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LC-ESI-MS profile, cytotoxic, antioxidant, insecticidal and antimicrobial activities of wild and in vitro propagated Tanacetum sinaicum Del. ex DC

Rasha A Attia*, Samih I El-Dahmy, Doaa D Abouelenein, and Afaf E Abdel-Ghani
[a] *Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, 44519 Zagazig, Egypt.*
*Corresponding author e-mail: rashaadelelfeqy@gmail.com
Tel./ Fax: 002-0552303266, **ORCID:** 0000-0002-0744-4005
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Running title: Chemistry and biological activities of Tanacetum sinaicum

Abstract

Tanacetum sinaicum (Asteraceae) is a rare herb growing wild in Southern Sinai mountains in Egypt. The aim of this work was to develop a comparative study between the chemical compositions of the wild plant and the micropropagated one, using HPLC− ESI− MS/MS technique. HPLC- ESI-MS/MS analysis resulted in the identification of 56 compounds including flavonoids, organic acids and their glycosides. Totally, 52 compounds were tentatively identified from the methanolic extracts of wild plant, 10 of them could also be detected in the methanolic extract of the micropropagated plant, in addition to 4 compounds that were detected only in the micropropagated plant. Methanolic extracts of the wild and micropropagated plants showed moderate cytotoxic activity against Hep-G2, HCT-116 and A-549 cell lines using MTT cell viability assay. The antioxidant activity using the DPPH free radical assay was investigated and the results indicated that the wild plant methanolic extract has better antioxidant activity compared to the in vitro plant extract. In addition, the insecticidal activity was measured using the immersion method and the methanolic extract of the micropropagated plant showed a higher mortality percentage than wild plant extract on culex pipiens. Also, results indicate that wild plant extract has mild antimicrobial activity against Gram positive bacteria Staphylococcus aureus and Bacillus subtilis and showed no activity on both fungi and Gram negative bacteria. while in vitro plant extract showed some activity against Gram positive bacteria Staphylococcus aureus and the Gram negative bacteria Escherichia coli.

Key words: *Tanacetum sinaicum*, HPLC− ESI− MS/MS**,** Cytotoxicity, Antioxidant, Insecticide, Antimicrobial.

Introduction

Genus *Tanacetum* (Asteraceae)s is represented by one species in the flora of Egypt, named *Tanacetum sinaicum* [Boulos, 2002]. It is a perennial, fragrant, herb with discoid, yellow capitulum with only tubular florets [Täckholm, 1974]. Many traditional uses were reported for this plant including the treatment of migraines, arthritis, bronchitis and fevers [Hegazy et al., 2015]. Pharmacological studies on the extracts, volatile oils, and chemical constituents of this plant confirmed its pesticide, antimicrobial, anti-inflammatory and cytotoxic activity [Rabeh, 2000; El-Shazly et al., 2002; Ibrahim et al., 2007; Youssef et al., 2007; Soltan and Zaki, 2009; Hegazy et al., 2013; Hegazy et al., 2015]. Important pharmacologically active secondary metabolites were isolated from this plant such as terpenoids [El-Sebakhy et al., 1986; El Sebakhy and El Ghazouly, 1986; Jakupovic et al., 1987; Abdel-Mogib et al., 1989; Mahmoud et al., 2007; Hegazy et al., 2015] and flavonoids [Eldin et al., 1985; Mosharrafa et al., 1994]. Chemical composition of the volatile oil and the *n*-Hexane extract of the plant were reported in

previous studies [Ateya, 1992; Rabeh, 2000; El-Shazly et al., 2002].

This study aimed to compare the constituent of the alcoholic extracts of wild and micropropagated plants using HPLC- ESI-MS. Additionally, the evaluation of some biological activities of the extracts were carried out.

2. Material and Methods

2.1 Plant

The aerial parts of *Tanacetum sinaicum* Del. ex DC; was collected in May 2014 from Wadi Elarbaeen, Sinai, Egypt. The plant was Identify by Prof. Dr. Azza El-Hadidy, Department of Botany, Faculty of Science, Cairo University. A voucher specimen was deposited in the Herbarium of Pharmacognosy Department, Zagazig University, Egypt.

2.2. Production of *in vitro* micropropagated plant

The *in vitro* micropropagated plants have been developed as previously described by the authors [Adel et al., 2021]. Briefly, sterilized seeds were germinated on Murashige and Skoog media (MS). Five weeks old explants were transferred to MS media with 0.5mg/L naphthalene acetic acid (NAA) and 1mg/L benzyl amino purine (BAP) for callus induction. Six weeks old callus were transferred to hormone free MS media, mature embryos germinated into cotyledonary stage, which developed into plantlets within 7-10 weeks.

2.3 High performance liquid chromatography-Mass spectrometry (HPLC- MS/MS):

The sample solutions of methanolic extracts of both wild and *in vitro* plants (2 months old) were prepared in a concentration of 100 µg/mL using HPLC grade methyl alcohol. The samples were filtered through a membrane disc filter (0.2 µm) prior to LC-ESI-MS analysis. UPLC-ESI-MS-MS analysis was carried out on a triple quadruple instrument (XEVO TQD) using both positive and negative ion acquisition modes. The instrument was coupled to mass spectrometer (MA01757, U.S.A). The chromatographic separation was performed on a reversed phase, C18 column, of dimensions 50 × 2.1 mm and particle size 1.7 µm (ACQUITY UPLC – BEH). The injection volume was set at 10 µL. The Mobile phase flow rate was adjusted at 0.2 mL/min. Mobile phase consisting of two eluents: A (H_2O with 0.1% formic acid) and B (MeOH with 0.1% formic acid). The following gradient elution program was applied: 10% B (0-2 min), 10-30% B (2-5 min), 30-70% B (5-15 min), 70-90% B (15-22 min), 90% B (22-225 min), 90-100% B (25-26 min), 100% B (26-29 min, and 10% B (29-32 min).

The analysis were carried out using both negative and positive ion modes as follows: 150 °C source temperature, 30 eV cone voltage, 3 kV capillary voltage, 440 °C desolvation temperature, 50 L/h cone gas flow, and 900 L/h desolvation gas flow. The scale of the mass spectra were adjusted between *m/z* 100–1000. The analysis was processed using the Maslynx 4.1 software and components were tentatively identified by comparing their retention time (Rt) and mass spectrum with reported data.

2.4 Cytotoxic activity

Three cancer cell lines including human hepatocellular carcinoma (HepG-2), human lung cancer (A-549) and colon carcinoma (HCT-116) obtained from VACSERA, Egypt were used to estimate the cytotoxicity of the tested extracts using cell viability assay (MTT) as previously described by Gomha et al., [2015].

