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MEDICINAL PLANTS

CHEMICAL CONSTITUENTS AND *IN VITRO* ANTICANCER, CYTOTOXIC, MUTAGENIC AND ANTIMUTAGENIC ACTIVITIES OF *ARTEMISIA DIFFUSA*

M. Taherkhani[1]

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Water-distilled essential oil from the leaves of Artemisia diffusa Krasch. ex Poljakov, collected from north east of Iran, was investigated for phytochemical constituents and anticancer, cytotoxic, mutagenic and antimutagenic activities. Camphor (28.30%), 1,8-cineole (21.03%) and β-thujone (14.20%) were the major components in this oil. The largest part of the leaf oil of *A. diffusa* was formed by oxygenated monoterpenes (75.58%). Cytotoxicity was measured using a modified MTT assay against Hela and lymphocyte cells. The IC_{50} shows that cytotoxicity of the oil with respect to human tumor cell line (IC_{50} = 16.34 μg/mL) is much higher than that for healthy human cells (IC_{50} = 4594.92 μg/mL). These results indicate low adverse side effects of the oil. The mutagenic and antimutagenic activities of the *A. diffusa* oil were evaluated by the Ames *Salmonella*/microsome assay, using the *Salmonella typhimurium* tester strains TA98 and TA100, with and without the presence of metabolic activation of rat liver (S9). The excellent anti-mutagenic effect was seen in 1.16 mg/plate against both strains of *S. typhimurium* TA100 and TA98, without the presence of S9 fraction.

Keywords: *Artemisia diffusa*, camphor, cytotoxicity, MTT assay, Ames *Salmonella*/microsome test, anti-mutagenic agent

INTRODUCTION

Artemisia plants belong to the important family Compositae (Asteraceae) [1]. Within this family, *Artemisia* genus is included into the tribe *Anthemideae* and comprises over 500 species [2]. The genus *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 [3, 4]. Among these species, Artemisia diffusa Krasch. ex Poljakov grows naturally in wide regions of Iran. Previously, the essential oil of *A. diffusa* has not been characterized with respect to its biological and pharmaceutical activities. Therefore, the aims of this study were to study the anticancer, cytotoxic, mutagenic and antimutagenic activities of the essential oil of *A. diffusa* from Iran.

[1] Department of Chemistry, Takestan Branch, Islamic Azad University, Takestan, Iran.
e-mail: mahtaherkhani@yahoo.com, mah.taherkhani@tiau.ac.ir

EXPERIMENTAL CHEMICAL PART

Plant Material

The aerial parts of *A. diffusa* were collected in June 2011 from Ahmad Abad and Zaman Abad, province of Khorassan, north east of Iran. Voucher specimens have been deposited at the herbarium of the research institute of Forests and Rangelands, Tehran, Iran.

Isolation of the Essential Oil

The leaves of *A. diffusa* were dried at room temperature for several days. Air-dried leaves of *A. diffusa* (100 g) were separately subjected to hydrodistillation for 3 h using a clevenger-type apparatus. After decanting and drying the distillate over anhydrous sodium sulfate, the essential oil was recovered with a yield of 1.31% (w/w).

Analysis of the Essential Oil

The composition of the essential oil obtained by hydrodistillation from the leaves of *A. diffusa* was analyzed by the gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) techniques. Identification

of the oil constituents was achieved by comparison of their mass spectra and retention indices with those reported in the literature and those of authentic reference samples [5].

Gas chromatography. GC analysis was performed on a Schimadzu 15A gas chromatograph equipped with a split/splitless injector (250°C) and a flame ionization detector (250°C). Nitrogen was used as a carrier gas at a flow rate of 1 mL/min. The capillary column was DB-5 (50 m × 0.2 mm; film thickness, 0.32 μm). The column temperature was kept at 60°C for 3 min, then heated to 220°C at a 5°C/min rate, and then kept constant at 220°C for 5 min. Relative percentages were calculated from peak areas by a Schimadzu C-R4A Chromatopac Data Processor without the use of correction factors.

