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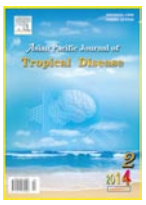

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Mutagenic and antimutagenic activities of *Artemisia absinthium* volatile oil by the bacterial reverse mutation assay in *Salmonella typhimurium* strains TA98 and TA100

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ABSTRACT

Objective: To investigate the mutagenic and antimutagenic activities of *Artemisia absinthium* L. (*A. absinthium*) essential oil by the bacterial reverse mutation assay in *Salmonella typhimurium* (*S. typhimurium*) strains.

Methods: Water-distilled essential oil of *A. absinthium* collected from Ardabil, North-Western Iran, was investigated for mutagenic and antimutagenic activities. In present study, the mutagenic and antimutagenic activities of *A. absinthium* oil were investigated by the bacterial revere mutation assay in *S. typhimurium* TA98 and TA100 strains with and without S9 (microsomal mutagenesis assay).

Results: The comparative mutagenicity effect was seen in 1.5 mg/plate by the bacterial reverse mutation assay in *S. typhimurium* TA98 strains, without S9 and the excellent antimutagenicity effect was seen in 1.5 mg/plate against *S. typhimurium* TA100, without S9.

Conclusions: The mutagenicity and antimutagenicity effects of the volatile oil of *A. absinthium* were seen without the presence of metabolic activation.

1. Introduction

The genus *Artemisia* has always been of great botanical and pharmaceutical interest and is useful in traditional medicines[1,2]. Among them, *Artemisia absinthium* L. (*A. absinthium*) is a species of wormwood, native to temperate regions of Eurasia and Northern Africa[3]. It grows naturally in wide regions of Iran. The bitter taste of wormwood is from sesquiterpene lactones (0.15%-0.40%). Absinthin, artabsin and guainolides are the main constituents, *A. absinthium* is bitter tonic, aromatic bitter, anthelmintic, stomachic, antiparasitic, antiseptic and choleretic, carminative, anti-inflammatory and mild antidepressant[4-6]. The effectiveness of wormwood as an aromatic bitter and its antimicrobial properties come from the bitter compounds and its essential oil. The oil of the plant can be used as a cardiac stimulant to improve blood circulation. Pure wormwood oil is very poisonous, but with proper dosage poses little or no danger[7]. This oil is a potential source of new agents for the treatment of leishmaniasis[8]. In the previous study, the composition of the leaf oil of *A. absinthium* was analyzed by gas chromatograph and gas chromatograph/mass spectrometer and 19 components were identified. 1,8-Cineole (36.46%), borneol (25.99%) and camphor (10.20%) were the main components in this oil[9]. Sabinene (17.56%) was the major constituent in volatile oil from the aerial part of *A. absinthium* collected from Turkey[10]. The leaf oil of *A. absinthium* indicated significant activity against *Candida albicans*. Bactericidal kinetics of the leaf oil of *A. absinthium* indicated that *Candida albicans* is the most vulnerable[10]. In our previous study, antioxidative properties of the leaf oil of *A. absinthium* was determined