The alcoholic extracts of the wild and micropropagated plants were dissolved in DMSO. The viability percentage of the cancer cells in each cell line were plotted against different concentrations of tested extracts and the IC_{50} was calculated for each of them [Gomha et al., 2015; Mosmann, 1983].

2.5 Insecticide activity

Culex pipiens mosquitoes were collected from Abu Rawash, Giza, Egypt. Mosquitoes were reared for many generations under controlled conditions in the insectariums of medical entomology, Department of Zoology, Faculty of Sciences, Al-Azhar University, Egypt. The insecticidal activity was estimated using the immersion method as previously reported [Wu et al., 2010]. In plastic cup, the larvae of *Culex pipiens* (2^{nd} instar) were treated with alcoholic extracts of the wild and micropropagated plants (At 100

µg/ml) with 10 ml of each extract. 10 ml of tap water were added in control tubes instead of the extract. 30 larvae was used in each treatment and repeated for three times. After 48 h from treatment, the numbers of live and dead larvae were counted. The mortality percent was calculated as follows: Mortality (%) = [Number of dead larva in treatment group - Number of dead larva in control group] ÷ Number of total tested larva × 100.

2.6 Antioxidant Assay

The antioxidant activity of alcoholic extracts of the wild and *in vitro* micropropagated plant was carried out at the Regional Center for Mycology and Biotechnology (Al- Azhar University) using the DPPH free radical scavenging assay. The DPPH radical was calculated according to [Yen and Duh, 1994].

2.7 Antimicrobial activity

Antifungal and antibacterial activities of the alcoholic extracts of the wild and the micropropagated plants were determined using the well diffusion method [Hindler et al., 1994]. The tested extracts were dissolved in DMSO (Oxoid, UK) at concentration of 1mg/ml. The tested organisms were subcultured on Sabouraud dextrose agar for fungi and nutrient agar for bacteria. The microorganisms used in this study were *Geotricum candidum* (RCMB 05097) and *Aspergillus fumigatus* (RCMB 02568) as fungi. *Escherichia coli* (RCMB 010052) and *Pseudomonas aeruginosa* (RCMB 010043) were used as Gram negative bacteria. Two Gram positive bacteria were used, *Bacillus subtilis* (RCMB 010067) and *Streptococcus Pneumonia* (RCMB 010010). Ketoconazole was used as a positive control for fungi while Gentamicin was used against Gram negative and Gram positive bacteria.

The plates were prepared in triplicates. Fungal cultures were incubated at 37 °C for 2-7 days while, bacterial cultures were incubated at 37 °C for 24 h. Antimicrobial activities were determined by measuring the zone of the inhibition (mm) formed around the well. Results were manifested in mean zone of inhibition (mm) ± standard deviation (SD). Minimal inhibitory concentration (MIC) was determined.

3. RESULTS AND DISCUSSION

HPLC− MS/MS in both positive and negative ion modes was used to analyze the total methanolic extracts of wild and *in vitro* plants of *T. sinaicum* Del. Totally, 56 secondary metabolites were tentatively identified, 34 of them were identified depending on the negative ionization mode, while the other 22 compounds were identified in the positive ionization mode. The identification of these compounds based on their MS^2 mass fragmentation of the precursor ion and comparison with the literature. The compounds were ordered according to their retention times (t_R). The identified secondary metabolites were summarized in Table (1).

3.1. HPLC- ESI-MS/MS

Phenolic acids and their derivatives

Phenolic acid derivatives included mainly as glycosides. The fragmentation of phenolic glycosides is started by the cleavage of the glycosidic linkage to give the fragment of the phenolic acid and the neutral mass loss of the sugar molecules followed by neutral mass losses of carboxylic, methyl or hydroxyl groups were useful in identification of some phenolic acid derivatives [Li et al., 2003].

Nineteen phenolic acids and their derivatives have been detected in the methanol extracts of wild and micropropagated *T. sinaicum* plants. Compound **35** with a protonated molecular ion peak at *m/z* 193 and a fragment ion at *m/z* 109 which is characteristic for **quinic acid** [Abu-Reidah et al., 2015]. Compound **2** exhibited a deprotonated ion at *m/z* 315 and a fragment at *m/z* 153 because of hexose moiety loss **(Figure 1)**, and conducted to be **protocatechuic acid hexoside** [Chen et al., 2011], or **genistic acid hexoside** [Yur et al., 2017]. Compounds **3** and **12** with *m/z* 297 and 367 $[M\text{-}H]^-$ respectively. Besides the production of MS^2 fragment ion at *m/z* 179. So, these compounds were tentatively assigned as **caffeic acid derivatives**. Compound **4** has been identified as **glucosyringic acid** , $[M - H]^-$ = 359 with MS^2 fragments at *m/z* 197 $[(M - H)^- Glc]^-$, 153 $[(M - H) - Glc - CO_2]^-$ and base peak of *m/z* 95 $[(M - H) - Glc - CO_2 - 2CH_3 - CO]^-$ [Li et al., 2016].

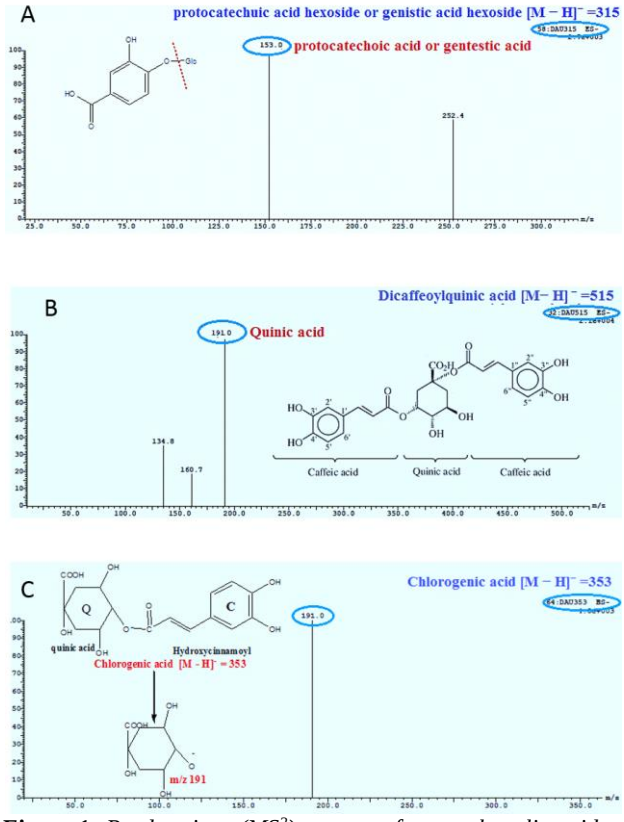

Figure 1: *Product ions (MS^2) spectra of some phenolic acid derivatives identified in methanol extracts of wild and in vitro T. sinaicum Del. in negative ion mode ESI⁻ MS. A: Protocatechoic acid; B: Dicaffeoyl quinic acid; C: Chlorogenic acid.*

