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ORIGINAL ARTICLE / ÖZGÜN MAKALE

GC-MS ANALYSIS OF ESSENTIAL OIL AND ANTICANCER ACTIVITIES OF EXTRACTS FROM DISCARDED LEAVES OF *NICOTIANA TABACUM* LINN.

KULLANILMAYAN NICOTIANA TABACUM LINN. YAPRAKLARINDAN ELDE EDİLEN UÇUCU YAĞIN GC-MS ANALİZİ VE EKSTRENİN ANTİKANSER AKTİVİTESİ

Samia HAJJAR[1,2], Ali JABER[1]*, Milad EL RIACHI[3], Fadi ABDEL SATER[2], Edmond CHEBLE[1]

[1]Laboratoire de Recherche et Développement des Médicaments et des Produits Naturels RDMPN, Faculty of Pharmacy, Lebanese University, 1500, Beirut, Lebanon.

[2]Laboratory of Cancer Biology and Molecular Immunology, Lebanese University, Faculty of Sciences, 1500, Hadath-Beirut, Lebanon.

[3]Department of olive and olive oil, Lebanese Agriculture Research Institute, 18021, Tal Amara, Lebanon.

ABSTRACT

Objective: *Cancer is still one of the most fatal diseases that threaten human health. Since the effectiveness of conventional cancer therapies based on chemotherapy, radiotherapy, cytotoxic drugs, and surgery is limited by their toxic effects, new therapies are needed. Secondary metabolites from plants exhibit good biological activities. The effect of solvent polarity and method of extraction on the yield of extraction, and the effect of the solvent extract on the biological activities of Nicotiana tabacum Linn. leaves extracts were evaluated. The essential oil extract of the leaves will be analyzed as well.*

Material and Method: *Samples of tobacco leaves, grown in Lebanon, were the subject of this work. Two methods of extraction in a series of solvents with decreasing polarities were performed. The antitumor activity was evaluated on breast adenocarcinoma MCF7 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. The chemical composition analysis was conducted using TLC and GC-MS for essential oil.*

Result and Discussion: *The Nicotiana tabacum extracts exhibit anticancer activities, and it is affected by the solvent used. Moreover, the polarity and method of extraction significantly affect the yield of extraction.*

* **Corresponding Author / Sorumlu Yazar:** Ali Jaber
e-mail / e-posta: ali.jaber.2@ul.edu.lb, **Phone / Tel.:** +9613451884

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These results required further investigations on this plant and must be tested on other cancer cell lines. The TLC analysis of different extracts lead us to suggest the presence of α-4,8,13-duvatriene-1,3-diol, β -4,8,13-duvatriene-1,3-diol and Z-abienol (Z-AB). Totally 19 volatile constituents were detected in N. tabacum and the main components were pentadecanal (47.71 %), biosol (15.88 %), solanone (11.06 %), thymol (5.52 %), damascenone (2.16 %), and β-Caryophyllene (2.19 %). As well as that many studies are required to determine the active principles and their mode of action.

Keywords*:* *Antitumor, essential oil, GC-MS, Nicotiana tabacum, solvent polarity*

ÖZ

Amaç: *Kanser, günümüzde hala insan hayatını tehdit eden en ölümcül hastalıklardan biridir. Kemoterapi, radyoterapi, sitotoksik ilaçlar ve cerrahiye dayalı geleneksel kanser tedavilerinin etkinliği, toksik etkilerden dolayı sınırlı olduğundan, yeni tedavilere ihtiyaç duyulmaktadır. Bitkilerden elde edilen sekonder metabolitler, iyi biyolojik aktivite göstermektedir. Çözücü polaritesi ve ekstraksiyon yönteminin, ekstraksiyonun verimi üzerine etkisi ve ekstraksiyonun Nicotiana tabacum Linn. ekstresinin biyolojik aktivitesi üzerine etkisi değerlendirilmiştir. Bunun yanında, yapraklardan elde edilen uçucu yağın analizi de gerçekleştirilmiştir.*

Gereç ve Yöntem: *Çalışmanın konusu, Lübnan'da yetişen tütün yapraklarından alınan örneklerdir. Azalan polaritede çözücü serisi ile iki ekstraksiyon yöntemi kullanılmıştır. Antitümör aktivite; meme adenokarsinoma MCF7 hücrelerinde, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) sitotoksisite testi ile değerlendirilmiştir. Ekstrenin kimyasal içeriği İTK ile, uçucu yağınki ise GC-MS ile değerlendirilmiştir.*

Sonuç ve Tartışma: *Nicotiana tabacum ekstreleri antikanser aktivite göstermiş ve çözücü türünün bu aktiviteyi etkilediği gözlenmiştir. Ayrıca, polarite ve ekstraksiyon yöntemi, ekstraksiyon verimini önemli ölçüde etkilemiştir. Bu sonuçlar doğrultusunda, bitki üzerine daha ileri çalışmalar yapılması ve başka kanser hücre serileri üzerinde testlerin yürütülmesi gerekmektedir. Farklı ekstrelerin İTK analizleri; α-4,8,13-duvatrien-1,3-diol, β -4,8,13-duvatrien-1,3-diol ve Z-abienol (Z-AB) bileşiklerinin varlığını göstermiştir. N. tabacum içeriğinde 19 uçucu bileşik tespit edilmiştir ve bunların arasında pentadekanal (47.71 %), biosol (15.88 %), solanon (11.06 %), timol (5.52 %), damasenone (2.16 %), and β-karyofilen (2.19 %) bileşikleri major olarak bulunmaktaır. Bunun yanı sıra aktif bileşiklerin ve etki biçimlerinin belirlenmesi için çok sayıda çalışmaya ihtiyaç vardır.*

Anahtar Kelimeler: *Antitümör, çözücü polaritesi, GC-MS, Nicotiana tabacum, uçucu yağ*

INTRODUCTION

Cancer has become one of the major diseases and problems that have caused predominant death, and it is considered the second cause of death after cardiovascular diseases. This disease is characterized by an uncontrolled multiplication of cells leading to the formation of malignant tumors, with the ability to be metastatic [1-3]. The typical cancer treatment is generally based on using chemotherapy, radiotherapy, cytotoxic drugs, and surgery [4, 5]. These conventional therapies are effective and can even cure many types of cancers including breast cancer, colon, pancreatic, testicular, ovarian, and certain lung cancers, but their effectiveness is often limited by toxic effects [6]. These toxic effects occur when healthy cells are damaged. Among these side effects, fatigue, anemia, bleeding, muscle pain, appetite loss, diarrhea and vomiting, sore throat, constipation, damage to the nervous system, memory and concentration problems, insomnia, and hair loss can be mentioned [4, 7].

