Available online at www.garmian.edu.krd

Passer Journal

Passer 5 (Issue 2) (2023) 237-242

http://passer.garmian.edu.krd/

GC-MS Profiling of Essential Oils from Aerial Parts of *Hypericum triquetrifolium* Turra using ITEX and HD Methods

Hoshyar A. Azeez[1*]

[1]*Department of Medical Laboratory Science, Sulaimani Polytechnic University, Sulaimani, Kurdistan Region, Iraq.*

Received 17 March 2023; revised 16 April 2023;
accepted 25 Apil 2023; available online 16 May 2023

DOI: 10.24271/PSR.2023.390199.1285

ABSTRACT

Hypericum (H.) triquetrifolium contains numerous bioactive molecules in the aerial parts. Such molecules have numerous biological activities, including antioxidant, anti-inflammatory, antidepressant, antimicrobial, antifungal, antiviral, and anticonvulsant compounds. Despite the presence of *H. triquetrifolium* in Iraq, no previous studies have examined the biochemical contents of plant in the region. This study is carried out to evaluate the variability of the essential oil profile of aerial parts of *Hypericum triquetrifolium* Turra. Samples were also analyzed using gas chromatography-mass spectrometry (GC-MS) with two different extraction methods, namely in-tube extraction (ITEX) dynamic headspace and hydrodistillation (HD). A total of thirty-three, forty-three, and thirty-nine compounds were identified in stem, leaves, and flowers, respectively. Monoterpene hydrocarbons were the main compounds in the essential oil extracted from different parts. The highest constituent detected in the ITEX/GC-MS in stem parts was alpha-pinene (20.47%). On the other hand, the predominated compound of the essential oil detected by the HD method was cubenol (26.72%). The essential oil extracted from leaves by ITEX and HD methods showed 43 compounds. ITEX/GC-MS detected 32 products in the essential oil of leaves; vubenol was the highest (23.64%), and Using ITEX and HD/GC–MS, a subset of 39 chemical components in *H. triquetrifolium* essential oil were detected in the flower. Twenty-five out of 39 compounds, were analyzed by ITEX/GC-MS. The main constituent was 3-methyl-nonane (27.76%). HD/GC-MS method was characterized by detecting 23 components in flower oil. The major chemical structure identified in the essential oil in flower by HD/GC-MS was the monoterpene hydrocarbon represented by alpha-pinene (18.39%). Our data indicates that the ITEX method is more accurate than HD in the separation of essential oil components in this plant.

Keywords: Hypericum triquetrifolium, ITEX/GC-MS, HD/GC-MS, Essential oil

1. Introduction

Several species belong to the *H.* genus is recognized to have a valuable worldwide effect as traditional medicinal plants. In particular, the species *H. triquetrifolium* is defined as a perennial herb, which is widely branched along most of the length, the inflorescence is yellow, pyramidal shape, and all aerial parts are covered with typical dark glands[1]. This species (*H. triquetrifolium*) is also characterized with a wide range of ecological amplitude that enable them to grow under various environmental conditions. This species is found in abundance along roads, as a weed in fields and meadows[2]. *H. triquetrifolium* is a very common plant In Iraq. It usually grows in the lower forest zone; in the moist steppe zone, and it is found on rocky mountain slopes, stony hillsides, and stepping plains, sometimes near streams, in vineyards, gardens, and fields, on sandstone, clay, loam, gravel[3]. The traditional medical uses of *H. triquetrifolium* are consequences of their contents with various bioactive molecules, including; naphthodianthrones, hypericin and pseudohypericin[4], hyperforin and adhyperforin[5], essential oils[6], xanthones[7], phenolic compounds (tannic acid, p-OH-benzoic acid, chlorogenic acid, and caffeic acid), procyanidins, and other water-soluble compounds[8], which have various biological values; namely antioxidant, anti-inflammatory, antidepressant, antimicrobial, antifungal, antiviral, and anticonvulsant activities.

Essential oils in various plant parts are the most important bioactive compounds characterized by their odorous principles. Such oils evaporate when they are exposed to the air. Essential oils are usually present in specialized secretory structures, including glandular hair, modified parenchyma cells and oil tube called vittae. These oils are mostly produced by either a direct function of the protoplasm through the decomposition of the resinogenous layer of the cell wall, or an indirect way by the hydrolysis of certain glycolysis[9].

* Corresponding author
E-mail address: hoshyar.azeez@univsul.edu.iq (Instructor).
Peer-reviewed under the responsibility of the University of Garmian.