Compound **5** exhibited the molecular ion of **caffeic acid hexose deoxyhexose** at *m/z* of 487 [M - H] ⁻. As well as a fragment ion at *m/z* 179 [(M-H) -162 -146] ⁻ due to the loss of the hexose and deoxyhexose moieties [Carrillo-López and Yahia, 2013]. Compound **6** with [M − H] ⁻ at m/z 313 was suggested to be methoxy benzoic acid ion at *m/z* 151 due to the loss of hexose moiety [(M – H) − 162] ⁻ [Fang et al., 2002]. Additionally, three **benzoic acid derivatives** were tentatively identified at *m/z* 255, 294 and 167 (compounds **15, 23** and **24;** respectively**)** as they showed benzoic acid fragment at *m/z* 121 [Lin et al., 2015].

Compound **7** with a parent ion at *m/z* 341 [M − H]⁻ and base peak at *m/z* 179 for caffeic acid through the loss of a hexose moiety [Chen et al., 2011]. Thus, compound **7** was tentatively deduced as **caffeoyl hexoside**.

Chlorogenic acids are constituted by a quinic acid unit esterified with a caffeic acid. Compound **8** is a **caffeoylquinic derivative** exhibited [M – H] ⁻ at *m/z* 353 [Agar et al., 2015]**.** Compound **9** gave a molecular ion peak at *m/z* 373 [M – H] ⁻. It was suggested to be **geniposidic acid**. The compound showed a base peak at *m/z* 211 [M – H– Glc] −) and *m/z* 167 [(M – H) – Glc − CO2] ⁻ [Li et al., 2016]. Compound **13** exhibited [M − H] ⁻ at *m/z* 473 was identified as **quinic acid derivative** due to the presence of quinic acid fragment at *m/z* 191 [Bakr et al., 2016]. Compound **14** which exhibited [M − H] ⁻ at *m/z* 515 was identified as **dicaffeoylquinic acid** [Echiburu-Chau et al., 2017; Simirgiotis et al., 2015; Simirgiotis et al., 2016].

Compound **18** at *m/z* 359 was characterised as **rosmarinic acid**. The MS^2 provided fragments at *m/z* 161 [(M − H) − caffeoyl − 2 H_2O] ⁻ as caffeoyl molecule and two water molecules were lost [Taamalli et al., 2015]. Compound **19** with a precursor ion at *m/z* 325 [M − H] ⁻ and a base peak at 119 was assigned as **p-coumaroyl-*O*-glucoside** [Li et al., 2016]. Moreover, compounds **31** and **32** showed deprotonated ion peaks at *m/z* 311 & 313, respectively and were tentatively identified as **caftaric acid** and **hydroxygallic acid derivative** by comparing their MS^2 with available literature [Chen et al., 2011].

Flavonoids

Eight flavonoid aglycones were identified in methanol extracts of wild and micropropagated plant. Compound **41** was identified as **myricetin** (3,3',4',5,5',7− hexahydroxy flavones) as it produced a precursor ion at *m/z* 319 [M + H] ⁺ and fragmentation ion at *m/z* 271 as three hydroxyl groups were lost and a fragment at *m/z* 223 corresponds to the flavone nucleus after the loss of six hydroxyl groups. Additionally, compound **22** showed [M − H] ⁻ at *m/z* 299 and the fragmentation ion at *m/z* 284 (base peak), indicating one methoxy group. So, compound **22** was tentatively deduced as **trihydroxy methoxy flavone** [Li et al., 2016].

Compound **25,** (M − H)⁻ at *m/z* 359, was characterized as **trihydroxy trimethoxy flavone** confirmed by the MS^2 fragment ions **(Figure 2)** with successive loss of methyl groups at *m/z* 344 [(M − H)⁻ CH_3] ⁻, 329 [(M − H) ⁻ $2CH_3$] ⁻ and 314 [(M − H) − $3CH_3$] ⁻ [Taamalli et al., 2015; Yur et al., 2017]. Similarly, Compound **26** with a deprotonated ion at *m/z* 329 was identified tentatively as

trihydroxy dimethoxy flavone [Yur et al., 2017].

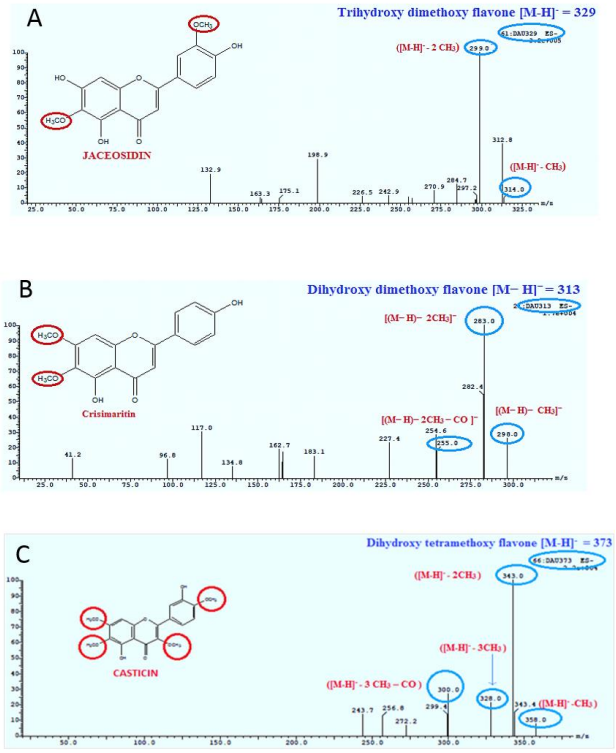

Figure 2: *Product ions (MS^2) spectra of some flavone aglycones identified in methanol extracts of wild and in vitro T. sinaicum Del. in negative ion mode ESI - MS. A: Trihydroxy dimethoxy flavones; B: Dihydroxy dimethoxy flavones; C: Dihydroxy trtramethoxy flavones.*

