RESEARCH ARTICLE

Editorial Process: Submission:12/22/2023 Acceptance:04/15/2024

In Vitro Antiviral and Anticancer Effects of *Tanacetum sinaicum* Essential Oil on Human Cervical and Breast Cancer

Abeer A Sheashea[1], Fatma A Ahmed[1], Emad El Zayat[2], Bassam W Ebeed[3], Mostafa H Elberry[4], Zeinab K M Hassan[4], Mohamed M Hafez[4]*

Abstract

Background: Cervical cancer has been linked to human papillomavirus (HPV) types 16 and 18. Essential oils (EOs) are vital natural products of plants with various therapeutic and biological properties. **Objectives:** The purpose of this study is to investigate and assess *Tanacetum sinaicum* essential oil's possible antiviral and anticancer properties, with a focus on its in vitro effects on human cervical cancer and human breast adenocarcinoma cell lines. **Materials and Methods:** *Tanacetum sinaicum* EO was extracted via hydrodistillation (HD) and characterized using gas chromatography-mass spectrometry (GC-MS). MTT assay was used to determine the cell viability of Hela (a human epithelial cervical cancer) and MCF-7 (human breast adenocarcinoma) cell lines. Quantitative real-time polymerase chain reaction (PCR) was utilized to assess the antiviral efficacy of EO against HPV-16 and 18, and anti-metastatic characteristics. The biological activity of EO was assessed using Autophage and Cell genotoxicity via the comet assay. **Results:** EO is mostly composed of chrysanthenyl acetate, thujone, and verbenol. The cell viability was reduced after 24 hours of incubation at doses from 100 to 400 µg/ml. Concentrations of 800 to 3,200 µg/ml significantly inhibit cell growth. After a 24-hour incubation period, doses ranging from 100 to 400 µg/ml reduced cell viability from 62 to 72%. Concentrations of 800 to 3,200 µg/ml significantly suppress cell growth by over 95%. In MCF7 and HeLa cell lines, EO lowered virus copy numbers in a dose-dependent manner, with higher concentrations of the oil inhibiting virus replication more effectively. EO treatment increased the number of autophagosomes/autolysosomes and acidic vesicular organelles in both cell lines. On the HeLa and MCF7 cell lines, EO demonstrated antiproliferative and antimetastatic effects. The results demonstrated that EO had dose-dependent genotoxic effects on both cancer cell lines, as evidenced by DNA damage. **Conclusion:** *Tanacetum sinaicum* EO is a prospective source of natural bioactive compounds that can be employed in pharmaceutical and medicinal applications due to its antiviral, antiproliferative, anti-metastatic and genotoxic properties.

Keywords: *Tanacetum sinaicum* essential oil- human papillomavirus type 16 and 18- antiviral efficacy and anti-cancer

Asian Pac J Cancer Prev, **25 (4)**, 1457-1471

Introduction

Viral infections have a considerable impact on public health, ranging from acute self-limiting illnesses to potentially fatal disorders such as cancer, which is recognized as a global public health issue. To date, viral infections are thought to account for nearly 12% of cancer cases. Human papillomavirus, a member of the papillomaviridae family, is a small non-enveloped icosahedral capsid with a circular double-stranded DNA genome of around 7,000-8,000 bp in length that is chromatinized.

The International Agency for Research on Cancer (IARC) divided HPVs into two categories based on their level of association with human malignancy: oncogenic high-risk HPVs (HR-HPVs), which are classified as group I carcinogens and are responsible for cervical cancer, oropharyngeal cancers, and ano-genital cancers, and non-cancerous low-risk HPVs (LR-HPVs), which are responsible for ano-genital and cutaneous warts. The oncogenic group consists of 14 HR-HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. HPV 16 and HPV 18 are the most carcinogenic forms and cause the majority of HPV-related malignancies worldwide [1, 2].

Medical plants are a source of a wide variety of natural products with specific medical properties that are continually being studied in order to develop new treatments. The bulk of pharmaceuticals are made from natural components [3]. EOs is secondary metabolites that play an important role in plant protection. They are primarily composed of volatile terpenes with a diverse chemical structure and a wide range of bioactivity.

*[1]Department of Medicinal and Aromatic Plants, Desert Research Center, Cairo, Egypt. [2]Department of Biotechnology, Faculty of Science, Cairo University, Cairo, Egypt. [3]Department of Zoology, Faculty of Science, Cairo University, Cairo, Egypt. [4]Department of Cancer Biology, National Cancer Institute, Cairo University, Cairo, Egypt. *For Correspondence: mohhafez_2000@yahoo.com*

Recent research has shown that cancer cell prevention and cytotoxicity can be achieved through the synergistic and antagonistic activity of specific essential oil major and minor components. EOs have been shown to target cancer cells and improve the efficacy of common chemotherapy drugs like paclitaxel and docetaxel [4, 5]. The Asteraceae family, known as the sunflower family, is one of the largest flowering plant families among essential oil-producing medicinal plants [6].

Tanacetum, a genus in the Asteraceae family, has over 200 species and is found in many countries in North America, Asia, and Europe [7]. Tanacetum is a well-known source of EOs, which are extracted from various parts of the plant, particularly aerial parts such as leaves, stems, and flowers. The EO yields varied significantly from 0.04% to 1.09% (v/w), depending on the species, harvested components, and abiotic and biotic variables [8-10]. Tanacetum was discovered to be a rich source of secondary and primary metabolites with a variety of therapeutic applications. The analysis of over 240 identified metabolites revealed that monoterpenes are the dominant metabolites (19%), followed by sesquiterpenes (18%), flavonoids (15%), phenolic acids (12%), and fatty acids and alkanes (9%) [11]. Tanacetum species have been used ethnopharmacologically to treat a variety of medical conditions, including diabetes, migraine, cholecystitis, dyspepsia, nausea, diarrhoea, hypertension, stomach pain and bloating, ringworms, and sexually transmitted infections. Crude extracts and isolated metabolites have revealed a range of biological activities [11].

Tanacetum sinaicum is native to Egypt, specifically south Sinai, and grows on the rocky slopes of Saint Catherine. It produces yellow tubular blooms and woolly leaves [12, 13]. T. sinaicum (pyrethrum santolinoides) is native to the Middle East and has long been used to treat migraines, fevers, stomach problems, arthritis, and bronchitis [14]. T. sinaicum has antiviral properties, according to one study [15], while other studies have found it to be anti-inflammatory [13, 16]. T. sinaicum essential oil was likely chosen as a candidate for cancer and viral prevention or treatment because of its promising preclinical data, unique chemical composition, and favorable safety profile.

The purpose of this study was to investigate the potential therapeutic applications of T. sinaicum essential oil by investigating its antiviral and anticancer activities, with a particular emphasis on human cervical cancer and human breast adenocarcinoma cell lines, in order to provide valuable insights into the effectiveness of this essential oil in combating these specific types of cancer.

Materials and Methods

This study was approved by the National Cancer Institute (NCI)-Cairo University's institutional review board (IRB) ethical committee under IRB No. 2109-302-046.

Plant material

Fresh Asteraceae family medicinal plant aerial parts were taken in St. Katherine Protectorate, South Sinai, Egypt, during the flowering stage in June 2022 and identified in Protectorate of St. Katherine- Egyptian Environmental Affairs Agency (EEAA), as: *Tanacetum sinaicum* (Fresen.) Delile ex K.Bremer& Humphries.

Preparation and analysis of the essential oil components

A Clevenger-type device was used to hydro-distill the plant sample's fresh aerial parts for 6 hours. Dimethyl sulfoxide (DMSO) (MercK, Germany) was used as a solvent to prepare varying amounts of the essential oil, with a final concentration of no more than 0.1% (v/v). Chemical analysis of the extracted essential oil was performed using a Gas Chromatography-Mass Spectrometry apparatus at the Laboratory of Medicinal and Aromatic Plants, National Research Centre, Egypt, according to the standards [17-19].

Cell lines and culture

The HeLa (human cervical cancer integrated with HPV type 18) and MCF7 (human breast adenocarcinoma integrated with HPV type 16) cancer cell lines were obtained from the NAWAH Centre. The two cell lines are tested for microbial contamination at the NAWAH centre using the Short Tandem Repeat assay. The cell lines were grown in DMEM (Merck, Germany), which contained 10% foetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% fungizone. (Merck, Germany). The cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2. According to the provided company, the cell lines are not contaminated with microbes or other cell lines. The mycoplasma test was performed using a qPCR detection kit (Cat. No. Mp0040, Sigma-Aldrich, MO, USA) in accordance with the manufacturer's instructions for mycoplasma detection in cell cultures. The kit's internal control discarded PCR inhibition. The PCR was carried out on an ABI 7500 real-time PCR (Applied Biosystem, Singapore, Asia).

Cell viability assay using MTT

The cell viability assay was designed to determine the viability of HeLa and MCF7 cell lines in response to various concentrations of EO. This evaluation was conducted by evaluating the activity of cellular oxidoreductase enzymes within viable cells that convert the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Merck, Germany) into its insoluble formazan form. Cell lines were seeded in 96-well plates with 1×10^4 cells per well and incubated for 24 hours. The medium was replaced with fresh medium containing different concentrations of EO: 6.25, 12.5, 25, 50, 100, 200, 400, 800, 1600, and 2300 µg/ml. Untreated cells were treated with DMSO. After 24 hours, 10 µl of MTT reagent was added to each well. The plates were then incubated for 4 hours at 37°C. After adding 100 µl of DMSO to each well, the amount of formazan was quantified at 570 nm using a Tecan (Infinite®F50) absorbance microplate reader and Magellan™ data analysis software Version 7.0. Percentage of cell proliferation was calculated relative to control wells designated as 100% viable cells, using the formula (A treated)/(A control) × 100.

Using GraphPad Prism® software Version 5.01

(GraphPad Software, Inc.), La Jolla, California, USA (Jamalzadeh, Ghafoori, Aghamaali, & Sariri, 2017), the IC50 values (a concentration of the EO inhibiting 50% of the cell growth) of the EO were calculated from a dose-response curve of the mean absorbance of cell viability (Y-axis) versus log tested oil concentrations (X-axis). The MTT assay was performed in three independent replicates (Figures 1-I and 1-II).

Integrated HPV type 16 and 18 detection by quantitative PCR (qPCR)

Quantitative PCR (qPCR) is a method used for detecting HPV and quantifying of viral load [20]. HeLa and MCF7 cell lines were seeded at 106 cells in 96 well plates and treated with different concentration of essential oil. After 24 hours of treatment, cells and culture supernatants from treated and untreated (control) cells were collected.

