Antioxidant properties of Zhumeria majdae Rech.f. & Wendelbo

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ABSTRACT

Zhumeria majdae Rech (ZM) has a limited geographic range in southern region of Iran (near Persian Gulf). The leaves of this plant are using as a herbal tea, antiseptic and analgesic agent. Ethanol extract of Zhumeria majdae leaves and polar fractions were screened for their reducing power, radical scavenging capacity, inhibition of lipid peroxidation and anti toxicity effect on non-immortalized fibroblasts. The total phenolic compounds of Zhumeria majdae extract and polar fractions were determined, in order to assess their antioxidant activities. Zhumeria majdae extract and polar fractions showed antioxidant potential but ethyl acetate fraction was more active. Also, the phenolic compounds of ethyl acetate fraction was more than crude extract and butanol fraction. Crude extract of Zhumeria majdae (12.5-25 µg/ml) was not toxic on human non-immortalized fibroblasts. This result may confirm the antitoxic effect of Zhumeria majdae.

Keywords: Zhumeria majdae, Labiatae, Antioxidant activity, Phenolic compounds, Anti-toxic effect.

INTRODUCTION

Oxidant compounds which are produced in metabolism of macromolecules cause damage DNA and proteins as well as lipids [1-3]. These damages may contribute to different diseases such as aging, cancer, cardiovascular diseases, immune system decline, brain dysfunction and cataracts. In these diseases agents such as superoxides, hydrogen peroxide, hydroxyl radical as well as nitrogen oxyradicals are responsible [3]. There are many antioxidant compounds which protect the cells against free radicals. These antioxidants include exogenous and endogenous, whether synthetic or natural have been used for the treatment of disorders attributed to free radical oxidative damages [4-6].

On the other hand, using of synthetic antioxidants has been suspected to cause or promote negative health effects. Hence there is a trend to substitute them with naturally antioxidant. Many other plant species besides fruit and vegetable were found to possess antioxidant properties [7-11].

The antioxidant activity of plant compounds, in the form of crude extract or isolated constituents have been widely considered [12-13]. Phenolic compounds have attracted considerable attention because they possess antioxidant activity. It has been reported that these compounds are more powerful antioxidant than vitamins C, E and β-carotene [14]. Consumption of phenolic compounds such as flavonoids and their potential significance as inhibition of oxidative stress has been the main subject of many investigations [12-13].

The aim of present study was to investigate the antioxidant properties and antitoxic effect ZM crude extract.

MATERIALS & METHODS:

Materials:

BHT (Butylated hydroxyl toluene), DPPH (2, 2- Diphenyl-1-picrylhydrazyl), gallic acid, MTT (2,5-Diphenyl tetrazolium bromide) were purchased from Sigma (ST. Louis, MO, USA). All other reagents were obtained from Merck Chem. Co. Human non-immortalized fibroblasts (HFFF-P16) obtained from Iran Pasteur Institute, Tehran, Iran.

Extraction and Fractionation of Plant Extract

Aerial parts of ZM were collected before flowering in March 2004, from Fin (80 km faraway from Bandar-e-abbas, Capital of Hormozgan...
Protein materials were identified by one of us (M.R.Moein). A voucher specimen (no.138) was deposited at the Herbarium of Faculty of Pharmacy, Shaheed Beheshti University of Medical Sciences, Tehran, Iran. Aerial parts of ZM (5000 g) were extracted with (ethanol 37% v/v) to give 1064 g of concentrated crude extract. The ethanol extract was suspended in MeOH 50% (500 cc) and extracted with n-Hexane (3× 500 cc). The residue was concentrated and dissolved in water and the aqueous was extracted with chloroform (3× 500 cc), ethyl acetate (3×500 cc) and n-buthanol (3× 500 cc), respectively.