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by three methods: the ferric-reducing antioxidant power, radical-scavenging capacity of the oil or bleaching of 2,20-diphenylpicrylhydrazyl (DPPH) and β-carotene-linoleic acid assay. The ferric reducing power of the essential oils was determined (10.67±0.45) gallic acid equivalent (mg/g). The leaf essential oil of *A. absinthium* reduced the concentration of DPPH free radical [(61.4±1.4)%, 10 mg/mL of essential oil], with an efficacy lower than that of reference oil Thymus x-porlock (69.3% inhibition). IC_{50} for DPPH radical-scavenging activity was 5.85 μg/mL. In β-carotene-linoleic acid test system, oxidation of linoleic acid was effectively inhibited by *A. absinthium* oil [(58.56±2.5)%, amount of essential oil 0.625 mg/mL]. The results suggest application of *A. absinthium* oil as a natural antioxidant agent. Total phenolic content of the leaf oil of *A. absinthium* was determined to be (168.86±9.50) μg gallic acid equivalent (mg/g). The results suggested application of *A. absinthium* oil as a natural antioxidant agent[10]. The large-scale use of essential oils requires accumulation of toxicological data on these substances. Essential oils contain a complex mixture of volatile and odorous compounds from secondary plant metabolism, and are widely used as fragrance components and flavouring additives[11]. Increasing human exposure to these compounds makes mutagenicity effect of these compounds very important to study in order to protect human population from their toxic and adverse effects[12]. There is a high correlation between mutagenicity and carcinogenicity. Ninety percent of the carcinogens were mutagenic. The Ames mutagenicity test is a short term bacterial reverse mutation assay specifically designed to detect a wide range of chemical materials that can produce genetic damage that leads to gene mutations. The *Salmonella* test can play a central role in a program of prevention to identify mutagenic chemicals and to aid in the development of non-mutagenic products to prevent future human exposure[13]. The aims of this study were to estimate the mutagenicity and antimutagenicity effects of the *A. absinthium* oil by the bacterial reverse mutation assay in *Salmonella typhimurium* (*S. typhimurium*) TA98 and TA100 strains with and without S9 (microsomal mutagenesis assay). A preliminary toxicity experiment is performed to determine the appropriate dose that can be tested.

2. Material and methods

2.1. General

The main equipment types used were a clevenger apparatus. Microbial and cell culture media and laboratory reagents were from Merck, Germany. Other chemicals were of analytical grade.

2.2. Plant material

The leaves of *A. absinthium* were collected from Namin, Province of Ardabil, after Heyran ghaut, in North-Western Iran in July 2011. Voucher specimens have been deposited at the Herbarium of the Research Institute of Forests and Rangelands, Tehran, Iran.

2.3. Isolation of the volatile oil

The aerial parts of *A. absinthium* were dried at room temperature for several days. Air-dried aerial parts of *A. absinthium* (120 g) was separately subjected to hydrodistillation using a clevenger-type apparatus for 3 h. After drying of the oil over anhydrous sodium sulfate, the oil was recovered. Results showed that volatile oil yield was 0.92% (w/w).

2.4. Preparation of metabolic activation system (S9 mixture)

The S9 metabolic activator was prepared just before use by adding: phosphate buffer (0.2 mol/L) 500 μL, deionised water 130 μL, KCl (0.33 mol/L) 100 μL, $MgCl_2$ (0.1 mol/L) 80 μL, S9 fraction 100 μL, glucose-6-phosphate (0.1 mol/L), 50 μL and nicotinamide adenine dinucleotide phosphate (0.1 mol/L) 40 μL. The mixture was kept on ice during testing. S9 fraction, the liver postmitochondrial supernatant of rats treated with the mixture phenobarbital/β-naphthoflavone to induce the hepatic microsomal enzymes, was purchased from Moltox[14].

2.5. Toxicity determination

Bacterial toxicity was determined based on the values in Table 1. For toxicity test, 12 mL of nutrient agar and 0.50 mL of metabolic activation (S9) mix or buffer (phosphate buffer 0.2 mol/L, pH 7.4), 0.01 mL of the test chemical dilution and 0.1 mL overnight culture of the *Salmonella* strain were added in tubes (Table 1). The contents of the test tubes were then mixed and poured onto the surface of glucose minimal agar plates (the glucose minimal agar, consisting of 1.5% agar, 0.02% $MgSO_4 \cdot 7H_2O$, 0.2% citric acid, 1% K_2HPO_4, 0.35% $NaNH_4HPO_4 \cdot 4H_2O$ and 2% glucose). The plates were inverted and placed in a 37 °C incubator for 48 h. The colonies are then counted and the results were expressed as the number of revertant colonies per plate. Comparisons of bacterial counts on test compound plates with bacterial counts on

control plates were used to determine growth inhibition[14].

Table 1

Toxicity determination.