Compound **28** was suggested to be **dihydroxy dimethoxy flavone** with $[M - H]^-$ = 313 confirmed by the MS^2 spectrum **(Figure 2)** that gave fragments at *m/z* 283 after two methyl groups loss from its parent ion [Simirgiotis et al., 2015; Wang et al., 2008]. Compound **29** was characterized as **dihydroxy tetramethoxy flavone** [Lin et al., 2015]. The compound has $[M - H]^-$ ion at *m/z* 373 and fragment ions in the MS^2 spectra at *m/z* 358 $[(M - H) - CH_3]^-$, 343 $[(M - H) - 2CH_3]^-$, 328 $[(M - H) - 3CH_3]^-$ and the fragment ion at *m/z* 300 $[(M - H) - 3CH_3 - CO]^-$ **(Figure 2).**

Compound **46,** *m/z* $[M + H]^+$ = 345, was characterized as **dihydroxy trimethoxyflavone**. This was confirmed by the MS^2 fragments at *m/z* 330 $[(M + H) - CH_3]^+$, 315$[(M + H) - 2CH_3]^+$, 300 $[(M + H) - 3CH_3]^+$ and 284 $[(M + H) - (CO + H_2O + CH_3^{\bullet})]^+$. Similarly, compound **49** produced a precursor ion at *m/z* 329 $[M + H]^+$, which was identified as **monohydroxy trimethoxy flavone** [Zhang et al., 2011].

Flavonoid -*O*− Glycosides

Compound **17 and 30** presented $[M - H]^-$ at 447 and 461, respectively. The MS/MS spectrum showed fragments at *m/z* 271 and 285, respectively (corresponds to the aglycon; naringenin and luteolin) were formed due to the loss of a glucuronyl $[(M - H)^- - 176]$ [Cuyckens and Claeys, 2004]. In accordance with these results, compound **17** was assigned to **naringenin-*7-O*- glucuronide** while, compound 30 was identified as **luteolin-7-*O*-glucuronide** [Li et al., 2016]

Compound **20** exhibited a deprotonated ion at *m/z* 461. MS^2 fragment at *m/z* 298 indicating the loss of a hexose molecule, suggesting that it was a chrysoeriol hexoside. Moreover, compound **21 (Figure 3)** with molecular ion at *m/z* 577 in negative mode, which produced the MS^2 base peak at 269 characteristic for apigenin, suggesting the compound to be **apigenin-7-*O*- rutinoside (isorhoifolin)** [Cao et al., 2016; Gattuso et al., 2006; Hossain et al., 2010; Yur et al., 2017].

Compound **37** with a protonated ion at *m/z* 565, produced MS/MS fragment at *m/z* 317 formed after elimination of malonyl− hexose unit. This compound has been identified tentatively as **isorhamnetin− *3−O−*malonyl hexoside** [Kumar, 2017; Martucci et al., 2014].

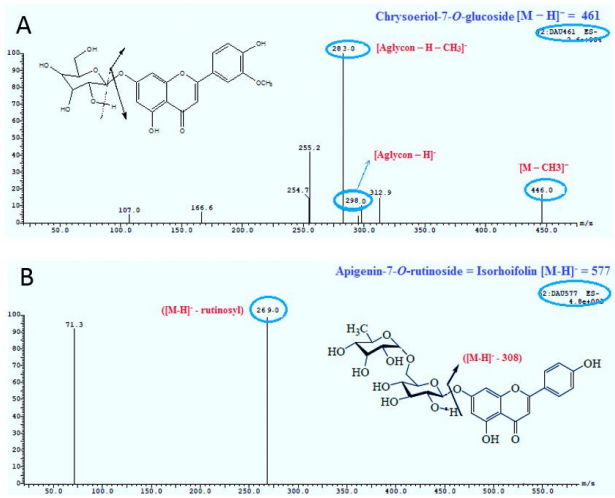

Figure 3: *Product ions (MS^2) spectra of some flavonoidal -O-glycosides identified in methanol extracts of wild and in vitro T. sinaicum Del. in negative ion mode ESI− MS. A: Chrysoeriol-7-O-glucoside; B: Apigenin-7-O-rutinoside.*

Compounds **47** was identified as **myricetin−*3−O−*rhamnosyl rhamnoside** depending on its $[M + H]^+$ at *m/z* 611, and MS/MS fragment ion at *m/z* 319 (confirmed as myricetin) formed after elimination of two

rhamnosyl moieties [(M+H) − 146 – 146] $^{+}$ [FoodDB, 2019; TMIC, 2019]. Similarly, Compound **52** was tentatively identified as **myricetin-3-*O*-rhamnoside**.

Flavonoid –*C*–Glycosides

Compound **1** with [M − H] $^{-}$ ion at *m/z* 431, produced a fragment at 311 in the MS^2. We tentatively identified this compound to be **apigenin-*C*-glucoside (Vitexin)**, which has also been identified previously by [Cao et al., 2016]. On the other hand, compound **16** was identified as **Apigenin 6, 8-di-C-glucoside**. It gave a molecular ion at *m/z* 593, which produced the ions at *m/z* 312 and 135 in negative mode [Cao et al., 2016].

Compound **38** identified as apigenin-6-*C*-pentoside-8-*C*-hexoside (**shaftoside or its isomer)** followed the characteristic fragmentation pattern of flavonoids *C*– glycosides **(Figure 4&5)** as reported by [Hassan et al., 2019]. Additionally, compound **39** was identified as **Chrysoeriol or diosmetin -*8*-*C*-glucoside**. It showed molecular ion [M + H] $^{+}$ at *m/z* 463 and MS/MS fragment ions at *m/z* 343 [(M + H) − 120] $^{+}$ and *m/z* 313 [(M − H) − 150] $^{-}$ **(Figure 4)** [Andersen and Markham, 2005; Sato et al., 1992].