In several species, the variability of the essential oil profile occurs by the effect of different factors, including developmental stages and plant organs maturation[10, 11], climate conditions[12], and soil texture[13]. Contents and compositions of essential oils in *Hypericum* plants are also affected by harvesting time[14, 15], the organ type, and the condition of biomass growth[16].

Several methods, such as hydrodistillation (HD), Solid Phase Microextraction (SPME) and The In-Tube Extraction Technique (ITEX), are used to extract essential oils from different parts of the plant. The quantitative and qualitative of extracting essential oils differ according to the extraction technique. The ITEX dynamic headspace has become a good alternative extraction method for a small amount of plant material in comparison with the classical one HD. The ITEX separates volatile compounds from a liquid or solid composition prior to GC analysis; and this feature is of importance for those samples that cannot be directly injected into the GC[17]. The main objective of the current study is to compare and evaluate variations of essential oils in *H. triquetrifolium* stems, leaves and flowers collected by ITEX and HD methods using GC-MS.

2. Materials and Methods

2.1 Plant Material

Stem, leaves, and flowers from 8-10 plants of *H. triquetrifolium* Turra were randomly collected during plant development from July – October in an experimental field located on the road in the Peramagrun mountain district of Sulaimania province in the North – East of Iraq at an altitude of (1150 m.a.s.l.). Stems, leaves, and flowers were individually separated for the biochemical studies.

In light of the morphological patterns described in Flora of Iraq, the plant was botanically identified by Dr. Saman A. Ahmad., Filed Crops Dept., College of Agricultural Engineering Sciences, Sulaimani University. Voucher specimens are kept at the Kurdistan Botanical Foundation Herbarium (ESSE 14682). Following the harvest of plant materials; they were air-dried at room temperature (22 ± 2 °C) for seven days, and followed by the identification of essential oil contents using ITEX / GC-MS and HD/GC-MS.

2.2 Isolation of the Essential Oil

2.2.1 ITEX Method

The current study was conducted at the Institute of Life Sciences (Institutul De Stiintele Vietii) at the University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca-Romania.

According to[17], the extraction of volatile oils was done using the ITEX technique which ten grams of the plant sample, including stems, leaves, and flowers were incubated at 60°C for 20 minutes. Thirty extraction strokes were investigated from the headspace phase of the vial. The volatile compounds were also adsorbed onto the ITEX fiber through thermal desorption. Therefore, an aliquot of the extracted volatiles was directly injected into the GC.

All other parameters were constantly maintained for all samples: syringe temperature was set at 60°C; agitation speed was set at 500 RPM; the final extraction volume was 1000 µL; extraction speed was set at 100 µL/second; temperatures of desorption and trap cleaning were at 200°C and 250°C respectively; and trap cleaning time was set at two minutes. The ITEX fiber used in this study was the ITEX-II Trap (G23) -SilicoNert 2000, Tenax TA 80/100 mesh, fiber. Following optimal incubation, a dose of 250 µL headspace sample was injected into the GC-MS.

2.2.2 Hydrodistillation method

The essential oil of the stems, leaves, and flowers of *H. triquetrifolium* was determined via hydrodistillation using a Clevenger-type apparatus. A quantity of 30 grams of dried samples were placed in 450 mL distilled water, and then placed on a heater adjusted at 60°C for 180 minutes since the distillation began. The volatile oil was dried over anhydrous sodium sulfate to remove water from the oil and stored in a sealed vial at 4°C until analysis[18].

2.3 Identification and Quantification by GC-MS

The current study was investigated at the Food Science and Technology Dept., USAMV-Cluj-Napoca-Romania. The analyses of samples were carried out using GC-MS (Shimadzu GC-MS QP-2010 model) equipped with an AOC-5000 autosampler (Combi PAL). A Zebrone ZB-5ms column of 50m x 0.32 mm i.d. and 0.25 µm film thickness was used for the analyses. The setup of the analysis was made by using injector temperature at 250 °C; pressure at 93.1 kPa; linear velocity of 44.0 cm/second; split ratio 1:200; carrier gas-helium; detector MS; ion source temperature 250.0 °C; interface temperature 250.0°C; MS mode EI, scan range 50-400u; scan rate 2000u/second. The program for column oven temperature was: 60 °C (3 minutes) to 160 °C at 3 °C/minute to 150 °C (10 minutes). Separated compounds were identified by comparing the detected mass spectra of our samples with their equivalent mass spectra libraries of NIST27 and NIST147.

