



Evaluation of Antioxidant, Cytotoxicity, and Genotoxicity Activity of *Ammoides pusilla* (Brot.) Breistr Oil for Breast Cancer

Priyanka A. Ahire¹, Dr Anjali M. Wanegaonkar², Vijeta C. Dhabre³,
Ashwini P. Salunkhe⁴, Manisha Y. Chaudhari⁵, Rahul K Verma⁶, Pallavi B. Bangar⁷,
Dr. Vilasrao J. Kadam⁸, Savita S. Patil (Pol)*

¹Assistant Professor, Bharati Vidyapeeth's College of Pharmacy, C.B.D Belapur, Navi Mumbai, Maharashtra

²Assistant Professor, Bharati Vidyapeeth's College of Pharmacy, C.B.D Belapur, Navi Mumbai, Maharashtra

³Associate Professor, Gahlot Institute of Pharmacy, Koparkhairane, Navi Mumbai, Maharashtra

⁴Assistant Professor, Shivnagar Vidya Prasarak Mandal's College of Pharmacy, Malegaon, Pune, Maharashtra

⁵Head of Department, Konkan Gyanpeeth Rahul Dharkar College of Pharmacy And Research Institute, Karjat, Maharashtra

⁶Assistant Professor, Bharati Vidyapeeth College of Pharmacy, C.B.D Belapur Navi Mumbai, Maharashtra

⁷Assistant Professor, St. Wilfred's Institute of Pharmacy - Panvel

⁸Principal, Bharati Vidyapeeth's College of Pharmacy, C.B.D Belapur, Navi Mumbai, Maharashtra

***Corresponding Author- Ms. Savita S Patil (Pol)**

savitabvcop@gmail.com

Article History: Received: 10/7/23

Revised: 18/7/23

Accepted: 27/7/23

Abstract: The unchecked growth of malignant cells in breast tissue is a hallmark of breast cancer, and the development and progression of the disease are both correlated with oxidative stress (ROS) activation. Essential oils from medicinal plants, known for their antioxidant and therapeutic properties, are being explored as alternatives. *Ammoides pusilla* (Brot.) Breistr, also known as Nûnkha, is a medicinal plant native to Morocco, belonging to the Apiaceae family, and used for generations in traditional medicine. This study focuses on the phytochemical characterization of *P. verticillata* essential oil (PVEO) from the province of Oujda, Morocco, for its therapeutic properties. Hydro-distillation was used to get the essential oil, and gas chromatography-mass spectrometry (GC-MS) was used to look at its volatile parts. The results revealed the presence of various aromatic and terpene compounds, with carvacrol being the most abundant compound. PVEO showed antioxidant properties in several tests, including β -carotene bleaching, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, and total antioxidant capacity (TAC). It also demonstrated cytotoxicity against MCF-7 and MDA-MB-231 breast cancer cell

lines, with higher selectivity for MDA-MB-231. The results reveal that *Ammoides pusilla* (Brot.) Breistr essential oil (PVEO) could be a promising natural alternative for the treatment of breast cancer,

Keywords: Breast cancer; oxidative stress; essential oil; *Ammoides pusilla* (Brot.) Breistr; phytochemical characterization; antioxidant activity; genotoxicity; cytotoxicity; alternative treatment.

DOI: 10.48047/ecb/2023.12.Si12.136

Introduction

Breast cancer is characterized by the uncontrolled proliferation of malignant cells in the breast tissue. Although it can affect both sexes, it mainly affects women. According to the latest GLOBOCAN 2020 statistics, breast cancer is the fifth leading cause of cancer mortality, with around 2.3 million new cases detected worldwide [1]. In 2020, around 2.26 million breast cancer cases were reported, with a 95% uncertainty interval ranging from 2.24 to 2.79 million [2]. Oxidative stress is thought to considerably influence the development and progression of breast tumors through various mechanisms. One such mechanism involves DNA damage in breast cells, which can be attributed to oxidative stress-induced damage [1]. The rich phytochemical composition of essential oils derived from medicinal and aromatic plants in terms of secondary metabolites is mainly responsible for their antioxidant activity against free radicals and their potential in treating various diseases, including cancer [2,3].

Other secondary metabolites may have harmful or toxic biological activities. For example, some plants produce toxic secondary metabolites. These compounds may be responsible for the poisoning of certain animals, or even toxic effects on humans [4]. For this reason, it is important to assess an essential oil's toxicity before using it. These essential oils are increasingly being explored as an alternative approach to mitigate the undesirable side effects of conventional treatments. Among these plants, *Ammoides pusilla* (Brot.) Breistr Duby., commonly known as "Nûnkha" in Morocco [5], is an endemic dicotyledonous plant belonging to the Apiaceae family. It naturally grows in arid, sandy regions of eastern Morocco and is extensively utilized in traditional Moroccan medicine [6]. This plant exhibits adaptations to challenging environmental conditions, such as drought and nutrient-poor soils. Its distinctive feature is the whorled arrangement of its leaves in circles around the stem. The flowers are small and typically pale yellow.

The remarkable ability of *P. verticillata* to thrive under various stresses contributes to the abundance of secondary metabolites it produces. These secondary metabolites are bioactive molecules responsible for its diverse biological and pharmacological activities [7]. The plant is renowned for its medicinal properties, and different parts such as leaves and stems are therapeutically utilized. It is commonly employed in the treatment of digestive system disorders, particularly gastric and intestinal issues. Furthermore, it possesses analgesic, diuretic, and anti-asthmatic potentials [8]. Notably, *P. verticillata* is highly valued for its potent stimulating action, as well as its antioxidant and antimicrobial properties [5]. Essential oils are increasingly being

explored as an alternative to alleviate the undesirable side effects of conventional treatments. The main objective of this study is to investigate the chemical components of *P. verticillata* essential oil (PVEO) by applying gas chromatography-mass spectrometry (GC/MS). In addition, we focus on predicting the toxicity of the essential oil using computational methods, and the genotoxicity of the essential oil was investigated using the comet assay. In addition, we evaluated the effects of the essential oil on two breast cancer cell lines, namely MCF7 and MDA-MB-231, in terms of cell toxicity. In addition, antioxidant activity was assessed utilizing the diphenyl-1-picrylhydrazyl (DPPH) scavenging assay anti-radical assay and β -Carotene Bleaching Assay and by calculating total antioxidant activity.

Materials and Methods

Plant Material and Essential Oil Extraction

The *Ammoides pusilla* (Brot.) Breistr plant specimens were obtained from the local market in the province of Oujda, located in eastern Morocco, during the spring season of 2022. Taxonomic determination procedures were conducted, and a voucher specimen (number HUMPOM17*) was deposited at the Faculty of Sciences, Mohammed 1st University, in Oujda City, Morocco. The essential oil was extracted from the aerial part of the plant using the hydro-distillation method, employing a modified Clevenger apparatus. The device was securely sealed and positioned once it had been properly loaded with water and the plant sample. The heat source was activated and precisely set to 100 °C, ensuring a consistent and stable extraction process. Throughout the extraction, strict control of heating conditions was maintained to achieve the highest quality extraction output.

Qualitative and Semi-Quantitative Analysis of PVEO

Phytochemical analysis of PVEO was carried out using a Shimadzu QP2010 mass spectrometer with electron impact ionization (70 eV), in line with the technique used in our study. The NIST library of mass spectra from the GC-MS data system was used to compare results [9,10]. The peak area was used to calculate the percentage composition.