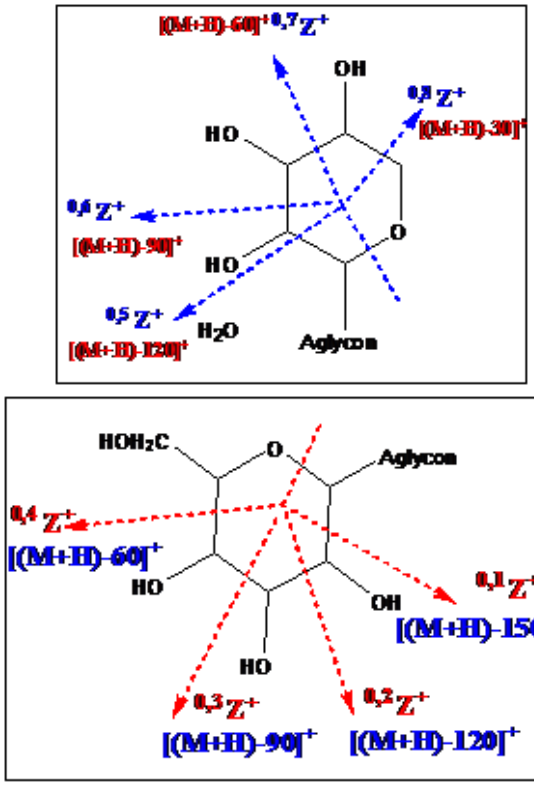

Figure 4: *C-Hexose and C-pentose fragmentations in ESI-MS/MS*

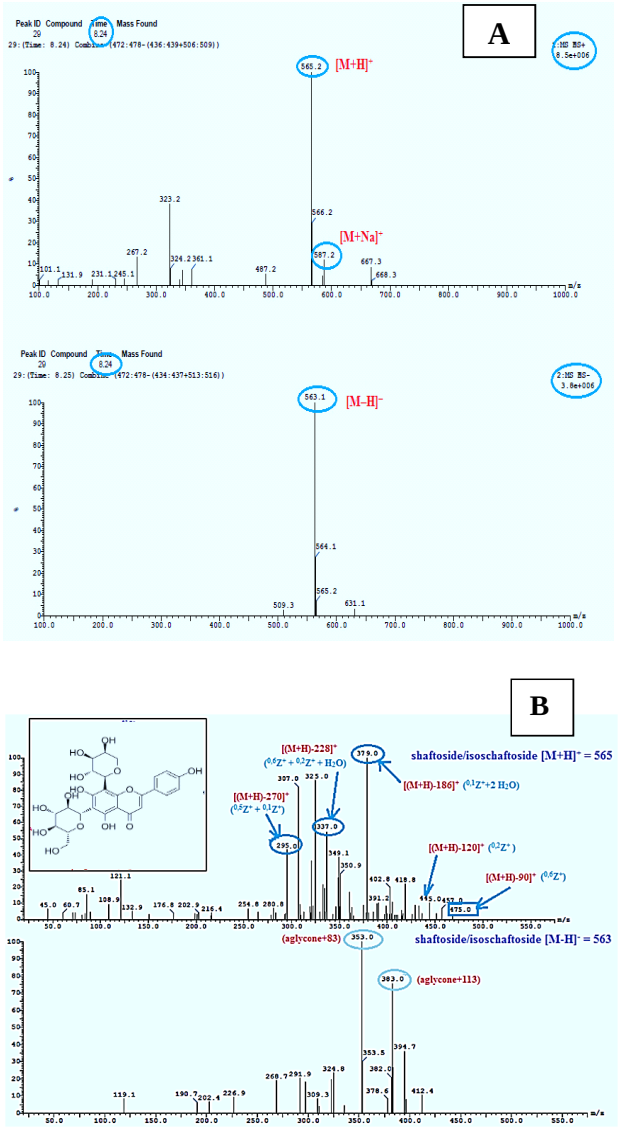

Figure 5: *ESI-MS and MS/MS spectra of shaftoside or its isomer in positive and negative ionization mode; A: Mass fragmentation pattern of Shaftoside /Isoschaftoside in positive and negative ionization modes, B: Product ions (MS^2) spectra of in positive and negative ion modes ESI– MS.*

Compound **40** with molecular ion [M + H] $^{+}$ at *m/z* 447 was suggested to be **Swertisin (Figure 6&7)**. The compound showed fragment ions at *m/z* 327 [(M + H) – 120] $^{+}$ and *m/z* 297 [(M + H) – 150] $^{+}$ revealing the presence of a hexose molecule at the 6, 8 or 3 position from *C*– type as shown in **Figure 4**. Ions at *m/z* 191 and 163 confirmed that the compound is flavone 6-*C*-glucoside as described by [Andersen and Markham, 2005; Sato et al., 1992]. The fragment at *m/z* 121 indicated a monohydroxylated B ring.

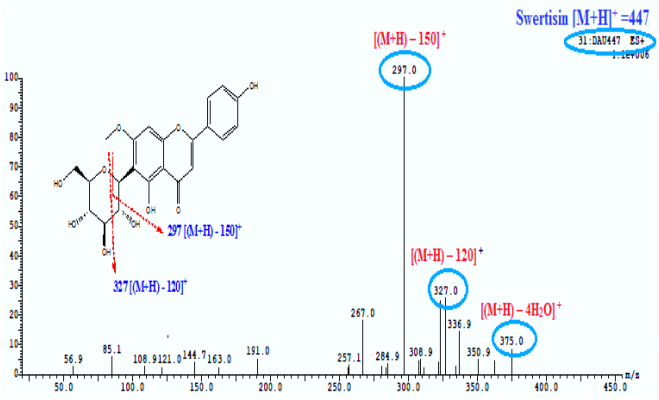

Figure 6: *Product ions (MS^2) spectra of swertisin in positive ion mode ESI− MS*

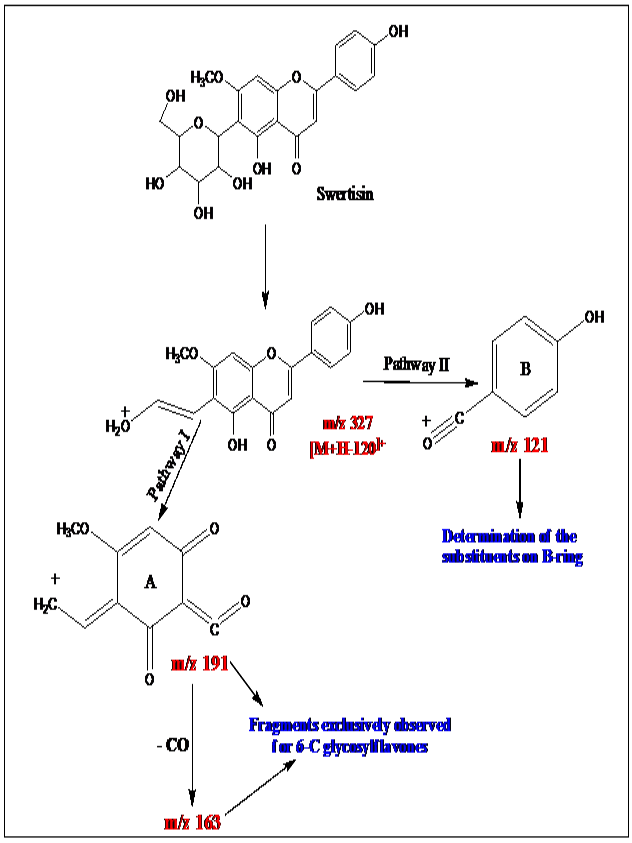

Figure 7: *The suggested LC-MS-MS fragmentation pattern of compound 40.*

Sesquiterpene Lactones

Six sesquiterpene lactones were tentatively identified in the tested extracts. Compound **42** with a precursor ion at *m/z* 301 $[M + H]^+$ and MS/MS fragment ions at *m/z* 228, 201, 196, 185, 183, 165, 155, 139, 131, 125, 110, 103, 95, 91, 71, 69, 57 and 45 was tentatively identified as **tanacetolide A** which was isolated previously from *T. sinaicum* [Hegazy et al., 2015].