Gas chromatography/Mass spectroscopy. GC/MS analysis was performed using a Hewlett-Packard 5973 instrument with an HP-5MS column (30 m × 0.25 mm; film thickness, 0.25 μm). The column temperature was kept at 60°C for 3 min, programmed to 220°C at a rate of 5°C/min,

and kept constant at 220°C for 5 min. The flow rate of helium as carrier gas was 1 mL/min. The mass spectra were taken at electron impact energy of 70 eV. The retention indices (RI) for all components were determined according to the van den Dool method, using *n*-alkanes as standards. The compounds were identified by comparison of their relative retention indices (RRI, DB5) with those reported in the literature and by comparison of their mass spectra with the Wiley library or with the published mass spectra [5].

TABLE 1. Composition of the Leaf Oil of A. diffusa Krasch. ex Poljakov

Compound	RI*	Content (%)
α-Thujene	926	0.18
Camphene	950	0.42
β-Pinene	974	0.22
Myrcene	983	0.12
1,8-Cineole	**1026**	**21.03**
α-Thujone	1100	4.63
β-Thujone	**1110**	**14.20**
Camphor	**1126**	**28.30**
Pinocarvone	1163	0.42
Borneol	1157	4.12
Terpinen-4-ol	1169	0.15
α-Terpineol	1179	0.25
trans-Carveol	1217	0.59
Thymol	1287	0.73
Carvacrol	1298	0.16
Eugenol	1356	0.82
cis-Jasmone	1394	0.18
Spathulenole	1572	0.77
Caryophyllene oxide	1576	0.29
Monoterpene hydrocarbons, 0.94		
Oxygenated monoterpenes, 75.58		
Sesquiterpene hydrocarbons, –		
Oxygenated sesquiterpenes, 1.06		
Total, 77.58		

* Retention indices (RI) were as determined on a DB-5 column using the homologous series of *n*-alkanes.

Chemical Composition of the Essential Oil

The composition of the essential oil obtained by hydrodistillation from the leaves of *A. diffusa* growing in Iran, as analyzed by GC and GC/MS, is listed in Table 1, where the percentage content and retention indices of components are given. In the leaf oil of *A. diffusa*, 19 components, representing 77.58% of the total composition, have been identified. Camphor (28.30%), 1,8-cineole (21.03%) and β-thujone (14.20%) were the main components in this oil. The other major components of the leaf oil of *A. diffusa* were α-thujone (4.63%) and borneol (4.12%). Thus, the leaf oil of *A. diffusa* consists of four monoterpene hydrocarbons (0.94%), thirteen oxygenated monoterpene (75.58%), and two oxygenated sesquiterpenes (1.06%). As can be seen from these data, the leaf oil of *A. diffusa* is rich in monoterpenes (76.52%). As it is shown in Table 1, the largest part of the essential oil of *A. diffusa* represents oxygenated monoterpenes (75.58%).

In previous study [6], the essential oil from the aerial parts of *A. diffusa* growing wildly in the northeast of Iran was analyzed by GC/MS. Twenty-six components were identified in the essential oil of *A. diffusa* (94.43% of the total composition of the oil). Camphor (25.5%), 1,8-cineole (25.0%), β-thujone (22.%) and α-thujone (6.93%) were the major components in the volatile oil of *A. diffusa*. In another investigation [7], the water-distilled essential oil from the aerial parts of *A. diffusa* Krasch. ex Poljakov growing wild in Iran was analyzed by GC and GC/MS. Among the twenty identified components constituting 91.3% of the oil, camphor (35.0%) and 1,8-cineole (25.7%) were found to be the major constituents. As can be seen from the above information, the essential oils from the leaves and total aerial parts of *A. diffusa* are rich in camphor and 1,8-cineole. In still another study [8], the results specifically indicated the inhibitory effects of the *A. diffusa* crude extracts and the fraction, which contains sesquiterpene lactones including tehranolide, on the developmental stages of *P. berghei* by decreasing parasitaemia.