Methods	Test 1 (mL)	Control 1 (mL)	Test 2 (mL)	Control 2 (mL)
Test strain (approximately 1/6×10^6 cells/mL)	0.10	0.1	0.10	0.1
Oil	0.01	–	0.01	–
Phosphate buffer (0.2 mol/L, pH 7.4)	–	0.1	0.50	0.6
S9 mix	0.50	0.5	–	–
Nutrient agar	12.00	12.0	12.00	12.0

2.6. Mutagenicity and anti-mutagenicity test

Mutagenic activity was evaluated by the *Salmonella*/microsome assay, using the *S. typhimurium* tester strains TA98 and TA100, with (+S9) and without (-S9) metabolization, using the preincubation method[15]. It was notable that the same number of bacteria were used in the preliminary toxicity assay as well as in the definitive mutagenicity assay[14].

Salmonella inoculated cultures 15-18 h prior to performing the experiment. Top agar melt supplemented with 0.05 mmol/L histidine and biotin and maintain at 43 °C to 48 °C. To the 13 mm×100 mm sterile glass tubes maintained at 43 °C, the following order with mixing after each addition was added (Figure 1). Each test should be performed using a single batch of reagents, media and agar[14].

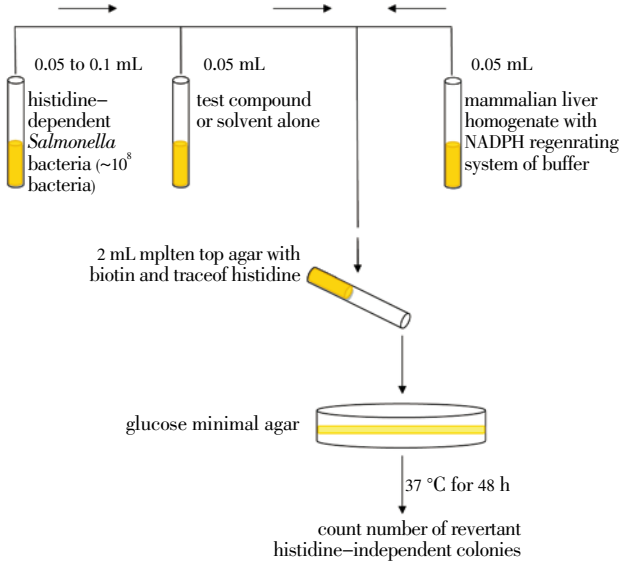

Figure 1. Diagram depicting the steps involved in the plate incorporation assay[14].

The top agar, consisting of 0.6% NaCl and 0.6% agar, was one of the most critical medium components in the Ames test because it contained the trace amount of histidine (0.05 mmol/L) for limited growth. It also contained biotin at a concentration of 0.05 mmol/L which was in excess of what was needed for the growth of the *Salmonella* strains. The effect of metabolic activation was studied using pre incubation. In condition without metabolic activation, 0.01 mL of each concentration of test ingredient, negative control or positive control was added to 0.5 mL of 0.1 mol/L phosphate buffer (pH 7.4) and 0.1 mL of each strain (approximately 1/6×10^6 cells/mL), and then incubated at 37 °C for 20 min. After shaking incubation, 2 mL 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 h and the revertant colonies were counted manually. In the presence of metabolic activation, 0.5 mL of freshly prepared S9 mix instead of 0.1 mol/L phosphate buffer (pH 7.4) was added to the incubation mixture. Other procedures were performed in the same way (Figure 1)[14].

All experiments were analyzed in duplicate (Tables 2-9). The colonies were then counted and the results were expressed as the number of revertant colonies per plate. The standard mutagens used as positive controls in experiments without the S9 mix were 2-nitrofluorene for TA98, sodium azide for TA100. In experiments with S9 activation, 2-aminoanthracene was used with TA98 and TA100 DMSO served as negative (solvent) control[14].

Table 2

Determination of mutagenic potency of *A. absinthium* oil by *S. typhimurium* strain TA100 without S9.

TA100	Positive control (mL)	Negative control (mL)	Test (mL)
Test strain (approximately 1/6×10^6 cells/mL)	0.1	0.1	0.10
Sodium azide (NaN_3) (50 μg/mL)	0.1	–	–
Test concentration (oil)	–	–	0.01
Phosphate buffer (0.1 mol/L, pH 7.4)	0.5	0.5	0.50
Top agar	2.0	2.0	2.00

Mutagenesis percentage was calculated using the following formula: (T/M)×100; T: the number of revertant colonies in the presence of volatile oil, M: the number of revertant colonies in the presence of mutagen.

