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Research Article

The Effect of *Helichrysum odoratissimum* (L.) Sweet on Cancer Cell Proliferation and Cytokine Production

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ABSTRACT
The cytotoxicity of *Helichrysum odoratissimum* against human epidermoid carcinoma (A431), malignant melanoma (A375), cervical epithelial carcinoma (HeLa) and human embryonic kidney (HEK-293) cells was investigated. The cytokine production and antioxidant activity was also determined. *H odoratissimum* was selected based on its traditional usage as a dressing for wounds. In this study the cytotoxicity was performed on skin and cervical cancer which are associated with the presence of wounds. *H. odoratissimum* showed inhibitory activity against A431 cells, with a fifty percent inhibitory concentration (IC_{50}) of 15.5±0.2 µg/ml, and a high DPPH inhibition with an IC_{50} of 5.13±0.07 µg/ml. A431 cells treated with the extract showed an increase in morphological characteristics associated with apoptosis. The extract induced IL-12 in U937 cells and inhibited IL-8 at the tested concentrations, with the highest levels being 12.4±7 pg/ml and 103±6.1 pg/ml respectively. IL-2 was inhibited by 56 % and 52 % in naive and PHA induced murine splenocytes respectively. Furthermore, 2 lipophilic fractions and 3 compounds were isolated from the extract. The lipophilic fractions showed relative cytotoxicity on A431 cells with IC_{50} values of 175±13.5 µg/ml and 61.3±0.16 µg/ml. The extract showed significant hepatoprotection at 25 µg/ml on HepG2 cells exposed to D-Galactosamine. This is the first report of the activity of *H. odoratissimum* ethanolic leaf stem extract on A431, A375, HeLa and HEK-293 cell lines as well as the isolation of two promising lipophilic fractions.

Keywords: *Helichrysum odoratissimum*; Cytotoxicity; Melanoma; Cervical cancer; Hepatoprotection; Interleukin; Light Microscopy.

INTRODUCTION

Approximately 27 million South Africans are reported to rely on traditional herbal medicine as a primary source of healthcare[1]. From the large plant diversity in South Africa, it has been estimated that approximately 3000 plant species are used for medicinal purposes[2]. According to the Cancer Association of South Africa, one in every six males and one in every seven females in South Africa will get cancer during their lifetime. In a recent study it has been predicted that the number of cancer cases in South Africa could increase by 78% by the year 2030[3].

Helichrysum odoratissimum (L.) Sweet (HO) is an aromatic herbaceous shrub from the Asteraceae family and is commonly known as 'Imphepho' in Xhosa and Zulu and 'Kooigoed' in Afrikaans. It flowers throughout the year producing bright yellow flowers, which gives rise to the genus name which refers to the flowers' golden colour. The genus *Helichrysum* comprises about 600 species which are largely distributed in Africa, Asia, Australia, Madagascar and Europe, of which 244 species occur in southern Africa[4]. The plant is reported to have many traditional usages for various ailments and is used culturally for religious purposes. The Zulus, a South African tribe, often use it to make smoking blends from the leaves and stems, which are used to induce a deep trance, during which sacred messages may be received from their ancestors[5]. The roots are used to treat coughs and colds and are also used as a cleanser. Furthermore, the leaf pulp is used as a dressing for treating wounds[6]. The flowers of the plant are made into a paste which is used for treating acne and pimples[4].

H. odoratissimum was selected for this study due to its traditional usage for treating wounds. In the present study the cytotoxicity of the extract was tested against skin cancer and cervical cancer which can both be associated with the presence of wounds. Thereafter, the mechanism of action was determined by noting various cytokines level of production and the cells morphological characteristics using microscopy.

MATERIALS AND METHODS

Chemicals and reagents

The A431 and HepG2 cells were obtained from European Collection of Cell Cultures (ECACC), England, UK. The A375 cells and HEK-293 cells were kindly donated by the Department of Biochemistry, University of Johannesburg and the Department of Molecular Medicine and Haematology from the University of the Witwatersrand

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respectively. The HeLa cells, U937 cells, cell culture medium and antibiotics were purchased from Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, RSA) Foetal bovine serum (FBS) was purchased from Separations (Pty) Ltd. (Randburg, Johannesburg, RSA). The Cell Proliferation Kit II (XTT) for the cytotoxicity assay was purchased from Roche Diagnostics (Pty) Ltd. (Randburg, Johannesburg, RSA). The reagent set B, BD Falcon™ ELISA plates, human interleukin 12 (IL-12) (p40), human interleukin 8 (IL-8), and mouse IL-2 ELISA kits were purchased from BD Biosciences, San Diego, CA, USA. All other chemicals and reagents were of analytical grade and were acquired from Sigma Chemicals Co. (St. Louis, MO, USA).

Plant material

The leaves and stems of *H. odoratissimum* were collected in Venda, an area in the Limpopo province, South Africa. The plant material was identified by Ms. Magda Nel, a taxonomist at the University of Pretoria,and a voucher specimen (PRU 96677) was deposited in the HGWJ Schweickerdt Herbarium, Pretoria, South Africa.

Preparation of extract

Plant material was shade dried for two weeks and then ground to a fine powder. The dried powder (5 kg) was macerated in distilled ethanol (7 L) and shaken for 48 hrs. The plant material was filtered using a Buchner funnel with Whatman No.1 filter paper. Thereafter, the filtrate was subjected to reduced pressure using a Büchi Rotavapor R-200. The extract was kept in a cold room until further use.

In vitro cytotoxicity assay

The human cervical epithelial carcinoma (HeLa), human embryonic kidney (HEK-293) and human hepatocarcinoma (HepG2) cell lines were maintained in culture flasks containing Eagle's Minimum Essential Medium (EMEM), whereas the human epidermoid carcinoma (A431) and human malignant melanoma (A375) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM). The human leukemic monocyte lymphoma (U937) cells and the mice splenocytes were maintained in RPMI 1640 and the latter was supplemented with 50µM β-mercaptoethanol. All cell lines were supplemented with 1% antibiotics (100 U/ml penicillin, 100µg/ml streptomycin and 250 µg/Lfungizone) and 10% heat-inactivated fetal bovine serum. The cells were grown at 37 °C in a humidified incubator set at 5% CO_2. Cells were sub-cultured every 2-3 days after the cells had formed a confluent monolayer.