EXPERIMENTAL BIOLOGICAL PART

Cytotoxicity Assay

The human cervical carcinoma Hela cell lines 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°C 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 mitochondria and cell viability [9]. Briefly, the cells (5×10^4) were seeded in each well containing 100 µL 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 [10]. 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 dye, which formed in the cells, was dissolved with 100 µL dimethyl sulfoxide (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}). All tests and analyses were run in triplicate and mean values were calculated.

Preparation of metabolic activation system (S9 mixture). The S9 metabolic activator was prepared immediately before use by adding phosphate buffer (0.2 M) 500 µL, deionized water 130 µL, KCl (0.33 M) 100 µL, $MgCl_2$ (0.1 M) 80 µL, S9 fraction 100 µL, glucose-6-phosphate (0.1 M), 50 µL, and NADP (0.1 M) 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 (PB/NF) to induce the hepatic microsomal enzymes, was purchased from Moltox [11].

Toxicity Determination

Bacterial toxicity was determined based on the values in Table 2. For the toxicity test, 12 mL of Nutrient agar and 0.50 mL of metabolic activation (S9) mix or Buffer (Phosphate buffer 0.2 M, 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. 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 were 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 [11].

Mutagenicity and Antimutagenicity Test

Mutagenic activity was evaluated by the *Salmonella*/microsome assay, using the *Salmonella typhimurium* tester strains TA98 and TA100, with (+S9) and without (–S9) metabolization, using the preincubation method. It is important that the same number of bacteria be used in the preliminary toxicity assay as well as in the definitive mutagenicity assay. *Salmonella* inoculated cultures were grown for 15 – 18 h prior to performing the experiment. Top agar melt was supplemented with 0.05 mM histidine and biotin and maintain at 43 – 48°C. To 13 × 100 mm sterile glass tubes maintained at 43°C, components were added in the following order with mixing after each addition. Each test should be performed using a single batch of reagents, media and agar. 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 (0.05 mM) for limited growth. It also contains biotin at a concentration of 0.05 mM which is in excess of what is needed for the growth of the *Salmonella* strains. Using pre incubation, we studied the effect of metabolic activation. 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 M phosphate buffer (pH 7.4) and 0.1 mL of each strain (approximately $1/6 \times 10^6$ 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 M phosphate buffer (pH 7.4) was added to the incubation mixture. Other procedures were performed in the same way (Fig. 1). All experiments were analyzed in dupli-

TABLE 2. Toxicity Determination

	Test 1	Control 1	Test 2	Control 2
Test strain (approximately $1/6 \times 10^6$ cells/mL)	0.1 mL	0.1 mL	0.1 mL	0.1 mL
Oil	0.01 mL	–	0.01 mL	–
Phosphate buffer (0.2 M, pH 7.4)	–	0.1 mL	0.5 mL	0.6 mL
S9 mix	0.5 mL	0.5 mL	–	–
Nutrient agar	12 mL	12 mL	12 mL	12 mL

TABLE 3. Determination of Mutagenic Potency of *A. diffusa* oil by *S. typhimurium* Strain TA100 without S9

TA100	Positive control	Negative control	Test
Test strain (approximately $1/6 \times 10^6$ cells/ml)	0.1 ml	0.1 ml	0.1 ml
Sodium azide (NaN_3) (50 µg/ml)	0.1 ml	–	-
Test concentration (oil)	–	–	0.01 ml
Phosphate buffer (0.1 M, pH 7.4)	0.5 ml	0.5 ml	0.5 ml
Top agar	2 ml	2 ml	2 ml