3. Results and Discussion

In the present research, different parts (stems, leaves and flowers) of *H. triquetrifolium* Turra with native distribution in Iraq were gathered and subjected to ITEX and HD/GC-MS analysis of their compositions of essential oils (Tables 1, 2 and 3 in the supplementary file). Our data exhibited that the separation and identification of the essential oil compounds analyzed by the ITEX/ GC-MS method is more sensitive than HD in all plant parts.

3.1 Essential oils profile in leaves

The essential oil extracted from leaves of *H. triquetrifolium* by ITEX and HD methods revealed the presence of 43 compounds (Figure 1). The ITEX/GC-MS detected 32 products, including the essential oil of leaves, which were categorized into five groups: three monoterpene aldehydes, twenty-four monoterpene hydrocarbons, one monoterpene alcohol, two sesquiterpenes and one monoterpene acetate while HD/GS-MS analyzed 16 compounds which distributed into four class: one monoterpene aldehydes, twelve monoterpene hydrocarbons, one monoterpene

alcohol and two sesquiterpenes (Figure 2). Nineteen components were detected in leaves, and considered as unique compounds. The considerable components of the essential oil of leaves collected by ITEX/GC-MS included (E)-2-hexenal (12.63%), 2,3,3-trimethyl-octane (11.36%), 2,2,3,3-tetramethyl-hexane (6.42%), alpha-pinene (5.75%), 7-methyl-pentadecane (9.79%) and undecane (6.15%). Cumene (7.5%), 3-methyl-nonane (6.85%), vubenol (23.64%), beta-humulene (6.70%) and cadalene (5.85%) were better extracted by the HD method. The insolubility of these compounds could explain their absence or presence in the extraction methods. Only; 2,3,3-trimethyl-octane, alpha-pinene, gamma-muurolene, delta cadinene, calamenene and 2,5-diphenyl-1,4-benzoquinone were obtained and detected by the two methods. The lowest component, namely cis-linalool oxide is possibly produced by the Maillard reaction that leads to the thermal degradation of carbohydrate[19]. Nevertheless; the availability of hydrocarbon compounds is considered as the characteristic product of *H. triquetrifolium*, which confirms the results of[20-22]. The leaves of *H. triquetrifolium* collected from Calabria were analyzed for their composition of essential oil, and they found that nonane (8%), beta-pinene (8%), alpha-pinene (13%), myrcene (16%), beta-caryophyllene (5%), germacrene-D (10%), sabinene (13%) and caryophellene oxide (5%) were predominated compounds which were partially different from our results. It has been identified that two compounds of essential oils extracted from leaves by the HD method, namely n-tetradecane (25.70%) and alpha-himachalene (27%) as major components, which is not in concurrence with our results[23]. The difference between our results and researchers; may be related to the condition of cultivation, time of sample collection and genotypical patterns of samples. The influence of environmental factors on the oil composition contents has already been reported and suggested that the variations in the essential oils profile of plant species can be affected by seasonal alteration, geographic sharing, and growth cycle[24].

