Mutagenic, Anti-Mutagenic and Cytotoxic Activities of Artediffusin (Tehranolide), in vitro, extracted from *Artemisia diffusa*

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ABSTRACT

**Background:** Artediffusin is a sesquiterpene lactone with an endoperoxide group which has been isolated from *Artemisia diffusa*. Artemisia has always been of great botanical and pharmaceutical interest and is useful in traditional medicines for the treatment of a variety of diseases and complaints. The aims of the present study were to evaluate the cytotoxic, mutagenic and anti-mutagenic activities of Artediffusin (Tehranolide) extracted from *Artemisia diffusa*.

**Methods:** Cytotoxicity was measured using a modified MTT assay on normal human lymphocytes and cancer cells. The mutagenic and anti-mutagenic activities of Artediffusin were evaluated using the *Salmonella typhimurium* tester strains TA98 and TA100 with and without metabolic activation S9.

**Results:** 28µg/ml concentration of Artediffusin inactivated 77.73±0.78% of HeLa cells activity and 5600µg/ml concentration of Artediffusin inactivated 28.79±1.82% of lymphocytes activity. The maximum percentage of anti-mutagenic activity of Artediffusin was observed in the strain of *S. typhimurium* TA98, with the presence of metabolic activation S9.

**Conclusion:** Artediffusin may be exploited as a natural anti-cancer and anti-mutagenic agent with low adverse side effects.

**Keywords:** 9-Methylfluorene; 1,9-Dimethylfluorene; Antimutagens; Cytotoxicity, Immunologic; *Salmonella Typhimurium*; Tehranolide.

INTRODUCTION

*Artemisia* which is categorized under Anthemideae tribe and Compositae (Asteraceae) family [1] has over 500 species. Among these, 34 have been reported in Iran, some of them are endemic [2]. The genus has always been of great botanical and pharmaceutical interest and is useful in traditional medicines for the treatment of a variety of diseases and complaints [3]. *Artemisia diffusa* Krasch. ex Poljakov which grows naturally in various regions of Iran, has been investigated chemically and monoterpenes and sesquiterpenes (especially sesquiterpene) lactones were found but among twenty identified components of the oil of *A. diffusa* (91.3%), camphor (35.0%) and 1,8-cineole (25.7%) were shown to be the major constituents [4].

The oil extracted by steam distillation of the aerial parts of *A. diffusa* from Semnan, Iran had been analyzed by GC and GC-MS. Twenty components were identified constituting 93.8% of the oil. The main components of the oil were camphor (57.5%), verbenone (13.8%) and 1,8-cineole (7.5%) [5]. While, the main components of other sample of the same species collected from the northeast of Iran were camphor (25.5%), 1,8-cineole (25.0%), β-thujone (22.0%) and α-thujone (6.93%) [6].

The extract of the aerial parts of *A. diffusa* afford a monoterpane lactone, namely filifolide A, an uncommon compound type in addition to several sesquiterpenoids [7]. The extract of the aerial parts of *A. diffusa* collected from Khorasan, northeast of Iran, afford several eudesmanolides and a new type of sesquiterpene lactone with an endoperoxide group (Artediffusin or Tehranolide) that probably has the same efficacy as the antimalarial drug Artemisinin [7].

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The antimalarial activity of the extract and the fraction which contains Artediffusin of *A. diffusa* has been recorded. The results indicated the inhibitory effects of the *A. diffusa* extracts and the fraction, which contains sesquiterpene lactones including Artediffusin, on the developmental stages of *Plasmodium berghei* by decreasing parasitaemia [8].

The aims of the present study were to evaluate the cytotoxic, mutagenic and anti-mutagenic activities of Artediffusin (Tehanolide) extracted from *A. diffusa*.

**MATERIALS AND METHODS**

**Plant collection, extraction and isolation**

The aerial parts of *A. diffusa* was collected from Ahmad Abad and Zaman Abad, Khorasan Province (northeast), Iran in April 2011 and were identified by an expert from the research institute of forests and rangelands (Vali-Aallah Mozaffarian, PhD). Voucher specimens were deposited at the institute’s herbarium.