Cytotoxicity was measured by the 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide salt (XTT) method using the Cell Proliferation Kit II. The method described by Berrington & Lall[7] was used to perform the assay. All cell lines, except the HepG2 cells, were seeded (100 µl) in a 96-well microtitre plate at a concentration of 1×10^5 cells/ml. The plate was then incubated for 24 h at 37 ºC and 5 % CO_2 to allow for cell adherence. To allow the U937 cells to differentiate into macrophages and to attach, phorbol-12-myristate 13-acetate (PMA) was added at a final concentration of 0.1 µg/ml. The extract was prepared to a stock solution of 20 mg/ml and added to the microtitre plate. Serial dilutions were made and the final concentration of the extract ranged from 1.6–200 µg/ml. The same concentrations were prepared for the pure compounds and lipophilic fractions which were tested on the A431 cells. The microtitre plate was incubated for a further 72 h. The control wells included vehicle-treated cells (2 % DMSO), a medium only control and the positive control, 'Actinomycin D', with concentrations ranging between 3.9×10^{-4} - 0.05 µg/ml. After the 72 h incubation period the XTT reagent (50 µl) was added to a final concentration of 0.3 mg/ml and the plate was then further incubated for another 2 h. The absorbance of the colour complex was read at 490 nm with a reference wavelength set at 690 nm using a BIO-TEK Power-Wave XS multi-well plate reader (A.D.P, Weltevreden Park, South Africa). The assay was performed in triplicate to calculate a fifty percent inhibitory concentration (IC_{50}) of the cell population.

DPPH free radical inhibition

The method of Berrington & Lall[7] was followed to determine the radical scavenging capacity (RSC) of the extract/lipophilic fractions. Stock solutions of Vitamin C, the positive control, and the extract/ lipophilic fractions were prepared at concentrations of 2 mg/ml and 10 mg/ml respectively. To each well in the top row of a 96-well plate, 200 µl of distilled water was added. To the rest of the wells 110 µl of distilled water was added. Twenty microlitres of extract/lipophilic fractions was added to the first top wells, in triplicate, followed by serial dilution with final concentrations ranging from 3.9-500 µg/ml for the extract and 0.781-100 µg/ml for Vitamin C. For the solvent control, ethanol was added instead of extract. Finally 90 µl of 0.04 M 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) ethanolic solution was added to each well, except for the negative control where distilled water was added instead. The plates were left in a dark room to develop for 30 min. Statistical analysis of the RSC of the extract was determined using a BIO-TEK Power-Wave XS multi-plate reader at a wavelength of 515 nm, using KC junior software. The IC_{50} value was calculated and the Vitamin C equivalent was calculated as follows: (IC_{50} of extract X 200 mg Vitamin C)/ IC_{50} of Vitamin C.

Nitric oxide inhibition

The method of Mayuret al.[8] was followed to determine the nitric oxide scavenging capacity of the extract/lipophilic fractions. Stock concentration of Vitamin C, the positive control, the extract and the lipophilic fractions were prepared at 10 mg/ml. Briefly, 90 µl of distilled water was added to the top row of a 96-well microtitre plate and 50 µl to the rest of the wells in the plate. To the top wells, 10 µl of plant extract/lipophilic fractions, positive control and ethanol solvent control were added, in triplicate, followed by serial dilution with final concentration ranging from 7.81-1000 µg/ml. Thereafter, 50 µl of sodium nitroprusside was added to all the wells and the plate was incubated under light at room temperature for 90 min. After the incubation period, 100 µl of Griess reagent was added to all the wells of the plate except for the negative colour control wells, where distilled water was added instead. The plates were read using a BIO-TEK Power-

Wave XS multi-well reader at a wavelength of 546 nm, using KC Junior software and the IC_{50} value was calculated.

Hydrophilic oxygen radical absorbance capacity (ORAC)

The assay was performed as described by Davalos et al[9]. Firstly 1 mg/ml stock solution of the extract was prepared in 75 mM phosphate buffer (pH 7.4). A pre-incubation mixture was prepared which contained the extract at 1 µg/ml and 0.5 µg/ml respectively, or Trolox, the positive control, at various concentrations or a blank containing 75 mM sodium phosphate buffer, and 70.2 nM of sodium fluorescein. A negative colour control was included to subtract the interference of the extract colour. The mixture was incubated at 37 °C for 10 min. After incubation, 12 mM of (2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) was added and mixed for 15 sec. The reaction was carried out for 90 min at 37 °C. The fluorescence measurements were read at 485 nm excitation and 520 nm emission filters. The activity of the extract was expressed as micro moles of Trolox equivalent per gram of extract.

Lipophilic oxygen radical absorbance capacity (ORAC)

The assay was performed as described by Hung et al[10]. A 1 mg/ml stock solution of the extract was prepared in acetone. Further dilutions of the extract were prepared in 7 % Methylated β-cyclodextrin DS-12 (RMCD). A pre-incubation mixture was prepared which contained the extract at 1 µg/ml and 0.5 µg/ml respectively or Trolox, the positive control, at various concentrations or a blank containing 75 mM sodium phosphate buffer and 75 nM of sodium fluorescein. A negative colour control was included to subtract the interference of the extract colour. The mixture was incubated at 37 °C for 10 min. Thereafter, 30 mM of AAPH was included and mixed for 15 sec. The reaction was carried out for 90 min at 37 °C. The fluorescence measurements were read at 485 nm excitation and 520 nm emission filters. The activity of the extract was expressed as micro moles of Trolox equivalent per gram of extract.

In vitro hepatoprotection effect and cytotoxicity in HepG2 cell line

The cytotoxicity of the extract on the liver hepatocellular carcinoma (HepG2) cells was determined prior to the hepatoprotective assay to determine the appropriate doses to be tested in the assay. The human HepG2 cells were seeded in a 96-well plate at a concentration of 3×10^4 cells/well. The cells were incubated overnight at 37 °C and 5 % CO_2 to allow the cells to adhere. Thereafter, the cells were treated with the extract at concentrations ranging from 3.125-100µg/ml and incubated for a further 24 h. Controls included the positive control Silymarin, at 12.5 µg/ml, a vehicle control (0.1% DMSO) and medium only as the blank. After 24 h incubation, the cells were treated with 30 mM of D-galactosamine (hepatotoxin) and incubated for a further 24 h. Thereafter, the supernatant was discarded and the cells were washed with phosphate buffer saline (PBS), and the fresh medium along with MTT was added and incubated for 1 h to allow the formation of formazan crystals. Finally, the medium was removed and the formazan crystals were dissolved using DMSO and the absorbance was measured at 570 nm. Percentage (%) hepatoprotection was calculated using the below equation:

$$\%\ hepatoprotection = \frac{\%\ cell\ viability\ of\ tested\ sample - 0\%\ control}{100\%\ control - 0\%control} X100$$

Where 0% control = % cell viability of D-Galactosamine (toxic inducer) and 100% control = % cell viability of medium and cells control.