Viral DNA and mRNA were extracted from both treated and untreated cells and supernatants using the Wizard® SV Genomic DNA Purification System (Promega, USA) and the QIAamp mRNA mini kit (Qiagen Inc., Valencia, CA), respectively, following the manufacturer's instructions. To extract mRNA, residue DNA was removed by incubating with 1 U of amplification grade DNAse I at room temperature for 15 minutes. A 1 µl aliquot of each mRNA sample was assayed by real-time PCR with primers and probe targeting GAPDH in parallel, without the reverse transcriptase procedure, to confirm a lack of contamination. Finally, the purified viral DNA and mRNA were quantified using Qubit Fluorometric (Thermo Fisher Scientific, Germany) and stored at -80°C until used.

The primers and probes were designed using primer 3 (v.0.4.0) Tool software to amplify and detect specifically E6 and E7 oncogenes of HPV16 and HPV18. The primers and probes' specificity against the human genome was determined using the online NCBI BLASTn tool (https://blast.ncbi.nlm.nih.gov). GAPDH primers were acquired from a previous study [21].

A standard curve was created by adding known amounts of plasmid DNA specific to the target gene being studied. Initially, a plasmid stock of 107 copies per microliter was diluted 1:50 in carrier E. coli DNA, resulting in a concentration of 2×10^5 copies/µl. Subsequently, serial ten-fold dilutions of carrier DNA were prepared, yielding a range from 2×10^5 copies/µl to 2 copies/µl. To ensure accuracy, the quantitative results were adjusted for cellularity with a measure of human genome equivalents before being expressed as the amount of HPV DNA per 1,000 cells.

To facilitate statistical analyses, DNA copy numbers were logarithmically transformed to base 10. When no viral DNA was detected by the quantitative assay, the samples were given a log10-transformed copy number of 0. It is worth noting that all samples were positive for GAPDH. Extracted viral DNA (100 ng) was subjected to qPCR using 2X Smart mix (Genetic Technologies SolGent, Korea) and 300 nM primers and probes for HPV-16 and HPV-18 in a 20 µl final volume. The results were analysed on an Applied Biosystems®7500 Fast Real-Time machine with 7500 software Version 2.3.

The amplifications were carried out using the following thermal cycling profile: incubation at 50°C for 2 minutes for one cycle, followed by incubation at 95°C for 10 minutes for one cycle, followed by 40 cycles of 30 seconds of denaturation at 95°C and 1 minute of annealing at 60°C for one minute. Each sample was analyzed in triplicate, and representative data sets are presented. The results were analyzed using the standard curve method. Data for three independent amplifications were expressed as mean ± SD.

Quantitative reverse-transcription (RT-qPCR) for HPV mRNA detection

HPV mRNA detection via RT-qPCR is used to identify transcriptionally active, high-risk HPV [22, 23]. These transcripts are linked to cellular genotoxic damage and changes in gene expression, which lead to cancer [24]. The primers used to detect integrated HPV 16 and 18 mRNA and free mRNA were commercially synthesized according to [25].

RT-qPCR was used to detect HPV 16 and 18 mRNA in cells and culture supernatants of the MCF7 and HeLa cell lines, respectively after treatment with different concentration of essential oil. The test was carried out with 2X OneStep Multiplex qRT-PCR Smart mix (Genetic Technologies SolGent, Korea) and analysed on an Applied Biosystems®7500 Fast Real-Time machine running 7500 software Version 2.3. The PCR reaction was done in 20 µL and contained 1X OneStep Multiplex qRT-PCR Smart mix for Taqman probe, 100 ng of template, 300 nM of forward and reverse primers, and probe.

The amplifications were performed with the following thermal cycling profile: incubation at 50°C for 20 min. for 1 cycle followed by incubation at 95°C for 10 min for 1 cycle followed by 40 cycles of 30 sec denaturation at 95°C, 1 min annealing at 60°C for 1 min. GAPDH was used as an internal control.

Autophagy assay

As a result, autophagy has emerged as a key target for drug discovery. Acridine orange (Merck, Germany) is a pH-dependent green fluorescent dye that accumulates in acidic organelles and glows bright red in autolysosomes. Despite its hydrophobic nature at neutral pH, it can induce the formation of acidic vesicular organelles in cultured cells [26]. The HeLa and MCF7 cell lines were seeded at a density of 5×10^5 cells per well into 6-well plates and incubated for 24 hours at 37°C with 5% CO_2. Following the initial 24-hour incubation, the wells were treated with the IC_{50} concentration as well as two lower concentrations of the EO, and the plates were incubated for another 24-hour period.

After treatment, the media was replaced with acridine orange at a final concentration of 5 µg/ml, and the plates were incubated in dark conditions for 15 minutes at 37°C. The stained cells were then washed twice with PBS, and 1ml of PBS was added before being analyzed with a Nikon Eclipse Ti-S fluorescence inverted microscope equipped with a 20x magnification objective lens. The microscope was outfitted with a blue light filter (B-2A)

with an excitation wavelength range of 450–490nm. Under this filter, green fluorescence indicated the presence of nuclei and cytoplasm, and red fluorescence indicated the formation of acidic vesicular organelles [27, 28]. The assay was carried out in three independent replicates.

In vitro scratch assay

The migration potential of HeLa and MCF7 cell lines after EO treatment was determined using a wound healing assay. HeLa and MCF7 cells (5×10^5) were cultured in 6-well plates and reached 70-80% confluence. Next, the cells were scratched with a 200-µl pipette tip. The cells were washed twice with PBS. The old culture medium was discarded and replaced with 2 ml of fresh culture medium containing IC_{50} and two lower concentrations of IC_{50} for the EO; with the exception of untreated wells, the fresh culture medium was free of EO.

Following treatment, the wounds were photographed using an Olympus LH50A inverted microscope with a 10x magnification and an AmScope MD35 digital eyepiece microscope camera before (0 time) and after (12, 24, and 48 hours incubation). Images were analyzed using AmScope software Version 4.11 to measure wound width before and after treatment in both treated and untreated cells [29]. The assay was performed in three independent replicates.

Cell genotoxicity by using single-cell gel electrophoresis (SCGE) (comet) assay

The comet assay is a method for assessing DNA damage in individual cells. After treating HeLa and MCF7 cell lines with the IC50 concentration and two lower concentrations below the IC50 for the EO, the cells were collected and the comet assay was performed using the procedures described by Singh, McCoy et al. (1988), [30] with minor modifications. Finally, all alkaline and neutral slides were analyzed using an Olympus CKX53 fluorescence inverted microscope with a magnification of 20x, an Olympus XC50 digital eyepiece microscope camera, and Olympus cell Sens Dimension software under a red light filter with excitation/emission at 493-620 nm.

The images were analyzed using TriTek Comet Score software Version 2.0 (automatic comet assay software) to assess DNA damage in comets based on the percentage of DNA in tail parameter. TriTek Comet Score software Version 2.0 automatically calculated the mentioned parameter as an average of 50 scores for the analysis. The assay was carried out in three independent replicates. It is recommended to investigate the effect of the tested essential oil on free virus.

Limitation of this study: In our next investigation, we will investigate the effect of sensational on additional viruses as well as non-integrated viruses in a tissue culture model, and we will employ flow cytometry to look for the LC3 protein marker for autophagy and the apoptotic pathway.

Statistical analysis

All values were expressed as mean ± standard deviation for three independent replicates for each assay. Data were analyzed by GraphPad Prism®software Version 5.01, La Jolla California USA using the one-way analysis of variance (ANOVA) followed by Tukey's test. Statistical differences were considered significant when the p-value was < 0.05.

Results

The percentage and identification of the essential oil components

Gas chromatography-mass spectrometry (GC-MS) analysis of EO revealed the presence of trans-chrysanthenyl acetate (33.67 %) and α-thujone (28.91 %) as the two major compounds as shown in Table 1.

Effect of T. sinaicum essential oil on cell viability

The MTT cell proliferation assay was used to determine the anti-proliferative effect of various EO concentrations on HeLa and MCF7 cell lines. Figure 1-I demonstrates that EO concentrations up to 50 µg/ml had no significant effect on HeLa cell viability compared to the control. Concentrations of 100, 200, and 400 µg/ml significantly reduced HeLa cell viability by 44, 54, and 80%, respectively, after 24 hours of incubation.

At concentrations of 800, 1,600, and 3,200 µg/ml, EO inhibits cell proliferation by more than 90%. EO

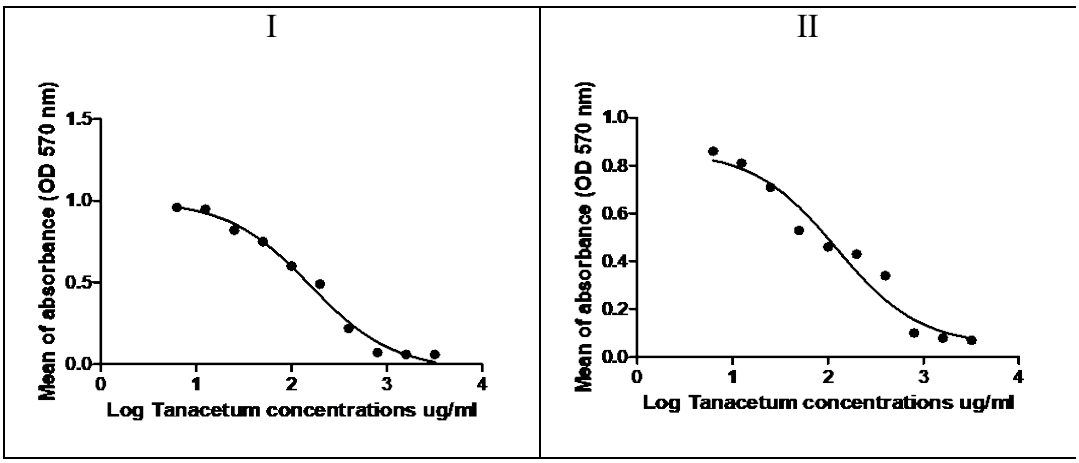

Figure 1. Dose-Response Curves for Different Concentrations of the Tested Essential Oil on Viability of HeLa and MCF7 Cancer Cell Lines by MTT Assay. Dose-response curves created by GraphPad Prism® software to determine the IC50 of all the tested essential oil where, (A) HeLa cell line (B) MCF7 cell line.