**Reducing Power Assay**

The reducing power of ZM crude extract and fractions were determined using the method described by Yen and Duh [19]. Two and half ml of various concentrations of ZM extract (12.5, 25, 50, 100 and 200 µg/ml) were made in 2.5 ml of 0.2 M phosphate buffer pH 6.6 containing 2.5 ml of 1% potassium ferrocyanide. The mixture was incubated at 50°C for 20 minutes. To 5 ml of this mixture, 2.5 ml of 10% TCA was added and centrifuged at 3000 g for 10 minutes. The upper layer mixed with 2.5 ml of distilled water containing 0.5 ml of ferric chloride 1%. The absorbance of this mixture was measured at 700 nm. The amount of absorbance showed the antioxidant activity of the samples [19]. The assays were carried out in triplicate and the results are expressed as mean ± standard deviation (SD). Gallic acid and BHT were used as standards.

**Radical Scavenging Activity**

The capacity to scavenge the stable free radical 2, 2 –diphenyl-1-picrylhydrazyl (DPPH) was determined in a modified assay [20]. Two hundred µl of a 100 mM solution of DPPH radical in methanol was mixed with various concentrations (12.5- 400 µg/ml) of ethanol extract of ZM (20 µl). The mixture was shaken vigorously and left to stand for 30 min in the dark. The DPPH radical inhibition was measured at 490 nm by using a microplate reader (model Panasonic Kx-P108 athos 2020) at 492 nm. Reading of all samples were performed immediately (t=0) and after 105 min of incubation [22]. The AOA coefficient (ACC) was given by the equation:

\[
ACC = \frac{[(A_{105}-A_{105})/(A_{0}-A_{105})]}
\]

Where “A” is the absorbance of the color formed in microplates wells, DPPH used as control (without plant extract), blank contains methanol.

**β-Carotene Bleaching Test**

A modified method described by Dapkevicius et al [21] was used: 1 mg of β-carotene was dissolved in 5 ml of chloroform and 25 µl of linoleic acid and 200 mg of tween 40 was added to 1 ml of this solution. After chloroform was evaporated at 40°C under vacuum, 50 ml of oxygenated pure water was added (oxygenation was performed by bubbling air through water for 15 min).

This emulsion was freshly prepared before each experiment. Stock solution of extract (0.1%) and antioxidant standard of (0.01%) BHT and or gallic acid were prepared in methanol. An aliquot of 250 µl of β-carotene/linoleic acid emulsion was distributed in each of the well of microplate plates and 30 µl of methanol crude extract and fractions were added. An equal amount of this extract and fractions were used for blank. The microplates were incubated at 55°C and absorbencies were measured using a microplate reader model (Panasonic Kx-P108 athos 2020) at 492 nm. Reading of all samples were performed immediately (t=0) and after 105 min of incubation [22].

A modified version of the formula of Chevolleau et al [23]. Where A, 105 and A, 0 are the absorbencies of the test and blank at zero and 105 min respectively and A, 105 is the absorbencies of blank at t=0 min.

**Determination of Total Phenolic Content**

The content of total phenolic compounds in ZM methanol extract and fractions were determined by folin-ciocalteu method [24]. For the preparation of calibration curve 1 ml aliquots of 0.024, 0.075, 0.105 and 0.3 mg/ml methanol gallic acid solutions were mixed with 5 ml folin-ciocalteu reagent (diluted ten –fold) and 4 ml (75 g/l) sodium carbonate. The absorption was read after 30 min at 20°C at 765 nm and the calibration curve was drawn. One ml methanol plant extract (10 g/l) was mixed with the same reagents as described above, and after 1 h the absorption was measured for the determination of plant phenolics. All determinations were performed in triplicate. Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) were calculated by the following formula:

\[
C = \frac{C \times v \times m}{m}
\]

Where: C is total content of phenolic compounds, mg/g plant extract, in GAE; c is the concentration of galic acid established from the calibration curve, mg/ml; v is the volume of extract, ml; m is the weight of pure plant methanol extract, g.