Light microscopy

Haematoxylin and eosin staining was used to analyse the morphological characteristics of the A431 cells after exposure to ½ × IC_{50} (7.5 µg/ml), IC_{50} (15 µg/ml) and 2 × IC_{50} (30 µg/ml) of the ethanol extract of *H. odoratissimum*. Light microscopy was performed to determine qualitatively the influence of the various concentrations of extract on the A431 cell morphology. A range of concentrations were tested, which included the IC_{50} value and these ranges represent a dose-response and significant effects on the cell numbers (anti-proliferative activity) were observed at these concentrations. Exponentially growing cells were seeded at 1×10^5 cells/ml in 6-well plates on heat-sterilized cover-slips and incubated for 24 h at 37 ºC and 5 % humidity, for cell adherence to occur. Thereafter, cells were exposed to 7.5 µg/ml, 15 µg/ml and 30 µg/ml of *H. odoratissimum* extract respectively as well as a vehicle-treated control (2 % DMSO), medium and cells only control and 0.025 µg/ml Actinomycin D control. Cells were further incubated for 72 h at 37 °C. Cells were then fixed in Bouin's fixative for 30 min and stained by standard haematoxylin and eosin staining procedures[11].

Cytokine production

The levels of cytokine production (IL-8, IL-12 and IL-2) from cell supernatants were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (BD Biosciences, San Diego, CA, USA). Briefly U937 cells and murine splenocytes were plated at a concentration of 2.5×10^5 and 5×10^5 cells/well in 24-well and 48-well plates respectively. The U937 cells, with additional 0.1 µg/ml PMA, and the murine splenocytes, were incubated for 24 h at 37 °C and 5% CO_2. The cells were treated with either various concentrations of *H. odoratissimum* extract plus PHA (2 µg/ml) or with different controls for 20 h and 48 h for the U937 and murine splenocytes respectively. Controls for the U937 cells included 20 µg/ml pentoxifylline and 5 µg/ml phytohemagglutinin (PHA); and the controls for the murine splenocytes included 0.1 % DMSO and 5 µg/ml PHA. After the incubation periods, the cells were centrifuged at 980 rpm for 5 min to collect the cell free supernatant and analyse the concentration of IL-8, IL-12 and IL-2.

Isolation of compounds from H. odoratissimum

Approximately 130 g of the ethanolic extract of *H. odoratissimum* was subjected to silica gel column chromatography with hexane:ethyl acetate mixtures of increasing polarity (100:0 to 0:100) followed by ethyl acetate :methanol mixtures with increasing polarity (100:0 to 0:100). In total 220 fractions were collected and similar

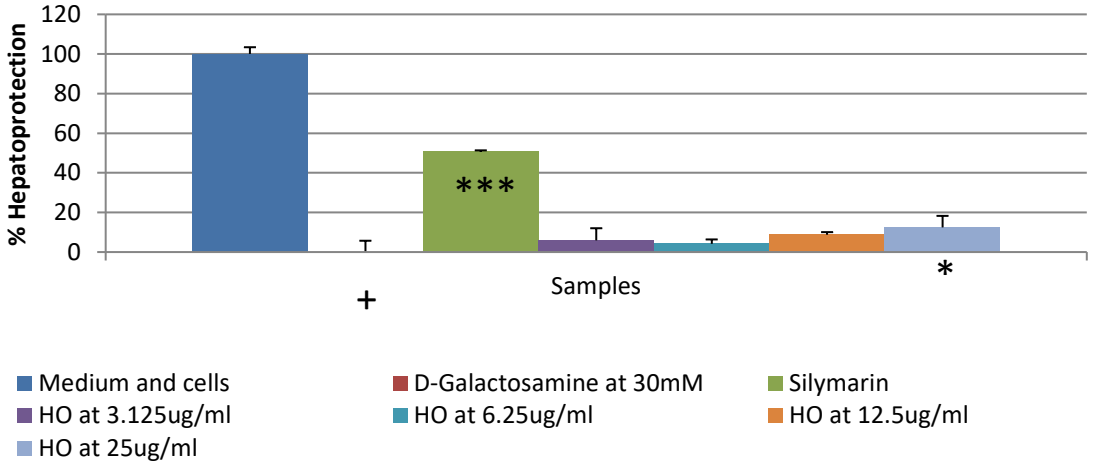

Figure 1: Cytoprotective effect of *Helichrysum odoratissimum* on D-Galactosamine treated Hep-G2 cells. Cells were treated with various concentrations of *H. odoratissimum* (3.125–100 µg/ml) plus D-Galactosamine (30 mM). The % hepatoprotection was measured after 24h treatment. Controls included Silymarin (12.5 µg/ml) as the positive control D-Galactosamine (30 mM) as the toxic inducer. Data was expressed as means ± SD (n = 3). **P*<0.05 and ****P*<0.001 compared with D-Galactosamine (+).