Table 3

Determination of mutagenic potency of *A. absinthium* oil by *S. typhimurium* strain TA100 with S9.

TA100	Positive control (mL)	Negative control (mL)	Test (mL)
Test strain (approximately 1/6×10^6 cells/mL)	0.1	0.1	0.10
2-aminoanthracene (1 μg/plate in DMSO)	0.1	–	–
Test concentration (oil)	–	–	0.01
S9 mix	0.5	0.5	0.50
Top agar	2.0	2.0	2.00

Table 4

Determination of mutagenic potency of *A. absinthium* oil by *S. typhimurium* strain TA98 without S9.

TA98	Positive control (mL)	Negative control (mL)	Test (mL)
Test strain(approximately 1/6×10^6 cells/mL)	0.1	0.1	0.10
2-nitrofluorene (1.5 μg/plate)	0.1	–	–
Test concentration (oil)	–	–	0.01
Phosphate buffer (0.1 mol/L, pH 7.4)	0.5	0.5	0.50
Top agar	2.0	2.0	2.00

Table 5

Determination of mutagenic potency of *A. absinthium* oil by *S. typhimurium* strain TA98 with S9.

TA98	Positive control (mL)	Negative control (mL)	Test (mL)
Test strain (approximately 1/6×10^6 cells/mL)	0.1	0.1	0.10
2-aminoanthracene (1 μg/plate in DMSO)	0.1	–	–
Test concentration (oil)	–	–	0.01
S9 mix	0.5	0.5	0.50
Top agar	2.0	2.0	2.00

Table 6

Determination of antimutagenic potency of *A. absinthium* oil by *S. yphimurium* strain TA100 without S9.

TA100	Positive control	Negative control	Test
Test strain(approximately 1/6×10^6 cells/mL)	0.1 mL	0.1 mL	0.1 mL
Sodium azide (NaN_3) (50 μg/μL)	200.0 μL	–	200.0 μL
Test concentration (oil)	–	–	10.0 μL
Phosphate buffer (0.1 mol/L, pH 7.4)	0.5 mL	0.5 mL	0.5 mL
Top agar	2.0 mL	2.0 mL	2.0 mL

Table 7

Determination of antimutagenic potency of *A. absinthium* oil by *S. typhimurium* strain TA100 with S9.

TA100	Positive control	Negative control	Test
Test strain (approximately 1/6×10^6 cells/mL)	0.1 mL	0.1 mL	0.1 mL
2-aminoanthracene (1 μg/μL in DMSO)	200.0 μL	–	200.0 μL
Test concentration (oil)	–	–	10.0 μL
S9 mix	0.5 mL	0.5 mL	0.5 mL
Top agar	2.0 mL	2.0 mL	2.0 mL

Table 8

Determination of antimutagenic potency of *A. absinthium* oil by *S. yphimurium* strain TA98 without S9.

TA98	Positive control	Negative control	Test
Test strain(approximately 1/6×10^6 cells/mL)	0.1 mL	0.1 mL	0.1 mL
2-nitrofluorene (1 μg/μL)	200.0 μL	–	200.0 μL
Test concentration (oil)	–	–	10.0 μL
Phosphate buffer(0.1 mol/L, pH 7.4)	0.5 mL	0.5 mL	0.5 mL
Top agar	2.0 mL	2.0 mL	2.0 mL

Table 9

Determination of antimutagenic potency of *A. absinthium* oil by *S. typhimurium* strain TA98 with S9.

TA98	Positive control	Negative control	Test
Test strain (approximately 1/6×10^6 cells/mL)	0.1 mL	0.1 mL	0.1 mL
2-aminoanthracene (1μg/μL)	200.0 μL	–	200.0 μL
Test concentration (oil)	–	–	10.0 μL
S9 mix	0.5 mL	0.5 mL	0.5 mL
Top agar	2.0 mL	2.0 mL	2.0 mL

Mutagenicity tests were determined based on the values in Tables 2-5. The number of colonies that had been grown on their own would be deducted from the numerator and denominator[14]. Anti-mutagenicity tests were determined based on the values in Tables 6-9.