Antioxidant Activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Scavenging Assay

The antioxidant activity of the essential oils was assessed by comparing them to a known antioxidant, ascorbic acid. Each concentration was tested in triplicate. The DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was prepared at a concentration of 0.1 mM. The extract concentrations were varied (0.2, 0.4, 0.5, 0.7, 0.8, and 1 mg/mL). To initiate the test, 2.5 mL of the DPPH methanolic solution was added to 0.5 mL of each extract concentration. After incubating the mixture in the dark for 30 min, the optical density was measured at 517 nm against a blank. Ascorbic acid was employed as a positive control [11]. The scavenging activity was subsequently calculated using the provided formula:

$$\text{Free Radical Scavenging (\%)} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

β -Carotene Bleaching Assay

In order to assess the antioxidant activity of PVEO, we conducted the β -carotene bleach-based assay, utilizing the methodology described by Elbouzidi et al. (2023) [12]. This assay was employed to determine the ability of PVEO to prevent β -carotene bleaching, serving as a measure of its antioxidant potential. For accurate and reliable results, the percentage of residual color was calculated using the following formula:

$$\text{Residual color \%} = \frac{\text{Initial OD} - \text{Sample OD} \times 100}{\text{Initial OD}}$$

Total Antioxidant Capacity (TAC)

Total antioxidant activity refers to PVEO's ability to neutralize free radicals and prevent oxidative damage in the body. It is measured in terms of vitamin C equivalent. Total antioxidant activity refers to PVEO's ability to neutralize free radicals and prevent oxidative damage in the body. It is measured in terms of vitamin C equivalent. TAC determination in our study was carried out using the phosphorus-molybdenum technique [13], in line with the technique used in the study by Elbouzidi et al. (2023) [12]. A standard curve was generated from vitamin C and the results obtained were expressed in terms of vitamin C equivalents [14,15].

Prediction of the Toxicity Analysis (Pro-Tox II)

The estimation of toxicity levels was performed using the Pro-Tox II online tool (https://tox-new.charite.de/protox_II/ accessed on 21 June 2023) [16]. This tool evaluates the chemical structure of each compound present in the essential oil and compares it with a comprehensive database of known toxic compounds. Based on this analysis, the tool predicts the potential toxicity or likelihood of adverse effects in humans or other organisms. The Pro-Tox II online tool provides valuable insights into various toxicological aspects, including the lethal dose 50 (LD₅₀), immunotoxicity, hepatotoxicity, mutagenicity, carcinogenicity, cytotoxicity, and toxicity class [17]. Utilizing these tools allowed for the assessment of potential therapeutic applications, as well as the identification and evaluation of toxicity risks associated with the compounds present in the *P. verticillata* essential oil (PVEO).

Genotoxic Effect

Blood Sample Collection and Treatment of Cells

Blood was taken from male Wistar rats via retro-orbital collection using heparin-containing tubes, following the administration of pentobarbital anesthesia. Fresh blood (2 mL) was then combined with PBS solution lacking Ca²⁺ or Mg²⁺ ions (composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4) at a volume of 2 mL. The resulting diluted blood mixture was exposed to the samples under investigation. To prepare different concentrations (5, 10, 20, and 40 μ g/mL) of PVEO, the oil was dissolved in dimethyl sulfoxide and added to PBS. Subsequently, 10 μ L of blood cells were incubated with PVEO for two hours at a temperature of 37 degrees Celsius. The final concentration of dimethyl sulfoxide in the media did not exceed 0.2%, and the negative control was exposed to an equivalent concentration of the solvent. For the genotoxicity assay, hydrogen peroxide (250 μ mol/L) was used as a positive control.

Comet Assay

Before conducting the alkaline comet assay [18]. Slight adjustments were made to the protocol originally established by Ouahhoud et al. (2022). Following the treatment, the suspension underwent centrifugation at 4500 g rpm for 10 min. Once the centrifugation was complete, the supernatant was discarded, and the pellet containing the leukocytes was reconstituted in 1 mL of PBS. Three successive washes were conducted. After the final centrifugation, the pellet was dissolved in LMP agarose (0.5% w/v in PBS solution), and the resulting mixture was applied to a slide that had been pre-coated with NMP agarose (1.5% w/v). The slides were placed in the lysis solution for 5 min (2.5 M NaCl, 0.1 M Na₂EDTA, 2 × 10⁻² M Tris, 0.3 M NaOH, 1% Sodium N-lauroyl-sarcosine, 10% DMSO and 1% Triton X-100), followed by immersion in the dark at a temperature of 4 °C for 1 h. Following the lysis period, the slides underwent a thorough wash with double-distilled water. Subsequently, the slides were positioned on horizontally aligned gel electrophoresis equipment and a freshly prepared solution (0.3 M NaOH and 10⁻³ M Na₂EDTA, pH = 13) was used. The DNA was then unraveled for a duration of 20 min. Migration was carried out under constant conditions of 300 mA current and 25 V voltage for a total of 20 min. It was essential to maintain a temperature of 4 °C throughout the entire migration and electrophoresis process. After migration, the slides were immersed in a neutralization buffer consisting of a Trizma solution at a concentration of 0.4 M and adjusted to a pH of 7.5 using HCl. The slides were allowed to remain in the buffer for a duration of five minutes. This neutralization process was repeated three times. After the electrophoresis and neutralization steps, the comets were visualized using the ethidium bromide method as described by Singh et al. (1988) [19].

Microscopic Observation

The ethidium bromide-stained slides were examined and imaged using a fluorescence microscope, specifically the ZOE Cell Imager. The observation was conducted using the red channel with excitation at 556/20 nm and emission at 615/61 nm. For quantitative analysis of DNA lesions, an image analyzer coupled with Comet Assay IV image analysis software was used. This software allows for the measurement of various parameters associated with DNA lesions [20]. Fifty cells were randomly chosen from each of the two replicates per sample for analysis.

Statistical Analysis

The statistical program GraphPad Prism 5.0 was employed to perform a one-way ANOVA for statistical data analysis. Tukey's honest significance test was utilized to compare differences between treatment groups with a significance level set at $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$.

Cytotoxic Activity against Breast Cancer Cell Lines

Assessing the effectiveness of novel natural cancer alternatives on cell lines is crucial in the research and advancement of new treatments. There are diverse methods employed to evaluate this effectiveness, with cell viability assays being the most commonly utilized, particularly the MTT test. The MCF-7 and MDA-MB-231 cell lines are of great significance in breast cancer

research. These cell lines are derived from breast tumors and exhibit distinct characteristics. The MCF-7 cell line represents hormone-dependent breast cancer, as it expresses estrogen and progesterone receptors. These cells tend to be less aggressive compared to the MDA-MB-231 cell line. The MCF-7 line is commonly employed to investigate mechanisms associated with the growth and progression of hormone-dependent tumors. On the other hand, the MDA-MB-231 cell line corresponds to triple-negative breast cancer, characterized by the absence of overexpression of estrogen, progesterone, and HER2 receptors [21].