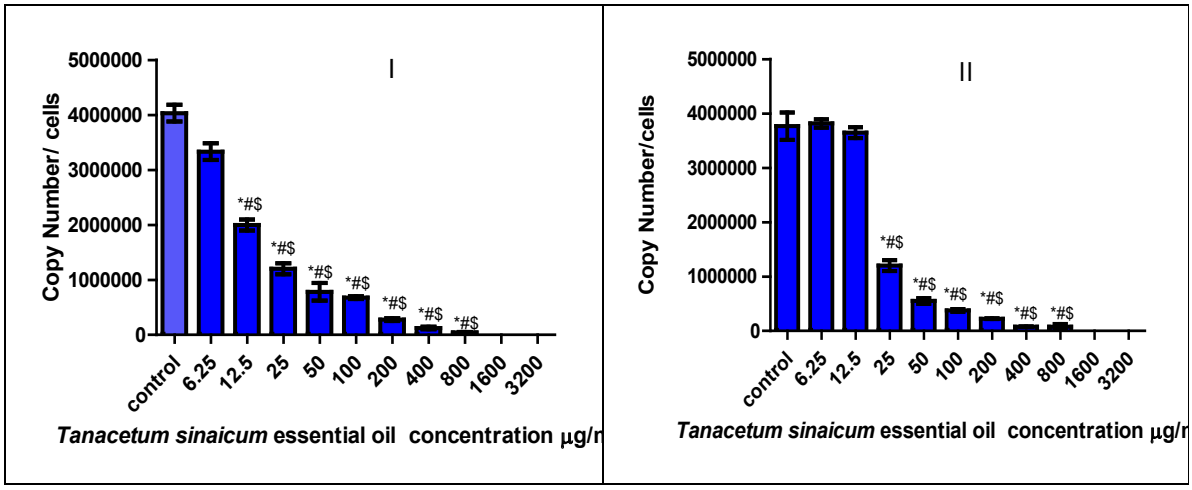

Figure 2. Effect of *Tanacetum sinaicum* EO on the Copy Number of Human Papillomavirus/Cells. Data are presented as mean ± SD (n = 3). *,# and $ indicate a significant change from untreated cells, 6.25 µg/ml and 12.5 µg/ml respectively, at $p < 0.05$ using ANOVA followed by Tukey test post-ANOVA test.

concentrations up to 50 µg/ml had no significant effect on the viability of MCF7 cells compared to the control. Concentrations of 100, 200, and 400 µg/ml significantly reduced MCF7 cell viability by 62, 65, and 72%, respectively, after 24 hours of incubation. Figure 1-II shows that EO concentrations of 800, 1,600, and 3,200 µg/ml inhibit cell proliferation by more than 95%.

Integrated HPV type 16 and 18 detection by Quantitative PCR (qPCR)

Figure 2 shows the results of the experiment, which looked at the effect of EO on virus copy numbers in MCF7 and HeLa cell lines at different concentrations. In the MCF7 cell line (Figure 2-I), treatment with EO up to 12.5 µg/ml did not significantly affect virus copy numbers compared to the untreated control group. Increasing the concentration of EO to 50, 100, 200, 400, and 800 µg/ml resulted in significant reductions in virus copy numbers. Specifically, virus copy numbers decreased from 4x106 copies per cell to $8x10^5$, $4x10^5$, $2x10^5$, $17x10^3$, and 10^3 copies per cell, as compared to the control group. At concentrations of 1,600 and 3,200 µg/ml, the virus remained undetectable. The difference in virus copy numbers was statistically significant when compared to the 6.25 µg/ml concentration of EO.

In the HeLa cell line (Figure 2-II), treatment with EO up to 50 µg/ml did not significantly impact virus copy numbers compared to the untreated control group. Increasing the concentration of EOs to 100, 200, 400, and 800 µg/ml resulted in significant reductions in virus copy numbers. Virus copy numbers decreased from $4x10^6$ copies per cell $4x10^5$, $2x10^5$, $17x10^3$, and 10^3 copies per cell, as compared to the untreated control group. Similar to MCF7 cells, the virus was undetectable at concentrations of 1,600 and 3,200 µg/ml. The difference in virus copy numbers was statistically significant when compared to the 6.25 µg/ml concentration of EO. These findings show that EO has a dose-dependent effect on virus copy

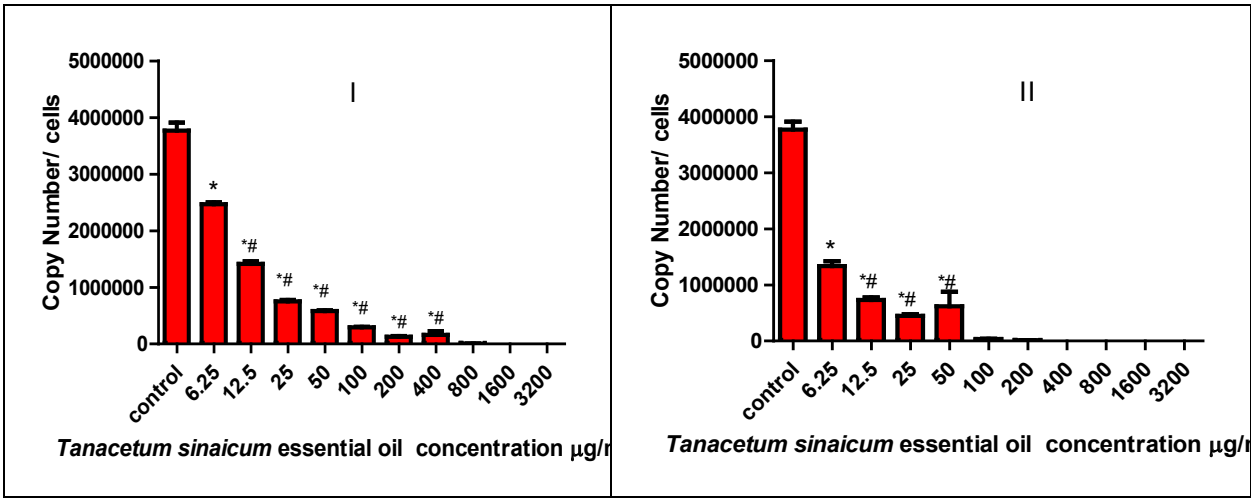

Figure 3. Effect of *Tanacetum sinaicum* EO on the Copy Number of Human Papillomavirus in Culture Supernatant. Data are presented as mean ± SD (n = 3). *,# and $ indicate a significant change from untreated cells, 6.25 µg/ml and 12.5 µg/ml respectively, at $p < 0.05$ using ANOVA followed by Tukey test post-ANOVA test.

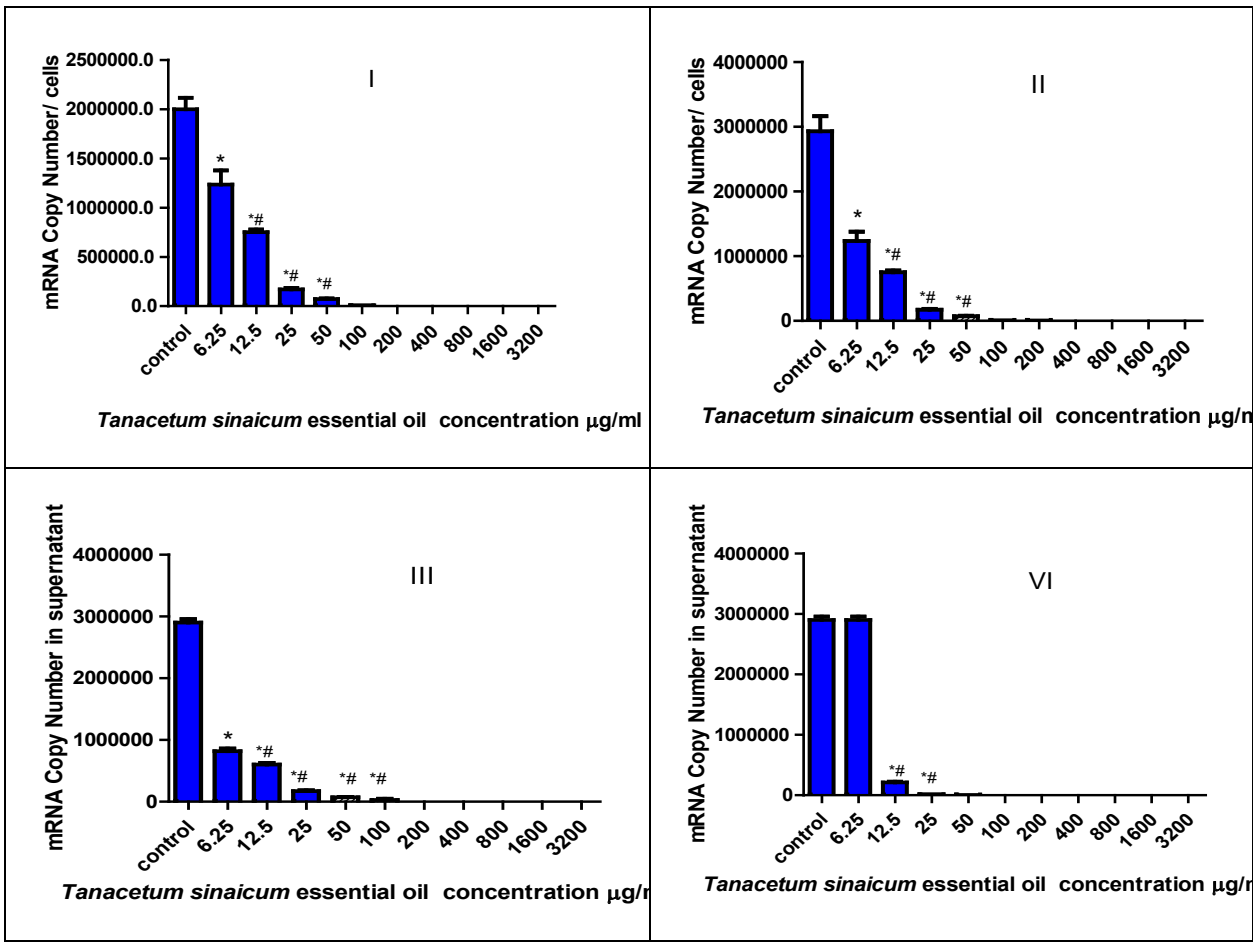

Figure 4. Effect of *Tanacetum sinaicum* EO on the Copy Number of Human Papillomavirus in Cells and Culture Supernatant. Data are presented as mean ± SD (n = 3). *,# and $ indicate a significant change from untreated cells, 6.25 µg/ml and 12.5 µg/ml respectively, at $p < 0.05$ using ANOVA followed by Tukey test post-ANOVA test.

