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ORIGINAL ARTICLE

In vitro cytotoxic and antioxidant activities of phenolic components of Algerian *Achillea odorata* leaves

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Abstract In this study, methanol extract from *Achillea odorata* was evaluated for its phenolic contents using Folin–Ciocalteu reagent, and antioxidant activity using: 1,1-diphenyl-2-picrylhidrazyl (DPPH) radical scavenging activity, reducing activity of H_2O_2 and ferric reducing power assay. The total phenolic content was determined as gallic acid (GAE) equivalent. Flavonoids and flavonols contents were determined as quercetin (QE) equivalents. The cytotoxicity of the plant extract was tested against three tumor cell lines: MCF-7, Hep2 and WEHI using 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphynyl tetrazolium bromide (MTT) assay. Preliminary screening was concluded in the presence of substances with large therapeutic values. The total phenolic content confirmed the presence of total phenolics in the extract and showed strong association with antioxidant activity. An important content of flavonoids and flavonols was also detected. The results of the antioxidant activities obtained indicate that *A. odorata* recorded a good capacity. For the cytotoxic activity, the results showed the plant extract significantly inhibited tumor cell growth and colony formation at various concentrations.

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1. Introduction

Herbal preparations have long been used as remedies for infectious and other diseases and they are used in primary health care in several countries (Sokmen et al., 1999). Since the prehistoric era, herbs have been the basis for nearly all medicinal therapy until synthetic drugs were developed in the nineteenth century (Exarchou et al., 2002). The preservative effect of

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many plant spices and herbs suggests the presence of antioxidative constituents in their tissues (Hirasa and Takemasa, 1998). Recently, interest has increased considerably in finding naturally occurring antioxidants to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (Sasaki et al., 2002). The antioxidative phytochemicals especially phenolic compounds found in vegetables, fruits and medicinal plants have received increasing attention for their potential role in prevention of human diseases. Phenolic compounds can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases (Anderson et al., 2001).

Yarrow (*Achillea L.*) is one of the youngest evolutionary genera of the Asteraceae family, which is present throughout the world. More than 100 species have been recognized in this genus (Goli et al., 2008; Rahimmalek et al., 2009). The use of yarrow for various medicinal purposes, for instance as a spasmolytic, a choleretic, a treatment for wounds and an anti-inflammatory agent, has made it an important medicinal plant (Benedek et al., 2007; Goli et al., 2008; Rahimmalek et al., 2009).

In Algeria, this aromatic plant has been used for their flavors in cooking and used in folk remedies as an appetizer, wound healer, diuretic, carminative or menstrual regulator. Leaves and flowers from *Achillea odorata* have been used for centuries for anti-inflammatory actions, such as rheumatism, skin inflammation and allergic rhinitis, wound healing and amelioration of diaphoresis and high blood pressure. The use of herbal teas from *A. odorata* especially against diseases of the gastrointestinal tract is quite common in folk medicine. *A. odorata* is also frequently used against diarrhea, abdominal pain and stomach ache in Algerian traditional medicine.

Since there is only a small number of reports in the literature studying the total phenolic content and no information about the biological activities of *A. odorata* leaves has been reported, the present study was designed to examine the efficacy of the methanol extract of *A. odorata* leaves as an antioxidant to find out new potential sources of natural antioxidants and to evaluate its antiproliferative effects on three tumor cell lines: WEHI, MCF-7 and Hep2.

2. Materials and methods

2.1. Plant material

A. odorata leaves were collected in May 2011 from Ouled Askar, Jijel (Algeria). After being thoroughly cleaned, the leaves of the plant were dried in the open air then in a Memmert type oven, at a temperature of 40 °C, until the stabilization of weight. Air-dried leaves were ground using an electric grinder type Sayona, model: SJ-Y8200R to obtain very fine powder. Only fractions less than 50 microns were used for extraction.