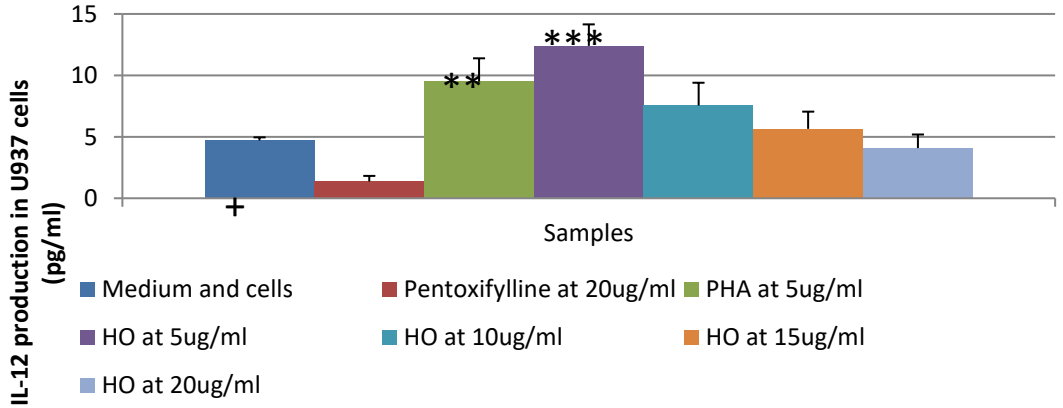

Figure 2: Effect of *Helichrysum odoratissimum* and controls on IL-12 production in U937 cells. U937 cells were treated with various concentrations of *H. odoratissimum* (5–20 µg/ml) plus PHA (2 µg/ml) to determine the production of IL-12 after 20 h. Controls included pentoxifylline (20 µg/ml) and PHA (5 µg/ml). Data shown are mean ± SD (n = 3). ***P*<0.01 and ****P*<0.001 compared with the medium and cells control (+).

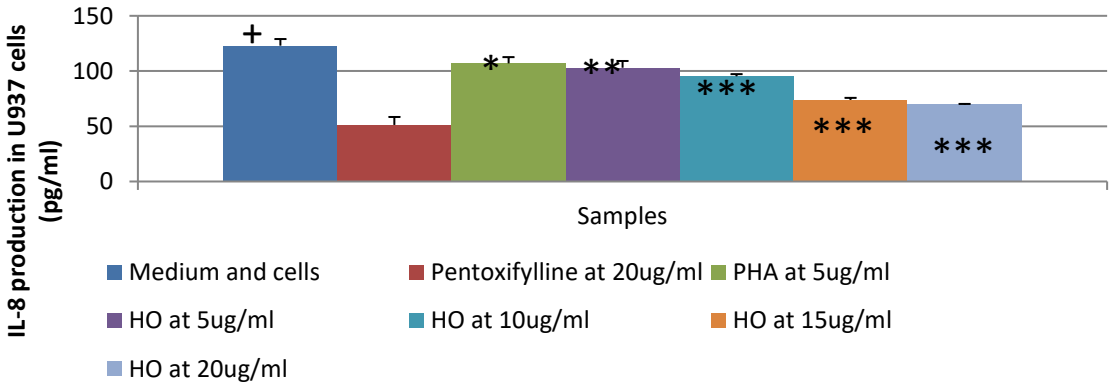

Figure 3: Effect of *Helichrysum odoratissimum* and controls on IL-8 inhibition in U937 cells. U937 cells were treated with various concentrations of *H. odoratissimum* (5–20 µg/ml) plus PHA (2 µg/ml) to determine the inhibition of IL-8 after 20 h. Controls included pentoxifylline (20 µg/ml) and PHA (5 µg/ml). Data shown are mean ± SD (n = 3). **P*<0.05, ***P*<0.01 and ****P*<0.001 compared with the medium and cells control (+).

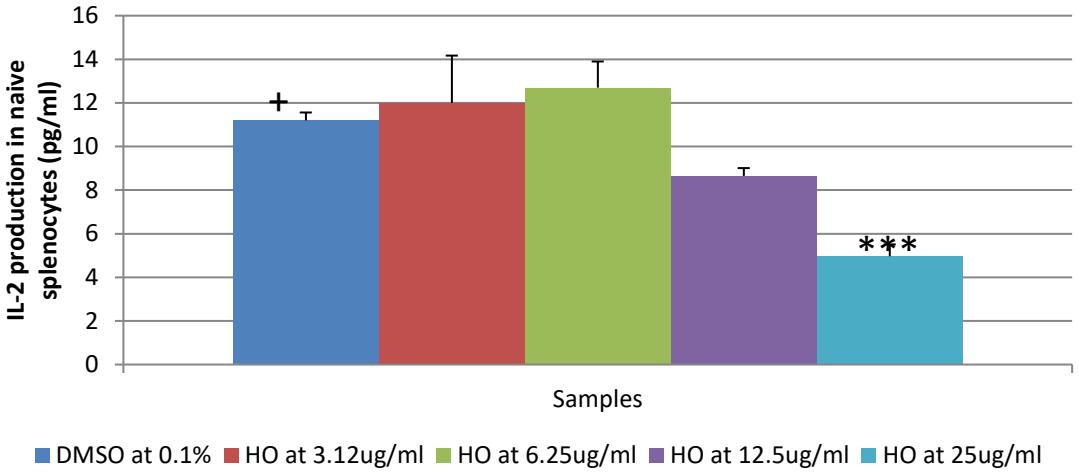

Figure 4: Effect of *Helichrysum odoratissimum* and control (DMSO at 0.1 %) on naive splenocytes. Naive splenocytes were treated with various concentrations of *H. odoratissimum* (3.12–25 µg/ml) to determine the levels of IL-2 after 48 h. Data shown are mean ± SD (n = 3). ****P*<0.001 compared with the DMSO (0.1 %) control (+).

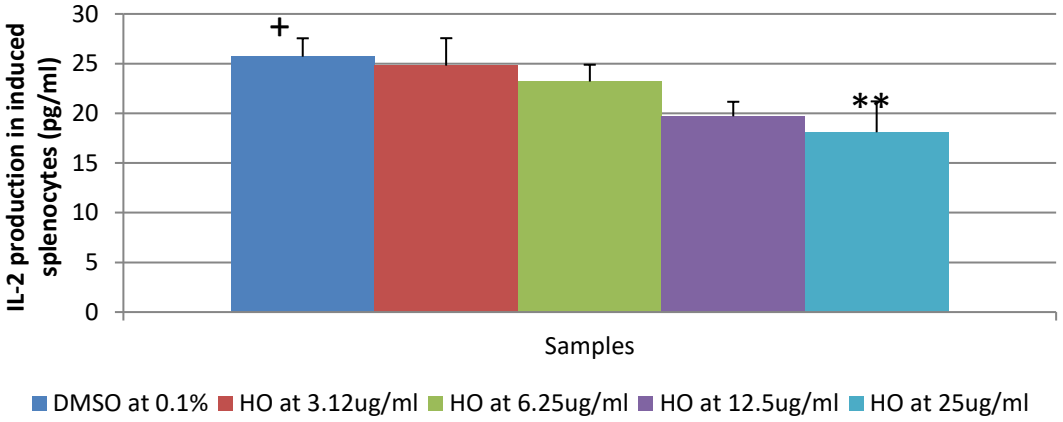

Figure 5: Effect of *Helichrysum odoratissimum* and control (DMSO at 0.1 %) on PHA induced splenocytes. Splenocytes were treated with various concentrations of *H. odoratissimum* (3.12–25 µg/ml) to determine the levels of IL-2 after 48 h. Data shown are mean ± SD (n = 3). ***P*<0.01 compared with the DMSO (0.1 %) control (+).