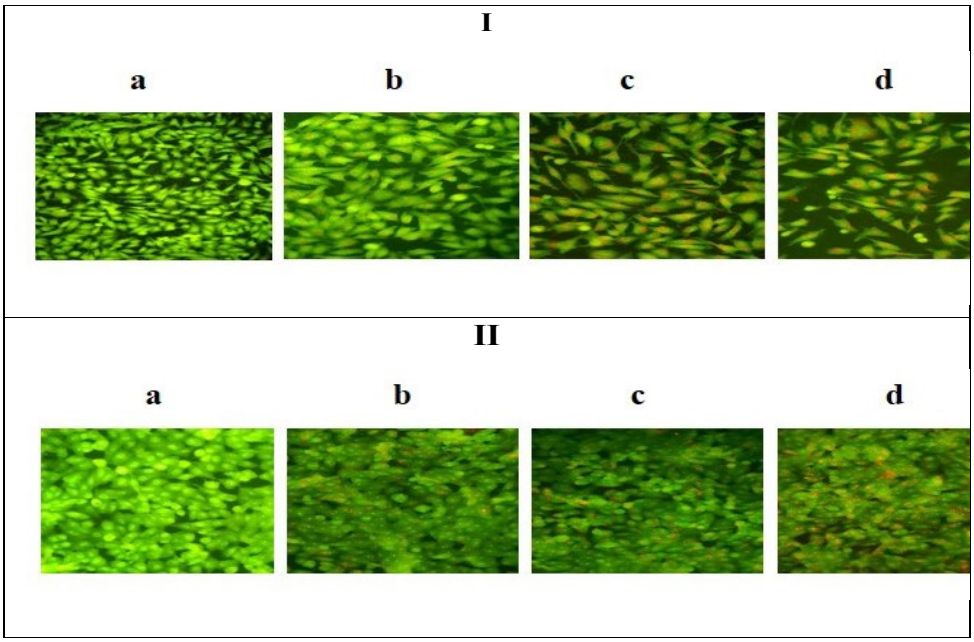

Figure 5. Effect of Different Concentrations of the EO on Autophagy Induction in HeLa and MCF7 Cell Lines. Fluorescence images of HeLa and MCF7 cell lines exposed to different concentrations of the EO stained with acridine orange after 24 hrs treatment under blue light excitation with magnification 20x. Red fluorescence indicated autophagic vacuoles, while green fluorescence indicated the presence of nuclei. Where, (I) HeLa cell line, A:Untreated cells, B:*Tanacetum sinaicum* 150 µg/ml, C:*Tanacetum sinaicum* 75 µg/ml and D: *Tanacetum sinaicum* 37.5 µg/ml and (II) MCF7 cell line, A:Untreated cells, B: *Tanacetum sinaicum* 120 µg/ml, C: *Tanacetum sinaicum* 60 µg/ml and D: *Tanacetum sinaicum* 30 µg/ml.

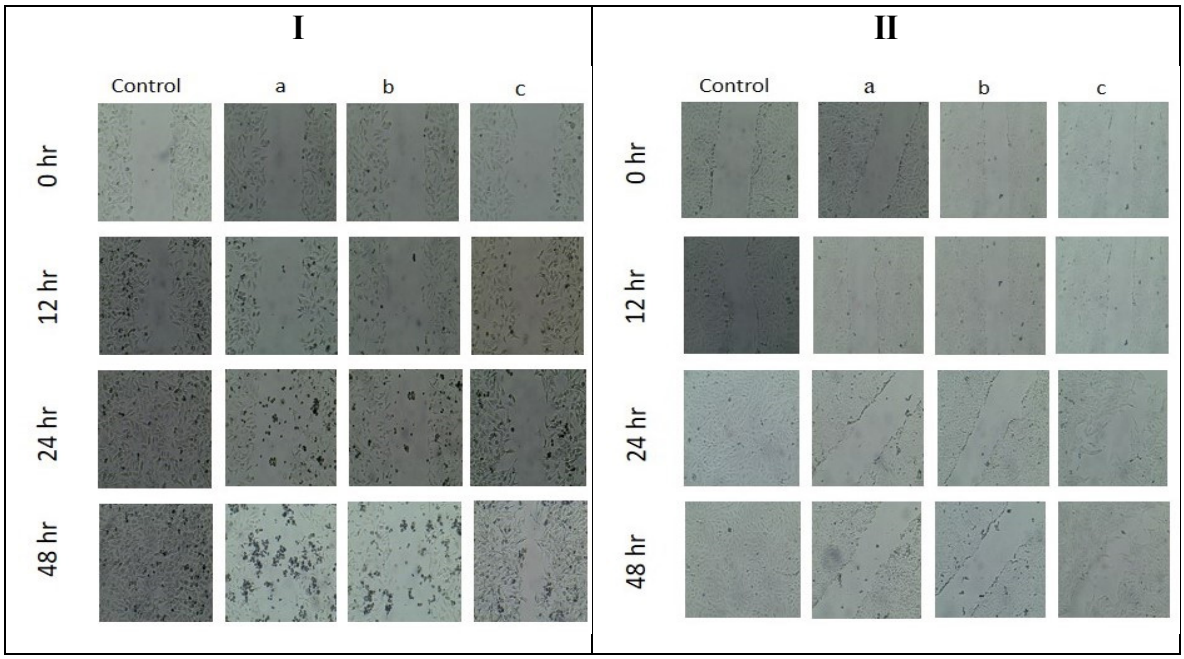

Figure 6. Effect of Different Concentrations of *Tanacetum sinaicum* EO on HeLa and MCF7 Cell Lines Migration. Cell monolayers were scratched and treated with different concentrations of EO for 12, 24 and 48 hrs.Untreated cells referred to control. Where, (I) HeLa cell line, A: *Tanacetum sinaicum* 150 µg/ml, B:*Tanacetum sinaicum* 75 µg/ml and C: *Tanacetum sinaicum* 37.5 µg/ml and (II) MCF7 cell line, A: *Tanacetum sinaicum* 120 µg/ml, B: *Tanacetum sinaicum* 60 µg/ml and C: *Tanacetum sinaicum* 30 µg/ml.

numbers in both the MCF7 and HeLa cell lines, with higher concentrations of the oil being more effective at suppressing viral replication.

Figure 3 depicts the results of an experiment that looked at the effect of EO on virus copy numbers in the culture supernatant of both MCF7 and HeLa cell lines at various concentrations. In the MCF7 cell line (Figure 3-I), EO at 6.25 µg/ml did not significantly affect virus copy numbers compared to the untreated control group. However, as the concentration of EO was incrementally increased to 12.5, 50, 100, 200, 400, and 800 µg/ml, virus copy numbers were significantly reduced. Virus copy numbers decreased from $4x10^6$ copies per cell to $1.4x10^6$, $7.5x10^5$, $5.8x10^5$, $2.9x10^5$, $12x10^3$, and $4.4x10^2$ copies, respectively, when compared to the control group. The virus was undetectable at concentrations as high as 1,600 and 3,200 µg/ml.

The virus copy numbers differed significantly from

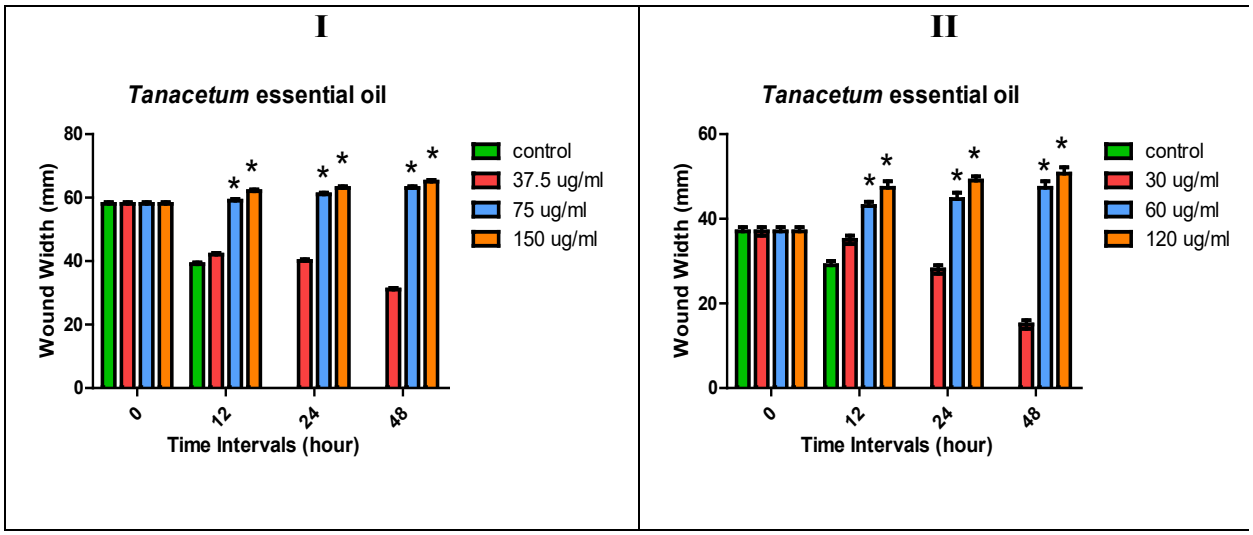

Figure 7. Measurement of Wound Width of HeLa and MCF7 Cell Lines after Treatment with Different Concentrations of *Tanacetum sinaicum* EO. The graphs created by GraphPad Prism® software by using one-way analysis of variance followed by Tukey's test were presented as wound width (mm) in relation to time intervals (hours) after treatment with different concentrations of EO compared with untreated cells (control). Error bars represented the mean ± SD of three independent replicates. The results were considered statistically significant as p-value was < 0.05 and (*) indicated statistically significant in relation to control. Where, (A) HeLa cell line and (B) MCF7 cell line.

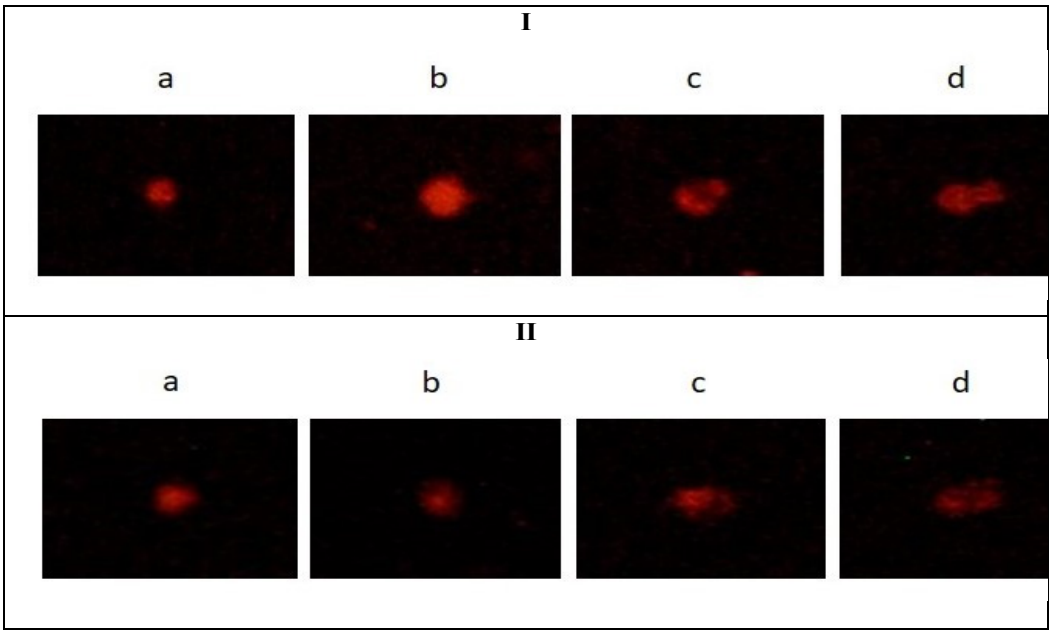

Figure 8. The Comets Obtained by the Alkaline and Neutral Versions of the Comet Assay in HeLa Cell Line. Fluorescence images of HeLa cell line exposed to different concentrations of the EO stained with Ethidium Bromide after 24 hrs treatment under red light excitation with magnification 20x. Where, (I)alkaline version, (II)neutral version, A:Untreated cells, B: *Tanacetum sinaicum* 37.5 µg/ml, C: *Tanacetum sinaicum* 75 µg/ml and D: *Tanacetum sinaicum* 150 µg/ml.