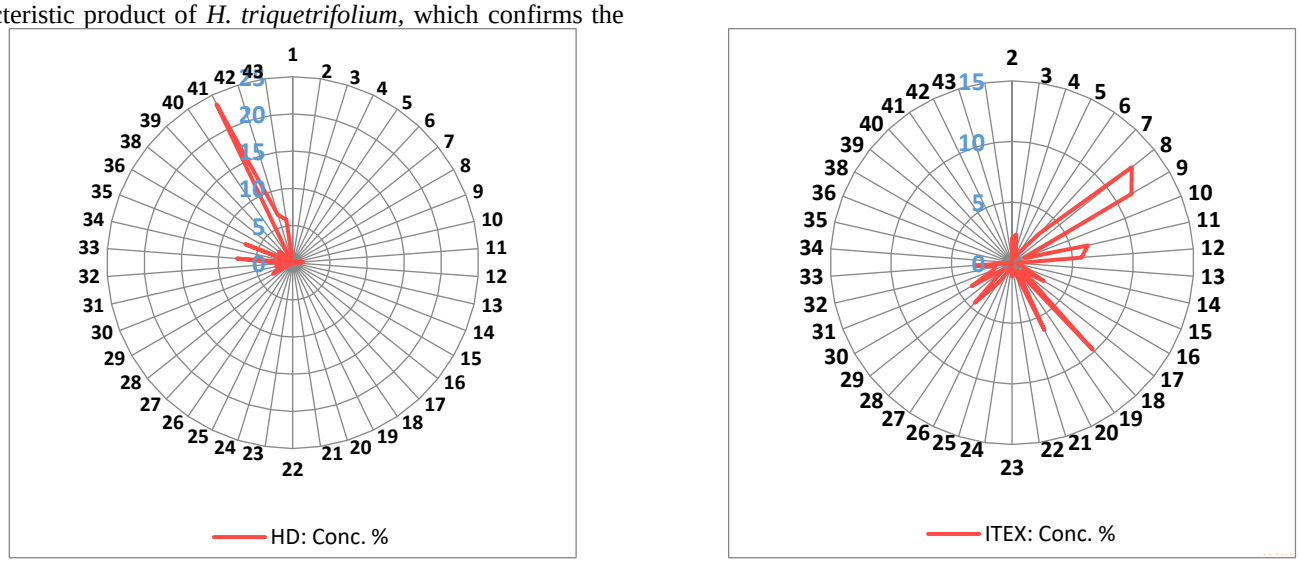

Figure 1: Chemical components of the essential oils in leaves of H. *triquetrifolium* extracted and detected by ITEX-GC/MS and HD/GS-MS.

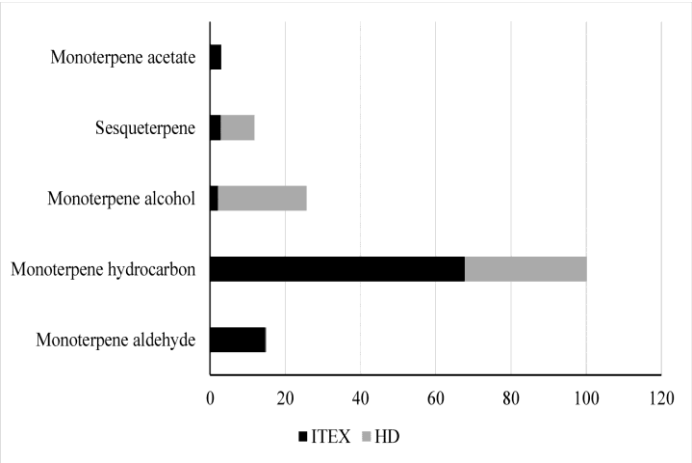

Figure 2: Class concentration of compounds extracted and detected by ITEX/GC-MS and HD/GC-MS of H. *triquetrifolium* leaves.

3.2 Essential Oils Composition in Stems

A total (of 21 and 15) oil products were separated from the stem by ITEX and HD methods, respectively (Figure 3). In the ITEX/GC-MS analysis, the 21 identified components were stored in four groups, including one monoterpene aldehyde, sixteen monoterpene hydrocarbons, one monoterpene ketone, and three monoterpene alcohols (Figure 4). Therefore, most compounds detected in the *Hypericum* volatile oil were monoterpene hydrocarbon compounds (89.81%). The main compounds detected by this method on the stems were alpha-pinene (20.47%), 2,4-dimethyl-hexane (11.52%), 2,3,3-trimethyl-octane (10.74%), camphene (9.79%) and undecane (9.28%) 1,3-dimethyl-benzene (5.62%). On the other hand, the number of compounds found in the HD/GC-MS was 15 in the stem. The identified products in this method were classified into three classes: monoterpene hydrocarbon (13 compounds), monoterpene alcohol (1 compound) and monoterpene ketone (1 compound) (Figure 2). The monoterpene hydrocarbon fractions were existing with a large amount of the essential oil (49.70%). Major compounds of the essential oil detected by this method were cubenol (26.72%), cumene (16.56%), 3-methyl-Nonane (7.87%), humulene (7.23%), and cadalene (6.67%). Also, the major oil components detected by the ITEX method in the stem are not present in oil products identified by the HD/GC-MS. The essential oil of *H. triquetrifolium* contains compounds of interesting biological properties. Previous studies stated that alpha-pinene is used as an anti-inflammatory, and in the treatment

of arthritic diseases[25,26]. Camphene exerted antitumor activity[27]. Therefore, humulene has anti-inflammatory activity[28].