fractions were combined according to thin-layer chromatographic (TLC) profile to obtain 18 major fractions. The first two fractions, Fr. A and Fr. B were obtained as pale yellow coloured oils. The two lipophilic fractions obtained during this study were designated as DP-HO-01 and DP-HO-02, respectively. Fraction F was subjected to another silica gel column with hexane:dichloromethane (DCM) mixtures of increasing polarity (100:0 to 0:100), which yielded compound **1**, tetracosane (DP/HO/03). Whereas, compound **2** (DP-HO-5) and **3** (DP-HO-6) were obtained during the column of fraction Q with the eluents as methanol:water mixtures of increasing polarity (100:0 to 0:100). Identification of the isolated compounds was done by Mass, ^{1}H and ^{13}C NMR spectroscopic data. Assignment of signals was facilitated by COSY, HSQC and HMBC experiments. The known compounds obtained in this study, Tetracosane (**1**) was identified based on the spectral analysis as well as comparison with respective literature reports[12]. However, compounds **2** and **3** were identified as inorganic components (clusters of KCl) based on the spectroscopic and physical analysis. All three compounds obtained in present study did not show significant cytotoxicity against the A431 cells. However, both the lipophilic fractions were found to exhibit relative cytotoxicity against the A431 cell line. Hence, it was decided to identify the constituents present in the lipophilic fractions. Both the samples DP-HO-01 and DP-HO-02 were submitted for GC-MS analysis.

Chemical composition of lipophilic fractions (GC-MS analysis)

The two samples DP-HO-01 and DP-HO-02 were analysed by gas chromatography coupled with a mass spectrometry (GC-MS) using an Agilent 6890N GC coupled directly to an Agilent 5975B MS. One microlitre of each sample was injected using a split ratio (20:1) with a CTC CombiPAL autosampler at an inlet temperature of 280 °C. The GC system equipped with a ZB-5MS Guardian (30 m, 0.25 mm ID, 0.25 um film thickness) ZB 7HG-G010-11 was used. The oven temperature programme was 70 °C for

Table 1: *In vitro* cytotoxicity, antioxidant activity, selectivity index and antioxidant equivalents of the *Helichrysum odoratissimum* extract.

Sample	HeLa	A431	A375	HEK-293	SI[b] HEK-293 vs HeLa	SI HEK-293 vs A431	SI HEK-293 vs A375	U937	DPPH[c]	NO[d]	Vitamin C equivalents in mg	H-ORAC Trolox equivalents in µmoles	L-ORAC
	IC_{50}[a]: (µg/ml) ± SD							IC_{50}: (µg/ml) ± SD					
H. odoratissimum	33.1±3.1	15.5±0.2	55.5±6.6	37.1±4.8	1.09	2.39	0.67	20.3±3.1	5.13±0.07	>1000	518	2542±34	3648±72
ActinomycinD[e]	0.005±0.001	0.04±0.001	0.035±0.001	0.003±0.001	0.6	0.08	0.09	-	-	-	-	-	-
Vitamin C[f]	-	-	-	-	-	-	-	-	1.98±0.01	285.9±25.6	-	-	-
PHA[g]	-	-	-	-	-	-	-	318±7.8	-	-	-	-	-
Pentoxifylline[h]	-	-	-	-	-	-	-	170±6.4	-	-	-	-	-
(DP-HO-01)		175±13.5	-	-	-	-	-	-	>500	104.7±15.2	NA	-	-
(DP-HO-02)		61.3±0.16	-	-	-	-	-	-	>500	78.42±9.56	NA	-	-
Tetracosane (1)		>200	-	-	-	-	-	-	-	-	-	-	-
DP-HO-05 (2)		>200	-	-	-	-	-	-	-	-	-	-	-
DP-HO-06 (3)		>200	-	-	-	-	-	-	-	-	-	-	-

[a]Fifty percent inhibitory concentration; [b]Selectivity index; [c]2,2-diphenyl-1-picrylhydrazyl; [d]Nitric oxide, [e]Positive control for cytotoxicity, [f] Positive control for antioxidant activity; [g] Positive control for cytokine production; [h] Control for cytokine production/ inhibition; [-] Not tested, [NA] Not applicable

the first 0.5 min, rising to 100 °C at a rate of 5 °C/min and held for 0.5 min and then rising to 240 °C at a rate of 5 °C/min and held for 1 min. Helium was used as a carrier gas at a constant flow of 1 ml/min. Spectra were obtained on electron impact at 70 eV, scanning from 25 to 650 m/z. Identification of the chemical compounds of the oil fractions was based on the GC retention time and computer matching of mass spectra with standards in the National Institute of Standards and Technology (NIST) Mass spectra (MS) (Version 2).

Molecular docking study

Molecular docking was performed using the GOLD program[13]. It uses genetic algorithm and considers ligand conformational flexibility and partial protein flexibility i.e. side chain residues[14]. The default docking parameters were employed for the docking study. It includes 100,000 genetic operations on a population size of 100 individuals and a mutation rate 95. The COX II crystal structure was taken from the protein data bank (pdb id: 3NT1)[15]. It has a crystal structure resolution of 1.73Å and contained the inhibitor known as naproxen. The structures of the small molecules were sketched using Chemdraw3D and minimized considering rmsd cut-off of 0.1Å. The docking protocol was set by extracting and re-docking the inhibitor in the crystal structure with rmsd<1.0Å. This was followed by docking of all molecules in the active site defined as 6Å regions around the co-crystal ligand in the COX II protein. Further, all molecules were evaluated for possible molecular interactions with the COX II active site residues using PyMol molecular visualizer[16].