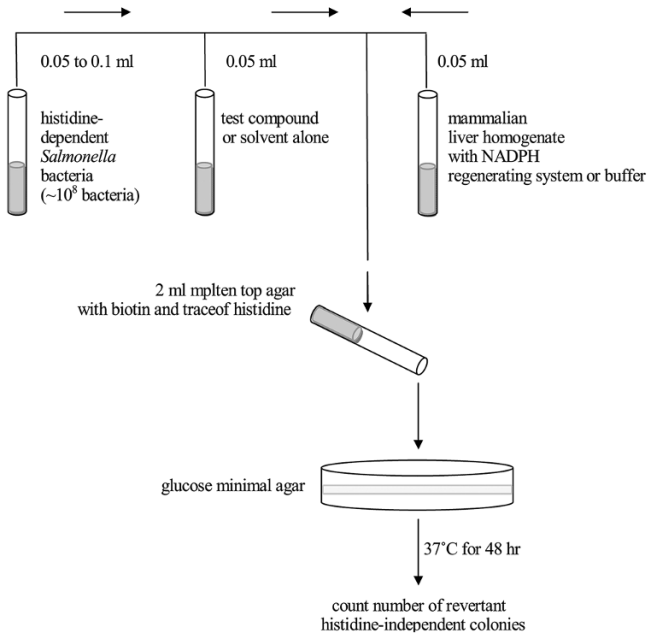

Fig. 1. Diagram depicting the steps involved in the plate incorporation assay [11].

cate (Tables 3 – 10). The colonies are then counted and the results are 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 and sodium azide for TA100. In experiments with S9 activation, 2-aminoanthracene was used with TA98 and TA100. DMSO served as negative (solvent) control [11].

Mutagenicity tests were determined based on the values in Tables 3 – 6.

Mutagenesis percentage was calculated using the following formula: $(T/M) \cdot 100$, where T is the number of revertant colonies in the presence of essential oil and M is the number of revertant colonies in the presence of mutagen. The number of colonies that had been grown on their own will be deducted from the numerator and denominator.

TABLE 4. Determination of Mutagenic Potency of *A. diffusa* oil by *S. typhimurium* Strain TA100 with S9

TA100	Positive control	Negative control	Test
Test strain (approximately $1/6 \times 10^6$ cells/ml)	0.1 ml	0.1 ml	0.1 ml
2-Aminoanthracene (1µg/plate in DMSO)	0.1 ml	–	-
Test concentration (oil)	–	–	0.01 ml
S9 mix	0.5 ml	0.5 ml	0.5 ml
Top agar	2 ml	2 ml	2 ml

TABLE 5. Determination of Mutagenic Potency of *A. diffusa* oil by *S. typhimurium* Strain TA98 without S9

TA98	Positive control	Negative control	Test
Test strain (approximately $1/6 \times 10^6$ cells/ml)	0.1 ml	0.1 ml	0.1 ml
2-Nitrofluorene (1.5 µg/plate)	0.1 ml	–	-
Test concentration (oil)	–	–	0.01 ml
Phosphate buffer (0.1 M, pH 7.4)	0.5 ml	0.5 ml	0.5 ml
Top agar	2 ml	2 ml	2 ml

TABLE 6. Determination of Mutagenic Potency of *A. diffusa* oil by *S. typhimurium* Strain TA98 with S9

TA98	Positive control	Negative control	Test
Test strain (approximately $1/6 \times 10^6$ cells/ml)	0.1 ml	0.1 ml	0.1 ml
2-Aminoanthracene (1µg/plate in DMSO)	0.1 ml	–	-
Test concentration (oil)	–	–	0.01 ml
S9 mix	0.5 ml	0.5 ml	0.5 ml
Top agar	2 ml	2 ml	2 ml

TABLE 7. Determination of Antimutagenic Potency of *A. diffusa* oil by *S. typhimurium* Strain TA100 without S9

TA100	Positive control	Negative control	Test
Test strain (approximately $1/6 \times 10^6$ cells/ml)	0.1 ml	0.1 ml	0.1 ml
Sodium azide (NaN_3) (50 µg/µl)	200 µl	–	200 µl
Test concentration (oil)	–	–	10 µl
Phosphate buffer (0.1 M, pH 7.4)	0.5 ml	0.5 ml	0.5ml
Top agar	2 ml	2 ml	2 ml

