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Cytotoxicity Assessment of *Malva Sylvestris* Crude Extract on Melanoma and Lymphoma Cell Lines

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Abstract

This article focus on the analysis of *M.sylvestris* methanolic leaves extract by GC-MS and the evaluation of its cytotoxic effect on two types of human cancerous cell lines which are melanoma and lymphoma cell lines. GC-MS analysis showed 29 peaks, the prominent peak was 22.942 peak area (RT 27.01): trans-Phytol; Cyclohexanol,5-methyl-2-(1-methylethyl)-,[1S-(1.alpha.2.beta.5.beta.)]; Dihydrogeraniol; Menthol,trans-1,3,trans-1,4; (+)-Isomenthol . The extract showed cytotoxicity against both melanoma and lymphoma cell lines and the cytotoxic effect was increased with the increasing of extract concentration, the cytotoxicity of the extract at 200 µL was 68.65% and 76.53% for lymphoma and melanoma cell lines respectively, for reference cell line the cytotoxicity of the extract was 7% at 200 µL. This implicit that *M.sylvestris* extract has minimum side effect on normal cells thus may be considered safe and potential candidate as anticancer agent.

Keywords-Lymphoma, melanoma, cytotoxicity, *Malva sylvestris*, maceration.

INTRODUCTION

Cancer is one of the lethal diseases it is characterized via the irregular cell proliferation. The very common reason for cancer is lifestyle changes, therefore there is urgent requirement to discover a better treatment for this lethal disease (According to World Health Organization) [1]. High fatality and incidence implicit it as a significant public health and economical issue that requires an effective way for prevention. Medicinal plants owns many advantages than chemical products because the compounds that are derived from plants are more tolerant with no toxic effect to the normal cells of the human body [2]. Several pharmacological roles of these plants compounds includes antioxidant, antimicrobial, antiviral, anticancer, antifungal and anti-parasitic roles [3]. The available classic curatives for cancer therapy are radiotherapy and chemotherapy both have diverse side effects such as neurological effect, cardiac effect, renal and pulmonary toxicity effect sorely affecting the healthiness of the person. Thus a substitutional method for treatment is in demand that include development of anticancer drug that is less toxic and more potent as compare to the already available drugs in the market. Many studies have been done on naturally occurring plant compounds known to own cytotoxicity effects as they demonstrate potentiality to destroy cancerous cells. On account of the advantages of medicinal plants they are on top of demand and various species have been scanned and selected for the accommodation of cancer medicines. Newly, there has been an elevated interest in the study of compounds from a plant source as anticancer compounds by the concerned scientists. Many studies have showed the role of these plants in prevention and therapy of cancer [4]. presently, near 25% of the drugs produced in all around the world are extracted from plants in a direct way or at least one of its active ingredient is from plant origin, According to world health organization 80% of the world's population depend on plants derived drugs for treatment [5], Also WHO promotes the herbal derived drugs addition in health care programs because these drugs are easy to access at a low cost within the reach of the common people and are time tested, thus counted to be much safer comparing to up to date synthetic drugs [6]. *Malva sylvestris* is one of the medicinal herbs that used in both food and medicine, this plant characterized by a perennial root, a juicy annual stem of 2-3 height, large heart-shaped seven-lobed leaves, and the Flowers is closely similar to that of honeysuckle [7]. Nowadays, the consumption of this plant is broad spread because modern researches have revealed the important medicinal properties of this plant such as; anti-ulcerogenic, antioxidant, anticancer, and anti-inflammatory [8; 9; 10; 11; 12; 13; 14; 15; 16; 17].

This research is aimed to investigate the cytotoxic activity of the crude methanolic extract of *M.sylvestris* leaves against two cancerous human cell lines which are melanoma and lymphoma.

MATERIALS AND METHODS

Collecting plant material and extract preparation

The plant material (leaves) of *M.sylvestris* were collected from Baghdad university gardens and identified by the herbarium in the department of biology/college of science/ university of Baghdad. The plant leaves was cleaned well, washed with tap water and then in distilled water, then wiped and dried in a ventilated cool place away from the sun. After drying the plant material were crushed using a grinder and kept until further usage. The raw plant extract is obtained by maceration which is leaving the powder of the plant leaves in prolonged contact with a solvent (methanol). The separation done by filtration, the powder was macerated for 24 hours at ambient temperature in a mixture of methanol and water (80/20, V/V), the extraction repeated three times with renewal of the solvent, then the macerates were combined then filtered by a filter paper and the solvent was removed from the filtrate under vacuum at 45^{o}C by a Rota vapor [18].