Since the current treatments usually have side effects, continued searching for a safer and more effective treatment is needed [8]. For many years' herbal medicines have been used and are still used in developing countries as the primary source of medical treatment [1, 8]. Researchers found that plants

are a great source for developing and producing new, effective, and safe anticancer drugs since it has been shown that they can prevent or reduce the incidence of cancer [9].

As part of the evaluation of Mediterranean endemic species, many studies were interested in the Lebanese *Nicotiana tabacum* (*N. tabacum*), commonly known as tobacco. *N. tabacum* is a perennial herbaceous plant, native to tropical and subtropical America and is now cultivated commercially worldwide [10, 11]. In Lebanon, Tobacco is a very important economic crop; moreover, 25,000 families benefit from the production of tobacco [10, 12]. Over 20 % of tobacco resources are discarded as processing waste [13] because only leaves are used in tobacco industries. The discarded tobacco leaves are scientifically valuable because of their content in bioactive compounds, such as polyphenols, flavonoids, proteins, and aromatic compounds [10, 14, 15]. Many studies have shown that constituents of *N. tabacum* have potential biological activities [16]. For instance, Wang et al. [15] identified the polyphenols in tobacco leaves and investigated their antimicrobial and antioxidant activities. The extraction of phytochemicals from the plant material is influenced by various factors including time, temperature, solvent concentration, and solvent polarity [17]. Moreover, many studies have shown that the biological activities of extracts obtained from plants are affected by solvent polarity [17-19].

The purpose of the recent study was to assess the anticancer activity of the total extracts from the waste of *N. tabacum* growing in Lebanon. The original Saada Six was the class of tobacco studied. It was named by the Regie's Saadiyat Laboratory, developed with a blend of Bulgarian and Azmirly tobacco, planting commenced in 1973 [10, 12]. In addition, the authors investigate whether the anticancer activity is affected by the nature of the solvent and the extraction method used. Extractions were performed using different solvents with different polarities, then the cytotoxic effect of extracts against MCF7 cells, an epithelial human breast cancer cell line was assayed using MTT assay. Furthermore, the components of essential oils from *N. tabacum* were analyzed by GC/MS.

MATERIAL AND METHOD

Materials and Reagents

Methanol (MeOH), ethyl acetate (EtOAc), petroleum ether (PE), dichloromethane (DCM), diethyl ether (Et_2O), hexane, Isopropanol, chloroform, and 3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich (USA). GC-grade *n*-hexane and analytical reagent grade anhydrous sodium sulfate (Na_2SO_4) were used. Samples were weighed using an analytical and numerical balance (Melter Toledo). The dried leaves were grinded using a POLYMIX (PX-MFC 90 D) grind mill. The extracts were concentrated using HEIDOLPH (Germany) rotavapor apparatus. Silica gel 60G F254 Thin Layer Chromatography plates were also purchased from Merck Co., Germany.

Plant Material

The *N. tabacum* plants were grown at the same periods, and harvested in Aitaroun (june 2019), southern Lebanon (33°07′N 35°28′E). The plant material was identified by Prof. Jean HABIB (Professor of Pharmacognosy at the Lebanese University). A voucher specimen (No. 1901) has been deposited at the Pharmacognosy Department, Faculty of Pharmacy of the Lebanese University. After separating the usable leaves to make smoking products (picked by hand at maturity), the remaining waste leaves are dried at room temperature for two weeks and then ground in a laboratory mill. The powders were stored at room temperature in well-closed bags until processing.

Extraction Procedures

The fresh leaves of *N. tabacum* were dried at room temperature and reduced to powder using a mill. To obtain the constituents of *N. tabacum*, a series of separately solid-liquid extractions using six solvents with different polarities (methanol 80 %, methanol, ethyl acetate, dichloromethane, diethyl ether, petroleum ether) were executed.

Two methods of extraction were applied. Briefly, in the first procedure, 10 g of leaves powder and 50 ml of each solvent were put in Erlenmeyer flasks (100 ml) and placed in an ultrasonic cleaning bath (VWR ULTRASONIC CLEANER) operating at a frequency of 35 kHz. Sonication was performed for 60 min. Bath temperature was monitored, and water is replaced almost every 10 min to maintain room temperature ± 2 ºC. In parallel, the second procedure was performed by mixing 10 g of plant material and 50 ml of each solvent in Erlenmeyer flasks (100 ml) and macerated using magnetic stirring for 24 hours.

At the end of each extraction procedure, the liquid extract was separated from the solid residue by filtration using Whatman paper. The solid residue was washed using 5 ml of fresh solvent used during extraction. The filtrates were collected the solvent was evaporated in a rotary vacuum evaporator at 40 °C. The resulting *N. tabacum* extracts (NTEs) were stored at 4 °C until use. Each extraction was carried out in triplicate.

Essential Oil's (EO) Content

Foremost, 100 g of the same batch of *N. tabacum* drying leaves were chopped into small pieces then carefully introduced into a 1000 ml round bottom flask containing 500 ml of distilled water. The hydro-distillation was carried out in a Clevenger-type distillation unit designed according to the British Pharmacopoeia specification [20]. After 3 hours of distillation, the EO was collected, and the oils were sealed and kept in dark glass vials in the refrigerator at 4º C for further analysis.

Determination of Percentage Yield (%)

The extraction yield was calculated according to the following equation (1):

$$Yield\ \% = \frac{W_2}{W_1} x\ 100 \quad \textbf{(Equation 1)}$$

Where W_1 is the dry weight of the used material and W_2 is the weight of collected extract after evaporation of the solvent.

Thin-Layer Chromatography

To compare qualitatively the constituents of different NTEs, several TLC (thin-layer chromatography) were applied using different mobile phases. TLC is performed on a sheet of aluminum foil coated with a thin layer of adsorbent silica gel, which is commercially available 60 F_{254}.

The different mobile phases that were used are: Hexane (100 %), Hexane/EtOAc (75/25 %), Hexane/EtOAc (50/50 %), EtOAc (100%), and isopropanol- chloroform -DCM- hexane (7/8/6/79 %). The crude samples of NTE were spotted onto the TLC plate as a single spot using capillary tubes. After spots drying, the plates were developed in a closed, filter paper-lined, saturated chamber, and the solvent front was allowed to ascend a minimum of 10 cm. The spots were visualized either under UV light and /or by heating plates sprayed with vanillin/sulfuric acid reagent. The retention factor of the compound is calculated as the following equation (2):

$$R_f = \frac{distance\ traveled\ by\ the\ compound}{distance\ traveled\ by\ the\ solvent} \quad \textbf{(Equation 2)}$$

Determination of Antitumor Activity

Cell Viability

The viability of the MCF7 cells obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) was assessed by MTT assay. MTT is reduced intracellularly in a mitochondrion-dependent reaction to yield insoluble formazan crystals. The ability of cells to reduce MTT indicates mitochondrial activity and serves as a measure of cell viability. Briefly, MCF7 cells, an epithelial human breast cancer cell line, were seeded in 96-well plates (10^4 cells/well). The following day, cells were treated with the different NTEs at concentrations ranging from 50 to 300 $\mu g.ml^{-1}$, and from 0.25 % to 1 % for methanolic extracts, for 48h. After 48 h,10 µL of MTT solution was added to each well of each plate. After 3 h of incubation at 37 °C, formazan crystals were solubilized with 100 µL of acidified isobutanol. The absorbance was measured spectrophotometrically with an ELISA microplate reader (ELISA reader/Biotech) at 595 nm wavelengths. The number of viable cells was directly correlated to the number of purple formazan crystals formed.