the 6.25 µg/ml concentration of EO. HeLa cell line (Figure 3-II) treated with EO up to 12.5 µg/ml did not show significant change in virus copy numbers compared to the untreated control group. However, increasing the concentration of EO to 50, 100, and 200 µg/ml significantly reduced virus copy numbers. Virus copy numbers decreased from $4x10^6$ copies to $6.1x10^5$, $3.5x10^4$, and $1.3x10^4$ respectively, when compared to the untreated control. At concentrations of 400-3,200 µg/ml, the virus was undetectable. The virus copy numbers differed significantly from the 6.25 µg/ml concentration of EO. These findings highlight that EO has a dose-dependent effect on virus copy number reduction in both the MCF7 and HeLa cell lines. Higher concentrations of the EO

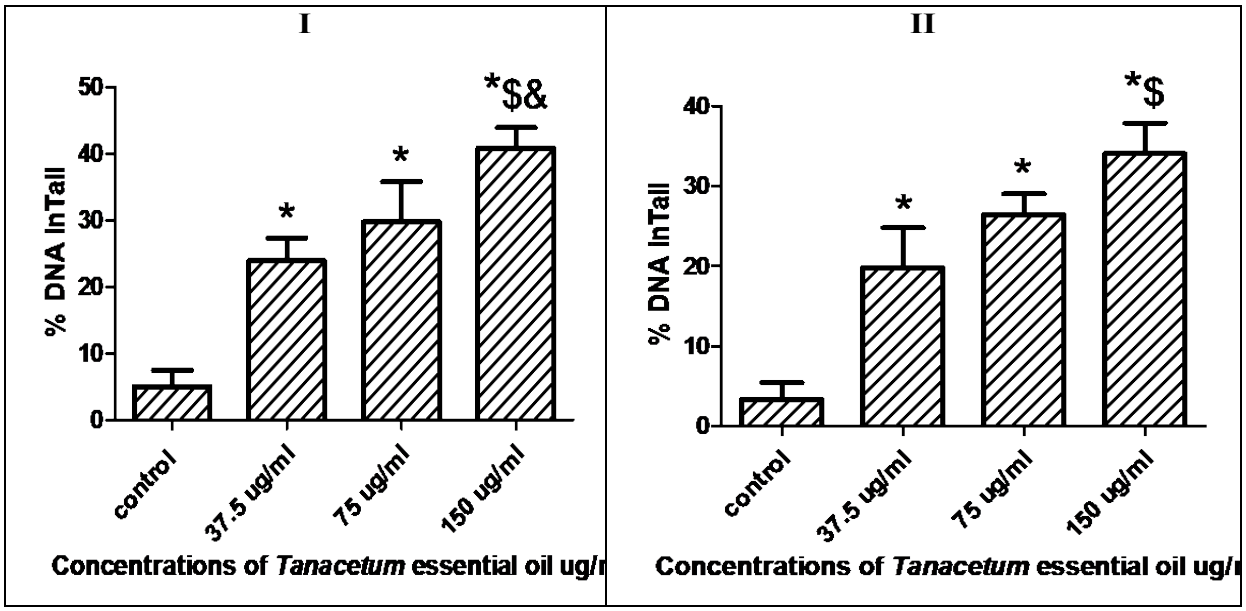

Figure 9. Evaluation of DNA Damage by the Comet assay in HeLa Cell Line. The graphs created by GraphPad Prism® software by using one-way analysis of variance followed by Tukey's test were presented as parameter of % DNA in tail in relation to different concentrations of EO compared with control. Where, (I)alkaline version, (II)neutral version. Error bars represented the mean ± SD of three independent replicates. The results were considered statistically significant as p-value was < 0.05. (*) indicated statistically significant in relation to control. ($) indicated statistically significant for 37.5 vs 150 µg/ml. (&) indicated statistically significant for 75 vs 150 µg/ml.

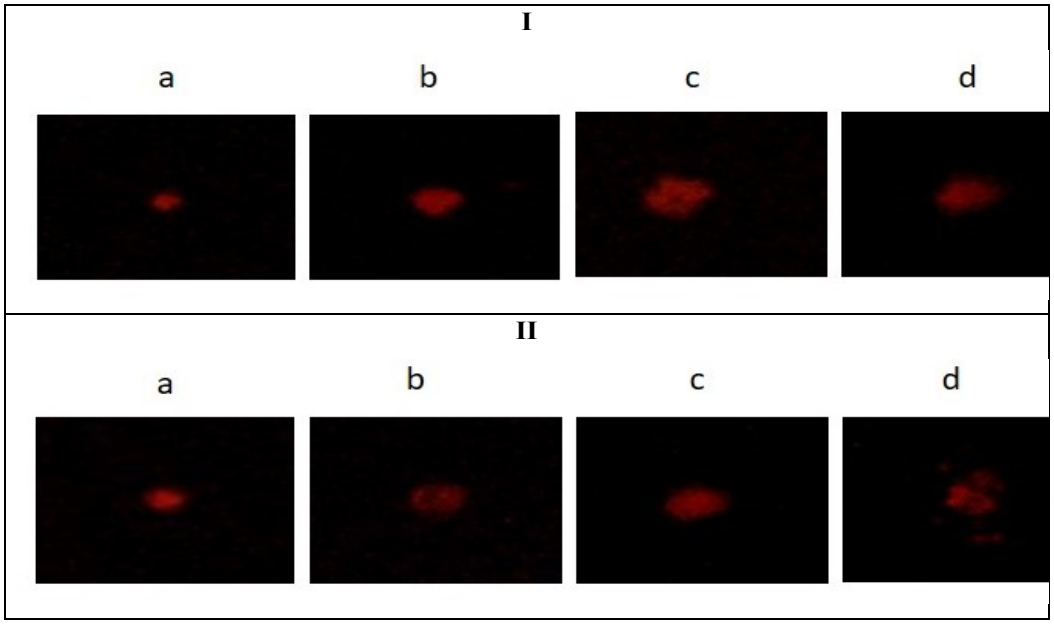

Figure 10. The Comets Obtained by the Alkaline and Neutral Versions of the Comet Assay in MCF7 Cell Line. Fluorescence images of MCF7 cell line exposed to different concentrations of all the tested EO stained with Ethidium Bromide after 24 hrs treatment under red light excitation with magnification 20x. Where, (I)alkaline version, (II)neutral version, A:Untreated cells, B: *Tanacetum sinaicum* 30 µg/ml, C: *Tanacetum sinaicum* 60 µg/ml and D: *Tanacetum sinaicum* 120 µg/ml.

are more effective at suppressing viral replication, as evidenced by the significant reductions observed.

Quantitative reverse-transcription (RT-qPCR) for HPV mRNA detection in cells

Figure 4 depicts the findings of an experiment that looked into the effect of EO on mRNA virus copy numbers in the cells and culture supernatant of both MCF7 and HeLa cell lines at various concentrations. In MCF7 cells (Figure 4-I), EO concentrations of 6.25, 12.5, 25, 50, and 100 µg/ml significantly reduced HPV-16 mRNA expression levels compared to the untreated control group.

Virus copy numbers decreased from 2x106 copies per cell to $1.2x10^6$, $7.5x10^5$, $1.7x10^4$, $7.1x10^3$ and $1.8x10^3$ virus mRNA copies, respectively, compared to the control group. In the HeLa cell line (Figure 4-II), treatment with

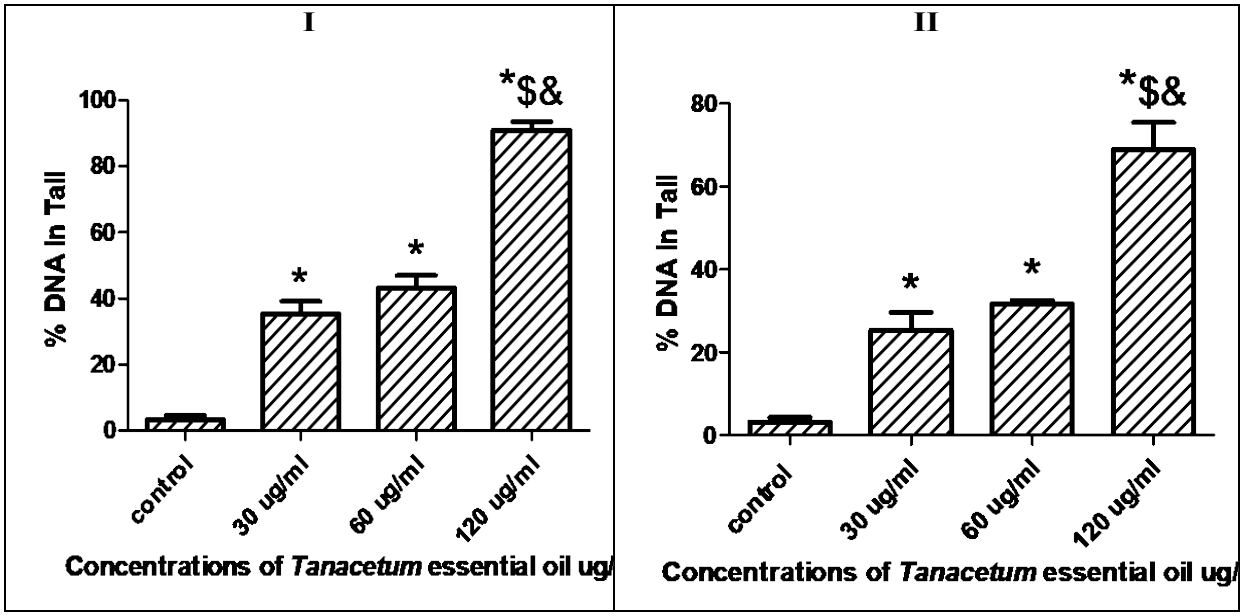

Figure 11. Evaluation of DNA Damage by the Comet Assay in MCF7 Cell Line. The graphs created by GraphPad Prism® software by using one-way analysis of variance followed by Tukey's test were presented as parameter of % DNA in tail in relation to different concentrations of EO compared with control. Where, (I)alkaline version, (II) neutral version. Error bars represented the mean ± SD of three independent replicates. The results were considered statistically significant as p-value was < 0.05. (*) indicated statistically significant in relation to control. ($) indicated statistically significant for 37.5 vs 150 µg/ml. (&) indicated statistically significant for 75 vs 150 µg/ml.