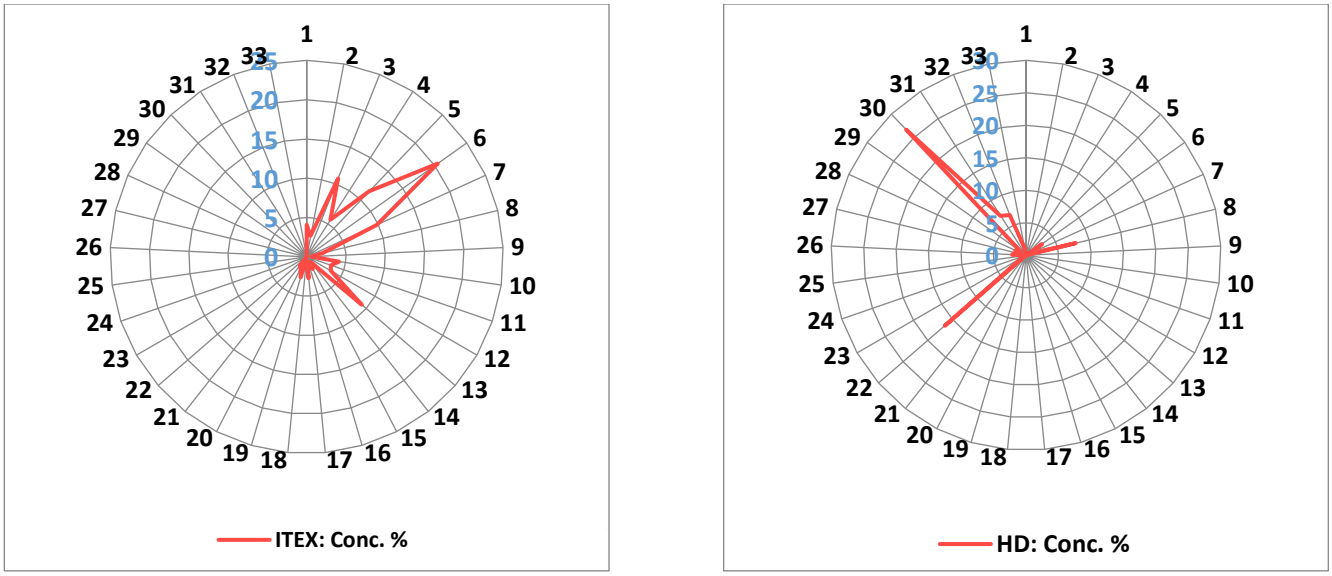

Figure 3: Constituents of the essential oils in stem of H. *triquetrifolium* Turra detected by ITEX-GC/MS and HD/GC-MS.

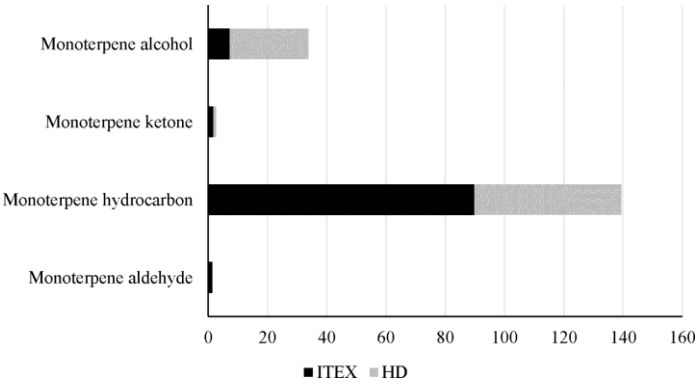

Figure 4: Class concentration of constituents isolated and detected by ITEX/GC-MS and HD/GC-MS of H. *triquetrifolium* stems.

3.3 Essential Oil Composition in Flowers

A subset of 39 chemical components in *H. triquetrifolium* essential oil were identified by ITEX and HD/GC–MS. Twenty-five out of 39 compounds were analyzed by ITEX/GC-MS (Figure 5). The chemical classes of compounds present in the oil of flowers were monoterpene aldehyde (2 compounds), monoterpene hydrocarbon (16 compounds), monoterpene ketone (2 compounds), monoterpene alcohol (3 compounds) and sesquiterpene (4 compounds) (Figure 6). The constituents identified in the essential oil of flowers by ITEX/GC-MS, being the principal components, are 3-methyl-nonane (27.76%), 2,3,3-trimethyl-octane (17.98%), 7-methyl-pentadecane (15.42%), alpha-pinene (14.44%), 2,4,6-trimethyl-octane (8.61%), and dodecane (6.99%) while the minor constituents in oil were delta-cadiene (0.10%), yalangene (0.11%), 2,3,5-trimethyl-decane (0.11) and 2-methyl-5-(1-methylethenyl)-2-cyclohexane-1-ol (0.11%). The HD/GC-MS method characterized 23 chemical components in oil of the flower (Table 2 and Figure 2). The major chemical structures identified in the essential oil of the flowers by HD/GC-MS were the monoterpene hydrocarbons represented by alpha-pinene (18.39%), 7-methyl-pentadecane (6.26%) and gamma-muurolene (6.25%) and sesquiterpene represented by caryophyllene oxide (10.84%) and germacrene D (4.17%). Two major compounds, namely alpha-pinene and 7-methyl-pentadecane were identified by two methods. The composition of the essential oil in the flowers showed 25 unique compounds.