**Cell Culture and Treatments**

Human non–immortalized fibroblast cell were bought from Pasteur institute, Tehran, Iran. The cells were grown at 37°C in humidified 5% CO₂, 95% air mixture in DMEM (Dulbecco’s modified Eagle medium) supplemented with 15% fetal calf serum (FCS) 1 ml-glutamine, 100 µg/ml penicillin-streptomycin [25]. The methanol extract of ZM was dissolved in dimethyl sulphoxide (DMSO).

Under this condition DMSO was neither toxic nor DNA damaging [25]. Extracts (12.5- 25 µM/ml) were added to the culture cells (32×10⁴ cells/ml) for 24 hours. BHT (12.5-25 µM) was used as a standard.
Table 1. IC₅₀ of Zhumeria majdae extract and fractions compared with BHT and gallic acid

<table>
<thead>
<tr>
<th>Antioxidant samples</th>
<th>IC₅₀ µg/ml</th>
</tr>
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<tbody>
<tr>
<td>BHT</td>
<td>63.17 ± 0.9</td>
</tr>
<tr>
<td>gallic acid</td>
<td>2.49 ± 0.6</td>
</tr>
<tr>
<td>Zhumeria majdae crude extract</td>
<td>&gt;400 ± 15</td>
</tr>
<tr>
<td>Zhumeria majdae ethyl acetate fraction</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>Zhumeria majdae butanol fraction</td>
<td>400 ± 12</td>
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Table 2. Antioxidant activity of Zhumeria majdae extract and fractions were compared with BHT and gallic acid as measured using the BCBT.

<table>
<thead>
<tr>
<th>Antioxidant sample</th>
<th>ACC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHT</td>
<td>868.6 ± 15.7</td>
</tr>
<tr>
<td>gallic acid</td>
<td>754.03 ± 44.28</td>
</tr>
<tr>
<td>Zhumeria majdae crude extract</td>
<td>-36.5 ± 5.2</td>
</tr>
<tr>
<td>Zhumeria majdae ethyl acetate fraction</td>
<td>295.2 ± 28.2</td>
</tr>
<tr>
<td>Zhumeria majdae butanol fraction</td>
<td>189.2 ± 27.37</td>
</tr>
</tbody>
</table>

BCBT: β-carotene test. *ACC: antioxidant activity coefficient by BCBT method given as ACC.

Table 3. Absorbance of different concentration of Zhumeria majdae crude extract using MTT test.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Viability of the cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Zhumeria majdae crude extract 12.5 µg/ml</td>
<td>68.7 ± 5.2</td>
</tr>
<tr>
<td>Zhumeria majdae crude extract 25 µg/ml</td>
<td>94.17 ± 3.14</td>
</tr>
<tr>
<td>BHT 12.5 µM/ml</td>
<td>54.17 ± 2.9</td>
</tr>
<tr>
<td>BHT 25 µM/ml</td>
<td>56.7 ± 5.84</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD of three experiments. Control contains fibroblast cells, MTT and cell culture.

Fig. 1: Reducing power of Zhumeria majdae extract and fractions compared with standards BHT and gallic acid (gallic acid is diluted 1:10). Each value is expressed as mean ± standard deviation (n=3).

Fig. 2: Radical (DPPH) scavenging activity of Zhumeria majdae extract and fractions compared with standards BHT and gallic acid (gallic acid is diluted 1:10).

acidified isopropanol and 20 µl of 3% (W/V) dodecyl sulphate in water [25]. The optical density of each sample was measured with a microplate spectrophotometer reader (model Panasonic Kx-P108 athos 2020) at 570 nm. Three replicates were performed for each sample.