Ground aerial parts of *A. diffusa* (900g) were extracted with Et$_2$O/MeOH/petroleum ether (1:1:1) (2×6l) at room temperature for 72h. Evaporation at reduced pressure was performed with furnished of crude extract (35g), which was suspended in EtOH (120ml), diluted with H$_2$O (300ml) and extracted successively with hexane (2×250ml) and CHCl$_3$ (3×250ml). Evaporation of the CHCl$_3$ extract at reduced pressure furnished of reside (13.5g), which was column chromatographed over silica gel (520mg, 70-230mesh) using hexane and increasing amounts of EtOAc (0-100%) and EtOAc/MeOH (9:1) to afford 32 fractions. The similar fractions were added together and the fractions were reduced to 20. $^{13}$C-NMR spectrum was taken from all fractions. Fractions 12 showed signals related to Tehranolide. Fractions 12 (120mg) was reunited and chromatographed again on silica gel (230-400mesh) to give 26 fractions. $^{13}$C-NMR spectrum was taken from all achieved fractions. Fractions 14 afforded Tehranolide. The structure of Tehranolide was elucidated by the 500MHz $^{13}$C-NMR and $^1$H-NMR Techniques. The most obvious characteristic of Tehranolide is 105 signal that related to C-5 attached to two oxygen in $^{13}$C-NMR.

**Cytotoxicity assay**

Cytotoxicity was measured using a modified MTT assay on normal human lymphocytes and cancer cells [9]. The human cervical carcinoma HeLa cell line (NCBI code No.: 115; ATCC No.: CCL-2) was obtained from Pasteur Institute, Tehran, Iran. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 1% (w/v) glutamine, 100U/ml penicillin and 100µg/ml streptomycin. The human normal healthy lymphocyte cell line (NCBI code No.: 124; ECACC No.: 91112124) was obtained from Pasteur Institute, Tehran, Iran. The cells were grown in RPMI 1640 supplemented with 10% FBS. They were cultured in a humidified atmosphere at 37°C in 5% CO$_2$. The cytotoxicity assay detected the reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by mitochondrial dehydrogenase, to the formazan blue product, which reflects the normal functioning of mitochondrial and cell viability [10]. Briefly, the cells ($5\times10^4$) were seeded in the wells containing 100µl of the RPMI medium supplemented with 10% FBS in a 96-well plate. After 24 hours adhesion, a serial of doubling dilution of the essential oil was added to triplicate wells over the range of 1.0-0.005µl/ml. The final concentration of ethanol in the culture medium was maintained at 0.5% (volume/volume) to avoid toxicity of the solvent [11]. After 2 days, 10µl of MTT (5mg/ml stock solution) was added, and the plates were incubated for an additional 4 hours. The medium was discarded, and the formazan blue formed in the cells was dissolved with 100µl dimethyl sulphoxide (DMSO). The optical density was measured at 490nm using a microplate ELISA reader DNM-9602G (Perlong Group; China). The cell viability curves were calculated in view of control cells incubated in the presence of 0.5% ethanol. Cytotoxicity was expressed as the
concentration of drug inhibiting cell growth by 50% (IC\textsubscript{50}).

**Toxicity assay**

The S9 metabolic activator was prepared by adding 500µl phosphate buffer (0.2M), 130µl deionised water, 100µl KCl (0.33M), 80µl MgCl\textsubscript{2} (0.1M), 100µl S9 fraction, 50µl glucose-6-phosphate (0.1M), and 40µl NADP (0.1M) just before use. The mixture was kept on ice during testing. S9 fraction, the liver post-mitochondrial supernatant of rats treated with the mixture phenobarbital/β-naphthoflavone (PB/NF) to induce the hepatic microsomal enzymes, was purchased from Moltox [12].

For the bacterial toxicity test, 12ml Nutrient agar, 0.5ml metabolic activation mix (S9), Phosphate buffer 0.2M (pH=7.4), 0.01ml of the test chemical dilution and 0.1ml overnight culture of the Salmonella strain were added to the tubes. The contents of the test tubes were then mixed and poured onto the surface of glucose minimal agar plates (1.5% agar, 0.02% MgSO\textsubscript{4}.7H\textsubscript{2}O, 0.2% citric acid, 1% K\textsubscript{2}HPO\textsubscript{4}, 0.35% NaN\textsubscript{2}HPO\textsubscript{4}.4H\textsubscript{2}O and 2% glucose). The plates were inverted and placed in 37°C incubator for 48 hours. The colonies were then counted, and the results were expressed as the number of reverted colonies per plate. Comparisons of bacterial counts on test compound plates with bacterial counts on control plates were used to determine the growth inhibition [12].