Analyses of Volatile Organic Compounds

One microliter of *N. tabacum* EO sample was diluted (1:100) with hexane and injected into the Gas chromatography-mass spectrometry (GC-MS) system. GC SHIMADZU QP2010 system was used to analyze the volatile compounds in the N. tabacum extract (without derivatization). DB-5MS (5 % Diphenyl / 95 % Dimethylpolysiloxane) capillary column having (30 m length, 0.25 i.d., film thickness 0.28 μm) and helium as carrier gas (1 ml/min, constant flow) was used for compound separation. The oven temperature was programmed from 65 ºC (2 min initial time) increased to 300 ºC at 10 ºC/min (isothermal for the final time). The actual temperature in the MS source reached 230 ºC, and the MS was operated in the electron impact mode at 70 eV ion source energy. The injector temperature was 250°C, while the injection volume was 1 μL and a total run of one hour is performed, mass detector scan range m/z = 50-550. Data receipt and processing were performed using Shimadzu GC-MS solution software. The detected compounds were tentatively identified, by MS spectral correlations using NIST08 (National Institute of Standards and Technologies, Mass Spectra Libraries), as well as published data.

Statistical Analysis

The analysis of data was carried out using GraphPad Prism version 5 for Windows (GraphPad Software, La Jolla California USA). Two-way analysis of variance (ANOVA) was used to determine if the extraction method and the solvent identity influence the extraction yield. Differences between the means of each condition and the control were explored using Paired t-test (Viability test). One-way ANOVA test followed by Dunnett's post- hoc test was used to determine the differences between means to study the effect of solvent polarity and method used on the extraction yield of extracts. Data are presented as means ± SEM. A probability value of less than 0.05 was regarded as statistically significant.

RESULT AND DISCUSSION

Extraction Yield

Figure 1 shows the effect of the polarity of the solvent and method of extraction on the yield of extraction. Values ranged from 1.3% for the NTE obtained using petroleum ether as a solvent to 21.7% for the extract obtained using methanol as a solvent, and maceration as the method of extraction. The yield of extraction obtained using methanol 80% as the extraction solvent and maceration as the method of extraction presents the highest yield.

Studies have shown that the quantity and quality of the yield of extraction are affected by the polarity, the solvent extract, and the method of extraction [17, 21]. In recent study, two methods of extraction in a series of solvents with decreasing polarities (MeOH 80%, MeOH, EtOAc, DCM, Et_2O,

PE) were conducted. One-way analysis of variance (ANOVA) of the results in this study had shown that the solvent identity, in other words, the polarity of the solvent, significantly affects the yield of extraction ($p= 0.0001<0.005$). Moreover, the extraction yield is also affected by the polarity of solvents, showing the highest yield to the lowest yield as per the following manner: MeOH 80%> MeOH> EtOAc> DCM> Et_2O> PE. This agrees with previous studies that also had shown that extraction in highly polar solvents resulted in a high extract yield compared to non-polar ones [17, 22]. Additionally, two-way *ANOVA* showed that the nature of the solvent and the method of extraction independently significantly affect the yield of extraction ($p= 0.0001<0.005$), also they interact and significantly affect the yield ($p<0.005$).

Furthermore, it showed that the solvent identity affects the yield more than the method of extraction. Therefore, this difference in the percentages of yield between the extracts is due to the polarity of the solvent and the method of extraction.

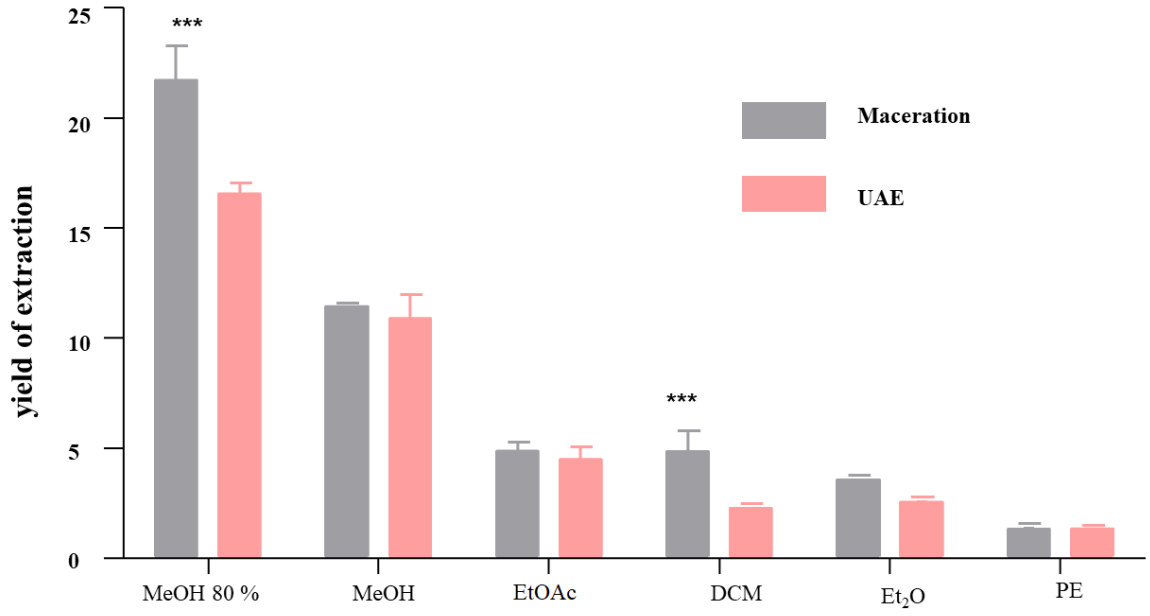


Figure 1. Effect of solvent polarity and method of extraction on the extraction yield of NTEs

Bars represent means ± SEM. The significant difference between means was represented by ***P value ≤0.0001. (n=3).