Cell Lines

The human mammary carcinoma cell lines MDA-MB-231 (a triple-negative breast cancer cell line lacking estrogen receptors—ER⁻) and MCF-7 cells (expressing estrogen receptors—ER⁺) were procured from the American Tissue Culture Collection (ATCC, Molsheim, France). These cell lines were cultivated under controlled conditions, including a 5% CO₂ atmosphere and a humidified temperature of 37 °C. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal calf serum (v/v) obtained from Dutscher, Brumath, France [22].

Determination of Cell Viability

The cells under investigation (1 × 10⁴ cells per well) were subjected to various concentrations (ranging from 6.25 to 200 µg/mL) of PVEO in a 96-well culture plate for 24, 48, and 72 h. PVEO was dissolved in dimethyl sulfoxide (DMSO) prior to exposure. Following this, the cells were treated with 20 µL of MTT reagent (0.5 mg/mL) at 37 °C for 4 h and rinsed thrice with 1 phosphate-buffered saline (PBS). Then, 150 µL of DMSO was added, and the absorbance of each sample was measured at 570 nm [23]. The experiments were replicated three times, with each experiment comprising three duplicate wells. GraphPad Prism version 8 software (GraphPad Software, La Jolla, CA, USA) was utilized to calculate the 50% inhibitory concentration (IC₅₀). Cisplatin, a chemotherapeutic drug, was employed as a positive control in the study [12,22].

Results and Discussion

Phytochemical Composition

The volatile compounds present in *Ammoides pusilla* (Brot.) Breistr, which contribute to its characteristic aroma and flavor, were subjected to analysis using gas chromatography-mass spectrometry (GC-MS). PVEO predominantly consists of terpenes and other aromatic compounds, and 23 volatile compounds were identified and quantified in PVEO, as presented in Figure 1 and Table 1. GC-MS analysis commonly reveals the presence of certain volatile compounds in *P. verticillata*. Additionally, it exhibits antioxidant, anti-inflammatory, and antifungal properties [24]. It also exhibits antioxidant, anti-inflammatory, and antifungal properties [25]. Another significant compound found in this oil is D-limonene (22.10%). γ -Terpinene (9.78%) and β -cymene (9.35%) are also identified as major terpenes in *P. verticillata* oil. α -Pinene and β -pinene, monoterpenes with a fresh, pine-like aroma, are detected as minor compounds (1.61% and 0.29%, respectively). Although several other compounds were detected in low quantities, such as (+)-2-carene, *p*-menth-1-en-4-ol, *p*-menth-1-en-8-ol, cinnamic

aldehyde, and 4,4,7a-trimethyl-2,4,5,6,7,7a-hexahydro-1H-inden-1-one, among others. It is important to note that these results differ from those obtained by Taibi et al. (2023) [5]. It is important to note that these results for essential oil extracted from *P. verticillata* from the Oujda region differ from those obtained by Taibi et al. (2023) [5], who analyzed the same plant from the Touissit region of Morocco. These variations can be attributed to environmental factors, the age of the plant, the period of the vegetative cycle, genetic factors and even differences in several abiotic factors between the two harvesting sites, such as temperature, altitude, humidity, air quality, which can be influenced by factors such as air pollution, industrial emissions and meteorological conditions, and the status of Touissit as a mining town with the presence and concentration of mineral elements in the soil that can influence plant growth [26,27]. The plant obtained from Oujda was gathered at the same time as the Touissit plant, albeit with a slight disparity in size and age. This resulted in a remarkable difference in the number and percentage of bioactive molecules; the Touissit PVEO contains only 12 constituents, with thymol (37.05%), D-limonene (22.97%), γ -terpinene (15.97%), *m*-cymene (12.14%), and carvacrol (8.49%) as the main constituents [5]. It is essential to emphasize that the biological activities of essential oils are generally the result of a complex, synergistic interaction between several chemical compounds present in the essential oil rather than an isolated compound [28].

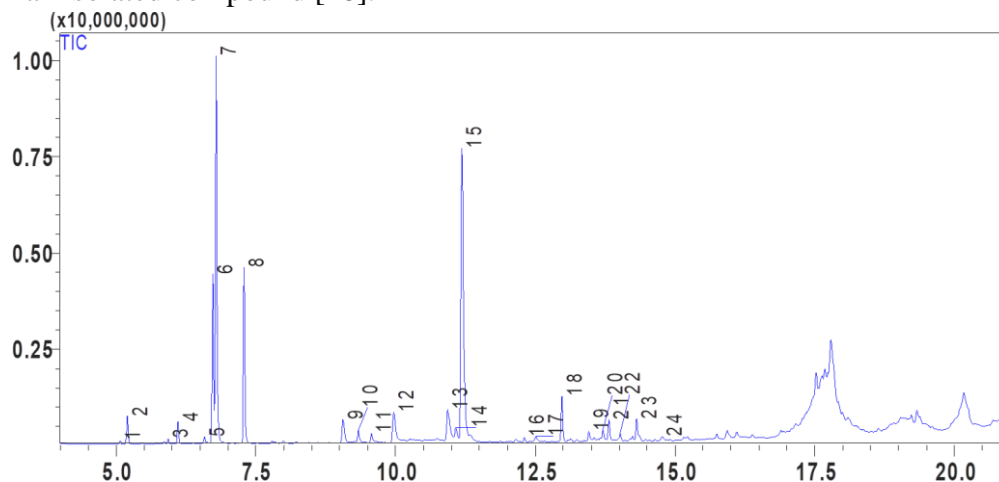


Figure 1. GC-MS chromatogram of *Ammoides pusilla* (Brot.) Breistr essential oil (PVEO). The peaks in the chromatogram correspond to the volatile compounds identified and quantified in Table 1

Table 1. Phytochemical Composition of PVEO

S.No.	Compound Name	Formula	Mol. Wt.	RT (min)	Peak Area (%)
1	α -Thujene	C ₁₀ H ₁₆	136.23	5.075	0.21
2	α -Pinene	C ₁₀ H ₁₆	136.23	5.204	1.61
3	β -Pinene	C ₁₀ H ₁₆	136.23	5.933	0.29
4	β -Myrcene	C ₁₀ H ₁₆	136.23	6.107	1.24
5	(+)-2-Carene	C ₁₀ H ₁₆	136.23	6.582	0.6

6	β -Cymene	C ₁₀ H ₁₄	134.22	6.732	9.35
7	D-Limonene	C ₁₀ H ₁₆	136.23	6.792	22.1
8	γ -Terpinene	C ₁₀ H ₁₆	136.23	7.29	9.78
9	<i>p</i> -Menth-4(8)-en-3-one	C ₁₀ H ₁₆ O	152.23	9.06	2.26
10	<i>p</i> -Menth-1-en-4-ol	C ₁₀ H ₁₈ O	154.25	9.334	0.94
11	<i>p</i> -Menth-1-en-8-ol 2	C ₁₂ H ₂₂ O	198.3	9.568	0.93
12	Cinnamic aldehyde 4,4,7a-Trimethyl	C ₉ H ₈ O	132.16	9.968	4.09
13	2,4,5,6,7,7a-hexahydro-1H-inden-1-one	C ₁₂ H ₁₈ O	178.27	10.934	4.34
14	Thymol	C ₁₀ H ₁₄ O	150.22	11.076	1.52
15	Carvacrol	C ₁₀ H ₁₄ O	150.22	11.186	29.82
16	Copaene	C ₁₅ H ₂₄	204.35	12.303	0.38
17	Caryophyllene	C ₁₅ H ₂₄	204.35	12.976	3.57
18	1,5,9,9-Tetramethyl-1,4,7-	C ₁₅ H ₂₄	204.35	13.457	0.85
19	Valencen	C ₁₅ H ₂₄	204.35	13.71	1.16
20	Germacrene D	C ₁₅ H ₂₄	204.35	13.817	1.56
21	α -Muurolene	C ₁₅ H ₂₄	204.35	14.018	0.82
22	Δ -Cadinene	C ₁₅ H ₂₄	204.35	14.313	1.96
23	Eicosanoic acid	C ₂₀ H ₄₀ O ₂	312.5	14.772	0.62
	Hydrocarbon monoterpenes				45.18
	Oxygenated monoterpenes				43.9
	Hydrocarbon sesquiterpenes				10.3
	Oxygenated sesquiterpenes				0.62
	Total identified (%)				100