Statistical analysis

IC₅₀ values were calculated by Matlab program. Means ± SD were calculated. The data were analyzed for statistical significance using one way ANOVA followed by Tukey post test. P values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Zhumeria majdae, a newly described plant was first collected by Miss Majda Zhumer in 1966. The plant was subsequently described as the first member of a new genus Zhumeria [17]. In this research, ZM extract and polar fractions were evaluated for their reducing power, free radical scavenging capacity, inhibition of lipid peroxidation, antitoxicity effect and determination of phenolic compounds. Reducing power was measured the absorbance at 700 nm after mixing the samples with ferric compounds; higher absorbance indicates the higher reducing power [14]. Figure 1 shows the reducing power of ZM extract and polar fractions as a function of their concentration. In this assay, the presence of reducer (i.e. antioxidants) causes the reduction of the Fe³⁺/ferricyanide complex to the ferrous form, blue complex [26]. The reducing power of the ZM extract and polar fractions increased with concentration. The reducing power of ZM extract and polar fractions were lower than BHT and gallic acid (antioxidant standards). But the reducing power of ethyl acetate fraction was more than crude extract and butanol fraction. It means that this fraction contains more antioxidant compounds. Previously, it was reported that the reducing power of plant extract might be due to their hydrogen donation ability [26].

The scavenging effects on DPPH radical were determined at 492 nm due to the reduction DPPH radical. The radical scavenging
activity of ZM extract and polar fractions were tested by using a methanol solution of the “stable” free radical, DPPH. Solution of DPPH exhibits a deep purple color which disappears when an antioxidant is added in the medium. In other words, antioxidant molecules can change the color of DPPH free radical to a colorless product [14]. Figure 2 depicts radical scavenging effects of ZM extract, its butanol and ethyl acetate fractions with standards of BHT and gallic acid in equivalent concentration. The IC50 of BHT and gallic acid were found to be 68 µg/ml and 2.49 µg/ml, respectively.

Table 2 showed that IC50 of ethyl acetate fraction is less than BHT. ZM crude extract and butanol fraction. It was reported that phenolic compounds inhibit the oxidation of the macromolecules by donation H atoms. The amount of these compounds are higher in polar fractions of plant extract [27, 28].

Inhibition of lipid peroxidation was determined using β-carotene; more inhibition of β-carotene bleaching indicates the higher antioxidant activity.

The results of β-carotene bleaching test illustrated in (Table 2). Crude extract of ZM couldn’t inhibit the β-carotene peroxidation. In other researches it was reported that a negative value in ACC showed the plant extract containing prooxidant activity [13, 29]. We observed that ZM crude extract is rich of fatty acids and waxes. It may be possible that these compounds involve in prooxidant activity of ZM crude extract.

The ethyl acetate and butanol fractions of ZM (may be due phenolic compounds) can inhibit β-carotene bleaching and there is a significant difference in ACC amount between these two fractions (p<0.05). The antioxidant activity coefficient (ACC) of ethyl acetate is more than butanol fraction (Table 2).

Anti-toxicity effect of crude extract was determined using MTT. MTT test is used for evaluation the viability of the cells. The viability of the fibroblasts cells in different concentration (12.5-25 µg/ml) of ZM extract was compared with control. Control contains fibroblast cells, cell culture, MTT reagent and its viability was equal 100. The viability of the cells in 12.5 and 25 µM of BHT was also shown (Table 3). The viability of the cells in 25 µg/ml of ZM crude extract was more than 12.5 µg/ml (Table 3). This result may confirm the usage of ZM leaves (in higher concentration) by people of Hormozgan Province without any reported toxic effect.

It had been reported that the antioxidant activity of plant extract is correlated with the amount of their phenolic compounds [12]. In fact, phenol such as BHT and gallate, are known to be effective antioxidants. The contents of total phenolic compounds in ZM crude extract, ethyl acetate and butanol fractions were 24.8±0.6, 44.5±4.2 and 34.7±2.5 mg/g, respectively. Between the amount of phenolic compounds of ethyl acetate fraction and crude extract a significant difference was observed (P<0.05).

The higher content of total phenolic compounds in ethyl acetate fraction of ZM might account for the better results found in their reducing power, DPPH radical scavenging and inhibition of β-carotene bleaching. In conclusion, polar fractions especially ethyl acetate fraction of ZM showed higher antioxidant activity than ZM crude extract.

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REFERENCES


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