2.2. Polyphenol extraction and concentration

The extraction of polyphenols was carried out at ambient temperature for 48 h by maceration in methanol–water 80/20 (v/v) at a solid–liquid ratio of 1/10 (w/v) with continuous stirring. The solutions are then filtered with filter paper (No. 1). The aqueous phase obtained underwent defatting with hexane, referring to the procedure of Yu et al. (2005). The covered methanol phase undergoes a concentration to dryness using a rotary evaporator (Heidolph, LABOROT4003) with a vacuum controller, at a temperature of 40 °C. The extraction yield was calculated as follows:

$$\text{Yield } (\%) = W1/W2 \times 100,$$

where W1 is the weight of extract after concentration and W2 is the weight of the dried powder of plant material. Concentrated extracts were stored in a refrigerator (4 °C) until analyzed.

2.3. Phenolic content of the extract

2.3.1. Total phenolic content

The amount of total phenolics was assessed using Folin–Ciocalteu reagent as described by Othman et al. (2007). 0.2 ml of sample was mixed with 1.5 ml of Folin–Ciocalteu reagent. The mixture was kept at room temperature for 5 min, and then 1.5 ml of 7.5% Na_2CO_3 solution was added. The final mixture was shaken and then incubated for 1 h in the dark at room temperature. The absorbance of all samples was measured at 750 nm using a Shimadzu UV mini 1240 spectrophotometer. Amounts of total phenols were calculated using a calibration curve for gallic acid. The results were expressed as mg of gallic acid equivalents (GAE) per g of crude extract. (CE) All samples were analyzed five times and results averaged.

2.3.2. Total flavonoid content

The flavonoids content in extracts was determined spectrophotometrically according to Djeridane et al. (2006) using a method based on the formation of a complex flavonoid–aluminum, having the maximum absorbance at 430 nm. Quercetin was used to make the calibration curve. 1.5 ml of diluted sample was mixed with 1.5 ml of 2% aluminum chloride solution. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 430 nm with a Shimadzu UV mini 1240 spectrophotometer and the flavonoids content was expressed in mg of quercetin equivalent (QE) per g of crude extract. The test is carried out in five replicates.

2.3.3. Flavonol content

The content of flavonols was determined by the modified colorimetric method described by Abdel-Hameed (2009). Quercetin was used to make the calibration curve.1 ml of 2% aluminum chloride and 3 ml of 5% sodium acetate were added to 1 ml of extract. The mixture is thoroughly homogenized and allowed to stand for 30 min at room temperature. The absorbance of all samples was measured at 440 nm using a Shimadzu UV mini 1240 spectrophotometer and the results are expressed in mg of quercetin equivalent per g of crude extract. Five repetitions are performed.

2.4. In vitro assay for cytotoxic activity (MTT assay)

2.4.1. Cell line and culture

MCF-7 (human breast adenocarcinoma), Hep2 (human epiglottis cancer) and WEHI (mouse leukemia) cell cultures were

obtained from Molecular Immunoparasitology laboratory, Yarmouk University (Irbid, Jordan). The cell lines were cultured in growth medium (RPMI-1640 medium, pH 7.4), supplemented with 10% fetal bovine serum (FBS) and antibiotics [(penicillin (100 units/ml) and streptomycin (100 μg/ml)]. The cell lines were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity.

2.4.2. Cytotoxicity assay

Microculture tetrazolium (MTT) assay (also known as mitochondrial reduction assay), a colorimetric assay developed by Takenouchi and Munekata (1998) was used to evaluate cell vitality. Briefly, the monolayers were trypsinized and the cells were seeded in 96-well plates at the density of 5×10^4 cell/well (100 μl/well) in a culture medium and incubated for 24 h at 37 °C, with 5% CO_2 in a humidified atmosphere. Later, the medium was removed and fresh growth medium containing different concentrations of plant extracts (5, 25, 50, 125, 250 and 500 μg/ml) were added separately. Stock solutions of extracts were obtained by dissolving samples in DMSO followed by filtration through syringe filters (pore diameter 0.2 mm). Stock solutions were further diluted in culture media to obtain necessary concentrations for the experiments. Simultaneously, the cytotoxicity of DMSO in the concentrations present in dilutions of stock solutions was evaluated. Control cells were supplemented only with a medium and colchicin was used as a positive control. The concentration of DMSO was kept at or below 0.1% in all experiments. After 68 h of incubation at 37 °C, 5% CO_2, medium was removed and 20 μl of 5 mg/ml MTT (pH 7.4) was added per well and cultivated for another 4 h. The supernatant fluid was removed and 100 μl DMSO was added per well and shaken for 15 min. The absorbance was then determined by an ELISA reader (Bio-Rad, USA) at a wavelength of 570 nm. The values are presented as means of duplicate analyses. The effect of extract on the proliferation of cancer cells was expressed as the % of cytoviability, using the following formula:

$$\%\,\text{cytotoxicity} = (A_{570}\,\text{control} - A_{570}\,\text{sample})/A_{570}\,\text{control} \times 100$$