Comparing the essential oil composition in this study and previous researches revealed some qualitative and quantitative differences. It is concluded that some notable compounds such as n-tetradecane (21.30%), alpha himachalene (14.20%), tricycline (10.70%) and cyclogeraniol acetate (7.60%) that were characteristics of a previous study (Jaimand K, Rezaee MB, Naderi M & Azadi R, Karimi SH 2012) were not identified in the present work. The flower of *H. triquetrifolium* from Calabria was studied for its composition of essential oil, and they found that nonane (15%), germacrene-D (13%), caryophyllene oxide (12%), beta-caryophyllene (11%), alpha-pinene (10%), myrcene (5%), beta-pinene (4%), sabinene (3%) were the predominant constituents[20]. These differences between our results and previous studies with regards to the composition of essential oil may be due to the condition of growth, time of sample collection and genotypes of underlying samples. Moreover, some authors stated that the difference of chemical compositions of essential oils observed in *H. perforatum* depending on the plant organs examined[29]. The essential oil of *H. triquetrifolium* contains some compounds owning interesting biological properties. Tricycline was found to be useful in the treatment of temporomandibular[30]. Caryophyllene oxide is considered an antifungal agent[31]. Previous studies communicated that the volatile oil having a large amount of α-humulene and germacrene D exhibited an exceptional cytotoxic influence to the human tumor cell line[32,33].

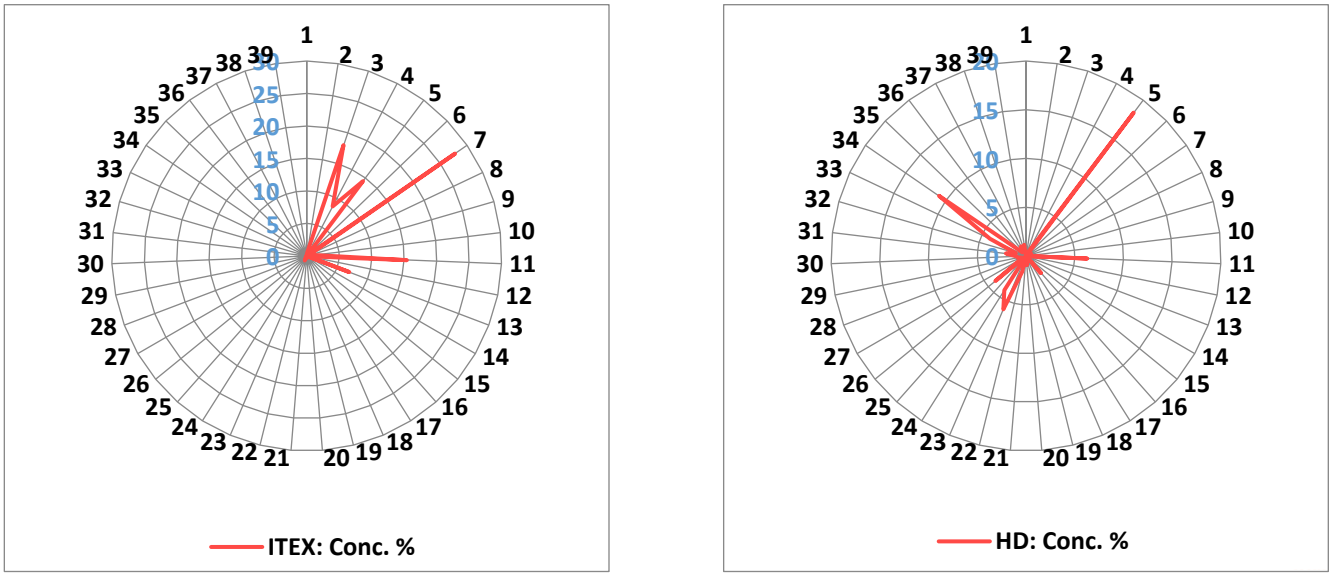

Figure 5: Chemical composition of the essential oils in flowers of H. *triquetrifolium* Turra extracted and detected by ITEX/GC-MS and HD/GC-MS.