TABLE 8. Determination of Antimutagenic Potency of *A. diffusa* oil by *S. typhimurium* Strain TA100 with S9

TA100	Positive control	Negative control	Test
Test strain (approximately $1/6 \times 10^6$ cells/ml)	0.1 ml	0.1 ml	0.1 ml
2-Aminoanthracene (1µg/µl in DMSO)	200 µl	–	200 µl
Test concentration (oil)	–	–	10 µl
S9 mix	0.5 ml	0.5 ml	0.5 ml
Top agar	2ml	2 ml	2 ml

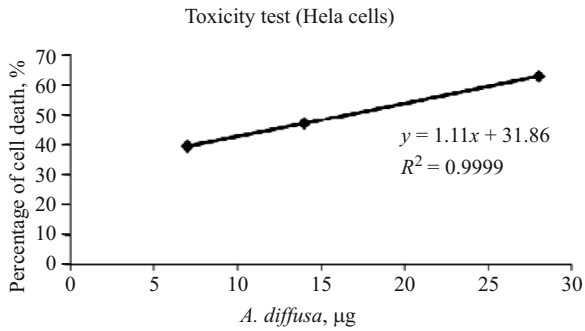

Fig. 2. Cytotoxicity tests on Hela cells.

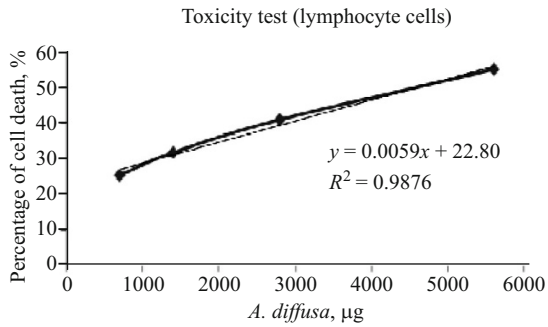

Fig. 3. Cytotoxicity test on lymphocyte cells

Antimutagenicity tests were determined based on the values in Tables 7 – 10.

RESULTS OF BIOLOGICAL ACTIVITY EVALUATION

Cytotoxicity Assay

Cytotoxicity was measured using a modified MTT assay [9]. The cytotoxic effects of *A. diffusa* oil were tested using human lymphocyte and Hela cells (Tables 11 and 12; Figs. 2 and 3) ($y = 1.11x + 31.86$; $R^2 = 0.9999$). At a concentration of 28 µg/ml, oil destructed Hela cells by 62.98 % (Table 11). At lower concentrations, the oil was tolerated by the cells and its 50% cytotoxic concentration was 16.34 µg/mL. The oil displayed good cytotoxic action towards the human tumor cell line. On the other hand, at a concentration of 5600 µg/mL, oil destructed lymphocyte cells by 55.11% (Table 12) ($y = 0.0059x + 22.89$; $R^2 = 0.9876$).

The IC_{50} values for Hela and lymphocyte cells were calculated to be 16.34 µg/mL and 4594.92 µg/mL, respectively. The IC_{50} shows that cytotoxicity of the oil with respect to human tumor cell line is much higher than that for healthy human cells. These results indicate low adverse side effects of the oil. Cancer chemoprevention is defined as the use of chemicals or dietary components to block, inhibit, or reverse the development of cancer in normal or preneoplastic tissue. A large number of potential chemopreventive agents have been identified, and they function by mechanisms directed at all major stages of carcinogenesis [12]. This makes the tested oil certainly deserve some further investigation. 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-

TABLE 9. Determination of Antimutagenic Potency of *A. diffusa* oil by *S. typhimurium* Strain TA98 without S9

TA98	Positive control	Negative control	Test
Test strain (approximately $1/6 \times 10^6$ cells/ml)	0.1 ml	0.1 ml	0.1 ml
2-Nitrofluorene (1 µg/µl)	200 µl	–	200 µl
Test concentration (oil)	–	–	10 µl
Phosphate buffer (0.1 M, pH 7.4)	0.5 ml	0.5 ml	0.5 ml
Top agar	2 ml	2 ml	2 ml