Chemical detection of secondary metabolites

Test for tannins (Braymer's test):

1ml of distilled water was added for diluting the extract sample and then two drops of ferric chloride was added to the sample. A transient greenish to black color indicates the presence of tannins [19].

Test for saponins (foam test):

Small quantity of the extract was diluted with 4ml of distilled water, then the mixture was shaken. Persistence of foam for 10 minutes indicates the presence of saponins [20].

Test for terpenoids (Salkwoski test):

The extract was mixed with 0.4ml of chloroform, then 0.6ml of concentrated H_2SO_4 was carefully added to the sample to form a layer. A reddish brown color at the interface is formed as a positive result for the presence of terpenoids [21].

Test for flavonoids (NAOH Test):

Few drops of sodium hydroxide solution added to the extract sample. Formation of intense yellow color, which turn colorless after the addition of acid indicates the presence of flavonoids [22].

Test for phenol (ferric chloride Test):

Addition of few drops of ferric chloride solution to the extract sample. Formation of bluish to black color indicates the presence of phenols [23].

Test for Alkaloids (Mayer's Test):

2% H_2SO_4 was added to 1ml of the extract sample and warmed for two minutes, then filtered and few drops of mayer's reagent were added. A creamy-white color precipitation is a positive test [24].

Gas chromatography-mass spectrum analysis (GC-MS)

The methanolic fraction of the extract analyzed via GC–MS (HP6890, USA) equipped with capillary silica HP5 column (30m×0.25mm i.d. film thickness 0.25 µm). The carrier gas was helium (1ml.min-1) and the injector temperature was of 280^0C, The temperature program was set as follows: 50^0 C hold for 2 min then raised in a ratio of 5^0C (min-1) until 300^0C then hold for 5 min. Mass spectrometer (HP 5973) was an electron impact (EI) type (70 eV) programmed from m/z 10 to m/z 400. The ion source and interface temperatures were set at 230 and 280^0 C respectively [25].

Maintenance of cell cultures

Cell lines were obtained from the Iraq biotech cell bank unit and maintained in RPMI-1640 supplemented with 10% Fetal bovine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged using Trypsin-EDTA reseeded at 50% confluence twice a week, and incubated at 37 °C [26].

Cytotoxicity assay

To determine the cytotoxic effect, the MTT cell viability assay was conducted on 96-well plates. Cell lines were seeded at 1 × 10^4cells/well. After 24 hours or a confluent monolayer was achieved, cells were treated with tested compound. Cell viability was measured after 72 hours of treatment by removing the medium, adding 28 µL of 2 mg/mL solution of MTT (and incubating the cells for 2.5 h at 37 °C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 130 µL of DMSO (Dimethyl Sulphoxide) followed by 37 °C incubation for 15 min with shaking [27]. The absorbency was determined on a microplate reader at 492 nm (test wavelength), the assay was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as the following equation:-

Cytotoxicity = A-B/A *100

Where A and B are the optical density of control and the optical density of test.

RESULTS AND DISCUSSION

Chemical detection

The maceration of 500 gm of the leaves powder in 5L of the solvent (80% methanol, 20% water) resulted in 50gm of the extract, the extraction ratio was 1-10 w/v. The chemical tests of the extract for the presence of the main secondary metabolites showed the presence of tannins,saponins,terpenoids, flavonoids,phenols but no alkaloids table(1). Shelbaya *et al.*, noticed that tannins, saponins, and flavonoids are present in the extracts of *M.sylvestris* leaves [28], our result also agree with the findings of Quave *et al.*, and Cutillo *et al.*, [29, 30] who confirmed the presence of terpenes and phenolic acids in *M.sylvestris* extract.