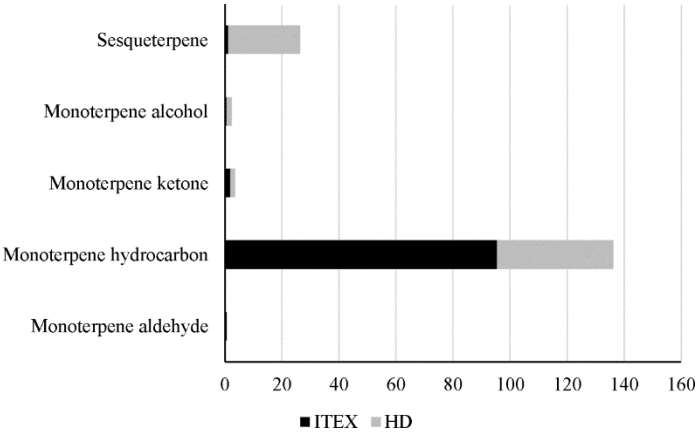

Figure 6: Class amounts of compounds extracted and detected by ITEX/GC-MS and HD/GC-MS of H. *triquetrifolium* flowers.

Conclusion

Results of the chemical composition of *H. triquetrifolium* were completely distinct from previous findings. This distinction might be an indication that the composition of essential oils is extremely altered by numerous parameters, namely seasons, growth stages and geographical distributions of samples. Previous detections by ITEX/GC-MS and HD/GC-MS have revealed qualitative and quantitative variations of the essential oil profile in different parts of *H. triquetrifolium*. We found that the ITEX method is more suitable technique than HD for the separation and identification of volatile compounds in all plant parts of *H. triquetrifolium.* The ITEX technique showed that the highest numbers of the essential oil constituents were detected in the leaves, flowers and stems, respectively.

Conflict of interests

None

Author Contribution and Funding Information

The Conceptualization, Methodology, Investigations, Data curation, Writing- Original draft, and Writing- Reviewing and Editing have been made by the corresponding author.

Funding

This study is financially supported by the Ministry of Higher Education and Higher Education, Iraq

References

1. Crockett, S.L. and N.K. Robson, *Taxonomy and chemotaxonomy of the genus Hypericum.* Medicinal and aromatic plant science and biotechnology, 2011. **5**(Special Issue 1): p. 1.
2. Azeez, H.A. and K.M. Ibrahim, *Hypericum Triquetrifolium callus cultures a potential source of phenolics and flavonoids.* JZS-Part A, Special Issue, 2014. **16**.
3. Guest, E., et al., *Flora of Iraq: Leguminales., edited by C.C. Townsend and Evan Guest. 1974. v. 4, pt. 1. Cornaceae to Rubiaceae (Hutchinson, 1959). pt. 2. Bignoniaceae to Resedaceai (Hutchinson, 1959), edited by C.C. Townsend and Evan Guest. 1980*. 1966: Ministry of Agriculture of the Republic of Iraq.
4. Çirak, C., et al., *Population variability of main secondary metabolites in Hypericum lydium Boiss.(Hypericaceae).* Iranian Journal of Pharmaceutical Research: IJPR, 2015. **14**(3): p. 969.
5. Cirak, C., et al., *Secondary metabolites of seven Hypericum species growing in Turkey.* Pharmaceutical biology, 2016. **54**(10): p. 2244-2253.
6. Azeez, H.A., *Comparison between Hypericum triquetrifolium leaves and derived calli in essential oil content.* Al-Nahrain Journal of Science, 2017. **20**(2): p. 123-130.
7. Tanaka, N. and Y. Takaishi, *Xanthones from Hypericum chinense.* Phytochemistry, 2006. **67**(19): p. 2146-2151.
8. Azeez, H.A. and K.M. Ibrahim, *Effect of Biotic Elicitors on Secondary Metabolite Production in Cell Suspensions of Hypericum triquetrifolium Turra.* Bulletin of the University of Agricultural Sciences & Veterinary Medicine Cluj-Napoca. Horticulture, 2013. **70**(1).
9. Fabiane, K.C., et al., *Physicochemical characteristics of the essential oils of Baccharis dracunculifolia and Baccharis uncinella DC (Asteraceae).* Revista Brasileira de Farmacognosia, 2008. **18**: p. 197-203.
10. Farhat, G., N. Affara, and H. Gali-Muhtasib, *Seasonal changes in the composition of the essential oil extract of East Mediterranean sage (Salvia libanotica) and its toxicity in mice.* Toxicon, 2001. **39**(10): p. 1601-1605.

11. Azeez, H.A., A.H. Ameen, and S.A. Faqe, *Variation in production of some bioactive molecules of Hypericum triqutrifolium Turra, during plant developmental stages.* 2017.

12. Medina-Holguín, A.L., et al., *Chemotypic variation of essential oils in the medicinal plant, Anemopsis californica.* Phytochemistry, 2008. **69**(4): p. 919-927.