Compound **43** was identified as **santamarin.** It showed a precursor ions at *m/z* 231 $[(M + H) – H_2O]^+$ and *m/z* 249 $[M + H]^+$ **(Figure 8)**. The MS^2 fragmentation pattern **(Figure 8)** showed characteristic fragments at *m/z* 233, 183, 119, 95 and 55. It was previously isolated from genus *Tanacetum* [Sanz and Marco, 1991].

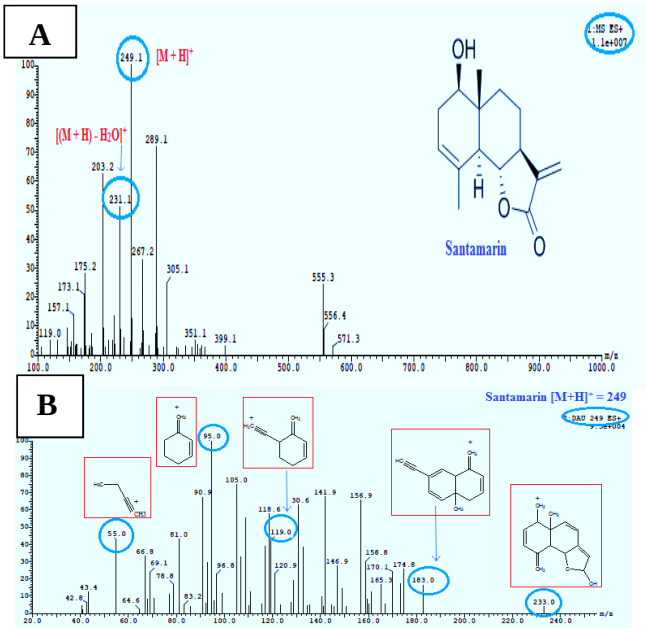

Figure 8: *ESI-MS and MS/MS spectra of santamarin in positive ion mode; A: Mass fragmentation pattern of santamarin in the positive ionization mode, B: Product ions (MS^2) spectra of santamarin in positive ion mode ESI− MS.*

Compound **45** was tentatively identified as **ketoplenolide B** [FoodDB, 2019; Sharma et al., 2016]. The ions detected for the compound were at *m/z* 273 $[M + Na]^+$ and 251 $[M + H]^+$. The MS^2 fragmentation pattern showed fragments at *m/z* 192, 155, 134, 109, 71, 59, 55.

Compound **51** was tentatively identified as **tanacetol B** [FoodDB, 2019; TMIC, 2019]. It gave precursor ions at *m/z* 319 $[M + Na]^+$, *m/z* 297 $[M + H]^+$. The precursor ion at *m/z* 397 showed MS^2 fragment ion at *m/z* 237, formed after neutral lose of acetate unit and product ion at *m/z* 195 formed after lose of isopropyl group as shown in **Figure 9** and Table (1). This compound was previously isolated from *T. vulgare* [Sanz and Marco, 1991].

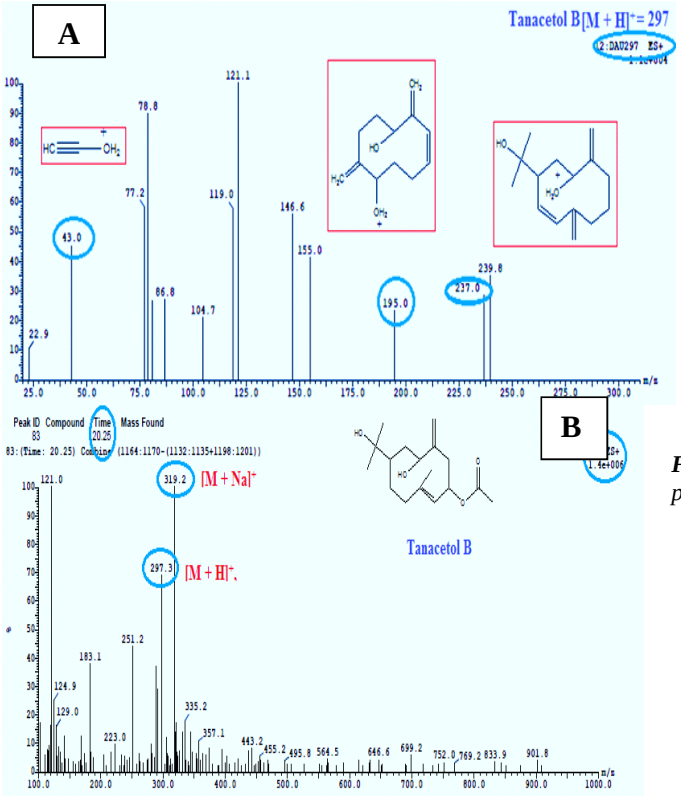

Figure 9: *ESI-MS and MS/MS spectra of tanacetol B in positive ion mode; A: Mass fragmentation pattern of tanacetol B in the positive ionization mode, B: Product ions (MS^2) spectra of tanacetol B in positive ion mode ESI− MS.*

Compounds **53** and **54** showed a precursor ions at *m/z* 285 and 267 $[M + H]^+$, respectively. Both have MS^2 spectra which formed product ions at *m/z* 109, 95, 85, 83, 81, 71, 71, 67, 57, 55, 43 and 41. Based on fragmentation pattern they were identified as **trihydroxy germacrenolide** and **dihydroxy germacradieneolide**; respectively [Mahmoud et al., 1994].

Sterols

Compound **55** and compound **56** showed protonated ion peaks at *m/z* 395 and 397, respectively after loss of water $[(M + H) - H_2O]^+$ which is common in unsaturated sterols [Khalaf et al., 2011; Mo et al., 2013]. They were tentatively identified as **stigmasterol** & ***β*-sitosterol,** respectively **[Duan et al., 2014]**.

Other Classes

Compound **36** possessed a precursor ion at *m/z* 153 $[M + H]^+$ **(Figure 10).** which was identified as **carvotanacetone, a** menthane monoterpenoid compound, according to [FoodDB, 2019; TMIC, 2019].