Thin-Layer Chromatography

In order to compare the chemicals contained in the NTEs obtained, a thin-layer chromatography analysis was executed. Briefly, appropriate volumes of extracts were applied on Silica G TLC plates using capillaries, then these plates were wetted in five different mobile phases systems. The four systems of mobile phases composed by a combination of hexane and ethyl acetate (0-25 %-50 %-75 % hexane) present similar results, represented by the TLC in Figure 2. TLC shows that extracts obtained using all

solvents in both methods of extraction except the petroleum ether (E, F) present similar compounds. Moreover, after migration, the TLC plates were sprayed with vanillin/sulfuric acid reagent, which transforms the color of the highest spot present in all solvents, except the petroleum ether, to blue. This colored transformation based on previous studies [23] indicates the presence of terpenoids or steroids. Additionally, it completely agrees with several studies that have demonstrated the presence of terpenoids and steroids in the extracts from *N. tabacum* leaves [24, 25]. It is worth noting that all the spots that appeared under 254 nm also came out under 366 nm, but under the latter wavelength additional spots were detected, specifically those that didn't migrate too much and remained near the spotting point.



Figure 2. TLC plate under UV 366 nm, using Hexane/ EtOAc as eluent

A: UAE, MeOH; B: maceration, MeOH; C: UAE, EtOAc; D: maceration, EtOAc; E: UAE, PE; F: maceration, PE; G: UAE, MeOH 80%; H: maceration, MeOH 80%; I: UAE, DCM; J: maceration, DCM; K: UAE, Et_2O; L: maceration, Et_2O.

Figure 3 shows the TLC plate observed under UV at 366 nm using a mobile phase composed of a combination of isopropanol-chloroform-dichloromethane-hexane (7:8:6:79). This new mobile phase has allowed the separation of more compounds compared to the first four ones. In comparison with a previous study to detect and quantify components in the leaf of *N. tabacum* [26], this study identify the presence of sucrose esters (SE) at R_f = 0.05 in extracts obtained using UAE methanolic extract and those obtained using EtOAc, PE, DCM, and Et_2O in both methods of extractions. Also, by comparison of the R_fs obtained in the literature using the same mobile phase system, the authors suggest the presence of α-4,8,13-duvatriene-1,3-diol (ADVT) at R_f = 0.22 in extracts obtained using EtOAc, DCM, and Et_2O. β -4,8,13-duvatriene-1,3-diol (BDVT) was identified at R_f = 0.25 in the methanolic extracts (MeOH, MeOH 80 %), and Z-abienol (Z-AB) at R_f = 0.63 in all extracts except extracts obtained using MeOH. This variety in compounds is due to the difference in polarity between solvents, and it will cause a difference in the biological activities of extracts.

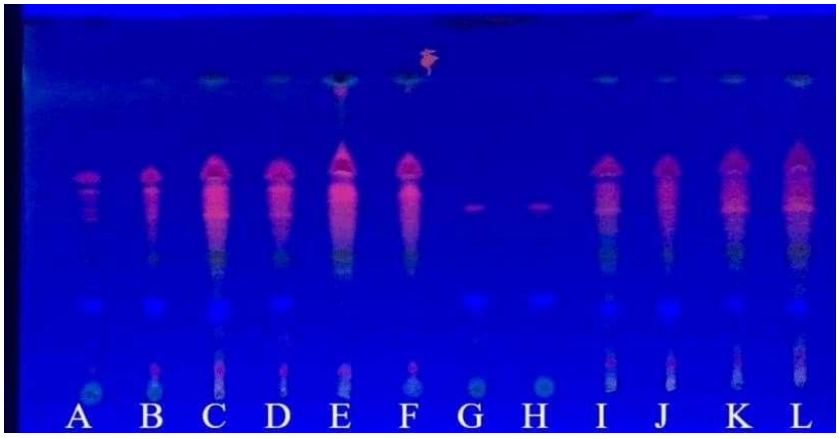


Figure 3. TLC plate under UV 366 nm, using isopropanol/chloroform/DCM/hexane as eluent

A: UAE MeOH; B: maceration MeOH; C: UAE EtOAc; D: maceration EtOAc; E: UAE PE; F: maceration PE; G: UAE MeOH 80%; H: maceration MeOH 80%; I: UAE DCM; J: maceration DCM; K: UAE Et_2O; L: maceration Et_2O.

Determination of Antitumor Activity

The effects of various NTEs were evaluated on breast adenocarcinoma MCF7 cells using MTT cytotoxicity assay. This is a colorimetric assay in which the yellow substrate MTT is reduced to purple formazan crystal produced only by succinate dehydrogenase enzymes in viable cells, that are metabolically active [10]. The amount of the formed formazan is proportional to the concentration of viable cells in the sample. MCF7 cancer cells were treated with different concentrations (50-300 $\mu g.ml^{-1}$) of various extracts and (0.25 %-1 %) of methanolic extracts for 48 h. The extract obtained using EtOAc and maceration as a method of extraction exerted no significant effect on cell viability in all tested conditions (Figure 4 A). All others extract exerted a significant effect on cell viability in all tested conditions (Figure 4). In the case of methanolic extracts (Figure 4-E and F), IC50 cannot be determined in this study because for all concentrations tested, the percentage of viability is less than 50. To determine the IC50 for the methanolic extracts, a smaller range of concentrations must be tested. In the case of extracts with DCM using UAE as the method of extraction (Figure 4 C), Et_2O using maceration as a method of extraction (Figure 4 D), and EtOAc using UAE as a method of extraction (Figure 4-B) IC50 are respectively 157,6 $\mu g.ml^{-1}$, 246.7 $\mu g.ml^{-1}$ and 238.9 $\mu g.ml^{-1}$. Based on the IC50s, extract obtained with DCM is more potent and caused more inhibition of cell viability than extracts obtained using Et_2O and EtOAc. The extracts that exhibit the higher potent inhibitory activity are the methanolic extracts (Figure 4-E and F) which are obtained using the most polar solvents. These findings are in agreement with previous studies, where flavonoid of tobacco leaves cultivated in Indonesia with concentrations of 160 $\mu g.ml^{-1}$ show a decrease of the MCF-7 cell viability of more than 50 %, with an IC50 value of 148.41 $\mu g.ml^{-1}$ [27]. Other studies, covering isolated compounds from *N. tabacum*, found weak to moderate inhibitory activities against MCF7 cell lines ranging from 6.2 to 44 μM [14, 28-31].

On the other hand, the nature of used solvent significantly affects the antitumor activity of extracts, which agrees with previous studies that have shown that the solvent affects the biological activities of extracts [16–19]. To see if more polar solvents exhibit better biological activities, more studies are needed, since DCM and Et_2O are less polar than EtOAc and exhibit better antitumor activity. Additionally, the best results (Figure 4) are promoted by the most polar solvents: MeOH, and MeOH 80 %.