**Mutagenicity and anti-mutagenicity test**

The mutagenic activity was evaluated by the Salmonella/microsome assay, using the Salmonella typhimurium tester strains TA98 and TA100, with (+S9) and without (−S9) metabolism, using the pre-incubation method [13]. It is important that the same number of bacteria was used in the preliminary toxicity assay as well as in the definitive mutagenicity assay [12]. Salmonella was inoculated in cultures 15-18 hours prior to performing the experiment. Top agar melt was supplemented with 0.05mM histidine and biotin and maintained at 43°C to 48°C. The following agents were added to the 13×100mm sterile glass tubes maintained at 43°C, mixing after each addition. Each test should be performed using a single batch of reagents, media and agar [12]. The top agar, consisting of 0.6% agar and 0.6% NaCl, is one of the most critical medium components in the Ames test, because it contains the trace amount of histidine (0.05mM) for limited growth. It also contains biotin at concentration of 0.05mM which is in excess of what is needed for the growth of the Salmonella strains. We studied the effect of metabolic activation using pre-incubation. In a condition without metabolic activation, 0.01ml of each concentration of the test ingredient, negative control or positive control was added to 0.5ml of 0.1M phosphate buffer (pH=7.4), and 0.1ml of each strain (approximately 1/6×10\textsuperscript{6}cells/ml), and then incubated at 37°C for 20min. After shaking incubation, 2ml of top agar was added to the incubation mixture according to the strains and then poured on to a plate containing minimal agar. The plates were incubated at 37°C for 48 hours, and the reverted colonies were counted manually. In the presence of metabolic activation, 0.5ml of freshly prepared S9 mix was added to the incubation mixture instead of 0.1M phosphate buffer (pH=7.4). All experiments were performed in triplicate. The colonies were then counted, and the results were expressed as the number of reverted colonies per plate. The standard mutagens used as positive controls in the experiments without the S9 mix were 2-nitrofluorene for TA98, and sodium azide for TA100. In experiments with S9 activation, 2-aminoanthracene was used with TA98 and TA100. DMSO was served as negative (solvent) control [12]. The percentage of mutations was calculated using \((T/M)\times10\textsuperscript{0}\) formula (T: The number of reverted colonies in the presence of mutagen). The number of colonies that had been grown up was deducted from the numerator and denominator.

Microbial and cell culture media, laboratory reagents and the mutagens (2-
Results

Cytotoxicity assay

28µg/ml concentration of Artediffusin inactivated 77.73±0.78% of HeLa cells activity; its 50% of cytotoxic activity was at 1.37µg/ml (y=1.0985x+48.494; r²=0.9889). 5600µg/ml concentration of Artediffusin inactivated 28.79±1.82% of lymphocytes activity; its 50% of cytotoxic activity was at 10932.9µg/ml (y=0.0039x+7.3618; r²=0.9874) (Table 1).

Table 1. Cytotoxicity assay of Artediffusin on HeLa and lymphocyte cells.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Viability (%)</th>
<th>Death (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa Cells (IC₅₀=1.37µg/ml) control</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>7 (µg/ml)</td>
<td>44.75±3.86</td>
<td>55.24±2.80</td>
</tr>
<tr>
<td>14 (µg/ml)</td>
<td>34.71±4.56</td>
<td>65.28±0.17</td>
</tr>
<tr>
<td>28 (µg/ml)</td>
<td>22.26±4.73</td>
<td>77.73±0.78</td>
</tr>
<tr>
<td>Lymphocyte Cells (IC₅₀=10932.9µg/ml) control</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1400 (µg/ml)</td>
<td>87.90±3.61</td>
<td>12.09±5.62</td>
</tr>
<tr>
<td>2800 (µg/ml)</td>
<td>80.67±8.42</td>
<td>19.32±0.91</td>
</tr>
<tr>
<td>5600 (µg/ml)</td>
<td>71.20±7.01</td>
<td>28.79±1.82</td>
</tr>
</tbody>
</table>

Mutagenicity and anti-mutagenicity test

1.128mg/plate concentration of Artediffusin was selected based on the preliminary toxicity test. Artediffusin showed excellent anti-mutagenic effects at the concentration of 1.128 mg/plate against chemical mutagens in Salmonella strains. The maximum percentage of anti-mutagenic activity of Artediffusin was observed in 1.128 mg/plate by the bacterial reverse mutation assay in the strain of S. typhimurium TA98, with the presence of metabolic activation S9 (Table 2).