2.5. Quantification of antioxidant activity

2.5.1. DPPH free-radical scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical that has been widely used as a tool to estimate the free-radical scavenging activity of antioxidants. The reduction capacity of the DPPH radical was determined by the decrease of absorbance induced by antioxidants, according to Brands-williams et al. (1995) with a few modifications. The reaction system consisted of 0.1 ml of extract and the standards (α-tocopherol and gallic acid) diluted to different concentrations (25, 50, 75, 100 and 125 μg/ml) and 2.9 ml of a 0,025 g/l DPPH in methanol. The mixture was shaken vigorously and left standing at room temperature in the dark for 30 min. The absorbance was measured at 515 nm against a blank. The ability to scavenge the DPPH radical was calculated using the following formula:

$$\text{DPPH scavenging effect } (\%) = (A_0 - A_1)/A_0 \times 100$$

where:

A_0: is the absorbance of the control at 30 min.
A_1: is the absorbance of the sample at 30 min.

Tests were carried out in triplicate.

2.5.2. Ferric reducing power assay

The ferric reducing antioxidant power assay, is based upon reduction of a ferric tripyridyltriazine ($Fe^3 + -TPTZ$) complex to the ferrous tripyridyltriazine ($Fe^2 + -TPTZ$) by a reductant under acidic conditions. Increase in absorbance of blue-colored ferrous form is measured at 700 nm. The reductive potential of the extract and the standard (α-tocopherol) was determined according to the method of Hseu et al. (2008). The different methanolic concentrations (1 ml, 25, 50, 75, 100 and 125 μg/ml) were mixed with phosphate buffer (1 ml, 0.2 M, pH 6.6) and potassium ferricyanide ($K_3Fe(CN)_6$; 1 ml, 1%). The mixture was then incubated at 50 °C for 20 min. Afterward, 1 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. Finally, the upper layer of solution (1.5 ml) was mixed with distilled water (1.5 ml) and ferric chloride ($FeCl_3$) solution (150 μl, 0.1% w/v), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The assay was carried out in triplicate.

2.5.3. Neutralization of the radical hydrogen peroxide (H_2O_2)

The ability of the hydro-methanol extract and standard (α-tocopherol) to neutralize the hydrogen peroxide was determined according to the method of Brands-williams et al. (1995) with some modifications. An aliquot of 2 ml extract or standards at different concentrations (25, 50, 75, 100 and 125 μg/ml) was mixed with 1.5 ml of 40 mM H_2O_2 prepared in 0.1 M phosphate buffer, pH = 7.4. A control was prepared containing only H_2O_2. After incubation for 10 min in the dark, the absorbance against a blank was recorded at 230 nm. The reducing percentage of H_2O_2 was calculated according to the following equation:

$$\text{Reducing percentage of } H_2O_2\ (\%) = (A_0 - A_1)/A_0 \times 100$$

such as:

A_0 is the absorbance of control and A is the absorbance of the extract.

The analyses were done in triplicate.

3. Results

3.1. Extraction yield

The extraction yield was 18.2% (18.2 g of dry extract/100 g of dry plant material).

3.2. Phytochemical study of the extract

Phenolic substances have been shown to be responsible for the antioxidant activity of plant materials. Therefore, the content of total phenol compounds, as determined by the Folin–Ciocalteu method, are reported as gallic acid equivalents by reference to standard curve ($y = 0.0024x + 0.0886$, $r^2 = 0.9142$). Total soluble phenolic constituents of the MeOH extract were found as 448.8 ± 1.04 mg GAE/g CE (Fig. 1). The results of this study on the *A. odorata* extract demonstrated that the plant extract was enriched in flavonoids

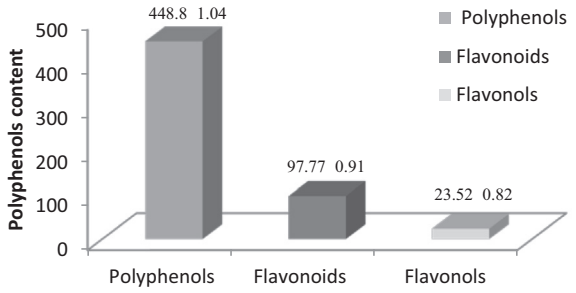

Figure 1 Content of the *Achillea odorata* extract in total polyphenols, flavonoids and flavonols.