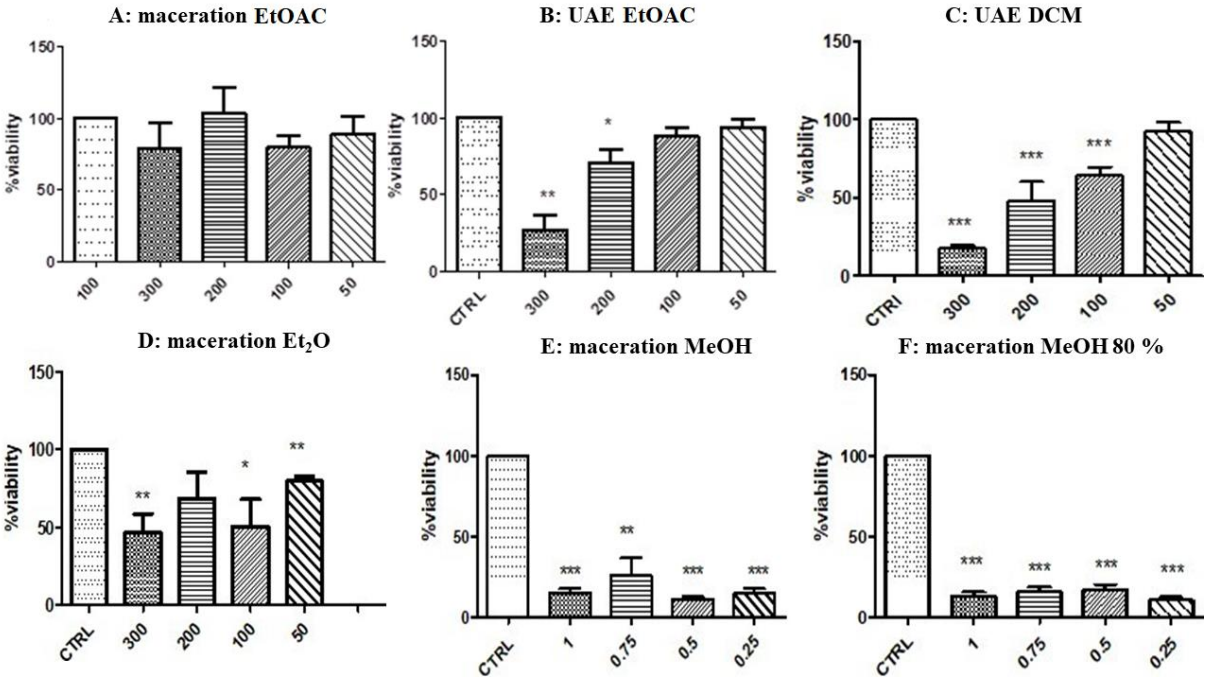


Figure 4. Effect of NTEs on MCF7 cell proliferation

Bar values are reported as mean +/- SEM of at least three independent experiments. Significant differences in expression are indicated by *(P<0.05), ** (P<0.01), and *** (P<0.001) as compared to control (untreated cells).

On the other hand, the essential oil from leaves of *N. tabacum* (EONts) was analyzed by GC–MS (Figure 5) to detect volatile, small, and non-thermolabile metabolites. The composition of EONt was determined and the % area of individual components is given in Table 1. Results showed the detection of 19 constituents from *N. tabacum* constituting 89.78 % of the total content. The main constituents (over 2 %) were pentadecanal (47.71 %), biosol (15.88 %), solanone (11.06 %), thymol (5.52 %), damascenone (2.16 %), and β-Caryophyllene (2.19 %).

The analysis of the available data about EONts compositions revealed that some compounds were found for the first time in EONt. While no precedent studies mention the presence of pentadecanal in EONts it has been shown that the leaf EO of Solanum macranthum, a plant from the same family (solanaceae) of *N. tabacum*, contains pentadecanal 28.1 % [32]. (E)-β-farnesene, carophylline and α-

terpineol were also detected between the volatile compounds from flowers of *N. tabacum* [33]. Moreover, damascenone, a carotenoid derived compound, is described as a powerful fruity-floral odor complex, was found in the work of Popova et al. [34]. In another paper, Popova et al. indicate the presence of solanone (0.48 %) in *N. tabacum* flue-cured Virginia type, β-caryophyllene (0.48 %) and β-farnesene (0.08 %) [35]. In the EO of the variety "Plovdiv 7" of Bulgarian oriental tobacco, nineteen volatile components were identified (92.3%), where (E)-phytol (53.4%) was the major compound followed by solanone (6.8%), β-damascenone (3.7 %) and β-caryophyllene (0.2 %) were also present [36].