Table 1. The Chemical Profile of Tanacetum sinaicium Essential Oil Using GC-MS.

No.	Compound Name	RT	Area %
1	Santolina triene	3.24	0.33
2	α-Pinene	3.76	1.31
3	Camphene	4.06	0.22
4	Sabinene	4.43	1.16
5	Yomogi alcohol	4.87	0.19
6	α-Humulene	5.24	0.94
7	o-Cymene	5.47	0.34
8	1,8 Cineol	5.56	5.44
9	Butanoic acid, 3-methylbut-2-enyl ester	5.93	0.26
10	α-Terpinene	6.05	1.89
11	α-Thujone	7.17	28.91
12	Cis-sabinol	7.99	1.06
13	Camphor	8.11	0.88
14	Verbenol	8.44	9.1
15	Umbellunone	8.59	3.71
16	Terpinen-4-ol	8.79	2.87
17	α-Terpineol	9.21	0.36
18	Trans-Chrysanthenyl acetate	9.61	33.67
19	Linalool oxide	10.66	0.23
20	Myrtenyl acetate	10.89	2.18
21	trans-2-Caren-4-ol	11.26	0.26
22	Thymol	11.57	2.82
23	Cis-Chrysanthenyl acetate	12.36	0.35
24	Germacene-D	14.69	0.2
25	Davanone	16.6	0.53
26	β-Eudesmol	18.15	0.79
	Terpenoids	48.34	
% of	Monoterpenes	36.94	
	Monoterpenes alcohol	9.29	
	Sesquiterpenes	1.93	
	Others	3.5	
% of total identified compounds		100	

RT, Retention time

EOs at concentrations up to 800 µg/ml resulted in a significant increase in HPV-18 mRNA expression levels compared to the control group. Treating the HeLa cell line with 6.25 and 800 µg/ml of EO reduced mRNA levels from $1.2x10^6$ to $5x10^2$.

Quantitative real time PCR was used to detect mRNA in the culture supernatants of MCF7 and HeLa cell lines (Figure 4). EO treatment at doses of 6.25, 12.5, 25, 50, and 100 µg/ml significantly reduced HPV-16 mRNA expression levels in MCF7 cells compared to the untreated control group. Virus copy numbers decreased from $2.9x10^5$ copies per cell in untreated cells to 8.1x104, $6.1x10^4$, $1.7x10^3$, $2.8x10^3$ and $3x10^3$ virus mRNA copies, respectively (Figure 4-III). HeLa cell line treated with EO concentrations up to 100 µg/ml showed significant changes in HPV-18 mRNA expression levels compared to the untreated control group. Treatment with 6.25 and 100µg/ml of EO decreased mRNA levels from 2.9x106 to $1.8x10^3$ in HeLa cells (Figure 4-VI).

Detection of acidic vesicular organelles with acridine orange staining (autophagy assay)

Figure 5 shows how EO induced autophagic concentration-dependent responses. After 24 hours of treatment, HeLa cells treated with EO at concentrations of 150, 75, and 37.5 µg/ml formed acidic vesicular organelles compared to untreated cells, as shown in Figure 5-I. This data revealed that EO induced autophagy by increasing the formation of acidic vesicular organelles in a dose-dependent manner after 24 hours of treatment. After 24 hours of treatment with EO at concentrations of 120, 60, and 30 µg/ml, MCF7 cells formed acidic vesicular organelles compared to untreated cells (see Figure 5-II). This data revealed that EO induced autophagy by increasing the formation of acidic vesicular organelles in a dose-dependent manner after 24 hours of treatment. In comparison to untreated cells, EO treatment increased the number of autophagosomes/autolysosomes and acidic vesicular organelles in both cell lines.

In vitro scratch assay

The treatment of HeLa cells with the highest two concentrations of EO resulted in a significant inhibitory migration effect with increasing wound width in correlation with increasing treatment incubation time when compared to untreated cells (control) (Figure 6-I). Our findings revealed that EO has an antiproliferative and anti-metastatic effect on the Hela cell line. The treatment with the lowest concentration of EO had no inhibitory migration effect, and wound width decreased as treatment incubation time increased. When comparing treated and untreated cells, the wound width did not reach full closure (Figure 7-I).

Concentrations of 75 and 150 µg/ml significantly increased wound width by 60 and 61% compared to the control, respectively. The effect was statistically significant when compared to untreated cells. There was no significant difference observed between 75 and 150 µg/ml (Figure 7-I). Similarly, treating the MCF7 cell line with the highest two concentrations of EO resulted in a significant inhibitory migration effect with increasing wound width in correlation with increasing treatment incubation time when compared to untreated cells (control) (Figure 6-II).

Our findings revealed that EO has anti-proliferative and anti-metastatic effects on the MCF-7 cell line. The treatment with the lowest concentration of EO had no inhibitory migration effect, and wound width decreased as treatment incubation time increased. When compared to the control, treated cells did not close the wound completely (Figure 7-II). Concentrations of 60 and 120 µg/ml significantly increased wound width compared to the control by 62% and 65%, respectively. The effect was statistically significant when compared to control. Figure 7-II shows no significant difference in wound width between 60 and 120 µg/ml.

Cell genotoxicity by using single-cell gel electrophoresis (SCGE) (comet) assay

(1) HeLa cell line

The alkaline version: After 24 hours, EO treatment induced DNA single strand breaks (SSBs) in a

dose-response manner compared to untreated cells, with the broken DNA migrating towards the anode and leaving different patterns of comet-like electrophoretic appearances (Figure 8-I). The percentage of DNA in tail parameter was used to assess induced DNA fragmentation and damage in comparison to untreated cells (Figure 9-I). At a concentration of 150 µg/ml, the percentage of DNA in tails increased by 87% compared to untreated cells, which was statistically significant. At 75 ug/ml, the percentage of DNA in the tail increased by 83% compared to untreated cells, which was statistically significant. At 37.5 µg/ml, the percentage of DNA in the tail increased by 79% compared to untreated cells, which was statistically significant. At 37.5 vs 150 µg/ml, the percentage of DNA in the tail increased significantly, as did 75 vs 150 µg/ml. However, at 37.5 vs 75 µg/ml, the difference was not significant.

The neutral version: After 24 hours, EO treatment induced DNA double strand breaks (DSBs) in a dose-response manner compared to untreated cells, where the broken DNA migrates towards the anode, leaving different patterns of comet-like electrophoretic appearances (Figure 8-II). The percentage of DNA in the tail parameter was used to assess induced DNA fragmentation and damage in comparison to untreated cells (Figure 9-II). At a concentration of 150 µg/ml, the percentage of DNA in tails increased by 93% compared to untreated cells, which was statistically significant. At 75 µg/ml, the percentage of DNA in the tail increased by 87% compared to untreated cells, which was statistically significant. At 37.5 µg/ml, the percentage of DNA in the tail increased by 83% compared to untreated cells, which was statistically significant. The percentage of DNA in the tail increased significantly at 37.5 vs 150 µg/ml, but not at 37.5 or 75 µg/ml.

(2) MCF7 cell line

The alkaline version: After 24 hours, EO treatment induced DNA single strand breaks (SSBs) in a dose-response manner compared to untreated cells, with the broken DNA migrating towards the anode and leaving different patterns of comet-like electrophoretic appearances (Figure 10-I). The percentage of DNA in the tail parameter was used to assess induced DNA fragmentation and damage in comparison to untreated cells (Figure 11-I). At a concentration of 120 µg/ml, the percentage of DNA in tails increased by 96% compared to untreated cells, which was statistically significant. At 60 µg/ml, the percentage of DNA in the tail increased by 92% compared to untreated cells, which was statistically significant. At 30 µg/ml, the percentage of DNA in the tail increased by 90% compared to untreated cells, which was statistically significant. The percentage of DNA in the tail increased significantly at 30 vs 120 µg/ml and 60 vs 120 µg/ml, but did not change significantly at 30 vs 60 µg/ml.

The neutral version: After 24 hours of treatment with EO, DNA double strand breaks (DSBs) were induced in a dose-response manner compared to untreated cells, where the broken DNA migrates towards the anode leaving different patterns of comet-like electrophoretic appearances (Figure 10-II). The percentage of DNA in the tail parameter was used to assess induced DNA fragmentation and damage in comparison to untreated cells (Figure 11-II). At a concentration of 120 µg/ml, the percentage of DNA in tails increased by 95% compared to untreated cells, which was statistically significant. At 60 µg/ml, the percentage of DNA in the tail increased by 90% compared to untreated cells, which was statistically significant. At 30 µg/ml, the percentage of DNA in the tail increased by 87% compared to untreated cells, which was statistically significant. The percentage of DNA in the tail increased significantly at 30 vs 120 µg/ml and 60 vs 120 µg/ml, but did not change significantly at 30 vs 60 µg/ml.

Discussion

Cancer is the second leading cause of death after heart disease, and is a growing global health concern. [31]. There are several physiological and biochemical carcinogens, including viral infections (for example, hepatitis B virus, which causes liver cancer, and HPV, which causes cervical cancer) [32, 33]. Secondary metabolites of plants, including flavonoids, tannins, alkaloids, and terpenoids, have significant anticancer properties. These phytoconstituents are crucial in chemotherapeutic drug investigations, such as paclitaxel, a plant alkaloid discovered in 1962 and commercially marketed as Taxol®, for breast and ovarian cancer treatment [4]. Because oxidation causes a variety of diseases, including cancer, the antioxidant activity of essential oils is critical [34, 35], and it may lead to the discovery of safe natural antioxidants [36]. The essential oil prevented further damage by neutralizing free radicals produced by hydroxyl radicals that damaged mitochondrial membranes [37-39].

Terpenoids, including D-limonene, have cancer-fighting properties and are low in toxicity. Parthenolide may lead to synthetic compounds with higher specificity and better pharmacokinetic properties. Semisynthetic diterpenoid taxanes, such as paclitaxel and docetaxel, are approved as cancer treatments and chemotherapeutic agents. Novel taxanes are being developed to reduce toxicity and enhance efficacy, with some showing promise for hormone-responsive cancer chemoprevention and chemotherapy [40].

Terpenoids were found in higher concentrations in the EO during the current study. In the current study, the first major component of EO is 33.67% chrysanthenyl acetate, followed by 28.91% -thujone and 9.10% verbenol. As a result, we can conclude that chrysanthenyl acetate, the primary oil ingredient, significantly contributes to the tested oil's antiviral activity. This is in contrast to the study of [41], which reported that the major compounds of the essential oil are trans-thujone, trans-2,7-dimethyl-4,6-octadien-2-ol, and cis-thujone. The composition may vary depending on the geographical area and harvesting period.

Thujones are commonly found in Tanacetum species that produce essential oils. They can be major components, such as β-thujone (up to 82%) in Tanacetum vulgare and α-thujone (up to 70%) in Tanacetum argyrophyllum, or minor components, such as β-thujone (3.6% in Tanacetum aucheranum and 1.1% in Tanacetum chiliophyllum) [42].