Antioxidant Activity

The antioxidant activity of PVEO was evaluated using the DPPH and β -Carotene bleaching tests and by determining total antioxidant capacity. The IC₅₀ value, representing the concentration of antioxidants required to decrease the concentration of free radicals by 50%, was determined in order to quantitatively evaluate the antioxidant potential. As shown in Table 2, PVEO exhibited notable inhibitory activity against lipid peroxidation and demonstrated the ability to reduce the stable DPPH radical to DPPH-H, with IC₅₀ values of $143.16 \pm 2.17 \mu\text{g/mL}$ and $258.11 \pm 2.12 \mu\text{g/mL}$, respectively. However, these antioxidant activities were lower than those observed for the positive controls, Butylated hydroxytoluene (BHT) and ascorbic acid, with IC₅₀ values ranging from $26.23 \pm 5.92 \mu\text{g/mL}$ to $234.89 \pm 2.36 \mu\text{g/mL}$, respectively. These results are consistent with previous studies indicating the significant antioxidant potential of PVEO [5,29]

Table 2. Free radical scavenging and antioxidant capacity of PVEO.

EO/Reference	TAC*	β -Carotene Bleaching Assay ($\mu\text{g/mL}$)	DPPH Scavenging Capacity IC_{50} ($\mu\text{g/mL}$)
PVEO	258.98 \pm 9.52	143.16 \pm 2.17	258.11 \pm 2.12
Ascorbic acid (AA)	-	-	234.89 \pm 2.36
Butylated hydroxytoluene (BHT)		26.23 \pm 5.92	

* TAC expressed in μg of ascorbic acid equivalents per milligram of essential oil.

The antioxidant potential of essential oils is often influenced by the interaction of their major components and the synergistic effects between major and minor components [30]. PVEO is rich in bioactive molecules, particularly carvacrol and D-Limonene, which contribute to its antioxidant power [31,32]. These compounds are known to exhibit direct antioxidant activity by scavenging free radicals and converting them into stable compounds. In addition, they can act as indirect antioxidants by boosting overall antioxidant status, essentially through non-enzymatic mechanisms. Antioxidants can also act directly by recycling or regenerating other antioxidants present in the body, such as vitamin E, vitamin C and glutathione. By acting in this way, they help maintain a high antioxidant status and protect cells against oxidative damage [27]. This high antioxidant activity may be indirectly involved in anticancer activity. It neutralizes free radicals, which harm human health and can damage cells and tissues, causing diseases such as cancer. These results prompted us to evaluate the anticancer activity of PVEO against breast cancer cell lines.

Prediction of Organ Toxicity and Toxicity Endpoints In Silico

Table 3 presents an evaluation of the toxicological properties of 23 compounds using the Pro-Tox II system. The compounds are numbered from **1** to **23** and assessed based on several toxicological parameters, including hepatotoxicity, carcinogenicity, cytotoxicity, immunotoxicity, mutagenicity, and predicted LD_{50} (median lethal dose). Additionally, each compound is assigned a probability of active toxicity [17]. Upon analyzing the data, variations in the toxicological properties of the compounds become apparent. Compounds **1**, **2**, **6**, **8**, **13**, **18**, and **22** exhibit relatively high probabilities for hepatotoxicity, indicating a potential for liver toxicity. Carcinogenicity is indicated by compounds **3**, **4**, **7**, **11**, **17**, **21**, and **22**, with moderate to high probabilities. Cytotoxicity is generally high across most compounds, with compounds **3**, **4**, **6**, **7**, **8**, and **18** demonstrating particularly high probabilities. Immunotoxicity is evident in compounds **3**, **6**, **7**, and **13**, with moderate to high probabilities. Mutagenicity is observed in compounds **3**, **6**, **7**, **8**, **11**, **12**, and **13**, each exhibiting varying probabilities. Notably, compounds **3**, **7**, and **12** are marked with an asterisk (*) to indicate active mutagenicity.

Table 3. Toxicological Evaluation of Compounds in PVEO using Pro-Tox II System. (1) α -Thujene, (2) α -Pinene, (3) β -Pinene, (4) β -Myrcene, (5) (+)-2-Carene, (6) β -Cymene, (7) D-Limonene, (8) γ Terpinene, (9) p-Menth-4(8)-en-3-one, (10) p-Menth-1-en-4-ol, (11) p-Menth-1-en-8-ol, (12) Cinnamic aldehyde, (13) 4,4,7a-Trimethyl-2,4,5,6,7,7a-hexahydro-1H-inden-1-one,

(14) Thymol, (15) Carvacrol, (16) Copaene, (17) Caryophyllene, (18) 1,5,9,9-Tetramethyl-1,4,7-cycloundecatriene, (19) Valencen, (20) Germacrene D, (21) α -Murolene, (22) Δ -Cadinene, (23) Eicosanoic acid. Note: Compounds with asterisk indicates an active toxicity

No.	Hepatotoxicity Carcinogenici		Cytotoxicity (Probability)	Immunotoxicity	Mutagenicity	Predicted LD ₅₀ (mg/kg)	Class
1	0.86	0.55	0.98	0.78	0.73	5000	V
2	0.86	0.6	0.99	0.93	0.75	3700	V
3	0.8	0.66	0.97	0.95 *	0.71 *	4700	V
4	0.77	0.6	0.99	0.98	0.75	5000	V
5	0.78	0.71	0.69	0.74	0.81	4800	V
6	0.87	0.67 *	0.98	0.98	0.89	2374	V
7	0.76	0.65	0.95	0.97 *	0.82 *	4400	V
8	0.83	0.6	0.98	0.92	0.82	2500	V
9	0.7	0.82	0.84	0.85	0.99	470	IV
10	0.8	0.72	0.99	0.83	0.88	1016	IV
11	0.72	0.76	0.9	0.64	0.99	2830	V
12	0.7	0.71	0.72 *	0.92	0.98	1850	IV
13	0.68	0.58	0.9	0.98	0.93	5000	V
14	0.75	0.6	0.93	0.99	0.89	640	IV
15	0.75	0.6	0.96	0.99	0.89	810	IV
16	0.97	0.57	0.85	0.78	0.99	5000	V
17	0.8	0.7	0.95	0.54 *	0.75	5300	V
18	0.82	0.76	0.79	0.97	0.87	3650	V
19	0.76	0.66	0.75	0.81	0.85	5000	V
20	0.8	0.73	0.83	0.80 *	0.87	5300	V
21	0.83	0.8	0.76	0.68	0.6	4400	V
22	0.82	0.75	0.69	0.66	0.68	4390	V
23	0.52	0.63	0.74	0.99	1	900	IV

Toxicity class explanation: IV: for substances that are harmful if swallowed (LD₅₀ ranging from 300, and 2000 mg/kg), Class V: for compounds that may be harmful if swallowed (LD₅₀ ranging between 2000, and 5000 mg/kg).