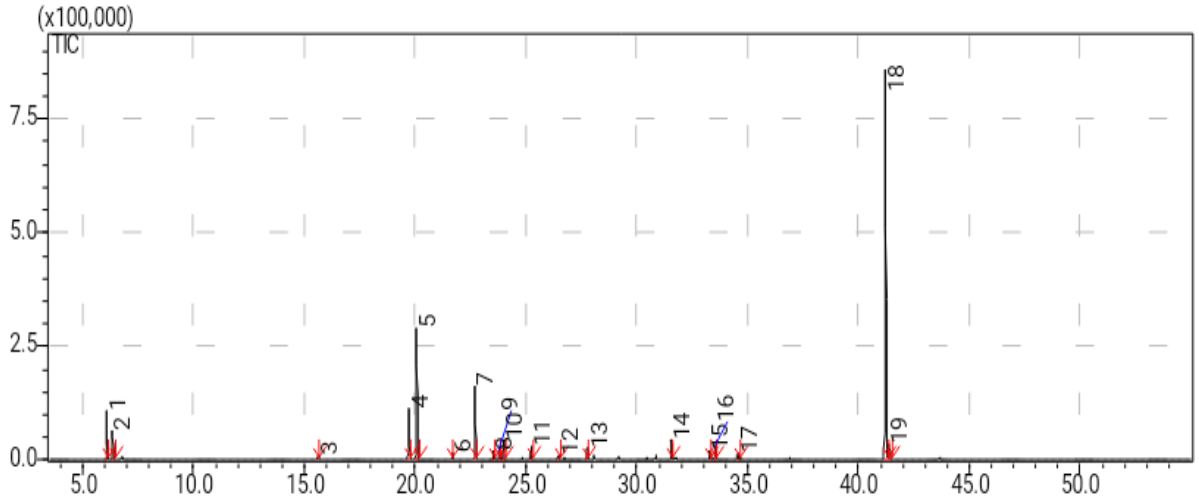


Figure 5. GC–MS chromatogram of essential oil from leaves of *N. tabacum*

Table 1. Chemical composition of the essential oils of *N. tabacum*

Compound	RT	Formula	% Area	Similarity (%)
α-Terpineol	15.633	$C_{10}H_{18}O$	0.7	59
Thymol	19.717	$C_{10}H_{14}O$	5.52	90
Biosol	20.067	$C_{10}H_{14}O$	15.88	80
Solanone	22.696	$C_{13}H_{22}O$	11.06	92
Damascenone	23.533	$C_{13}H_{18}O$	2.16	69
(E)-β-Farnesene	23.8	$C_{15}H_{24}$	0.97	93
β-Caryophyllene	25.233	$C_{15}H_{24}$	2.19	93
Trifluoroacetyl-lavendulol	26.45	$C_{12}H_{17}F_3O_2$	1.5	69
Germacrene -d	27.775	$C_{15}H_{24}$	0.5	93
But-2-enoic anhydride	28.067	$C_8H_{10}O_3$	0.79	87
Pentadecanal	41.083	$C_{15}H_{30}O$	47.71	68
Cyclodecanone	41.367	$C_{10}H_{18}O$	0.8	83

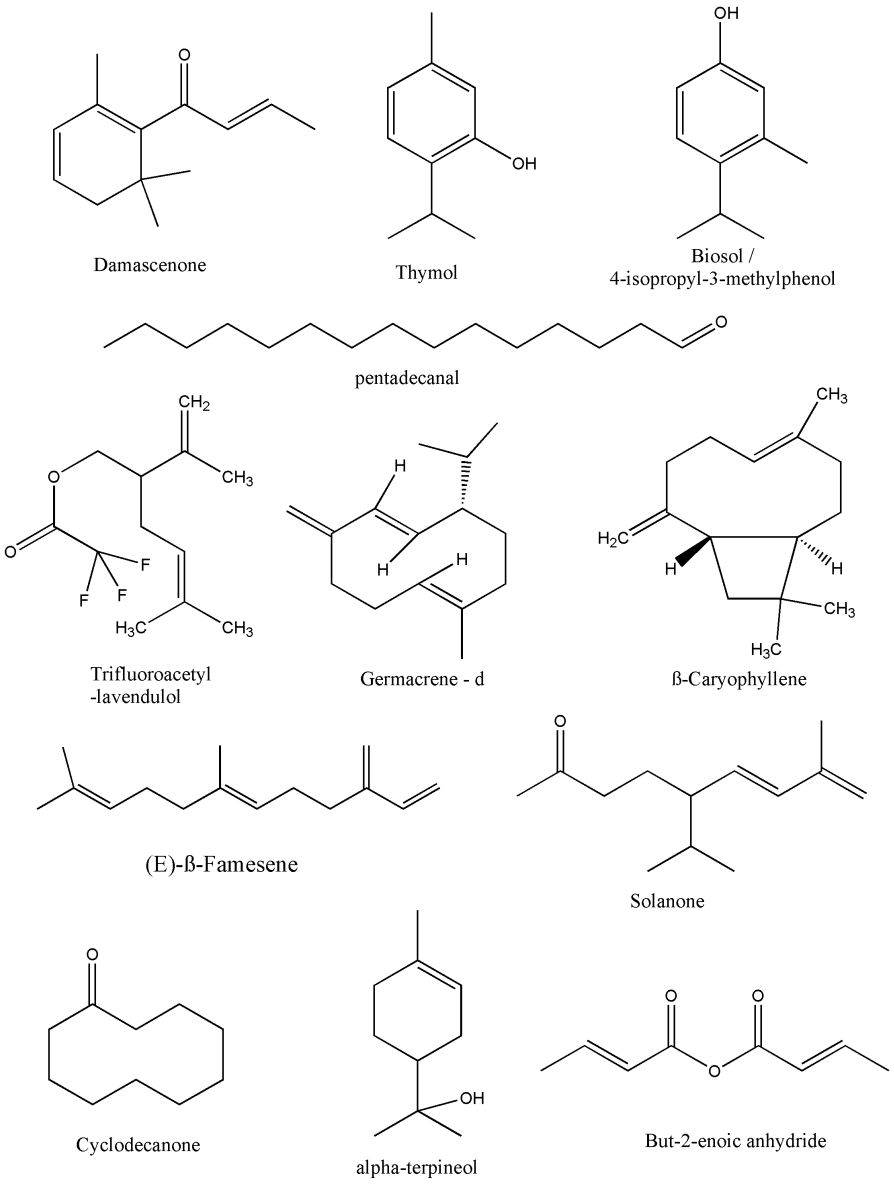


Figure 6. Chemical structures of compounds found in *N. tabacum* EO's

Among these, many compounds are biologically active compounds and others have aroma characteristics. Solanone, Trifluoroacetyl-lavenduliol and damascenone are the major contributor of *N. tabacum* aroma. Thymol, (E)-β-Famesene, own antimicrobial activity [38]. While the sesquiterpene "germacrene-d" is known as an antiproliferative agent against leukemia and melanoma [39]. Another sesquiterpene "β-Caryophyllene" has anticancer activity [40].

Differences in bioactivity and essential oils content from previous studies on tobacco from different varieties and origins [34, 41-44] were probably due to the genetic factor (variety) as well as to abiotic factors influencing plant development and metabolism and the uniqueness of SAADA 6. Since tobacco plants are thermophilic crop plants originating from tropical regions they are sensitive to the change of temperature [45].

According to the present study, results have demonstrated that discarded leaves from *N. tabacum* present anticancer activities and those activities are affected by the solvent used. The anticancer activities are attributed to the bioactive compounds present in *N. tabacum* leaves. The observed variation between extracts is due to the quantity and variety of compounds that can each solvent extract. All these results suggest that *N. tabacum* leaves can be a rich source of bioactive compounds that exhibit antitumor activities. Further studies are required to identify the active compounds and their mechanism of action. TLC and GC-MS analysis are conducted to identify the content of extract and some compounds have been identified by comparison to the literature and library. Also, these extracts should be tested on other cancer cell lines and normal cell lines. Furthermore, *in vivo* evidence for their biological activities is needed. Therefore, more research should be conducted to determine the potential utility of recycling waste tobacco. So, the authors hope to transform the tobacco plant from the source of cancer to a source of anticancer drugs.