3. Results

The concentration of the active constituents was seasonally and geographically different and some genotypes were characterised by particularly high contents of active volatile oil constituents. Oxygenated monoterpenes (99.51%) were the main components in the leaves of *A. absinthium* collected from Iran. 1,8-Cineole (36.46%), borneol (25.99%), camphor (10.20%) were the main components in this oil[10]. The large scale use of essential oils required accumulation of toxicological data on these substances. There was a relationship between mutagenesis and carcinogenesis. The Ames assay is commonly used to detect mutagenic and antimutagens activities and is a widely accepted method for identifying different chemicals and drugs that can cause gene mutations. In this study, the concentration of the oil (1.5 mg/plate) was selected based on a preliminary toxicity test. The colonies were counted to Determine the mutagenic and antimutagenic potencies of *A. absinthium* oil (Figure 2). Mutagenesis and antimutagenesis percentage of *A. absinthium* oil was calculated and listed in Table 10.

Table 10

Percent of mutagenicity and antimutagenicity of *A. absinthium* oil to *S. typhimurium* (TA98, TA100) with and without S9.

S. typhimurium strains	Percent of mutagenicity (%)	Percent of antimutagenicity (%)
TA100-S9	3.06	84.62
TA100+S9	7.50	62.50
TA98-S9	46.74	44.32
TA98+S9	30.00	82.76

Dilution: 1.5 (mg/plate). -S9: without S9, +S9: with S9.

Under these experiments, the comparative mutagenicity effect was seen in 1.5 mg/plate by the bacterial reverse mutation assay in *S. typhimurium* TA98 strains, without S9 and the excellent antimutagenicity effect was seen in 1.5 mg/plate against *S. typhimurium* TA100, without S9. In other words, *A. absinthium* oil had significant antimutagenic activity against sodium azide in the TA100 strain without S9.

Figure 2. Colonies counting in the Ames test.

4. Discussion

The Ames mutagenicity assay is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemicals that can produce genetic damage that leads to gene mutations. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the *Salmonella* tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence are able to form colonies[14].

In this study, the comparative mutagenicity effect of *A. absinthium* oil was seen in 1.5 mg/plate by the bacterial reverse mutation assay in *S. typhimurium* TA98 strains,

without S9. When this oil was added to the plate, the number of revertant colonies per plate was increased. The excellent antimutagenicity effect was seen in 1.5 mg/plate against *S. typhimurium* TA100, without S9. The mutagenicity and antimutagenicity activities of the essential oil of *A. absinthium* were seen without the presence of metabolic activation. Previously, Neffati *et al.* investigated the mutagenic and antimutagenic activities of the two oils by the *S. typhimurium*/microsome assay, with and without addition of an extrinsic metabolic activation system[16]. The oils showed no mutagenicity when tested with *S. typhimurium* strains TA98 and TA97. On the other hand, they showed that each oil had antimutagenic activity against the carcinogen Benzo (a) pyrene (B [a] P) when tested with TA97 and TA98 assay systems[16]. In another investigation, Nakasugi *et al.* examined antimutagens from *Artemisia argyi*[17]. The methanol extract strongly reduced the mutagenicity of 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), when *S. typhimurium* TA98 was used in the presence of the rat liver microsomal fraction. The four isolated flavones from the extract of *Artemisia argyi* were studied regarding their antimutagenic mechanisms with preincubation methods of the modified Ames test. The results suggested that all isolated flavones were desmutagens which directly inactivated Trp-P-2 or inhibited its metabolic activation[17]. In the present research, the antimutagenicity effect was seen against *S. typhimurium* TA100, without the presence of metabolic activation. Based on this results, the antimutagenicity activity of the essential oil of *A. absinthium* can cause by phenolic compounds in this oil.

Conflict of interest statement

I declare that I have no conflict of interest.

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