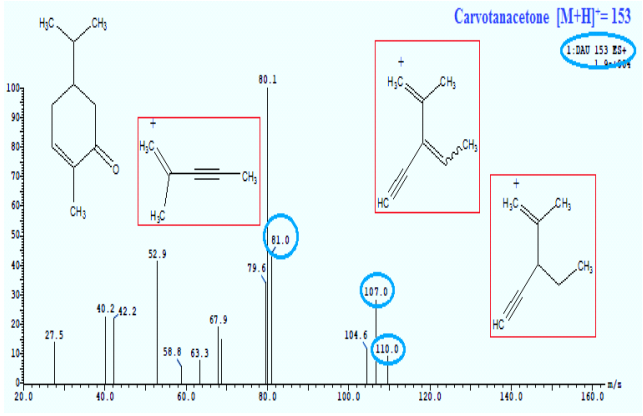

Figure 10: *Product ions (MS^2) spectrum of carvotanacetone in positive ion mode ESI+MS.*

Compound **10** was identified as **gallocatechin**. It possessed a deprotonated ion at *m/z* 305, and MS^2 spectrum diagnostic product ion at *m/z* 225 according to [Hossain et al., 2010]. Compound **11** exhibited a deprotonated ion peak at *m/z* 387 **(Figure 11)** was identified as **medioresinol** [Yur et al., 2017].

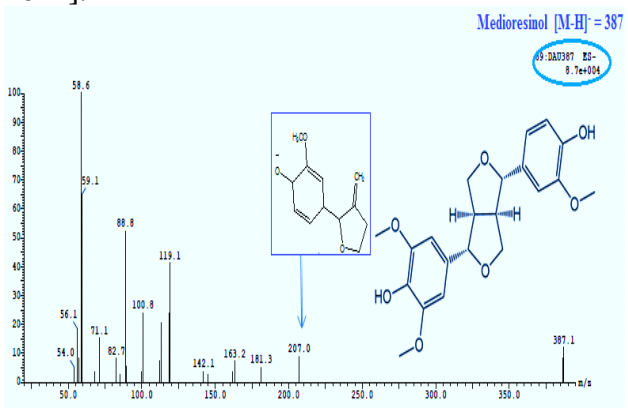

Figure 11: *Product ions (MS^2) spectrum of medioresinol in negative ion mode ESI− MS.*

Compound **44** and 48 showed a protonated ion at *m/z* 397 and 224, which were identified as hexacosanoic acid **(cerotic acid) and pellitorine, respectively** [FoodDB, 2019; TMIC, 2019] **(Figure 12).**

Compound **27 and 33** exhibited molecular ion peaks at *m/z* 433 and 447, respectively in negative mode. They were deduced as **geniposide** which is a terpenoidal glycoside and **cuneataside C, respectively** [Li et al., 2016].

Compound **50, ethyl palmitate,** showed a precursor ion at *m/z* 285 $[M + H]^+$ and a characteristic fragments at *m/z* 169, 141 and 71 [FoodDB, 2019; Luginbühl et al., 2016].

Compound **34**, **asperulosidic acid,** showed precursor ions at *m/z* 431 $[M-H]^-$ and a base peak at *m/z* 191 due to the lose of glucose unit, water and acetic acid molecules $[(M-H)-Glc-H_2O-CH_3COOH]^-$ [Li et al., 2016].

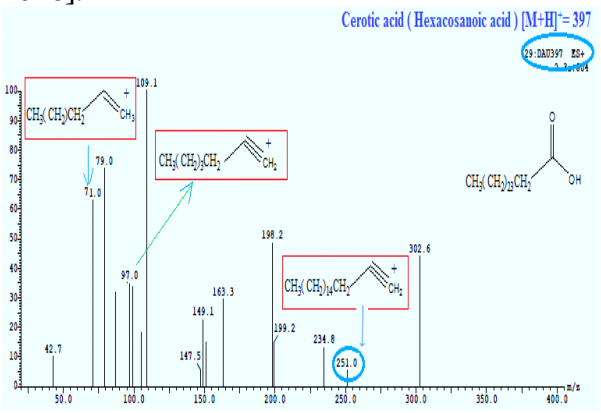

Figure 12: *Product ions (MS^2) spectrum of cerotic acid in positive ion mode ESI^+MS.*

3.2 Cytotoxic activity

A few previous studies reported the cytotoxicity of *T. sinaicum*. Its cytotoxic activity was related to sesquiterpene lactones content as tanacetonic acid [Ibrahim et al., 2007] [Youssef et al., 2007]. To the best of our knowledge, this is the first time to investigate cytotoxicity of the plant against different cancer cell lines using MTT assay. Cytotoxicity of the methanolic extract of the wild and micropropagated plants were evaluated against Hep-G2, A-549 and HCT-116 using MTT cell viability assay **(Figure 13 A&B)**. The IC_{50} values of the methanolic extract of the wild plant against Hep-G2, A-549 and HCT-116 were found to be 48.7 ±2.1, 54.6 ±3.8 and 39.6 ± 1.\ µg/ml; respectively, while those of the micropropagated plant were 30 ± 2.1, 46 ± 3.2 and 57.3 ± 3.9 µg/ml; respectively. This means that both tested extracts gave moderate cytotoxic activities against cell lines under investigation.

3.3 Insecticidal activity:

Mosquitoes can play a chief role in parasitic and viral diseases transmission. Volatile oils and extracts from plant origin could be alternative to synthetic insecticides because of their efficacy and safety [Soleimani-Ahmadi et al., 2017]. The volatile oil and different extracts of the wild *T. sinaicum* were reported to have a marked larvicidal activity against *culex pipiens* mosquito larvae [Hifnawy M. S. et al., 2000; Adel et al., 2021]. So, it was interesting in the current work to compare the insecticidal activity of both methanolic extracts of the wild and micropropagated plants. By comparing the mortality percentages of the methanolic extract of both wild and micropropagated plants on *culex pipiens*, the micropropagated plant showed higher mortality percentages 50, 25 and 66.7 % for different insect stages (larval, pupal and adult stages, respectively) in comparison with wild plant **(Figure 13 C)**.