(97.77 ± 0.91 mg QE/g CE) by reference to standard curve ($y = 0.0087x + 0.1121$, $r^2 = 0.9608$), and flavonols (23.52 ± 0.82 mg QE/g CE) by reference to standard curve ($y = 0.004x + 0.2241$, $r^2 = 0.9603$).

3.3. In vitro cytotoxicity

Cellular proliferation was estimated by the MTT assay using MCF-7, Hep2 and WEHI cells treated with varying concentrations of plant extract or positive control (colchicin) for 72 h. As shown in Table 1, *A. odorata* polyphenols were active against all the three cancer cell lines tested and inhibited cellular proliferation in a dose-dependent manner for the three cell lines studied. The extract showed good antiproliferative activity on WEHI and hep2 cell lines. However, on MCF7 the extract showed a moderate inhibition of proliferation. The percentage of dead cells in the case of MCF-7, Hep2 and WEHI was found in the order of 42.90%, 61.54% and 81.13%, respectively, for the extract concentration of 50 µg/ml which was better than that of colchicin which was 42.04%, 60.83% and 81.01% respectively for the same concentration. No cytotoxic effect of DMSO in the concentrations present in dilutions of stock solutions was determined (Table 2).

3.4. Antiradical power

Antioxidants either transfer an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character. The results of the scavenging ability of the methanolic extract and the standards expressed in percentage are illustrated by Fig. 2. Plant extract and standards (gallic acid and α-tocopherol)

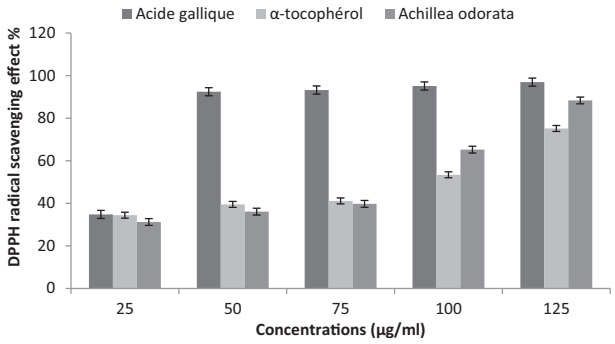

Figure 2 DPPH radical scavenging effect against the methanol extract of *A. odorata* and standards.

quenched DPPH radicals in a dose-dependent manner. At 100 and 200 µg/ml, the extract exhibited a high activity (65.24 and 88.34, respectively) which is better than that of α-tocopherol (53.39 and 75.1734, respectively).

3.5. Ferric reducing power assay

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of different concentrations of the methanolic extract. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Fig. 3 depicts the reductive effect of the extract. Similar to the antiradical activity, the reducing power of the extract increased with increasing dosage.

3.6. Neutralization of the radical hydrogen peroxide (H_2O_2)

The percentages of inhibition of H_2O_2 of the extract and standard at different concentrations tested are shown in Fig. 4. From the curves plotted the percentage of H_2O_2 scavenger increases with the concentration of the extract. The extract exhibited a high activity (89;97%) at 125 µg/ml, which is comparable with that of α-tocopherol

4. Discussion

The extraction of bioactive compounds can be described as a mass transport phenomenon where solids contained in plant structures migrate into the solvent, up to their equilibrium

Table 1 Cytotoxic activity of *Achillea odorata* on MCF-7, Hep2 and WEHI cancer cells. Results are mean ± SD.