13. Zheljazkov, V.D., et al., *Peppermint productivity and oil composition as a function of nitrogen, growth stage, and harvest time.* Agronomy Journal, 2010. **102**(1): p. 124-128.

14. Guedes, A.P., et al., *Variation of the essential oil content and composition in leaves from cultivated plants of Hypericum androsaemum L.* Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques, 2004. **15**(3): p. 146-151.

15. Tahir, N.A.-R., et al., *Exploring of bioactive compounds in essential oil acquired from the stem and root derivatives of Hypericum triquetrifolium callus cultures.* Natural product research, 2019. **33**(10): p. 1504-1508.

16. Guedes, A.P., et al., *Essential oils from plants and in vitro shoots of Hypericum androsaemum L.* Journal of agricultural and food chemistry, 2003. **51**(5): p. 1399-1404.

17. Salanţă, L.-C., et al., *Determination of the volatile compounds from hop and hop products using ITEX/GC-MS technique.* J Agroaliment Process Technol, 2012. **18**: p. 2.

18. Bousbia, N., et al., *Comparison of two isolation methods for essential oil from rosemary leaves: Hydrodistillation and microwave hydrodiffusion and gravity.* Food chemistry, 2009. **114**(1): p. 355-362.

19. Jerković, I., et al., *Comparison of hydrodistillation and ultrasonic solvent extraction for the isolation of volatile compounds from two unifloral honeys of Robinia pseudoacacia L. and Castanea sativa L.* Ultrasonics sonochemistry, 2007. **14**(6): p. 750-756.

20. Bertoli, A., et al., *Volatile constituents of the leaves and flowers of Hypericum triquetrifolium Turra.* Flavour and fragrance journal, 2003. **18**(2): p. 91-94.

21. Petrakis, P.V., M. Couladis, and V. Roussis, *A method for detecting the biosystematic significance of the essential oil composition: The case of five Hellenic Hypericum L. species.* Biochemical Systematics and Ecology, 2005. **33**(9): p. 873-898.

22. Hosni, K., et al., *Comparative analysis of the essential oils of Hypericum triquetrifolium Turra. Extracted by ultrasound, hydrodistillation and Soxhlet/dynamic headspace.* Medicinal and Aromatic Plant Science and Biotechnology, 2011. **5**(1): p. 100-104.

23. Jaimand, K., et al., *Chemical composition of the essential oils of six Hypericum species (Hypericaceae) from Iran.* Journal of Medicinal plants and By-product, 2012. **1**(1): p. 7-11.

24. Rowshan, V., A. Bahmanzadegan, and M.J. Saharkhiz, *Influence of storage conditions on the essential oil composition of Thymus daenensis Celak.* Industrial crops and products, 2013. **49**: p. 97-101.

25. Neves, Â., et al., *Screening of five essential oils for identification of potential inhibitors of IL-1-induced NF-κB activation and NO production in human chondrocytes: characterization of the inhibitory activity of α-pinene.* Planta medica, 2010. **76**(03): p. 303-308.

26. Russo, E.B., *Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects.* British journal of pharmacology, 2011. **163**(7): p. 1344-1364.

27. Girola, N., et al., *Camphene isolated from essential oil of Piper cernuum (Piperaceae) induces intrinsic apoptosis in melanoma cells and displays antitumor activity in vivo.* Biochemical and Biophysical Research Communications, 2015. **467**(4): p. 928-934.

28. Passos, G.F., et al., *Anti-inflammatory and anti-allergic properties of the essential oil and active compounds from Cordia verbenacea.* Journal of ethnopharmacology, 2007. **110**(2): p. 323-333.

29. Crockett, S.L., *Essential oil and volatile components of the genus Hypericum (Hypericaceae).* Natural product communications, 2010. **5**(9): p. 1934578X1000500926.

30. Cascos Romero, J., et al., *The use of tricyclic antidepressants in the treatment of temporomandibular joint disorders: systematic review of the literature of the last 20 years.* 2009.

31. Yang, D., et al., *Use of caryophyllene oxide as an antifungal agent in an in vitro experimental model of onychomycosis.* Mycopathologia, 2000. **148**(2): p. 79-82.

32. Sylvestre, M., et al., *Chemical composition and anticancer activity of leaf essential oil of Myrica gale L.* Phytomedicine, 2005. **12**(4): p. 299-304.

33. Setzer, W.N., et al., *Leaf oil compositions and bioactivities of abaco bush medicines.* Pharmacologyonline, 2006. **3**: p. 794-802.