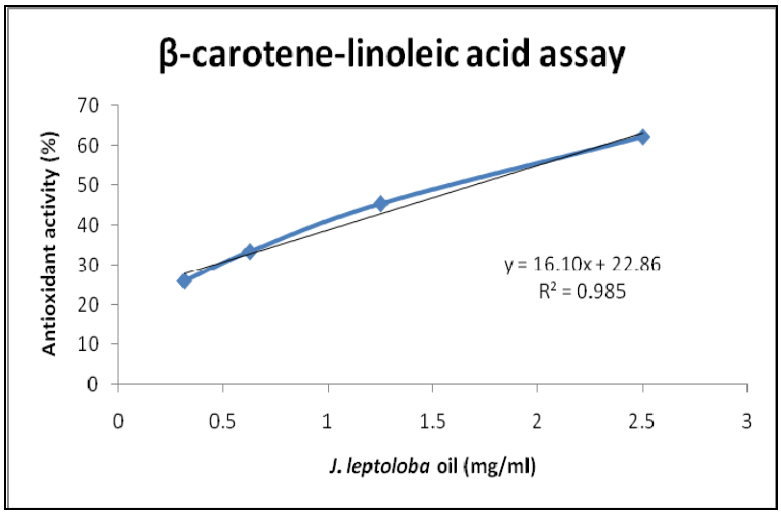

Fig. 4 β-carotene-linoleic acid test

Table 2 Cytotoxicity assay of *Jurinea leptoloba* DC. essential oil on HeLa and lymphocyte cells

Oil Dilutions (µg/ml)	% Viable HeLa cells	% HeLa cells Death
control	100	0
70	77.37 ± 3.16	22.62
140	65.74 ± 6.73	34.25
280	51.93 ± 5.28	48.07
IC_{50} (µg/ml)	290.76	
Oil Dilutions (µg/ml)	% Viable Lymphocyte cells	% Lymphocyte cells Death
control	100	0
700	80.07 ± 1.18	19.92
1400	74.41 ± 2.38	25.58
2800	50.42 ± 5.98	49.57
IC_{50} (µg/ml)	2900.97	

The essential oil of *Jurinea leptoloba* DC. has shown 55.43±0.59% (26 mg/ml of oil) inhibition of DPPH activity with an IC_{50}= 24.50 mg/ml (y = 2.9373x - 21.97; R^2 = 0.9701) (Fig. 3).

The radical scavenging effect of *Jurinea leptoloba* DC. essential oil was performed in the presence of BHT, BHA and Trolox as standards (Table 1). In β-carotene-linoleic acid test system, oxidation of linoleic acid was effectively inhibited by *J. leptoloba* essential oil (62.01±2.45%, amount of essential oil 2.5 mg/ml) (y = 16.109x + 22.862; R^2 = 0.9851) (Fig. 4).

Antioxidant activity percentage of *J. leptoloba* essential oil determined by β-carotene bleaching test revealed less potential than both standards BHT and BHA (Table 1).

Cytotoxicity Assay

Cytotoxicity was measured using a modified MTT assay. The cytotoxic effects of *Jurinea leptoloba* DC. essential oil were tested using lymphocyte and HeLa cells (Table 2). At a concentration of 280 µg/ml, oil destructed HeLa cells by 48.07% (Table 2), at lower doses, the oil was tolerated by the cells and its 50% cytotoxic concentration was 290.76 µg/ml (y = 0.1179x+15.719, R^2=0.9805) (Fig. 5). On the other hand at a concentration of 2800 µg/ml, oil destructed lymphocyte cells by 49.57% (y = 0.0145x+7.9358, R^2=0.9768) (Fig. 6). At lower doses, the oil was still toxic to the HeLa cells. The oil displayed moderate cytotoxic action towards the human tumor cell line.

Discussion

Total Phenolic Content

The phenolic assay involving an electron-transfer reaction evaluated by using Folin-Ciocalteu reagent. The results show that this essential oil has a relatively good phenolic content. The total phenol content (TPC) measures both types of antioxidants, hydrophobic and hydrophilic form complexes with Fe^{2+}. Phenols and flavonoids are known to inhibit lipid peroxidation by quenching lipid peroxy radicals and reduce or chelate iron in lipoxygenase enzyme and thus prevent initiation of lipid peroxidation reaction [13].