GC-MS analysis

The identification of biochemical compounds of the cold methanolic extract of *M.sylvestris* leaves was analyzed using GC-MS. The mass spectra of the obtained compounds has compatibility with those of standards available in the mass spectrum data-base. The cold methanolic extract showed 29 peaks in the GC-MS chromatogram which were characterized and listed based on their retention time (RT) and their peak area (Area %) as shown in table(2). The prominent peaks are: trans-Phytol; Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1.alpha.,2.beta.,5.beta.)]; Dihydrogeraniol; Menthol, trans-1,3,trans-1,4; (+)-Isomenthol 22.942 (27.01) , alpha.-Linolenic acid; Dihomo-.gamma.-linolenic acid; (9E,12E,15E)-9,12,15-Octadecatrien-1-ol; cis,cis,cis-7,10,13-Hexadecatrienal; Gamolenic Acid 23.224 (15.38), Tetracontane, 3,5,24-trimethyl; Tritetracontane; Di-tert-dodecyl disulfide; Sulfurous acid, pentadecyl 2-propyl ester; Sulfurous acid, 2-propyl tetradecyl ester 26.435 (7.35), Tritetracontane; Tetratetracontane; Heptadecane; Hexadecane; Sulfurous acid, butyl heptadecyl ester 22.419 (6.55), Heptadecane; 2,6,10,15-Tetramethylheptadecane; Norphytan; Heneicosane; Cetane 28.421 (5.15), Linolenic acid, methyl ester; 11,14,17-Eicosatrienoic acid, methyl ester; Linolenic acid; Methyl (Z)-5,11,14,17-eicosatetraenoate; Methyl 8,11,14-heptadecatrienoate 22.801 (4.93), Pentadecylic acid; Tridecylic acid; Eicosanoic acid; Palmitic acid; Myristic acid 21.390 (4.31), Methyl (4Z,7Z,10Z,13Z,16Z,19Z)-4,7,10,13,16,19-docosahexaenoate; cis-5,8,11,14,17-Eicosapentaenoic acid, trimethylsilyl ester; cis-5,8,11,14,17-Eicosapentaenoic acid, methyl ester; Methyl eicosa-5,8,11,14,17-pentaenoate; 5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z) 27.750 (4.13), Heneicosane; Tetracosane; Heptadecane; 2,6,10,15-Tetramethylheptadecane; 7-Methyl-octadecane 29.730 (3.52), Hexadecyl iodide; Pentadecane, 8-hexyl; Tetratetracontane; 2,6,10,15-Tetramethylheptadecane; Icosane 30.246 (2.92).

Table (1) Chemical detection the maethanolic extract of *M.sylevestris*

Methanolic extract	Tannins	Saponins	Terpenoids	Flavonoids	phenols	Alkaloids
	+	+	+	+	+	-