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AUTHOR CONTRIBUTIONS

Concept: *A.J., F.A.S., E.C.*; Design: *A.J., F.A.S., E.C.*; Control: *A.J., M.E.R., F.A.S., E.C.*; Materials: *S.H., A.J., M.E.R., F.A.S., E.C.*; Data Collection and/or processing: *S.H., A.J.;* Analysis and/or interpretation: *S.H., A.J., M.E.R., F.A.S., E.C.*; Literature review: *S.H., A.J.;* Manuscript writing: *S.H., A.J.*; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Wang, H., Khor, T.O., Shu, L., Su, Z., Fuentes, F., Lee, J-H., Kong, A-N.T. (2012). Plants against cancer: A review on natural phytochemicals in preventing and treating cancers and their druggability. *Anti-Cancer Agents in Medicinal Chemistry, 12*, 1281-1305. [CrossRef]

2. Ochwang'i, D.O., Kimwele, C.N., Oduma, J.A., Gathumbi, P.K., Mbaria, J.M., Kiama, S.G. (2014). Medicinal plants used in treatment and management of cancer in Kakamega County, Kenya. *Journal of Ethnopharmacology*, *151*, 1040-1055. [CrossRef]

3. Greenwell, M., Rahman, P.K.S.M. (2015). Medicinal plants: Their use in anticancer treatment. *International Journal of Pharmaceutical Sciences and Research*, *6*, 4103-4112. [CrossRef]

4. Aiello, P., Sharghi, M., Mansourkhani, S.M., Ardekan, A.P., Jouybari, L., Daraei, N., Peiro, K., Mohamadian, S., Rezaei, M., Heidari, M., Peluso, I., Ghorat, F., Bishayee, A., Kooti, W. (2019). Medicinal plants in the prevention and treatment of colon cancer. *Oxidative Medicine and Cellular Longevity*, *2019*, e.2075614. [CrossRef]

5. O'Reilly, M.S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W.S., Flynn, E., Birkhead, J.R., Olsen, B.R., Folkman, J. (1997). Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. *Cell*, *88*, 277-285. [CrossRef]

6. The American Cancer Society medical and editorial content team Chemotherapy Side Effects. Retrived from https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types/chemotherapy/chemotherapy-side-effects.html. Accessed date 09.08.2021.

7. Cragg, G.M., Newman, D.J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology*, *100*, 72-79. [CrossRef]

8. Sivaraj, R., Rahman, P.K.S.M., Rajiv, P., Narendhran, S., Venckatesh, R. (2014). Biosynthesis and characterization of Acalypha indica mediated copper oxide nanoparticles and evaluation of its antimicrobial and anticancer activity. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *129*, 255-258. [CrossRef]

9. Bassam, H. (2019). Plants and Cancer Treatment, *Medicinal Plants - Use in Prevention and Treatment of Diseases*, Bassam Abdul Rasool Hassan, Intech Open. Retrieved from https://www.intechopen.com/chapters/70522. Accessed date 09.08.2021.

10. Jaber, A., Soukariyeh, R., Khalil, A., Abdel-Sater, F., Cheble, E. (2020). Biological activities of total oligomeric flavonoids enriched extracts of nicotiana tabacum from eight lebanese regions. *International Journal of Pharmaceutical Sciences Review and Research, 6*, 70-77. [CrossRef]

11. Rawat, A., Mal, R. (2013). Phytochemical properties and pharmcological activities of Nicotiana tabacum. *Indian Journal of Pharmaceutical and Biological Research 1*, 74-82. [CrossRef]

12. Régie Libanaise des tabacs et tombacs Tobacco's economic contribution. In: REGIE LIBANAISE Tab. TOMBACS. Retrieved from https://www.rltt.com.lb/Article/31/tobaccos-economic-contribution/en. Accessed date 09.08.2021.

13. Sharma, Y., Nagar, A., Srivastava, N., Dua, D. (2016). Antibacterial activity, phytochemical screening and antioxidant activity of stem of nicotiana tabacum. *International Journal of Pharmaceutical Sciences and Research, 7*, 1156-67. [CrossRef]

14. Zhang, X., Gao, H., Zhang, L., Liu, D., Ye, X. (2012). Extraction of essential oil from discarded tobacco leaves by solvent extraction and steam distillation and identification of its chemical composition. *Industrial Crops and Products, 39*, 162-169. [CrossRef]

15. Wang, H., Zhao, M., Yang, B., Jiang, Y., Rao, G. (2008). Identification of polyphenols in tobacco leaf and their antioxidant and antimicrobial activities. *Food Chemistry, 107*, 1399-1406. [CrossRef]

16. Ameya, G., Manilal, A., Merdekios, B. (2017). In vitro antibacterial activity and phytochemical analysis of Nicotiana tabacum L. extracted in different organic solvents. *Open Microbiologie Journal, 11*, 352-359. [CrossRef]

17. Nawaz, H., Shad, M., Rehman, N., Andaleeb, H., Ullah, N. (2020). Effect of solvent polarity on extraction yield and antioxidant properties of phytochemicals from bean (Phaseolus vulgaris) seeds. *Brazilian Journal of Pharmaceutical Sciences, 56*, e.17129. [CrossRef]

18. Thouri, A., Chahdoura, H., El Arem, A., Omri Hichri, A., Ben Hassin, R., Achour, L. (2017). Effect of solvents extraction on phytochemical components and biological activities of Tunisian date seeds (var. Korkobbi and Arechti). *BMC Complementary and Alternative Medicine, 17*, 248. [CrossRef]

19. Nawaz, H., Aslam, M., Muntaha, S.T. (2019). Effect of solvent polarity and extraction method on phytochemical composition and antioxidant potential of corn silk. *Free Radicals and Antioxidants, 9*, 5-11. [CrossRef]

20. British Pharmacopoeia Commission. (1980). British Pharmacopoeia, Her Majesty's Stationery Office, Atlantic House, Holborn Viaduct, London.

21. Wakeel, A., Jan, S.A., Ullah, I., Shinwari, Z.K., Xu, M. (2019). Solvent polarity mediates phytochemical yield and antioxidant capacity of *Isatis tinctoria*. *PeerJ Life and Environment, 7*, 1-19. [CrossRef]

22. Karabegović, I.T., Veljković, V.B., Lazić, M.L. (2011). Ultrasound-assisted extraction of total phenols and flavonoids from dry tobacco (Nicotiana tabacum) leaves. *Natural Product Communications, 6*, 1855-1856. [CrossRef]

23. Pyka, A. (2014). Detection progress of selected drugs in TLC. *BioMed Research International*, 732078. [CrossRef]

24. Jijhotiya, A., Singhal, D.M., Goyal, D.S. (2017). Preliminary phytochemical screening of leaves extracts of *Nicotiana tabacum*. *International Journal of Engineering Technology Science and Research, 4*(6), 56-61.