Compounds with higher LD₅₀ values generally indicate lower toxicity. The LD₅₀ values in the table range from 470 mg/kg to 5300 mg/kg. Compounds with LD₅₀ values below 2000 mg/kg are classified as Class IV, indicating harmful substances if swallowed, while compounds with LD₅₀ values between 2000 mg/kg and 5000 mg/kg fall under Class V, suggesting potential harm if swallowed.

Overall, the results indicate that most of the compounds are considered safe based on their toxicological evaluations. However, it is important to acknowledge that certain compounds exhibit potential risks in terms of carcinogenicity, cytotoxicity, immunotoxicity, and mutagenicity. This information is valuable for assessing the potential hazards associated with these compounds and can serve as a foundation for further research and the implementation of appropriate safety measures, particularly in drug development processes.

Genotoxic Effect

Genotoxicity refers to the ability of an agent to damage DNA and cause genetic mutations, which can have implications for health, including cancer development. When a genotoxic agent comes into contact with the cells of an organism, it can cause alterations in the DNA sequence, DNA strand breaks or other genetic damage. This damage can lead to mutations that can have various effects, such as the development of cancers or other genetic diseases [33]. For this reason, it is necessary to test the genotoxicity of each natural product before its pharmaceutical use.

The objective of the current study was to examine the genotoxic impact of the Essential Oil (PVEO) on rat leukocytes. The findings from our study demonstrated that the application of PVEO at concentrations ranging from 5 to 40 $\mu\text{g/mL}$ did not result in any DNA damage (Figure 2). This was evident by the absence of significant changes in the percentage of DNA in the comet tail and the tail moment of DNA, when compared to the negative control. Based on these findings, PVEO can be considered non-genotoxic within the tested concentrations. It is important to emphasize that genotoxicity testing serves as an initial assessment of the safety profile for pharmaceuticals, substances, or nutraceutical [34]. The evaluation of various forms of DNA damage, encompassing single- and double-strand breaks, oxidative damage, as well as DNA-protein interactions, was carried out using the comet assay, also referred to as Single Cell Gel Electrophoresis (SCGE). This assay involved the utilization of agarose microgel electrophoresis. This well-established and precise microscopic technique is utilized for the analysis of selected prokaryotic and eukaryotic cells, both *in vitro* and *in vivo* [35–38]. In our study, we employed the alkaline method of the comet assay, which specifically detects single-strand breaks and alkalisable sites in DNA [34]. Chemical composition analysis of PVEO revealed the presence of major constituents such as carvacrol, D-limonene, beta-cymene, and terpinene. Previous studies have reported that carvacrol, at doses ranging from 81 to 810 mg/kg bw, did not induce *in vivo* genotoxicity or oxidative DNA damage in rat stomach and liver tissues [31]. Similarly, D-limonene, at concentrations below 10,000 μM , did not exhibit genotoxic effects on lymphocytes and V79 cells [39-53].

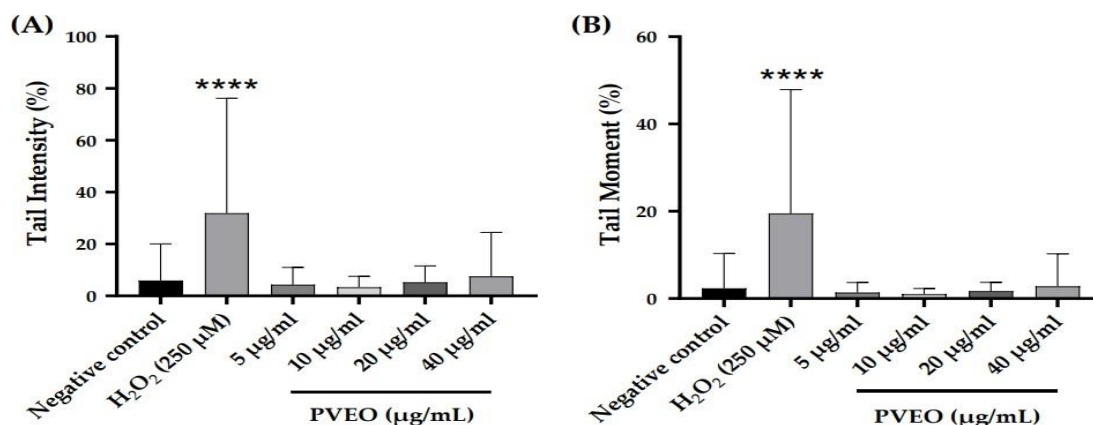


Figure 2. Evaluation of the effect of different concentrations of PVEO on (A) percentage of tail intensity and (B) DNA tail moment in rat leukocytes. The data are presented as mean \pm SEM (50 cells \times 2). **** $p < 0.0001$ compared to the negative control group.

Furthermore, it has been reported that D-limonene, when used at concentrations below 10,000 μM , does not exhibit genotoxic effects on lymphocytes and V79 cells [40]. Additionally, thymol and terpinene, when present at concentrations below 0.1 mM, do not induce an increase in DNA strand breakage. However, at a higher concentration of 0.2 mM, a significant rise in DNA damage is observed. Interestingly, when thymol, terpinene, and carvacrol are present in lower quantities alongside genotoxic agents like 2-amino-3-methylimidazo [4,5-f]-quinoline (IQ) and mitomycin C (MMC), they significantly reduce the DNA strand breaks caused by these agents [41]. Considering these findings, it is plausible to suggest that PVEO may also exhibit antigenotoxic effects at appropriate concentrations.

The Anticancer Effect of PVEO on Breast Cancer Cell Lines

We assessed, for the first time, the anticancer potential of PVEO on two human breast cancer cell lines, namely MCF-7 and MDA-MB-231. The selectivity indexes and IC_{50} levels of PVEO were assessed and are presented in Figure 3 and Table 3. The IC_{50} value represents the concentration of PVEO required to inhibit 50% of the cancer cell growth, with the standard deviation (SD) provided to account for experimental variability (Table 4). In the MCF-7 breast cancer cell line, the IC_{50} value of PVEO was determined to be $78.44 \pm 5.23 \mu\text{g/mL}$, indicating that this concentration was necessary to achieve the desired 50% inhibition of cell growth. Similarly, for the MDA-MB-231 cell line, the IC_{50} value of PVEO was found to be $19.72 \pm 4.81 \mu\text{g/mL}$. These results highlight the potential of PVEO as an anticancer agent, particularly in the context of breast cancer treatment.