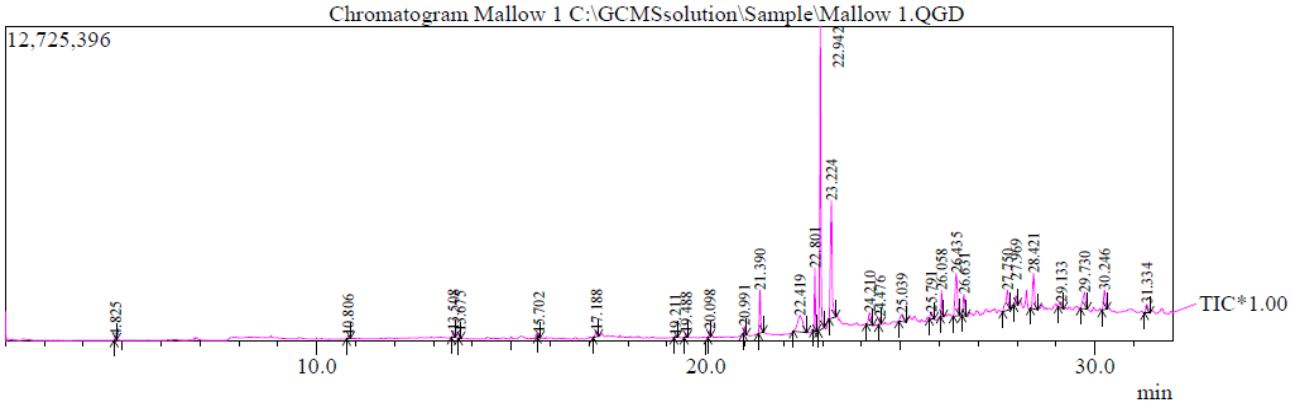

Figure 1. GC-MS analysis of *M.sylvestris* methanolic extract

Table (2) GC-MS analysis of *M.sylvestris* methanolic extract

Peak No.	Compound Name	R.T.	Area %
1	2-methyl-N-(2-methylpropylidene); N-Butylidene-1-butanamine; Ethanimine,N-t-butyl; 2-Piperidinemethanol; 2-Ethyl-1-hexanol	4.825	0.34
2	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one; 2,4,5-Trimethyl-1,3-dioxolane; 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furanone; 2,4-Dimethyl-1,3-dioxane; 1,2,4,5-Tetramethyl-1,2,4,5-tetraazinane	10.806	0.29
3	2-Methoxy-4-vinylphenol; 2,6,2',6'-Tetramethylazobenzene; Benzoic acid, 4-ethyl; 6-Phenyltetrahydropyran-2,4-dione; 1,3-Methano-5bH-cyclobuta[cd]pentalen-5b-ol, octahydro	13.508	0.65
4	2,2,4,4,6,8,8-Heptamethylnonane; 2,2,4,4-Tetramethyloctane; Octadecane, 2,2,4,15,17,17-hexamethyl-7,12-bis(3,5,5-trimethylhexyl); 2,2,4,4,5,5,7,7-Octamethyloctane; Tridecane,2,2,4,10,12,12-hexamethyl-7-(3,5,5-trimethylhexyl)	13.675	0.43
5	trans-Z-.alpha.-Bisabolene epoxide; .alpha.-Limonene diepoxide; cis-Z-.alpha.-Bisabolene epoxide; 3-Methyl-4-(phenylthio)-2-prop-2-enyl-2,5-dihydrothiophene 1,1-dioxide;	15.702	0.30
6	Methyl .beta.-d-galactopyranoside; .alpha.-Methylglucoside; .alpha.-Methyl-D-mannoside; .alpha.-D-Galactopyranose methyl glycoside; Methyl .beta.-D-glucopyranoside	17.188	0.83
7	Cyclobutanecarboxylic acid, 2-ethylcyclohexyl ester; 2-Ethyl-3-oxo-4-pyrrolidin-2-ylidene-butyronitrile; Cyclohexanecarboxylic acid, 2-ethylcyclohexyl ester; 5-Methyl-2-pyrimidone; Octanoic acid, 2-isopropoxyphenyl ester	19.211	0.19
8	p-Menthane, 1,2:8,9-diepoxy; cis-Z-.alpha.-Bisabolene epoxide; 1,2-Dihexylcyclopropene-3-carboxylic acid; 5-Isopropenyl-2-methyl-7-oxabicyclo[4.1.0]heptan-2-ol; 6-tert-Butylbicyclo[2.2.1]heptan-2-ol	19.488	0.37
9	3,7,11,15-Tetramethyl-2-hexadecen-1-ol; (8Z)-14-Methyl-8-hexadecen-1-ol; 9-Eicosyne; Oleyl Alcohol; 1,19-Eicosadiene	20.098	0.49
10	Methyl 5,9,13-trimethyltetradecanoate; Methyl 10-methyldodecanoate; Methyl 10-methylheptadecanoate; Cerotic acid methyl ester; Methyl 18-methylicosanoate	20.991	0.58
11	Pentadecylic acid; Tridecylic acid; Eicosanoic acid; Palmitic acid; Myristic acid	21.390	4.31
12	Tritetracontane; Tetratetracontane; Heptadecane; Hexadecane; Sulfurous acid, butyl heptadecyl ester	22.419	6.55
13	Linolenic acid, methyl ester; 11,14,17-Eicosatrienoic acid, methyl ester; Linolenic acid; Methyl (Z)-5,11,14,17-eicosatetraenoate; Methyl 8,11,14-heptadecatrienoate	22.801	4.93