The current study used a cell proliferation test to determine the anti-proliferative effect of various EO concentrations on cell line growth. Concentrations up

to 50 µg/ml did not affect the viability of the HeLa and MCF7 cell lines. After 24 hours incubation, doses ranging from 100 to 400 µg/ml significantly reduced the viability of the HeLa cell line. Cell growth is significantly reduced at concentrations of 800-3200 µg/ml. After a 24-hour incubation period, doses ranging from 100 to 400 g/ml significantly reduced the viability of the HeLa cell line by 62-72%. Concentrations ranging from 800 to 3200 µg/ml significantly reduce cell growth by over 95%.

Lampronti, Saab, and colleagues, [43] investigated the antiproliferative effect of essential oils extracted from Magnoliophyta plants on the human erythroleukemic K562 cell line [43]. They discovered that plant-derived EOs had the most intriguing biological action in inhibiting K562 cell proliferation. Satureja hortensis and Satureja montana contain active ingredients such as α-pinene, β-terpenene, 4-terpineol, α-terpineol, and caryophyllene, which have anti-proliferative activity with IC50 values ranging from 329-98 µm/ml. Another study discovered that the active ingredient eugenol found in cloves, nutmeg, basil, cinnamon, and bay leaves has an anti-proliferative effect on cancer cell lines and animal models [44]. Citrus aurantifolia volatile components inhibited the apoptosis-mediated growth of human colon cancer cells, according to Patil, Jayaprakasha, et al. [45].

The current study looked at how different EO concentrations affected virus copy numbers in cell lines. EO reduced virus copy numbers in MCF7 and HeLa cell lines in a dose-dependent manner, with higher concentrations of EO inhibiting virus replication more effectively. Increasing the EO concentration from 50 to 800 µg/ml reduced virus copy number from $4x10^6$ to 1,000 copies per cell in MCF7 cells. The virus remained undetectable at doses of 1600 and 3200 µg/ml. Increasing the concentration of EO from 100 to 800 µg/ml significantly reduced virus copy numbers in the HeLa cell line. At doses of 16,00 and 3,200 µg/ml, virus copy numbers decreased from 4x106 to 103 copies per cell, rendering the virus undetectable. This minor variation in cytotoxic activity could be attributed to the different behaviours of each cell line, with MCF7 being the most sensitive. This finding is consistent with those of [41], who discovered that EO has cytotoxic activity against human lung cancer (A-549), human hepatocellular carcinoma (HepG-2), and colon carcinoma (HCT-116) cell lines. The oil's IC50 values for Hep-G2, HCT-116, and A-549 were 51, 61.3, and 76.1 µg/ml, respectively.

Plant metabolites with antiviral properties are used in antiviral medications to target chemicals on virion surfaces that are responsible for virus identification, adsorption, and penetration into cells while causing no harm to host cells. EOs can inhibit viral protein production, post-translational processing, and virions formation. In the current study, EO at various concentrations was tested for its effect on mRNA viral copy counts in cells and culture supernatants from both cell lines. Increasing EO concentration from 6.25 to 100 µg/ml in the MCF7 cell line decreased HPV-16 expression levels and virus mRNA copies from $2x10^6$ to $1.8x10^3$ copies per cell. In HeLa cells, EO concentrations from 6.25 to 800 µg/ml decreased mRNA levels from $1.2x10^6$ to $5x10^2$. A study discovered that EOs components can interfere with viral entry via viral envelope breaking, capsid disintegration, or suppression of viral binding to host cell receptors, resulting in antiviral effects and viral replication inhibition [46].

In current study, qRT-PCR was used to detect mRNA in culture supernatant. EO concentration was gradually increased from 6.25 to 100 µg/ml in MCF7 cell line to reduce HPV-16 mRNA expression levels. Increasing EO concentration from 6.25 to 100 µg/ml in the HeLa cell line decreases HPV-16 mRNA levels from $2.9x10^6$ to 1.8x103. The EO may function by attaching to virion surface particles, preventing target cell identification and thus reducing viral copy number. Alternatively, it may inhibit receptors on the host cell surface, preventing virus entry or viral nucleic acid synthesis. Nucleic acids, proteins, viral RNA replicases, and reverse transcriptase have all been identified as appealing target sites for the action of these phytochemicals [47].

Autophagy is a cellular defence mechanism in which autophagosomes engulf cellular proteins and acidic vesicular organelles, digest them in lysosomes, and recycle them to maintain cellular metabolism. Depending on the type of stress and the cell involved, this mechanism responds to stress by promoting survival or cell death [48]. In this study, EO activated autophagy by increasing the production of acidic vesicular organelles in both cell lines after 24 hours of treatment. In both cell lines, EO treatment resulted in an increase in the number of autophagosomes/autolysosomes and acidic vesicular organelles compared to controls.

The autophay assay was used for the first time to evaluate the EO's pro-apoptotic effects on HeLa and MCF7 cell lines. The results showed that different EO concentrations induced oxidative stress, which triggered the formation of acidic vesicular organelles in a dose-dependent manner after 24 hours of treatment, as revealed by acridine orange staining. A recent study [49] found that thujone has promising effects against glioblastoma multiforme (GBM) cells, reducing viability and invasive potential in a dose-dependent manner. The thujone monoterpene compound inhibited carcinoma cell proliferation and promoted apoptosis in GBM cells, most likely by inducing oxidative stress. In another study, the monoterpene eucalyptol induced autophagy in cytokine-induced killer (CIK) cells [50].

Cell migration is an important feature of cancer metastasis because it allows cancer cells to invade neighbouring tissues and spread to other body parts, thereby promoting metastases [51]. The current study found that EO has an antiproliferative and anti-metastatic effect on the Hela cell line. Compared to untreated cells, concentrations of 75 and 150 µg/ml significantly improved wound width. EO treatment at concentrations of 60 and 120 µg/ml significantly inhibited migratory activity in MCF7 cells, leading to increased wound width and incubation duration.

This study found that EO has anti-proliferative and anti-metastatic properties on the MCF7 cell line. The in vitro scratch assay was used for the first time to assess the EO's migratory effects on HeLa and MCF7 cell lines. This result is completely consistent with the findings of

[52], who discovered that 1,8-cineole monoterpenes at high concentrations significantly reduced cell migration and exerted anti-metastatic effects on NSCLC A549 cells after 48 hours of treatment.

In the current study, the genotoxic effects of the investigated EO on HeLa and MCF7 cell lines were assessed using the alkaline and neutral versions of the comet assay. The results showed that different doses of EO caused dose-dependent DNA damage after a 24-hour treatment compared to untreated cells. Previous researches have shown that thujone monoterpenens compounds are both genotoxic and antigenotoxic, depending on the cell line type, experimental design, and concentration used [53-55]. Because the second main ingredient in the essential oil was from the monoterpenens class, it is assumed that the essential oil's genotoxic potential was related to the activity of the chemicals associated with this chemical class.

In conclusion, finally, the study found that *Tanacetum sinaicum* EO has strong antiviral activity against integrated human papillomavirus type 16, but weak antiviral activity against type 18. It also promotes autophagy, inhibits cell migration, and causes DNA damage in the HeLa and MCF7 cancer cell lines.

Author Contribution Statement

MMH, AAS, ZKH, and FAA.: conceptualization, methodology and resources, investigation and validation; data analysis; supervision and funding acquisition; writing—original draft; review and editing. EEZ, BWE, and MHE participated in some experiments. All authors read and approved the published version of the manuscript.

Acknowledgements

The current study was funded by the Science and Technology Development Fund (STDF), Project ID: 41907.

References

1. Kombe Kombe AJ, Li B, Zahid A, Mengist HM, Bounda GA, Zhou Y, et al. Epidemiology and burden of human papillomavirus and related diseases, molecular pathogenesis, and vaccine evaluation. Front Public Health. 2020;8:552028. https://doi.org/10.3389/fpubh.2020.552028.
2. Haręża DA, Wilczyński JR, Paradowska E. Human papillomaviruses as infectious agents in gynecological cancers. Oncogenic properties of viral proteins. Int J Mol Sci. 2022;23(3). https://doi.org/10.3390/ijms23031818.
3. Nasim N, Sandeep IS, Mohanty S. Plant-derived natural products for drug discovery: Current approaches and prospects. Nucleus (Calcutta). 2022;65(3):399-411. https://doi.org/10.1007/s13237-022-00405-3.
4. Mugao LG, Gichimu BM, Muturi PW, Mukono ST. Characterization of the volatile components of essential oils of selected plants in kenya. Biochem Res Int. 2020;2020:8861798. https://doi.org/10.1155/2020/8861798.
5. Fürstenberg-Hägg J, Zagrobelny M, Bak S. Plant defense against insect herbivores. Int J Mol Sci. 2013;14(5):10242-97. https://doi.org/10.3390/ijms140510242.
6. Verdi LG, Brighente II, Pizzolatti M. The baccharis genus (asteraceae): Chemical, economic and biological aspects. Quimica Nova. 2005;28:85-94.
7. Öksüz S. Sesquiterpenoids and other constituents from tanacetum cilicium. Phytochemistry. 1990;29(3):887-90. https://doi.org/https://doi.org/10.1016/0031-9422(90)80039-J.
8. Khatib S, Sobeh M, Faraloni C, Bouissane L. Tanacetum species: Bridging empirical knowledge, phytochemistry, nutritional value, health benefits and clinical evidence. Front Pharmacol. 2023;14:1169629. https://doi.org/10.3389/fphar.2023.1169629.
9. Aldayarov N, Tulobaev A, Salykov R, Jumabekova J, Kydyralieva B, Omurzakova N, et al. An ethnoveterinary study of wild medicinal plants used by the kyrgyz farmers. J Ethnopharmacol. 2022;285:114842. https://doi.org/10.1016/j.jep.2021.114842.
10. El-Shazly A, Dorai G, Wink M. Composition and antimicrobial activity of essential oil and hexane-ether extract of tanacetum santolinoides (dc.) feinbr. And fertig. Z Naturforsch C J Biosci. 2002;57(7-8):620-3. https://doi.org/10.1515/znc-2002-7-812.
11. Täckholm, v. And m. Drar, students' flora of egypt. (no title), 1974.
12. Hegazy ME, Hamed A, Mohamed T, Debbab a, Nakamura b, Matsudac c, et al. Anti-inflammatory sesquiterpenes from the medicinal herb tanacetum sinaicum. RSC Advances. 2015. https://doi.org/10.1039/C5RA07511.
13. Móricz Á M, Häbe TT, Böszörményi A, Ott PG, Morlock GE. Tracking and identification of antibacterial components in the essential oil of tanacetum vulgare l. By the combination of high-performance thin-layer chromatography with direct bioautography and mass spectrometry. J Chromatogr A. 2015;1422:310-7. https://doi.org/10.1016/j.chroma.2015.10.010.
14. Soltan MM, Zaki AK. Antiviral screening of forty-two egyptian medicinal plants. J Ethnopharmacol. 2009;126(1):102-7. https://doi.org/10.1016/j.jep.2009.08.001.
15. Hegazy ME, Matsuda H, Nakamura S, Yoshikawa M, Pare P. Two egyptian herbal medicines, tanacetum sinaicum and pulicaria undulata with nitric oxide production inhibitory effects in raw264.7 macrophage cells. Planta Medica. 2013;79. https://doi.org/10.1055/s-0033-1352133.
16. Jennings WA, Shibamoto TA. Part A-analytical considerations. Qualitative Analysis of Flavor and Fragrance Volatiles by Glass Capillary Gas Chromatography. 1980:1-27.
17. Stenhagen E, Abrahamsson S, McLafferty FW. Registry of mass spectral data.1974.
18. Adams RP. Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. Allured publishing corporation; 2001.
19. Coser J, Boeira Tda R, Fonseca AS, Ikuta N, Lunge VR. Human papillomavirus detection and typing using a nested-pcr-rflp assay. Braz J Infect Dis. 2011;15(5):467-72. https://doi.org/10.1016/s1413-8670(11)70229-x.
20. Bordigoni A, Motte A, Tissot-Dupont H, Colson P, Desnues C. Development and validation of a multiplex qpcr assay for detection and relative quantification of hpv16 and hpv18 e6 and e7 oncogenes. Sci Rep. 2021;11(1):4039. https://doi.org/10.1038/s41598-021-83489-2.
21. Mirghani H, Amen F, Moreau F, Guigay J, Ferchiou M, Melkane AE, et al. Human papilloma virus testing in oropharyngeal squamous cell carcinoma: What the clinician should know. Oral Oncol. 2014;50(1):1-9. https://doi.org/10.1016/j.oraloncology.2013.10.008.
22. Bishop JA, Lewis JS Jr, Rocco JW, Faquin WC. Hpv-related squamous cell carcinoma of the head and neck: An update on testing in routine pathology practice. Semin