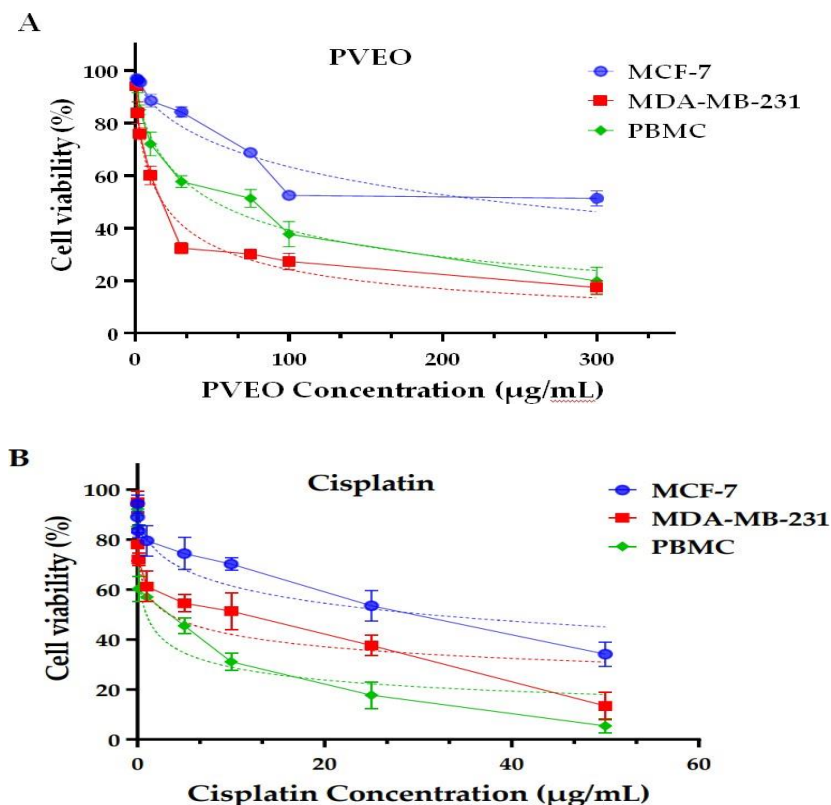


Figure 3. Assessment of Cell Viability in MCF-7, MDA-MB-231, and PBMC Cells Treatment with PVEO (A) and Cisplatin (positive control) (B), Following 72-h Using the MTT Assay.

Treatments	IC50 Value \pm SD ($\mu\text{g/mL}$) *			Selectivity Index (SI) **	
	MDA-MB-231	MCF-7	PBMC	MCF-7	MDA-MB-231
PVEO	19.72 \pm 4.81	78.44 \pm 5.23	311.83 \pm 8.21	3.97	15.8
Cisplatin	14.30 \pm 4.22	4.17 \pm 2.38	31.65 \pm 5.41	7.221	2.21

* The mean values from three independent experiments were used for the calculation, and the results are presented as means with corresponding standard deviations. ** The selectivity index was determined by calculating the ratio of the IC50 values of peripheral blood mononuclear cells (PBMC) to the IC50 values of tumor cells.

To evaluate the selectivity of PVEO towards cancer cells in comparison to non-cancerous cells, the selectivity index (SI) was calculated. The SI is determined by taking the ratio of the IC50 values of peripheral blood mononuclear cells (PBMC), representing non-cancerous cells, to the IC50 values of the tumor cells (MCF-7 and MDA-MB-231). For PVEO, the selectivity index for MCF-7 cells was calculated to be 3.97, indicating that the IC50 value for PBMC was approximately four times higher than that for MCF-7 cells. This suggests that PVEO exhibits some degree of selectivity towards MCF-7 cells. Similarly, for MDA-MB-231 cells, the

selectivity index was determined to be 15.80, indicating a higher selectivity towards this specific breast cancer cell line. In comparison, the widely used anticancer drug cisplatin was included in the analysis for reference. The IC₅₀ values for cis-platin on MCF-7 and MDA-MB-231 cells were 4.17 2.38 µg/mL, and 14.30 4.22 µg/mL, respectively. These results provide insight into the selectivity of PVEO and its potential as an anticancer agent, demonstrating its favorable selectivity towards breast cancer cells compared to non-cancerous cells.

The selectivity indexes for cisplatin were determined to be 7.57 for MCF-7 cells and 2.21 for MDA-MB-231 cells. Overall, these results indicate that it exhibits selectivity towards both MCF-7 and MDA-MB-231 breast cancer cell lines, with higher selectivity observed for MDA-MB-231 cells. This suggests that the essential oil of *P. verticillata* may have a greater affinity for the cellular subtype ER- (estrogen receptor negative) compared to the ER+ (estrogen receptor positive) subtype of breast cancer. The selectivity indexes further demonstrate that PVEO displays relatively higher toxicity towards cancer cells compared to non-cancerous peripheral blood mononuclear cells (PBMCs). These findings emphasize the need for further investigation into the potential therapeutic applications of PVEO as a selective treatment for breast cancer. By exhibiting selectivity towards cancer cells, PVEO holds promise as a potential targeted therapy for breast cancer, particularly in the context of ER- subtypes.

Conclusions

In conclusion, the findings of this study highlight the rich chemical composition of *Ammoides pusilla* (Brot.) Breistr essential oil (PVEO), characterized by the presence of various ter-penes and aromatic compounds including carvacrol, D-Limonene, γ-Terpinene, β-Cymene, and 4,4,7a-Trimethyl-2,4,5,6,7,7a-hexahydro-1H-inden-1-one. Moreover, the essential oil demonstrated strong antioxidant activity. The cytotoxicity experiments demonstrated dose-dependent impacts on both cell lines, where the MDA-MB-231 cell line exhibited heightened sensitivity. Furthermore, the results indicate that the PVEO exhibits selectivity towards both MCF-7 and MDA-MB-231 breast cancer cell lines, with a greater selectivity observed for MDA-MB-231 cells. These findings suggest that the PVEO may possess a stronger affinity for the ER- (estrogen receptor negative) cellular subtype compared to the ER+ (estrogen receptor positive) subtype of breast cancer. Importantly, no genotoxic effects were observed, and there is even evidence to suggest a potential antigenotoxic effect at certain concentrations. Based on these findings, PVEO holds promise as a natural bioactive substance for preventive and therapeutic applications, offering potential benefits without associated toxicity or adverse effects.

References

1. Klaunig, J.E. Oxidative stress and cancer. *Curr. Pharm. Des.* 2018, 24, 4771–4778.
2. Bencheikh, N.; Radi, F.Z.; Fakchich, J.; Elbouzidi, A.; Ouahhoud, S.; Ouasti, M.; Bouhrim, M.; Ouasti, I.; Hano, C.; Elachouri, M. *Ethnobotanical, Phytochemical, Toxicological, and Pharmacological Properties of Ziziphus lotus (L.) Lam.: A*