14	trans-Phytol; Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1.alpha.,2.beta.,5.beta.)]; Dihydrogeraniol; Menthol, trans-1,3,trans-1,4; (+)-Isomenthol	22.942	27.01
15	.alpha.-Linolenic acid; Dihomo-.gamma.-linolenic acid; (9E,12E,15E)-9,12,15-Octadecatrien-1-ol; cis,cis,cis-7,10,13-Hexadecatrienal; Gamolenic Acid	23.224	15.38
16	Tetratetracontane; Tritetracontane; 2,6,10,15-Tetramethylheptadecane; n-Heptadecane; Eicosane	24.210	2.28
17	Hexanoic acid, 2-dimethylaminoethyl ester; 4-Butylbenzoic acid, 2-dimethylaminoethyl ester; Phenylacetic acid, 2-dimethylaminoethyl ester; Fumaric acid, 2-dimethylaminoethyl octadecyl ester; 3-Phenylpropionic acid, 2-dimethylaminoethyl ester	24.476	0.53
18	Heneicosane; Stearyl iodide; Tetracosane; Cetyl iodide; Heptadecane	25.039	1.80
19	Octadecyl chloride; Valtrate; 1-Octadecanesulphonyl chloride; Palmityl chloride; Sulfurous acid, octadecyl 2-propyl ester	25.791	1.05
20	Hexanoic acid, 2-dimethylaminoethyl ester; 3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester; Octanoic acid, 2-dimethylaminoethyl ester; Fumaric acid, 2-dimethylaminoethyl nonyl ester; 3-Phenylpropionic acid, 2-dimethylaminoethyl ester	26.058	2.07
21	Tetracontane, 3,5,24-trimethyl; Tritetracontane; Di-tert-dodecyl disulfide; Sulfurous acid, pentadecyl 2-propyl ester; Sulfurous acid, 2-propyl tetradecyl ester	26.435	7.35
22	Eicosane; Tritetracontane; Tetratetracontane; Hexatriacontane; Heneicosane	26.631	2.81
23	Methyl (4Z,7Z,10Z,13Z,16Z,19Z)-4,7,10,13,16,19-docosahexaenoate; cis-5,8,11,14,17-Eicosapentaenoic acid, trimethylsilyl ester; cis-5,8,11,14,17-Eicosapentaenoic acid, methyl ester; Methyl eicosa-5,8,11,14,17-pentaenoate; 5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)	27.750	4.13
24	Linolenic acid; Linolenic acid, methyl ester; Ethyl 9,12,15-octadecatrienoate; n-Propyl 9,12,15-octadecatrienoate; 7-Propylidene-bicyclo[4.1.0]heptane	27.969	1.11
25	Heptadecane; 2,6,10,15-Tetramethylheptadecane; Norphytan; Heneicosane; Cetane	28.421	5.15
26	4,6-Cholestadien-3.beta.-ol; Ethyl iso-allocholate; Stigmasta-5,22-dien-3-ol, acetate, (3.beta.); Cholestan-19-ol, 5,6-epoxy-3-fluoro-, acetate, (3.beta.,5.alpha.,6.alpha.); 4,6-Cholestadiene-3-one, 2,4-dinitrophenylhydrazone	29.133	1.23
27	Heneicosane; Tetracosane; Heptadecane; 2,6,10,15-Tetramethylheptadecane; 7-Methyl-octadecane	29.730	3.52
28	Hexadecyl iodide; Pentadecane, 8-hexyl; Tetratetracontane; 2,6,10,15-Tetramethylheptadecane; Icosane	30.246	2.92
29	4-Methyltetradecane; 3-Formylamino succinimide; Decane, 2,3,4-trimethyl; N-(2-Cyanoethyl)-N'-methylpiperazine; 4-Methyldodecane	31.334	1.40