TABLE 10. Determination of Antimutagenic Potency of *A. diffusa* oil by *S. typhimurium* Strain TA98 with S9

TA98	Positive control	Negative control	Test
Test strain (approximately $1/6 \times 10^6$ cells/ml)	0.1 ml	0.1 ml	0.1 ml
2-Aminoanthracene (1µg/µl)	200 µl	–	200 µl
Test concentration (oil)	–	–	10 µl
S9 mix	0.5 ml	0.5 ml	0.5 ml
Top agar	2 ml	2 ml	2 ml

TABLE 11. Cytotoxicity Assay of *A. diffusa* oil on Hela Cells

Oil dilutions (µg/mL)	% Viable cells	% Cell death
Control	100	0
7	60.28 ± 4.93	39.71
14	52.72 ± 2.81	47.28
28	37.01 ± 0.86	62.98
IC_{50} (µg/mL)	16.34	

TABLE 12. Cytotoxicity Assay of *A. diffusa* oil on Lymphocyte Cells

Oil dilutions (µg/mL)	% Viable cells	% Cell death
Control	100	0
700	74.50 ± 2.47	25.49
1400	68.16 ± 8.25	31.83
2800	58.96 ± 2.94	41.04
5600	44.88 ± 2.94	55.11
IC_{50} (µg/mL)	4594.92	

TABLE 13. Percent of Mutagenicity (M) and Antimutagenicity (A) of *A. diffusa* oil to *S. typhimurium* (TA98, TA100) with and without S9

Dilution (mg/plate)	Percentage mutagenicity (M)			
1.16	TA 100 M-S9	TA 100 M+S9	TA 98 M-S9	TA 98 M+S9
	9.18	25.00	10.87	14.29
	Percentage antimutagenicity (A)			
	TA 100 A-S9	TA 100 A+S9	TA 98 A-S9	TA 98 A+S9
	80.77	41.66	73.86	27.59

translational modification of cellular proteins, induce or inhibit some hepatic detoxifying enzymes. So, essential oils may induce very different effects in prokaryotes and eukaryotes [13]. 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 antineoplastic chemotherapy is concerned. This further forms a firm base for future research.

Mutagenicity and antimutagenicity test. The Ames assay is commonly used to detect mutagenic and antimutagenic, activities and is a widely accepted method for identifying different chemicals and drugs that can cause gene mutations. In this research, the concentration of the oil (1.16 mg/plate) was selected based on a preliminary toxicity test. The colonies were counted to determine the mutagenic and antimutagenic potencies of *A. diffusa* oil. Mutagenesis and antimutagenesis percentage of *A. diffusa* oil was calculated and listed in Table 13. The maximum percentage of mutagenicity was seen in 1.16 mg/plate by the bacterial reverse mutation assay in both strains of *S. typhimurium* TA100 and TA98, with the presence of metabolic activation (S9). On the other hand, the excellent antimutagenicity effect was seen in 1.16 mg/plate against both strains of *S. typhimurium* TA100 and TA98, without presence of metabolic activation (S9).

The large-scale use of essential oils requires accumulation of toxicological data on these substances. There is a relationship between mutagenesis and carcinogenesis. 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 [11].

Previously, there was not research on the mutagenic and anti mutagenic effect of the essential oil of *A. diffusa* against *S. typhimurium*. In this study, the comparative mutagenicity effect of *A. diffusa* oil was seen in 1.16 mg/plate by the bacterial reverse mutation assay in both strains of *S. typhimurium*, with the presence of S9 fraction. While the excellent antimutagenicity effect was observed at the same concentration against both *Salmonella* strains in the absence of S9. In general, the results suggested that the essential oil of *A. diffusa* shows a better antimutagenic effect in the absence of metabolic activation.

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