- Comprehensive Review. *Pharmaceuticals* 2023, 16, 575.
3. Addi, M.; Elbouzidi, A.; Abid, M.; Tungmunnithum, D.; Elamrani, A.; Hano, C. An Overview of Bioactive Flavonoids from Citrus Fruits. *Appl. Sci.* 2022, 12, 29.
 4. Madariaga-Mazón, A.; Hernández-Alvarado, R.B.; Noriega-Colima, K.O.; Osnaya-Hernández, A.; Martínez-Mayorga, K. Toxicity of secondary metabolites. *Phys. Sci. Rev.* 2019, 4, 20180116.
 5. Taibi, M.; Elbouzidi, A.; Ou-Yahia, D.; Dalli, M.; Bellaouchi, R.; Tikent, A.; Roubi, M.; Gseyra, N.; Asehrou, A.; Hano, C.; et al. Assessment of the Antioxidant and Antimicrobial Potential of *Ammoides pusilla* (Brot.) Breistr Duby Essential Oil from Eastern Morocco: An In Vitro and In Silico Analysis. *Antibiotics* 2023, 12, 655.
 6. Bencheikh, N.; Elbouzidi, A.; Kharchoufa, L.; Ouassou, H.; Merrouni, I.A.; Mechchate, H.; Es-Safi, I.; Hano, C.; Addi, M.; Bouhrim, M.; et al. Inventory of medicinal plants used traditionally to manage kidney diseases in north-eastern Morocco: Ethnobotanical fieldwork and pharmacological evidence. *Plants* 2021, 10, 1966.
 7. Pappalardo, H.D.; Toscano, V.; Puglia, G.D.; Genovese, C.; Raccuia, S.A. *Cynara cardunculus* L. as a multipurpose crop for plant secondary metabolites production in marginal stressed lands. *Front. Plant Sci.* 2020, 11, 240
 8. Belkhodja, H.; Belhouala, K.; Nehal, S. Phytochemical Screening and Evaluation of the Antiarthritic Potential of *Ammoides pusilla* Aqueous Extract on Freund's Adjuvant-Induced Rheumatoid Arthritis. *Pharm. Sci.* 2020, 27, 170–182.
 9. Kadda, S.; Belabed, A.; Loukili, E.H.; Hammouti, B.; Fadlaoui, S. Temperature and extraction methods effects on yields, fatty acids, and tocopherols of prickly pear (*Opuntia ficusindica* L.) seed oil of eastern region of Morocco. *Environ. Sci. Pollut. Res.* 2022, 29, 158–166.
 10. Zrouri, H.; Elbouzidi, A.; Bouhrim, M.; Bencheikh, N.; Kharchoufa, L.; Ouahhoud, S.; Ouassou, H.; El Assri, S.; Choukri, M. Phytochemical analysis, antioxidant activity, and nephroprotective effect of the *Raphanus sativus* aqueous extract. *Mediterr. J. Chem.* 2021, 11, 84.
 11. Ouahabi, S.; Loukili, E.H.; Elbouzidi, A.; Taibi, M.; Bouslamti, M.; Nafidi, H.-A.; Salamatullah, A.M.; Saidi, N.; Bellaouchi, R.; Addi, M. Pharmacological Properties of Chemically Characterized Extracts from Mastic Tree: In Vitro and In Silico Assays. *Life* 2023, 13, 1393.
 12. Elbouzidi, A.; Taibi, M.; Ouassou, H.; Ouahhoud, S.; Ou-Yahia, D.; Loukili, E.H.; Aherkou, M.; Mansouri, F.; Bencheikh, N.; Laaraj, S. Exploring the Multi-Faceted Potential of Carob (*Ceratonia siliqua* var. *Rahma*) Leaves from Morocco: A Comprehensive Analysis of Polyphenols Profile, Antimicrobial Activity, Cytotoxicity against Breast Cancer Cell Lines, and Genotoxicity. *Pharmaceuticals* 2023, 16, 840. [CrossRef]
 13. Chaudhary, S.; Chandrashekar, K.S.; Pai, K.S.R.; Setty, M.M.; Devkar, R.A.; Reddy, N.D.; Shoja, M.H. Evaluation of antioxidant and anticancer activity of extract and

- fractions of *Nardostachys jatamansi* DC in breast carcinoma. *BMC Complement. Altern. Med.* 2015, 15, 50.
14. Prieto, P.; Pineda, M.; Aguilar, M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Anal. Biochem.* 1999, 269, 337–341.
 15. Elbouzidi, A.; Ouassou, H.; Aherkou, M.; Kharchoufa, L.; Meskali, N.; Baraich, A.; Mechchate, H.; Bouhrim, M.; Idir, A.; Hano, C.; et al. LC-MS/MS Phytochemical Profiling, Antioxidant Activity, and Cytotoxicity of the Ethanolic Extract of *Atriplex halimus* L. against Breast Cancer Cell Lines: Computational Studies and Experimental Validation. *Pharmaceuticals* 2022, 15, 1156.
 16. Kandsi, F.; Lafdil, F.Z.; Elbouzidi, A.; Bouknana, S.; Miry, A.; Addi, M.; Conte, R.; Hano, C.; Gseyra, N. Evaluation of Acute and Subacute Toxicity and LC-MS/MS Compositional Alkaloid Determination of the Hydroethanolic Extract of *Dysphania ambrosioides* (L.) Mosyakin and Clemants Flowers. *Toxins* 2022, 14, 475.
 17. Banerjee, P.; Eckert, A.O.; Schrey, A.K.; Preissner, R. ProTox-II: A webserver for the prediction of toxicity of chemicals. *Nucleic Acids Res.* 2018, 46, W257–W263.
 18. Ouahhoud, S.; Khoulati, A.; Kadda, S.; Bencheikh, N.; Mamri, S.; Ziani, A.; Baddaoui, S.; Eddabbeh, F.-E.; Lahmass, I.; Benabbes, R.; et al. Antioxidant Activity, Metal Chelating Ability and DNA Protective Effect of the Hydroethanolic Extracts of *Crocus sativus* Stigmas, Tepals and Leaves. *Antioxidants* 2022, 11, 932.
 19. Singh, N.P.; McCoy, M.T.; Tice, R.R.; Schneider, E.L. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 1988, 175, 184–191.
 20. Strubbia, S.; Lyons, B.P.; Lee, R.J. Spatial and temporal variation of three biomarkers in *Mytilus edulis*. *Mar. Pollut. Bull.* 2019, 138, 322–327.
 21. Bartucci, M.; Morelli, C.; Mauro, L.; Ando, S.; Surmacz, E. Differential insulin-like growth factor I receptor signaling and function in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. *Cancer Res.* 2001, 61, 6747–6754.
 22. Jlizi, S.; Lahmar, A.; Zardi-Bergaoui, A.; Ascrizzi, R.; Flamini, G.; Harrath, A.H.; Chekir-Ghedira, L.; Jannet, H. Ben Chemical composition and cytotoxic activity of the fractionated trunk bark essential oil from *tetraclinis articulata* (Vahl) mast. growing in tunisia. *Molecules* 2021, 26, 1110.
 23. Yang, C.; Chen, H.; Chen, H.; Zhong, B.; Luo, X.; Chun, J. Antioxidant and Anticancer Activities of Essential Oil from Gannan Navel Orange Peel. *Molecules* 2017, 22, 1391.
 24. Memar, M.Y.; Raei, P.; Alizadeh, N.; Aghdam, M.A.; Kafil, H.S. Carvacrol and thymol: Strong antimicrobial agents against resistant isolates. *Rev. Med. Microbiol.* 2017, 28, 63–68.
 25. Silva, E.R.; de Carvalho, F.O.; Teixeira, L.G.B.; Santos, N.G.L.; Felipe, F.A.; Santana, H.S.R.; Shanmugam, S.; Quintans Júnior, L.J.; de Souza Araújo, A.A.; Nunes, P.S.