Cytotoxicity evaluation

The cytotoxicity assay showed that the methanolic extract of *M.sylvestris* leaves is active on both melanoma and lymphoma cell lines at all the tested concentrations (10, 50,100,150,200 µL) and the cytotoxic effect increased with increasing the extract concentration, the extract was cytotoxic to melanoma in a higher rate (76.53%) than lymphoma (68.65%) at the same concentration (200 *M*l) of the extract , also there was a minimal cytotoxic effect against normal cell line reached to 7% at the same concentration.

Table (3) The cytotoxicity of *M.sylvestris* extract on lymphoma cell line

No.	Extract Concentration µL	Cytotoxicity	Standard Error (+,-)
1	10	10	1.528
2	50	23.27	0.617
3	100	42.67	1.545
4	150	60.33	3.210
5	200	68.65	1.925

Table (4) The cytotoxicity of *M.sylvestris* extract on melanoma cell line

No.	Extract Concentration µL	Cytotoxicity	Standard Error +,-
1	10	11.67	1.453
2	50	25.33	2.728
3	100	45.77	2.751
4	150	68.23	4.931
5	200	76.53	2.881

Table (5) The cytotoxicity of *M.sylvestris* extract on reference cell line

No.	Extract Concentration µL	Cytotoxicity	Standard Error +,-
1	10	4.433	0.480
2	50	5	0.577
3	100	5.333	0.333
4	150	6.167	0.441
5	200	7	0.577

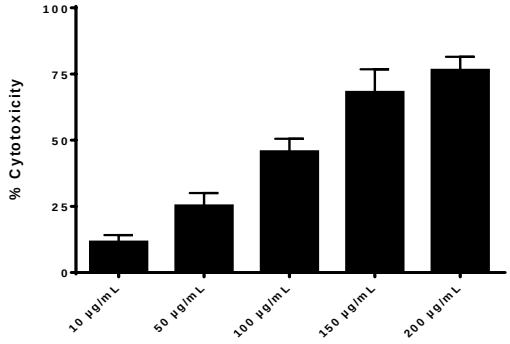

Figure2. Cytotoxicity on melanoma

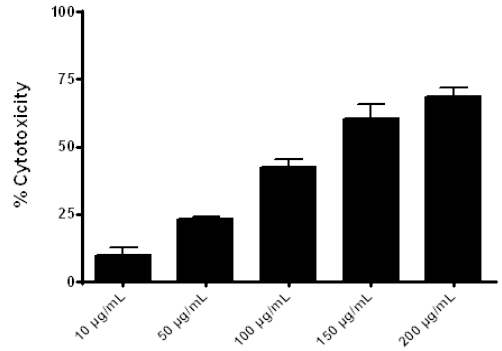

Figure3. Cytotoxicity on lymphoma

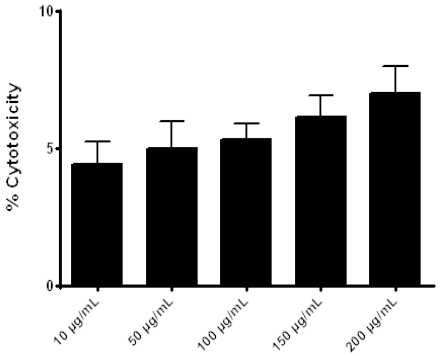

Figure 4. Cytotoxicity on reference cell line

Our result agrees with the findings of Alesiani Daniela *et al.*, [31] who found that *M.sylvestris* extract has a cytotoxic effect on B16 and A375 cell lines. In B16 cells, the extract showed an activity of 61% and 97% for 1:200 and 1:40 dilution, respectively. For A375 cells, the 1:10 dilution the reduction of the cell proliferation was 58%, the 1:40 dilution reduction of 85%, there biological assay showed for the first time that *M. sylvestris* extract is cytotoxic to cancer cell lines. In a study done by Razavi *et al.*, [32] the viability of McCoy cells was reduced by the methanol extract of *M.sylvestris* flowers and leaves. In another study done by Cagla bozkurt *et al.*, {33} relative cytotoxicity against Hella cell line with IC50 value of 29.8 mg/ml. All mentioned studies including this research showed that *M.sylvestris* extract possess a cytotoxic effect against different cancerous cell lines.

CONCLUSION

The GC-MS analysis prominent compounds were trans-Phytol; Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1.alpha.,2.beta.,5.beta.)]; Dihydrogeraniol; Menthol, trans-1,3,trans-1,4; (+)-Isomenthol. The extract of *M.sylvestris* is cytotoxic to both melanoma and lymphoma cell lines 76.53% and 68.56% respectively, minimum cytotoxicity against normal cell line (reference) was 7%, these findings give a perception that this plant is as a good candidate for the manufacture of a new anticancer drug of a natural source.

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