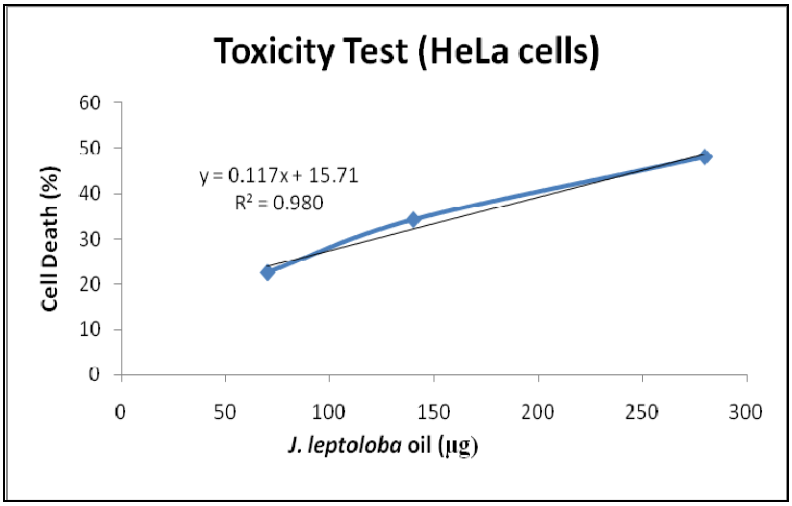

Fig. 5 Cytotoxicity effect on HeLa cells

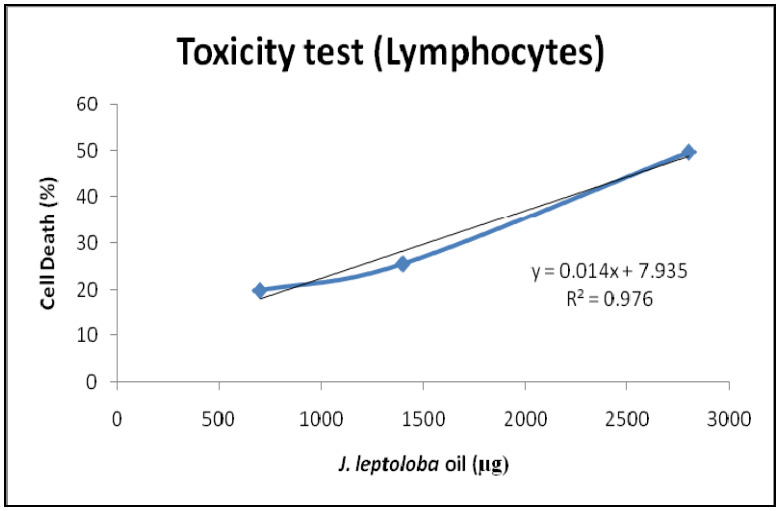

Fig. 6 Cytotoxicity effect on lymphocytes

Previously, no studies have been reported on the biological activities of the essential oil of *J. leptoloba*.

Antioxidant Activity

J. leptoloba oil exhibited a dose-dependent scavenging of DPPH radicals and 24.50 mg of the oil was sufficient to scavenge 50% of DPPH radicals/ml. The DPPH radical scavenging is independent of substrate polarity. DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule [14]. A significant correlation was shown to exist between the phenolic content and with DPPH scavenging capacity for each sample. The DPPH radical scavenging activity of *J. leptoloba* oil was found less potential than the standards BHT and BHA.

Results such as the relative abundance of phenolic compounds, and the significant correlations that existed between phenolic content and antioxidant capacity, as measured by β-carotene or DPPH scavenging methods, would appear to be highly consistent with corresponding results presented by previous researches [15]. Numerous reports indicated good correlation between the RSA and the concentration of phenolic compounds measured by Folin-Ciocalteu method. A great number of simple phenolic compounds as well as flavonoids can act as antioxidants, however, their antioxidant power depends on some important structural prerequisites, particularly on the number and the arrangement of hydroxyl groups, the extent of structural conjugation and the presence of electron-donating and electron-accepting substituents on the ring structure [16].

Cytotoxicity Assay

The IC_{50} values for HeLa and lymphocyte cells were calculated to be 290.76 µg/ml and 2900.97 µg/ml, respectively. The IC_{50} shows that cytotoxicity of the oil towards human tumor cell

line is much higher than that required for human healthy cells. These results indicate low adverse side effects of the oil. Cancer chemo prevention is defined as the use of chemicals or dietary components to block, inhibit, or reverse the development of cancer in normal or pre neoplastic tissue. A large number of potential chemo preventive agents have been identified, and they function by mechanisms directed at all major stages of carcinogenesis [17]. Essential oil constituents have a very different mode of action in bacterial and eukaryotic cells. For bacterial cells they are having strong bactericidal properties, while in eukaryotes they modify apoptosis and differentiation, interfere with the post-translational modification of cellular proteins, induce or inhibit some hepatic detoxifying enzymes. So, essential oils may induce very different effects in prokaryotes and eukaryotes [18]. In spite of the limitations of all *in vitro* studies with respect to *in vivo* impact, the present results are very promising as far as anti-neoplastic chemotherapy is concerned. This further forms a firm base for future research. From the mentioned results, it can be concluded that the essential oil of *J. leptoloba* may be exploited as a antioxidant and anticancer agent.

Conclusion

From the mentioned results, it can be concluded that the essential oil of *J. leptoloba* could be a natural radical scavenger and antioxidant agent. The cytotoxicity of the essential oil of *J. leptoloba* towards human tumor cell line is much higher than that required for human healthy cells. These results indicate low adverse side effects of this oil.

Acknowledgements

I wish to thank the Islamic Azad University's (Takestan Branch) research deputy office for the sanction of research grant to conduct the current research (Grant No. TIAU: 50155). The author would like to thank sincerely professor Rustaiyan for assistance in the botanical information. I would like to thank sincerely professor Iraj Rasooli in the medicinal plant research center of Shahed university for scientific assistance. I would also like to thank Dr. Mozaffarian for help in collecting plant material.

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