Diagn Pathol. 2015;32(5):344-51. https://doi.org/10.1053/j.semdp.2015.02.013.
23. Robinson M, Schache A, Sloan P, Thavaraj S. Hpv specific testing: A requirement for oropharyngeal squamous cell carcinoma patients. Head Neck Pathol. 2012;6 Suppl 1(Suppl 1):S83-90. https://doi.org/10.1007/s12105-012-0370-7.
24. Wu MZ, Li WN, Cha N, Tian LX, Zhang YI, Wu X, et al. Diagnostic utility of hpv16 e6 mrna or e7 mrna quantitative expression for cervical cells of patients with dysplasia and carcinoma. Cell Transplant. 2018;27(9):1401-6. https://doi.org/10.1177/0963689718788521.
25. Thomé MP, Filippi-Chiela EC, Villodre ES, Migliavaca CB, Onzi GR, Felipe KB, et al. Ratiometric analysis of acridine orange staining in the study of acidic organelles and autophagy. J Cell Sci. 2016;129(24):4622-32. https://doi.org/10.1242/jcs.195057.
26. Wang H, Zhao Z, Lei S, Li S, Xiang Z, Wang X, et al. Gambogic acid induces autophagy and combines synergistically with chloroquine to suppress pancreatic cancer by increasing the accumulation of reactive oxygen species. Cancer Cell Int. 2019;19:7. https://doi.org/10.1186/s12935-018-0705-x.
27. Abdullah ML, Al-Shabanah O, Hassan ZK, Hafez MM. Eugenol-induced autophagy and apoptosis in breast cancer cells via pi3k/akt/foxo3a pathway inhibition. Int J Mol Sci. 2021;22(17). https://doi.org/10.3390/ijms22179243.
28. Abdullah ML, Hafez MM, Al-Hoshani A, Al-Shabanah O. Anti-metastatic and anti-proliferative activity of eugenol against triple negative and her2 positive breast cancer cells. BMC Complement Altern Med. 2018;18(1):321. https://doi.org/10.1186/s12906-018-2392-5.
29. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175(1):184-91. https://doi.org/10.1016/0014-4827(88)90265-0.
30. Reddy L, Odhav B, Bhoola KD. Natural products for cancer prevention: A global perspective. Pharmacol Ther. 2003;99(1):1-13. https://doi.org/10.1016/s0163-7258(03)00042-1.
31. Tabor E. Pathogenesis of hepatitis b virus-associated hepatocellular carcinoma. Hepatol Res. 2007;37 Suppl 2:S110-4. https://doi.org/10.1111/j.1872-034X.2007.00172.x.
32. Lee JY, Li J, Yeung ES. Single-molecule detection of surface-hybridized human papilloma virus DNA for quantitative clinical screening. Anal Chem. 2007;79(21):8083-9. https://doi.org/10.1021/ac071159y.
33. Halliwell B, Gutteridge JM. The antioxidants of human extracellular fluids. Arch Biochem Biophys. 1990;280(1):1-8. https://doi.org/10.1016/0003-9861(90)90510-6.
34. Paz-Elizur T, Sevilya Z, Leitner-Dagan Y, Elinger D, Roisman LC, Livneh Z. DNA repair of oxidative DNA damage in human carcinogenesis: Potential application for cancer risk assessment and prevention. Cancer Lett. 2008;266(1):60-72. https://doi.org/10.1016/j.canlet.2008.02.032.
35. Poulsen HE, Prieme H, Loft S. Role of oxidative DNA damage in cancer initiation and promotion. Eur J Cancer Prev. 1998;7(1):9-16.
36. Stadler RH, Markovic J, Turesky RJ. In vitro anti- and pro-oxidative effects of natural polyphenols. Biol Trace Elem Res. 1995;47(1-3):299-305. https://doi.org/10.1007/bf02790130.
37. Sakihama Y, Cohen MF, Grace SC, Yamasaki H. Plant phenolic antioxidant and prooxidant activities: Phenolics-induced oxidative damage mediated by metals in plants. Toxicology. 2002;177(1):67-80. https://doi.org/10.1016/s0300-483x(02)00196-8.
38. Azmi AS, Bhat SH, Hanif S, Hadi SM. Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: A putative mechanism for anticancer properties. FEBS Lett. 2006;580(2):533-8. https://doi.org/10.1016/j.febslet.2005.12.059.
39. Kamran S, Sinniah A, Abdulghani MAM, Alshawsh MA. Therapeutic potential of certain terpenoids as anticancer agents: A scoping review. Cancers (Basel). 2022;14(5). https://doi.org/10.3390/cancers14051100.
40. El-Dahmy SI, Abdel-Ghani AE, Attia RA, Abouelenein DD. Chemical composition and biological activities of the essential oil of *Tanacetum sinaicum* Del. grown in Egypt. Journal of Pharmacognosy and Phytochemistry. 2019;8(4):326-31.
41. Zámboriné Németh É, Thi Nguyen H. Thujone, a widely debated volatile compound: What do we know about it? Phytochemistry Reviews. 2020;19(2):405-23. https://doi.org/10.1007/s11101-020-09671-y.
42. Lampronti I, Saab AM, Gambari R. Antiproliferative activity of essential oils derived from plants belonging to the magnoliophyta division. Int J Oncol. 2006;29(4):989-95.
43. Jaganathan SK, Supriyanto E. Antiproliferative and molecular mechanism of eugenol-induced apoptosis in cancer cells. Molecules. 2012;17(6):6290-304. https://doi.org/10.3390/molecules17066290.
44. Patil J, Jayaprakasha G, Murthy K, Tichy S, Chetti M, Patil B. Apoptosis-mediated proliferation inhibition of human colon cancer cells by volatile principles of citrus aurantifolia. Food Chemistry. 2009;114:1351-8. https://doi.org/10.1016/j.foodchem.2008.11.033.
45. de Sousa DP, Damasceno ROS, Amorati R, Elshabrawy HA, de Castro RD, Bezerra DP, et al. Essential oils: Chemistry and pharmacological activities. Biomolecules. 2023;13(7). https://doi.org/10.3390/biom13071144.
46. Sarowska J, Wojnicz D, Jama-Kmiecik A, Frej-Mądrzak M, Choroszy-Król I. Antiviral potential of plants against noroviruses. Molecules. 2021;26(15). https://doi.org/10.3390/molecules26154669.
47. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The role of autophagy in cancer: Therapeutic implications. Mol Cancer Ther. 2011;10(9):1533-41. https://doi.org/10.1158/1535-7163.Mct-11-0047.
48. Pudełek M, Catapano J, Kochanowski P, Mrowiec K, Janik-Olchawa N, Czyż J, et al. Therapeutic potential of monoterpene α-thujone, the main compound of thuja occidentalis l. Essential oil, against malignant glioblastoma multiforme cells in vitro. Fitoterapia. 2019;134:172-81. https://doi.org/10.1016/j.fitote.2019.02.020.
49. Liu H, Sun W, Zhu H, Guo J, Liu M, Xu S. Eucalyptol relieves the toxicity of diisobutyl phthalate in ctenopharyngodon idellus kidney cells through keap1/nrf2/ho-1 pathway: Apoptosis-autophagy crosstalk and immunoregulation. Fish Shellfish Immunol. 2022;130:490-500. https://doi.org/10.1016/j.fsi.2022.09.056.
50. Weigelt B, Peterse JL, van 't Veer LJ. Breast cancer metastasis: Markers and models. Nat Rev Cancer. 2005;5(8):591-602. https://doi.org/10.1038/nrc1670.
51. Rodenak-Kladniew B, Castro MA, Crespo R, Galle M, García de Bravo M. Anti-cancer mechanisms of linalool and 1,8-cineole in non-small cell lung cancer a549 cells. Heliyon. 2020;6(12):e05639. https://doi.org/10.1016/j.heliyon.2020.e05639.
52. Mitić-Culafić D, Zegura B, Nikolić B, Vuković-Gacić B, Knezević-Vukcević J, Filipic M. Protective effect of linalool, myrcene and eucalyptol against t-butyl hydroperoxide induced genotoxicity in bacteria and cultured human cells. Food Chem Toxicol. 2009;47(1):260-6. https://doi.org/10.1016/j.fct.2008.11.015.

53. Vuković-Gacić B, Nikcević S, Berić-Bjedov T, Knezević-Vukcević J, Simić D. Antimutagenic effect of essential oil of sage (salvia officinalis l.) and its monoterpenes against uv-induced mutations in escherichia coli and saccharomyces cerevisiae. Food Chem Toxicol. 2006;44(10):1730-8. https://doi.org/10.1016/j.fct.2006.05.011.
54. Nikolić B, Mitić-Ćulafić D, Vuković-Gačić B, Knežević-Vukčević J. Modulation of genotoxicity and DNA repair by plant monoterpenes camphor, eucalyptol and thujone in escherichia coli and mammalian cells. Food Chem Toxicol. 2011;49(9):2035-45. https://doi.org/10.1016/j.fct.2011.05.015.