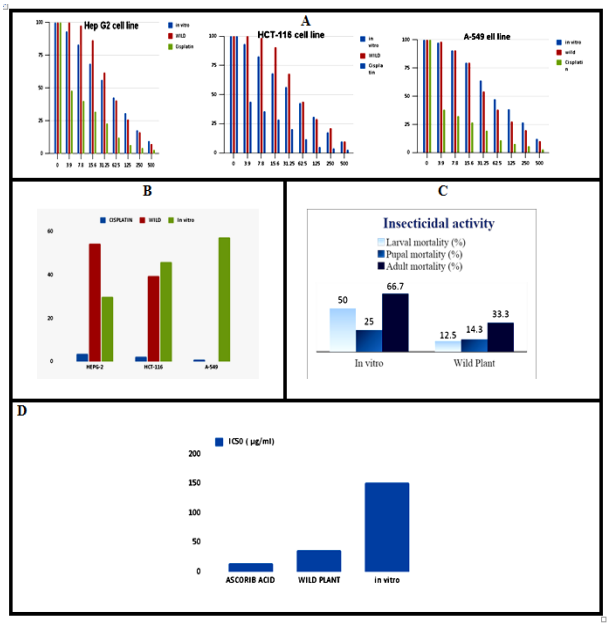

Figure 13 Biological activities of of T. sinaicum.
A: *Inhibitory activity of methanolic extracts of wild and in vitro T. sinaicum against Hep-G2, HCT-116 and A-549 cell line cells ;* ***B:*** *Half-maximal inhibitory concentration (IC_{50}) of methanolic extracts of in vivo and in vitro T. sinaicum against Hep-G2, HCT-116 and A-549 cell line cells.;* ***C:*** *Mortality (%) of methanolic extracts of in vivo and in vitro T. sinaicum against culex pipiens mosquito;* ***D:*** *DPPH radical scavenging activity IC_{50} % (µg/mL) of wild and in vitro T. sinaicum.*

We suppose that the insecticidal activity of both extracts of the wild and *in vitro* propagated plants could be due to pellitorine compound as it was previously reported that pellitorine is one of the many compounds known for its insecticidal and fungicidal activities [Lee, 2005].

3.4 Antioxidant activity

The antioxidant activity of the methanolic extracts of the wild and micropropagated plants using the DPPH free radical assay was carried out. The IC_{50} values of the extracts of wild and micropropagated plants were 36.8 and 150.9 µg/ml; respectively

and this means that wild plant extract is more effective as antioxidant than the micropropagated plant extract **(Figure 13 D)**. Antioxidant activity of the plant may be due to flavonoids [Ibrahim et al., 2007] and phenolic compounds [Marzouk et al., 2016] which previously reported in the plant.

3.5 Antimicrobial activity

Antifungal and antibacterial activities of methanolic extracts of the wild and micropropagated plants were shown in (**Table 2**). Results indicated that wild plant extract has mild antimicrobial activity against Gram positive bacteria *Bacillus subtilis* (MIC 1.25 mg/ml) and *Staphylococcus aureus* (MIC 2.5 mg/ml) while, no activity on tested fungi and Gram negative bacteria. The extract of the micropropagated plant showed some activity against Gram positive bacteria *Staphylococcus aureus* (MIC 10 mg/ml) and the Gram negative bacteria *Escherichia coli* (MIC 2.5 mg/ml) in comparison to standard drugs, gentamicin (MIC 0.004 mg/ml) and ketoconazole (MIC 0.1 mg/ml).

4. Conclusion

The secondary metabolites of the alcoholic extracts of wild and micropropagated plants of *T. sinaicum* Del were analyzed using HPLC–MS/MS in negative and positive ion modes. 56 secondary metabolites were identified. 52 compounds from the methanolic extracts of the plant were tentatively identified, 10 of which could also be detected in the methanolic extract of the *in vitro* propagated plants. In addition to 4 compounds that were detected only in the *in vitro* propagated plants. The identified compounds include organic acids, flavonoids and their glycosides and sesquiterpene lactones.

Methanolic extracts of the wild and *in vitro* propagated plants showed moderate cytotoxic activity against Hep-G2, A-549 and HCT-116 cell lines using MTT cell viability assay. The antioxidant activity was investigated using the DPPH assay and the results explained that the wild plant methanolic extract has better antioxidant activity compared to the micropropagated plant extract. In addition, the methanolic extract of the micropropagated plant showed a higher insecticidal activity than wild plant extract on *culex pipiens*. Also, results indicate that wild plant extract has some antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* and showed no activity on tested fungi and gram negative bacteria. In addition, micropropagated plant showed some activity against *Staphylococcus aureus* and *Escherichia coli*.

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بروفيل مواد الأيض الثانوية الفعالة لخلاصة نبات التاناسيتم سينايكوم البرى والمنمى معمليا بإستخدام كروماتوجرافيا السائل عالى الكفاءة مع مطياف الكتلة ونشاطها على سمية الخلايا وكمضادات للأكسدة ومضادات للميكروبات

رشا عطية, سميح الدهمي, دعاء أبو العينين, عفاف عبد الغني

قسم العقاقير- كلية الصيدلة- جامعة الزقازيق 44519 الزقازيق- مصر

تاناسيتم سينايكوم هو عشب معمر نادر ينمو في جبال جنوب سيناء (مصر). الهدف من هذا العمل هو تطوير دراسة مقارنة بين نواتج الأيض الثانوية للنباتات البرية والنباتات المكثرة في المختبر باستخدام جهاز فصل السوائل عالي الآداء و المرتبط بمطياف الكتلة (HPLC- ESI-MS / MS) حيث أتاحت هذه التقنية التعرف على 56 مركبًا بما في ذلك الأحماض العضوية وجليكوسيداتها وفلافونويدات. إجمالاً ، تم تحديد 52 مركبًا مبدئيًا من المستخلصات الميثانولية للنباتات البرية ، تم اكتشاف 10 منها أيضًا في المستخلص الميثانولي للنبات المكثر معمليا، بالإضافة إلى 4 مركبات تم اكتشافها فقط في النبات المكثر معمليا. أظهرت المستخلصات الميثانولية للنباتات البرية و المكثرة معمليا نشاطًا سامًا للخلايا معتدلاً ضد خطوط الخلايا Hep-G2 و HCT-116 و A-549باستخدام اختبار قابلية بقاء الخلية (MTT). كما تم فحص النشاط المضاد للأكسدة باستخدام مقايسة الجذور الحرة DPPH وأظهرت النتائج أن المستخلص الميثانولي للنباتات البرية له نشاط أفضل كمضاد للأكسدة مقارنة بالمستخلصات النباتية المختبرية. بالإضافة إلى ذلك ، تم قياس فعالية المبيدات الحشرية باستخدام طريقة الغمر وأظهر المستخلص الميثانولي للنبات المكثرة معمليا نسبة وفيات أعلى من مستخلص النباتية البرية على ناموسة *culex pipiens* . كما أشارت النتائج إلى أن مستخلصات النباتات البرية لها نشاط مضاد للميكروبات ضد البكتيريا موجبة الجرام Staphylococcus aureus و Bacillus subtilis ولم تظهر أي نشاط على الفطريات والبكتيريا سالبة الجرام. بينما أظهرت المستخلصات النباتية المكثرة معمليا بعض النشاط ضد بكتيريا Staphylococcus aureusموجبة الجرام والبكتيريا سالبة الجرام Escherichia coli