Statistical analysis

The presented data is expressed as the mean ± SD (n=3). Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test (cytokines and hepatoprotective activity) using the GraphPad Prism statistical software. Difference with $*P<0.05$, $**P<0.01$ and $***P<0.001$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Table 2: Chemical composition of lipophilic fraction DP-HO-01 isolated from the ethanolic extract of *H. odoratissimum*

RT	Ref	Library/ID	Area %
7.7121	10973	Cyclooctanone	1.65
10.9152	16336	2,7-dimethyl-2,6-Octadiene	1.39
17.138	62218	Acridine-9-carbaldehyde	3.31
19.0782	71353	Caryophyllene oxide	9.75
19.4521	59849	Alpha-Caryophyllene	1.48
19.7202	17040	3,5-Dimethylcyclohex-1-ene-4-carboxaldehyde	4.91
20.08	71333	Cedren-13-ol	1.59
22.0696	113466	Heptadecyl-oxirane	1.91
23.5583	34719	3a,7a-dimethyl-hexahydro-2(3H)-Benzofuranone	3.21
24.0522	59797	Caryophyllene	2.24
24.4261	14389	2-ethyl-1,4-dimethyl-benzene	2.26
24.7365	104262	6,10,14-trimethyl-2-pentadecanone	3.06
26.0347	51209	3-phenyl-3-methylbutanoic acid methyl ester	1.31
26.451	105639	Hexadecanoic acid methyl ester	1.77
26.966	14426	1-isopropyl-3-methylbenxene	18.32
29.5341	99423	3-Methyl-N-naphthalen-1-yl-benzamide	2.16
29.6188	121114	9,15-Octadecadienoic acid methyl ester	2.49
29.7528	122311	12-Octadecenoic acid methyl ester	2.41
30.4796	52814	2-(2-octenyl)-cyclopentanone	1.20
30.8394	52910	7-Tetradecyne	1.06
30.9452	128659	9,12,15-Octadecatrienoic acid ethyl ester	1.38
32.6949	118744	3,7,11,16-tetramethyl-hexadeca-2,6,10,14-tetraen-1-ol	4.30
32.8783	72901	2-Nerolidol	3.39

[RT] Retention Time; [Ref] Reference

In vitro cytotoxicity

H. odoratissimum was tested for its anticancer activity (cytotoxicity) using the XTT colorimetric assay, which is based on the ability of viable cells to reduce the yellow water soluble XTT into an insoluble formazan product[17]. The plant extract was tested for its *in vitro* cytotoxicity against HeLa, A431, A375 and HEK-293 cells. The isolated compounds and two lipophilic fractions were also tested for cytotoxicity against A431 cells, as the extract showed the highest inhibitory activity toward this cell line [Table 1]. The ethanolic extract of *H. odoratissimum* showed potential as an anti-cancer agent against A431 cells [Table 1]. The IC_{50} values used to determine the selectivity index (SI) values, indicated selectivity of the extract against cancerous cells versus the non-cancerous HEK-293 cells[18]. *H. odoratissimum* showed the highest selectivity index for A431 cells. In a similar study a 1:1 ratio of chloroform: methanol leaf and stem extract of *H. odoratissimum* also showed activity against human breast adenocarcinoma (MCF-7) cells with an IC_{50} value of 7.4 ± 0.7 µg/ml. On the glioblastoma (SF-268) cells, moderate activity was observed with an IC_{50} value of 48.2 ± 1.4 µg/ml[6].

Antioxidant activity

The extract showed strong DPPH radical scavenging activity, which was comparable to that of the positive control, Vitamin C. The extract, however showed low NO scavenging activity [Table 1]. The lipophilic fractions isolated from the extract showed opposite antioxidant activity with that of the extract. The lipophilic fractions were not able to inhibit the DPPH free radical, however the lipophilic fractions were able to inhibit NO activity, and showed lower IC_{50} values than that of Vitamin C. *H. odoratissimum* was also evaluated for both hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC). The antioxidant activity of the extract was expressed as micro moles of Trolox equivalent per gram of sample. The hydrophilic (H-ORAC) and lipophilic (L-ORAC) value of the extract was found to be 2542 ± 34 µmoles and 3648 ± 72 µmoles of Trolox equivalents per gram of extract respectively [Table 1].

Isolation of compounds from H. odoratissimum

During isolation the ethanol extract yielded two pale yellow coloured lipophilic fractions (DP-HO-01 and DP-HO-02) and three pure compounds; namely tetracosane (1) and two cluster of KCl (DP-HO-05 and DP-HO-06). The chemical compositions of each of the lipophilic fractions are shown in Tables 2 and 3. Previously many chemical constituents have been reported from the flowers of *H. odoratissimum* such as monoterpene oxides, p-methone, pulegone and 1,8-cineole[19]. A total of 54 constituents were identified and quantified from the sample DP-HO-01. The main components have been listed in Table 2. Similarly, 43 chemical constituents were identifiedfrom the sample DP-HO-02. The main constituentsare listed in Table 3. The most prevalent compounds found in DP-HO-01 were 1-isopropyl-3-methylbenzene, with 18.32 % of the total area and Caryophyllene oxide, with 9.75% of the total area. Furthermore, β-caryophylleneoxide has been reported to have inhibitory effects against HeLa, HepG2, human lung cancer (AGS), human gastric cancer (SNU-1) and human stomach cancer (SNU-16) cells with IC_{50} values of 13.55, 3.95, 12.6, 16.79 and 27.39 µM respectively[20].