Concentrations (µg/ml)	5	25	50	125	250	500	Colchicin
MCF-7	38.92 ± 0.64	38.92 ± 0.64	42.90 ± 0.36	45.66 ± 0.36	47.04 ± 0.28	48.52 ± 0.5	42.04 ± 0.43
Hep2	32.82 ± 0.12	48.08 ± 0.29	61.54 ± 0.126	62.29 ± 0.93	65.02 ± 0.21	65.61 ± 0.21	60.83 ± 0.04
Wehi	63.19 ± 1.34	78.40 ± 1.54	81.13 ± 0.48	82.63 ± 0.32	83.34 ± 0.28	84.77 ± 0.26	81.01 ± 0.06

Table 2 Cytotoxic activity of *DMSO* on MCF-7, Hep2 and WEHI cancer cells. Results are mean ± SD.

Concentrations (µg/ml)	5	25	50	125	250	500
MCF-7	0.05 ± 0.07	0.05 ± 0.5	0.07 ± 0.18	0.15 ± 0.07	0.20 ± 0.28	0.25 ± 0.07
Hep2	0.05 ± 0.33	0.05 ± 0.16	0.11 ± 0.25	0.17 ± 0.33	0.20 ± 0.04	0.23 ± 0.25
Wehi	0	0	0	0	0	0

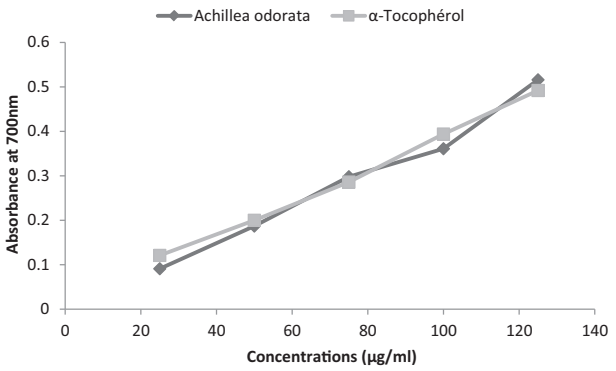

Figure 3 Reducing power of *A. odorata* extract and α-tocopherol at different concentrations.

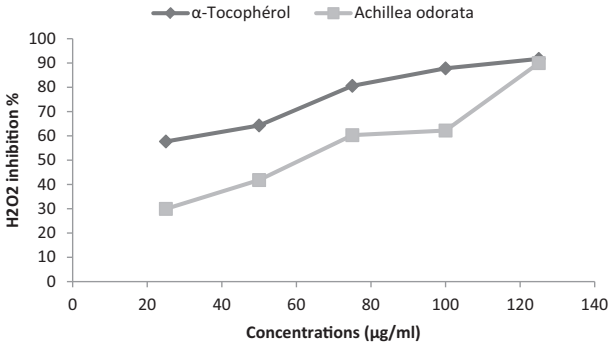

Figure 4 H_2O_2 scavenger effect by crude extracts and standard.

concentration. Mass transport phenomenon can be increased by heating, changes in concentration gradients, and under the influence of new technologies such as ultrasonics, high pressure, and pulsed electric field (Corrales et al., 2008).

The extraction of phenolic compounds from plant material is influenced by their chemical structure, method of extraction, the size of the particles forming the sample, the time and the conditions of storage as well as the present of interferents (Naczk and Shahidi, 2004). The solvent is also one of the parameters that can affect the extraction of polyphenols (Trosznska et al., 2002).The extraction may be carried out by several solvents as water, methanol, ethanol and acetone. Otherwise, the aqueous solvents give the best yields of extraction than the absolute solvents (Spigno et al., 2007).

There is abundant evidence that a great number of medicinal plants contain chemical compounds exhibiting antioxidant and anticancer properties. With this respect a particular interest has been given to plant polyphenols. All the phenolic classes have the structural requirements of free radical scavengers and have potential as food antioxidants (Bandoniene and Murkovic, 2002), so phenolic compounds are attracting considerable interest in the field of chemistry, food and medicine.

Experimental investigations demonstrated that many naturally occurring agents and plant extracts have shown antioxidant and anticancer potential in a variety of bioassay systems and animal models, having relevance to human disease (Aziz et al., 2003). Among them, phenolic compounds which are powerful antioxidants were considered as interesting molecules against anti-inflammatory, antimutagenic and anticancer activities (Vuorela et al., 2005). In fact, the anticancer activity of *A. odorata* has not been shown in the literature and the important antioxidant activity in this species related to their phenolic compounds prompted us to investigate their anticancer capacity.