- Pharmacological effects of carvacrol in in vitro studies: A review. *Curr. Pharm. Des.* 2018, 24, 3454–3465.
26. Hayani, M.; Bencheikh, N.; Ailli, A.; Bouhrim, M.; Elbouzidi, A.; Ouassou, H.; Kharchoufa, L.; Baraich, A.; Atbir, A.; Ayyad, F.Z.; et al. Quality Control, Phytochemical Profile, and Antibacterial Effect of *Origanum compactum* Benth. Essential Oil from Morocco. *Int. J. Plant Biol.* 2022, 13, 546–560.
 27. Argane, R.; Benzaazoua, M.; Bouamrane, A.; Hakkou, R. Valorisation des rejets miniers du district Pb-Zn de Touissit-Boubker (région orientale-Maroc). *Environ. Ingénierie Développement* 2014, 66, 38–44.
 28. Tariq, S.; Wani, S.; Rasool, W.; Shafi, K.; Bhat, M.A.; Prabhakar, A.; Shalla, A.H.; Rather, M.A. A comprehensive review of the antibacterial, antifungal and antiviral potential of essential oils and their chemical constituents against drug-resistant microbial pathogens. *Microb. Pathog.* 2019, 134, 103580.
 29. Tomi, P.; Bouyanzer, A.; Hammouti, B.; Desjobert, J.-M.; Costa, J.; Paolini, J. Chemical composition and antioxidant activity of essential oils and solvent extracts of *Ptychotis verticillata* from Morocco. *Food Chem. Toxicol.* 2011, 49, 533–536.
 30. Boulaghmen, F.; Chaouia, C.; Saidi, F. Composition chimique et propriétés antioxydante et antimicrobienne de l'huile essentielle d'*Origanum floribundum* Munby. *Phytothérapie* 2019, 17, 249–258.
 31. Sharifi-Rad, M.; Varoni, E.M.; Iriti, M.; Martorell, M.; Setzer, W.N.; del Mar Contreras, M.; Salehi, B.; Soltani-Nejad, A.; Rajabi, S.; Tajbakhsh, M.; et al. Carvacrol and human health: A comprehensive review. *Phyther. Res.* 2018, 32, 1675–1687.
 32. Shah, B.; Mehta, A. In vitro evaluation of antioxidant activity of D-Limonene. *Asian J. Pharm. Pharmacol.* 2018, 4, 883–887.
 33. Doak, S.H.; Manshian, B.; Jenkins, G.J.S.; Singh, N. In vitro genotoxicity testing strategy for nanomaterials and the adaptation of current OECD guidelines. *Mutat. Res. Toxicol. Environ. Mutagen.* 2012, 745, 104–111.
 34. Wilson, A.; Grabowski, P.; Elloway, J.; Ling, S.; Stott, J.; Doherty, A. Transforming early pharmaceutical assessment of genotoxicity: Applying statistical learning to a high throughput, multi end point in vitro micronucleus assay. *Sci. Rep.* 2021, 11, 2535.
 35. Hosseinzadeh, H.; Sadeghnia, H.R. Effect of safranal, a constituent of *Crocus sativus* (Saffron), on methyl methanesulfonate (MMS)-induced DNA damage in mouse organs: An alkaline single-cell gel electrophoresis (Comet) assay. *DNA Cell Biol.* 2007, 26, 841–846.
 36. Tice, R.R.; Strauss, G.H. The single cell gel electrophoresis/comet assay: A potential tool for detecting radiation-induced DNA damage in humans. *Stem Cells* 1995, 13 (Suppl. 1), 207–214.
 37. Fairbairn, D.W.; Olive, P.L.; O'Neill, K.L. The comet assay: A comprehensive review. *Mutat. Res. Genet. Toxicol.* 1995, 339, 37–59.
 38. Tice, R.R.; Andrews, P.W.; Hirai, O.; Singh, N.P. The single cell gel (SCG) assay: An

- electrophoretic technique for the detection of DNA damage in individual cells. *Biol. React. Intermed. IV Mol. Cell Eff. Their Impact Hum. Health* 1991, 283, 157–164.
39. Pal N, Mandal S, Shiva K, Kumar B. Pharmacognostical, Phytochemical and Pharmacological Evaluation of *Mallotus philippensis*. *Journal of Drug Delivery and Therapeutics*. 2022 Sep 20;12(5):175-81.
 40. Singh A, Mandal S. Ajwain (*Trachyspermum ammi* Linn): A review on Tremendous Herbal Plant with Various Pharmacological Activity. *International Journal of Recent Advances in Multidisciplinary Topics*. 2021 Jun 9;2(6):36-8.
 41. Mandal S, Jaiswal V, Sagar MK, Kumar S. Formulation and evaluation of carica papaya nanoemulsion for treatment of dengue and thrombocytopenia. *Plant Arch*. 2021;21:1345-54.
 42. Mandal S, Shiva K, Kumar KP, Goel S, Patel RK, Sharma S, Chaudhary R, Bhati A, Pal N, Dixit AK. Ocular drug delivery system (ODDS): Exploration the challenges and approaches to improve ODDS. *Journal of Pharmaceutical and Biological Sciences*. 2021 Jul 1;9(2):88-94.
 43. Shiva K, Mandal S, Kumar S. Formulation and evaluation of topical antifungal gel of fluconazole using aloe vera gel. *Int J Sci Res Develop*. 2021;1:187-93.
 44. Ali S, Farooqui NA, Ahmad S, Salman M, Mandal S. *Catharanthus roseus* (sadabahar): a brief study on medicinal plant having different pharmacological activities. *Plant Archives*. 2021;21(2):556-9.
 45. Mandal S, Jaiswal DV, Shiva K. A review on marketed *Carica papaya* leaf extract (CPLE) supplements for the treatment of dengue fever with thrombocytopenia and its drawback. *International Journal of Pharmaceutical Research*. 2020 Jul;12(3).
 46. Mandal S, Vishvakarma P, Verma M, Alam MS, Agrawal A, Mishra A. *Solanum Nigrum* Linn: An Analysis Of The Medicinal Properties Of The Plant. *Journal of Pharmaceutical Negative Results*. 2023 Jan 1:1595-600.
 47. Vishvakarma P, Mandal S, Pandey J, Bhatt AK, Banerjee VB, Gupta JK. An Analysis Of The Most Recent Trends In Flavoring Herbal Medicines In Today's Market. *Journal of Pharmaceutical Negative Results*. 2022 Dec 31:9189-98.
 48. Mandal S, Vishvakarma P, Mandal S. Future Aspects And Applications Of Nanoemulgel Formulation For Topical Lipophilic Drug Delivery. *European Journal of Molecular & Clinical Medicine*.;10(01):2023.
 49. Chawla A, Mandal S, Vishvakarma P, Nile NP, Lokhande VN, Kakad VK, Chawla A. Ultra-Performance Liquid Chromatography (Uplc).
 50. Mandal S, Raju D, Namdeo P, Patel A, Bhatt AK, Gupta JK, Haneef M, Vishvakarma P, Sharma UK. Development, characterization, and evaluation of *rosa alba* l extract-loaded phytosomes.
 51. Mandal S, Goel S, Saxena M, Gupta P, Kumari J, Kumar P, Kumar M, Kumar R, Shiva K. Screening of *catharanthus roseus* stem extract for anti-ulcer potential in wistar rat.

- 52.** Shiva K, Kaushik A, Irshad M, Sharma G, Mandal S. Evaluation and preparation: herbal gel containing thuja occidentalis and curcuma longa extracts.