Table 3: Chemical composition of lipophilic fraction DP-HO-02 isolated from the ethanolic extract of *H. odoratissimum.*

RT	Ref	Library/ID	Area %
15.5434	60076	(−)-Alloaromadendrene	1.39
16.8134	60025	*β*-selinene	1.80
19.0922	71350	Caryophyllene oxide	20.61
21.1242	71364	Isoaromadendrene epoxide	2.75
23.1349	15383	(+)-p-Mentha-2,8-diene	1.29
23.269	59916	4 methylene-2,8,8 trimethyl-2-vinyl-bicyclo nonane	1.22
25.3009	69940	6-(p-Tolyl)-2-methyl-2-heptenol	1.15
25.7454	14398	5-ethyl-m-xylene	1.15
28.3982	110586	Phthalic acid mono-2-ethylhexyl ester	6.75
28.4194	115986	Phthalic acid, butyl pent-2-en-4-yn-1-yl ester	6.98
28.7721	154651	Docosanoic acid methyl ester	11.44
31.3755	78671	Dodecanal dimethyl acetal	3.16
32.4409	140316	Nonadecanoic acid, ethyl ester	1.21
32.7654	102833	Z,E-3,13-Octadecadien-1-ol	6.28
33.097	122436	Heneicosane	2.83
33.1394	34905	1-(2-Isopropyl-5-methylcyclopentyl)ethanone	2.13
33.9366	148953	1,22-Docosanediol	15.06

[RT] Retention Time; [Ref] Reference

Caryophyllene oxide was also the most prevalent compound in DP-HO-02 with 20.61 % of the total area. The two lipophilic fractions showed the highest toxicity on A431 with IC_{50} values of 175 ± 13.5 µg/ml and 61.3 ± 0.16 µg/ml respectively. The three compounds showed less toxicity than that of the lipophilic fractions with all the IC_{50} values >200 µg/ml [Table 1].

In vitro hepatoprotection effect and cytotoxicity in HepG2 cell line

HepG2 cells treated with various concentrations of the extract (1.56–100 µg/ml) after 24 h treatment showed high cell viability (low cytotoxicity) at each concentration. Therefore, the hepatoprotective activity of the extract was tested from 3.125–100 µg/ml to determine whether the extract was able to protect the cells against the toxic inducer D-Galactosamine (30 mM). The extract showed low cytotoxicity against the HepG2 cells at concentrations ranging between 1.56-100 µg/ml after 24 h treatment with the extract. The extract showed significant (P<0.05) hepatoprotection at a concentration of 25 µg/ml. Extract concentration 50-100 µg/ml are not depicted in the graph as negative values were obtained. Silymarin, a well-known hepatoprotective agent was used as the positive control, it showed significant increase (P<0.001) in cell viability against D-galactosamine-induced toxicity with a protection of 52 % obtained at 12.5 µg/ml [Figure 1].

Cytokine production

The extract was able to significantly induce (P<0.001) IL-12 at a concentration of 5 µg/ml and was further able to significantly inhibit IL-8 production at all the tested concentrations (P<0.01 and P<0.001) in U937 cells [Figure 2 & 2.2]. The controls and the extract were tested at non-toxic concentrations as determined by a cytotoxicity assay. The PHA control showed an increase in IL-12 and pentoxifylline showed an inhibition [Figure 2]. The results obtained for pentoxifylline corresponded with the results obtained in the study performed by Moller et al.[21] where pentoxifylline was able to increase IL-12 levels in lipopolysaccharide (LPS) stimulated PBMCs. A study by Karimi et al[22] isolated dendritic cells and exposed the cells to various concentrations of a 70 % ethanol root extract of *Cichorium intybus.* At 0.1 µg/ml and 1 µg/ml, the extract was able to increase IL-12 to 280.6±26.58 pg/ml and 195.5±16.88 pg/ml respectively when compared to that of the DMSO control (88.58±23.87 pg/ml). These results were similar to those in the present study where at a lower concentration of extract a higher production of IL-12 was observed.

At a concentration of 50 µg/ml and 100 µg/ml the extract showed 28 % and 66 % cytotoxicity respectively on naive murine splenocytes whereas on PHA induced murine splenocytes it showed 54 % and 89 % cytotoxicity respectively after 48h treatment. Therefore, the IL-2 production was not tested at these concentrations. The extract was tested at a range of concentrations (3.125–25 µg/ml) to determine the effect on IL-2 production in both naive and PHA induced murine splenocytes. The extract showed no significant activity to increase the production of IL-2, however at a concentration of 25 µg/ml the extract showed significant inhibitory activity on both naive and induced murine splenocytes (P<0.0001) and (P< 0.01) [Figure 4 & 5].

Light microscopy

The A431 cells showed signs of apoptotic cell death when viewed under light microscopy after treatment with various concentrations of the extract [Figure 4]. During light microscopy it was noted that control cells propagated in growth medium [Figure 4] and vehicle-treated controls [Figure 4b] showed normal signs of cell division and the presence of interphase cells. Cells (A431) treated with the extract of *H. odoratissimum* showed an increase in morphological characteristics associated with apoptosis. Cells exposed to 15 µg/ml and 30 µg/ml of extract showed no normal cell proliferation and signs of cell death started to appear. Typical characteristics of apoptosis, such as apoptotic body formation and cell shrinkage at 15 µg/ml as well as condensed nucleus and cell debris with low cell viability at 30 µg/ml [Figure 4 & 5], were viewed. Cells