Several plant species rich in polyphenols in particular flavonoids are reported to have disease preventive and therapeutic properties. This observation is of particular importance since flavonoids are ingredients of many vegetables and fruits and the association of vegetable and fruit consumption with reduced cancer risk has been reported (Kanadaswami et al., 2005; Ramos, 2007; Ren et al., 2003). In addition, the *in vitro* data support findings that a mixture consisting of these sterol compounds exerted cytotoxic activity against human lung and breast cancer cells (Sundarraj et al., 2012). Some phenolics may also alter hormone production and inhibit aromatase to prevent the development of cancer (Zhao et al., 2007). Cytotoxic activity recorded in the present study is in accordance with this finding, since the phytochemical evaluation indicates the presence of flavonoids with promising activity. The cytotoxic activity of active plants is probably due to the presence of flavonoids. From this study, it is evident that the methanolic extract from *A. odorata* has strong dose dependent *in vitro* cytotoxic activity against the three cell lines studied.

The antioxidant activity of the plant extract is probably due to its phenolic contents. It is well known that phenolic compounds are constituents of many plants, and they have attracted a great deal of public and scientific interest because of their health promoting effects as antioxidants (Hollman and Katan, 1999). The phenolic compounds exhibit considerable free radical scavenging activities, through their reactivity as hydrogen- or electron-donating agents, and metal ion chelating properties (Rice-Evans et al., 1996). The extracts were investigated regarding their composition by different colorimetric techniques, such as the content of total phenolic compounds by the Folin–Ciocalteu assay and flavonoids by $AlCl_3$ reagent.

DPPH is one of the compounds that possessed a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Yamaguchi et al., 1998). Further it is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability. DPPH is a free radical compound and has been used widely to test the free radical-scavenging ability of various samples. Antioxidants react with DPPH, reducing a number of DPPH molecules equal to the number of available hydroxyl groups.

Several authors previously confirmed that gallic acid and α-tocopherol are powerful antioxidants, particularly with high antiradical activity (Gülçin et al., 2004). According to the present work, the methanolic extract of *A. odorata* leaves showed a powerful antiradical activity, since it showed a high scavenging ability which was better than that of α-tocopherol. That activity may be due to the presence of polyphenolic compounds in the extract.

Many epidemiological studies showed that phenolic compounds have beneficial effects on human health due to their antioxidant activity. Since a good correlation between antioxidant activities and reducing power has been proved, reducing power assay has been used extensively to measure the total antioxidant power in some plant extracts (Benzie and Strain, 1999).

Various mechanisms, including reducing capacity, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging have been claimed to explain the antioxidant activities. The reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity (Hsu et al., 2006).

Except for superoxide radical, hydroxyl radical is considered to be a highly potent oxidant, which can react with all biomacromolecules functioning in living cells. Previous studies of the antioxidant activity of various natural plant derived biomolecules have suggested that the scavenging activity for hydroxyl radicals was not due to direct scavenging but due to inhibition of hydroxyl radical generation by chelating ions such as Fe^{2+} and Cu^{+} (Diplock, 1997).

Due to this high reactivity of hydroxyl radicals, measurements based on scavenging hydroxyl radicals, such as the non site-specific method, are not accurate measurement of oxidative protection of an antioxidant molecule *in vivo* (Halliwell and Gutteridge, 1999). This is because the radical is more likely to be scavenged by direct reaction with other surrounding molecules before it can attack its target molecule.

5. Conclusion

Concluding, this is the first report concerning cytotoxicity and antioxidant activities of *A. odorata* methanolic extracts. According to data obtained from the present study, the extract was found to be an effective antioxidant in different *in vitro* assays including DPPH radical, reducing power and hydrogen peroxide scavenging activities when it is compared to standard antioxidant compounds such as gallic acid and α-tocopherol. In addition, it has been demonstrated that the *A. odorata* leaves is a potential antiproliferative agent. The antioxidant and antiproliferative activities of *A. odorata* leaves might be due to the synergistic action of bioactive compounds present in it. Further studies will be needed to identify the active compounds that confer the antioxidant and/or anticancer activities of the *A. odorata* extract. Once such compounds are identified, the mechanisms by which they exert their effects can begin to be characterized.

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