Table 2. Percentage of Mutagenesis and anti-mutagenesis activity of Artediffusin against Salmonella typhimurium strains TA98 and TA100, with (+S9) and without (-S9) metabolization.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TA100 M-S9</th>
<th>TA100 M+S9</th>
<th>TA98 M-S9</th>
<th>TA98 M+S9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutagenicity</td>
<td>8.16</td>
<td>50.00</td>
<td>2.17</td>
<td>1.43</td>
</tr>
<tr>
<td>Anti-Mutagenicity</td>
<td>73.08</td>
<td>66.67</td>
<td>78.41</td>
<td>86.20</td>
</tr>
</tbody>
</table>

Discussion

The IC₅₀ values for HeLa and lymphocyte cells were calculated to be 1.37µg/ml and 10932.9µg/ml, respectively. This suggests that the cytotoxicity of Artediffusin on human cancer cells is much higher than that of normal human lymphocytes. The results indicate low adverse side effects of Artediffusin on the human cells. Cancer chemoprevention 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. They function by mechanisms directed at all major stages of carcinogenesis [14]. Therefore, Artediffusin is reported to be cytotoxic against HeLa cells.

Previously the essential oil of Artemisia absinthium (Asteraceae) from north-western Iran was investigated for its cytotoxicity using the MTT assay. The 50% cytotoxic concentrations were found to be 48.59µg/ml and 5456.07µg/ml for Hela cells and lymphocytes, respectively [15]. Based on previous studies, the immunotherapeutic effectiveness of Tehranolide was investigated by direct intra-tumor injection. Analysis of immune response showed that intra-tumor injection of Tehranolide decreased the rate of tumor growth compared to the control group [16]. Experiments have shown that, Molt-4 cells, a human leukemia cell line and human breast cancer cells are more susceptible to the cytotoxic effect of Tehranolide than their normal counterparts [17]. Finally, we found Artediffusin as an anti-cancer agent with few side effects in the present study.

There is a relationship between mutagenesis and carcinogenesis [18]. The Ames test is used to detect mutagenic and anti-mutagens activities and is a widely accepted method for identifying different chemicals and drugs that can cause gene mutations. In general, Artediffusin was not shown tangible mutagenic effect, except in the strain of S. typhimurium TA100 at the presence of metabolic activation.
S9. In the presence of chemical mutagens, Artediffusin showed resistance against these mutagens and anti-mutagenic effects in both strains of \textit{S. typhimurium} TA100 and TA98. From the mentioned results, it can be concluded that the anti-mutagenic activity of Artediffusin was higher in the presence of chemical mutagens than its mutagenic activity in non-selective medium. To our knowledge, there was no research carried out on the mutagenic and anti-mutagenic effect of Artediffusin before.

Based on previous studies, the excellent antimitogenic effect of the volatile oil of \textit{Artemisia absinthium} was seen in 1.5mg/plate against \textit{S. typhimurium} TA100, without the presence of metabolic activation [19]. In another investigation, the mutagenic and antimutagenic effects of the oils from \textit{Asteraceae} (\textit{Artemisia campestris} and \textit{Artemisia herba-alba}) were investigated by the Ames method, with and without addition of an extrinsic metabolic activation. The oils showed no mutagenicity when tested with \textit{S. typhimurium} strains TA98 and TA97. The authors showed that both oils had antimutagenic activity against the carcinogen Benzo (a) pyrene when tested with TA97 and TA98 assay systems [20]. Thus, Artediffusin can act as anti-mutaenic agent with low adverse side effects.

Despite of the limitations of all \textit{in vitro} investigations when compared to \textit{in vivo} studies, the obtained results are very promising as far as anti-neoplastic chemotherapy is concerned. This forms a valuable base for future investigations.

**CONCLUSION**

Artediffusin may be exploited as a natural anti-cancer and anti-mutagenic agent with low adverse side effects.

**ACKNOWLEDGMENT**

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