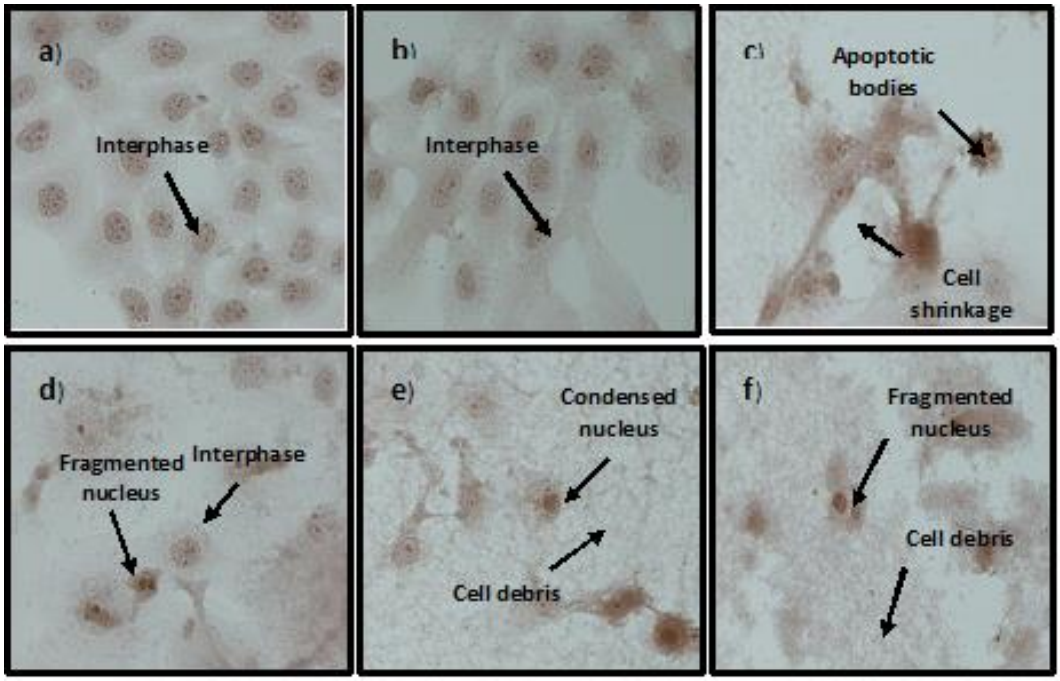

Figure 6: *Helichrysum odoratissimum* induced apoptosis in A431 cells after 72h treatment. Haematoxylin and eosin staining of A431 cells (a) medium-only control (b) vehicle-treated control (c) 15 µg/ml *H. odoratissimum* (d) 7.5 µg/ml *H. odoratissimum* (e) 30 µg/ml *H. odoratissimum* and (f) Actinomycin D at 20X magnification, after 72 h of exposure.

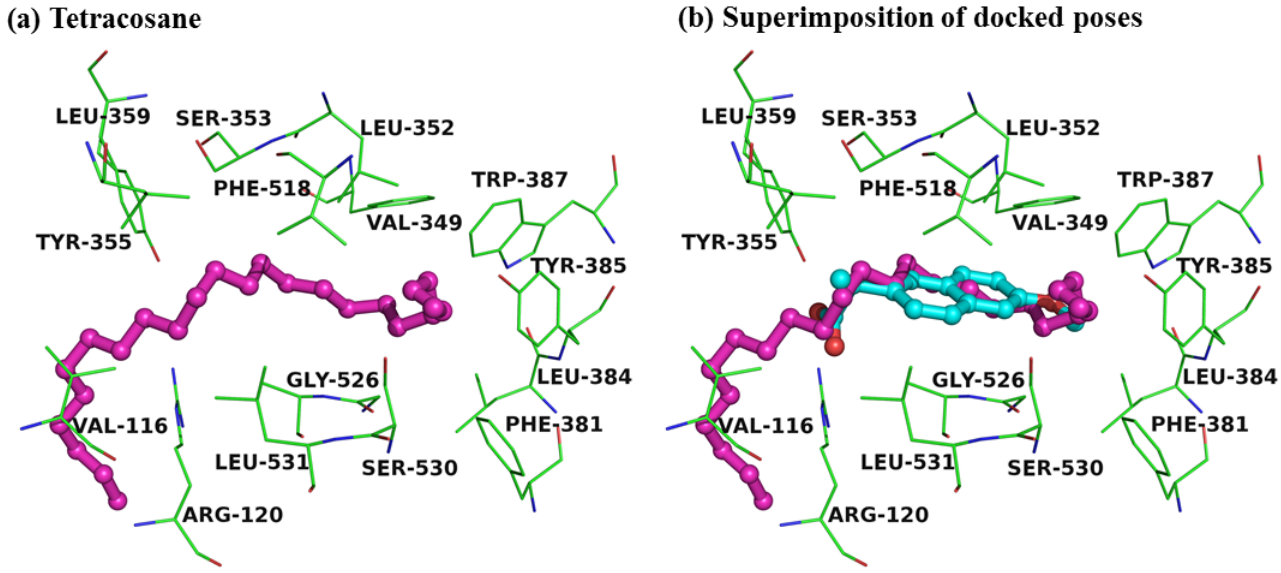

Figure 7: (a) Docked pose of tetracosane in the active site of COX II. (b) Comparison of tetracosane docked pose with naproxen orientation in 3NT1.

exposed to 7.5 µg/ml showed signs of cell death such as fragmented nucleus as well as signs of cell division [Figure 4d]. In cells exposed to Actinomycin D, a great amount of cell death and total loss of cell viability was observed with signs of compacted and fragmented nucleus [Figure 4]. In a similar study by Berrington & Lall.[7]acetone extracts were prepared using the aerial parts of *Laurus nobilis* and *Origanum vulgare* respectively. The influence of the extract on the morphology of HeLa cells was observed at IC_{50} (34 µg/ml) and $2\times IC_{50}$ (69 µg/ml) for *Laurus nobilis* and IC_{50} (126 µg/ml) and $2\times IC_{50}$ (253 µg/ml) for *Origanum vulgare* respectively. It was evident that the cell density decreased when treated at these concentrations and signs of cytoplasmic shrinking, hypercondensed chromatin, membrane blebbing and apoptotic bodies were evident[7].

Molecular docking analysis

Tetracosane is a long carbon chain molecule consisting of twenty four carbons. It was docked in the COX II active site defined as 6Å around the co-crystal ligand naproxen. Tetracosane fitted well in the cavity at the same site where naproxen binds and showed a fitness score of ~36. It did

not make H-bond interactions due to lack of electronegative atoms. However, tetracosane did make van der Waals interactions with surrounding residues [Figure 5]. The docked pose of tetracosane was compared with the orientation of naproxen in the crystal structure, 3NT1 [Figure 5]. Due to the enormous flexibility of the single bonded carbon chain, tetracosane occupied the active site cavity with an orientation similar to naproxen in the crystal structure. Naproxen, naphthalene moiety made van der Waals interactions with surrounding hydrophobic residues Val349, Tyr385, Phe518, Gly526 and Leu531. In addition to these residues, the aliphatic chain in tetracosane made van der Waals interactions with Val116, Tyr355, Phe381 and Leu384. Therefore, tetracosane showed a large network of van der Waals interactions which supports the observed fitness score.

CONCLUSION

H. odoratissimum showed activity against the A431 cell line, with a SI of 2.39 which is higher than that of the positive control with an SI of 0.08. The antioxidant activity of the extract showed high DPPH scavenging activity, but low nitric oxide scavenging activity. However, the lipophilic fractions showed low DPPH inhibitory activity and high NO scavenging activity. Furthermore, the extract showed signs characteristic of apoptosis. Lastly the extract was able to inhibit the production of IL-8 and promote the production of IL-12 in U937 cells. This is the first report of the activity of *H. odoratissimum* ethanolic leaf stem extract on A431, A375, HeLa and HEK-293 cell lines as well as the isolation of two promising lipophilic fractions from the extract.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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