25. Oeung, S., Nov, V., Ung, H., Roum, K., Yin, V., Keo, S., Chea, S. (2017). Phytochemical analysis of different extracts of leaves of *Nicotiana tabacum* L. of Cambodia. *Asian Journal of Pharmacognosy, 1*, 18-26. [CrossRef]

26. Lawson, D.R., Danehower, D.A. (1989). Quantitative thin-layer chromatography of *Nicotiana tabacum* leaf surface components. *Journal of Chromatography A, 463*, 429-440. [CrossRef]

27. Kusumawardani, B., Febi, Q.N., Rosidah, M., Azis, D.A., Puspitasari, E., Nugraha, A.S. (2020). Cytotoxic potential of flavonoid from *Nicotiana tabacum* leaves on MCF-7 human breast cancer cells. *Indonesian Journal of Cancer Chemoprevention, 11*, 97-102. [CrossRef]

28. Shang, S., Shi, J., Tang, J., Jiang, J., Zhao, W., Zheng, X., Lei, P., Han, J., Wang, C., Yuan, D., Yang, G., Chen, Y., Miao, M. (2019). New isolates from leaves of *Nicotiana tabacum* and their biological activities. *Natural Product Research, 33*, 1577-1583. [CrossRef]

29. Zhou, M., Zhou, K., Lou, J., Wang, Y-D., Dong, W., Li, G-P., Jian, Z-Y., Du, G., Yang, H-Y., Li, X-M., Hu, Q-F. (2015). New biphenyl derivatives from the leaves of *Nicotiana tabacum* and their cytotoxic activity. *Phytochemistry Letters*, *14*, 226-229. [CrossRef]

30. Chen, J., Leng, H., Duan, Y., Zhao, W., Yang, G., Guo, Y., Chen, Y., Hu, Q. (2013). Three new flavonoids from the leaves of oriental tobacco and their cytotoxicity. *Phytochemistry Letters, 6*, 144-147. [CrossRef]

31. Jassbi, A.R., Vafapour, M., Shokrollahi, A., Firuzi, O., Zare, M., Chandran, J.N., Schneider, B., Baldwin, I.T. (2017). Cytotoxic activity of two cembranoid diterpenes from Nicotiana Sylvestris against three human cancer cell lines. *The Open Bioactive Compounds Journal, 5*, 1-8. [CrossRef]

32. Essien, E.E., Ogunwande, I.A., Setzer, W.N., Ekundayo, O. (2012). Chemical composition, antimicrobial, and cytotoxicity studies on *S. erianthum* and *S. macranthum* essential oils. *Pharmaceutical Biology, 50*, 474-480. [CrossRef]

33. Loughrin, J.H., Hamilton-Kemp, T.R., Andersen, R.A., Hildebrand, D.F. (1990). Headspace compounds from flowers of *Nicotiana tabacum* and related species. *Journal of Agricultural and Food Chemistry, 38*, 455-460. [CrossRef]

34. Popova, V., Ivanova, T., Prokopov, T., Nikolova, M., Stoyanova, A., Zheljazkov, V.D. (2019). Carotenoid-related volatile compounds of tobacco (*Nicotiana tabacum* L.) essential oils. *Molecules, 24*(19), 3446. [CrossRef]

35. Popova, V., Ivanova, T., Stoyanova, A., Nikolova, V., Hristeva, T., Zheljazkov, V.D. (2020). GC-MS Composition and olfactory profile of concretes from the flowers of four Nicotiana species. *Molecules, 25*, 2617. [CrossRef]

36. Popova, V., Ivanova, T., Stoyanova, A., Georgiev, V., Hristeva, T., Nikolova, V., Docheva, M., Nikolov, N., Damyanova, S. (2018). Phytochemicals in leaves and extracts of the variety "Plovdiv 7" of Bulgarian oriental tobacco (Nicotiana tabacum L.). *Trends in Phytochemical Research, 2*(1), 27-36. [CrossRef]

37. Babushok, V.I., Linstrom, P.J., Zenkevich, I.G. (2011). Retention indices for frequently reported compounds of plant essential oils. *Journal of Physical and Chemical Reference Data, 40*, 043101. [CrossRef]

38. Candelaria-Dueñas, S., Serrano-Parrales, R., Ávila-Romero, M., Meraz-Martínez, S., Orozco-Martínez, J., Ávila-Acevedo, J.G., García-Bores, A.M., Cespedes-Acuña, C.L., Peñalosa-Castro, I., Hernandez-Delgado, T. (2021). Evaluation of the antimicrobial activity of some components of the essential oils of plants used in the traditional medicine of the Tehuacán-Cuicatlán Valley, Puebla, México. *Antibiotics, 10*, 295. [CrossRef]

39. Dutra, R.C., Pittella, F., Dittz, D., Marcon, R., Pimenta, D.S., Lopes, M.T.P., Raposo, N.R.B. (2012). Chemical composition and cytotoxicity activity of the essential oil of *Pterodon emarginatus. Revista Brasileira de Farmacognosia, 22*, 971-978. [CrossRef]

40. Francomano, F., Caruso, A., Barbarossa, A., Fazio, A., La Torre, C., Ceramella, J., Mallamaci, R., Saturnino, C., Iacopetta, D., Sinicropi, M.S. (2019). β-Caryophyllene: A sesquiterpene with countless biological properties. *Applied Sciences 9*, 5420. [CrossRef]

41. Popova, V., Ivanova, T., Stoyanova, A., Nikolova, V., Hristeva, T., Gochev, V., Yonchev, Y., Nikolov, N., Zheljazkov, V.D. (2020). Terpenoids in the essential oil and concentrated aromatic products obtained from *Nicotiana glutinosa L.* leaves. *Molecules*, *25*, 30. [CrossRef]

42. Alagić, S., Selekcija, I.S., Palić, R., Stojanović, G., Nikolić, M. (2002). Chemical composition and antimicrobial activity of the essential oil of the oriental tobacco yaka. *Journal of Essential Oil Research*, *14*, 230-232. [CrossRef]

43. Popova, V., Ivanova, T., Nikolova, V., Stoyanova, A., Docheva, M., Hristeva, T., Damyanova, S., Nikolov, N. (2017). Biologically active and volatile compounds in leaves and extracts of nicotiana alata link & otto from Bulgaria. *Journal of Pharmaceutical Sciences and Research, 9*, 2045-2051. [CrossRef]

44. Palic, R., Stojanovic, G., Alagic, S., Nikolic, M., Lepojevic, Z. (2002). Chemical composition and antimicrobial activity of the essential oil and CO_2 extracts of the oriental tobacco, Prilep. *Flavour and Fragrance Journal*, *17*, 323-326. [CrossRef]

45. Yang, L.Y., Yang, S.L., Li, J.Y., Ma, J.H., Pang, T., Zou, C.M., He, B., Gong, M. (2018). Effects of different growth temperatures on growth, development, and plastid pigments metabolism of tobacco (Nicotiana tabacum L.) plants. *Botanical Studies*, *59*, 5. [CrossRef]