Comparison between *Hypericum triquetrifolium* Leaves and Derived Calli in Essential Oil Content

Hoshyar A. Azeez
Department of Biology, College of Sciences, Sulamani University, Sulaimaniyah-Iraq.
Corresponding Author: hoshyar.azeez@univsul.edu.iq

Abstract

The analysis of essential oil using the in-tube extraction technique (ITEX) from the plant leaves and derived calli (fresh calli, dry calli and cell suspension culture) of *Hypericum triquetrifolium* Turra., initiated from leaves. The plant grows wild in Kurdistan region of Iraq. Studied parameters were determined using in-tube extraction coupled with gas chromatography - mass spectrometry system (ITEX/ GC-MS). A total of 33 compounds were identified as essential oils in leaves, the dominant constituents were measured such as Hexenal, (E) (12.63%), Octane, 2,3,3-trimethyl (11.36%), Pentadecane, 7-methyl- (9.7%), Undecane (6.15%) and alpha. -Pinene (5.75%), while the analysis of fresh calli derived from leaves showed 22 types of essential oil; Dodecane (23.78%), Nonane, 3-methyl- (10.45%), Limonene (9.68%), Furan, 2-pentyl-(9.11%), Toluene (8.18%) and Undecane (7.45%). On the other hand, 21 oil components were found in dry calli; the major compounds were identified as Limonene (17.18%), Undecane (12.21%), Beta.-Myrcene (5.51%) and Toluene (4.93%). However, only 23 oil components were determined in cell suspension culture, the main essential oils were; Undecan (42.92%), Octane, 2,4,6-trimethyl (13.71%), Oxirane, 2-(1,1-dimethylethyl)-3-methyl (9.84%), Limonene (6.69%) and Toluene (2.98%).
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Keywords: *H. triquetrifolium*, ITEX, GC-MS, Lemonene, Undecan.

Introduction

Plants are a main source of new products with medicinal value in drug development. Today, several active compounds derived from different plant parts are important natural drugs and used in worldwide [1]. The genus Hypericum belongs to the Clusiaceae family, encompasses 450 different species, but only sixteen species are found in flora of Iraq [2]. *Hypericum triquetrifolium* Turra is an herbaceous perennial plant and one of the Iraqi wild species of Hypericaceae which is distributed in the north and north-west of the country, the local Arabic name for this species is Roja and the Kurdish name is Swrnatik [3]. According to the literature, this species contains grate groups of phytochemical like phenolics such as tannic and caffeic acid, flavonoids like rutin and hypersoid [4], hypericin, and psudohypericn [5], and hyperforin [6], essential oil [7] and three new phloroglucinol derivatives named hyperscabrins A, B, and C, were determined the first time by [8].

The essential oil is defined as the product obtained from different plant organs (roots, stems, leaves, flowers, fruits and seeds) by various methods of distillation such as, hydrodistillation, steam distillation or dry distillation or by other suitable mechanical process. Volatile compounds found only in 10% of the plant kingdom which are produced and stored in plants in special secretory structures, such as glands, secretory hairs, secretory ducts, secretory cavities or resin ducts [9]. This product is highly complex mixtures of valuable volatile compounds like terpenes, terpenoids and aliphatic derivatives generally characterized by strong odour [10].

Essential oils play very important role inside the plant like defense system and signaling processes, for example, this type of secondary metabolites are participate in plant defense against microorganisms such as bacteria, fungi, different types of plant viruses, insects and herbivorous, attraction of the insects for pollination and fruit dispersing by animals, regulate water relation and allelopathic interactions [11,12,13]. Also, they are valuable active compounds intervene as raw material in many industrial fields, like agronomic, food, cosmetics, pharmaceutical, and perfume industries [14]. The production of secondary metabolites from undifferentiated

plant cells and callus cultures has been studied intensively since the 1960's. Indeed, successful protocols of cell suspension cultures can offer a repeatable method to produce secondary metabolites from elite mother plants with easily controlled conditions and with a continuous supply of material [15].

Previous work was carried out on this species in Iran which revealed a wide range of volatile oil obtained by hydrodistillation from the aerial parts of *H. triquetrifolium* Turra. They were analyzed using GC and GC-MS. Components (97.1% of the total composition) were detected as volatile oils. Germacrene-D (21.7%), β-caryophyllene (18.3%), β-cadinene (6.4%), *trans*-β -farnesene (4.3%), α-humulene (3.8%), β-selinene (3.7%), γ-cadinene (3.3%) and *trans*-phytol (3.2%) were found to be the major constituents [7]. Thus, the aim of the current study was to study the quantitative and qualitative valuable essential oils in plant leaves, fresh, dry calli and cell suspension cultures initiated from leaves.

Materials and Methods

Plant collection and identification

Leaves of *H. triquetrfolium* Turra intact plant were randomly collected from a dry rocky place within the Gllazarda mountain district of Sulaimania province, Iraq (35°21'49.35"N, 45°29'37.31 E; 1100 m.a.s.l.) in September and identified by the taxonomist Dr. Saman A. Ahmad., Filed Crops Dept., Faculty of Agricultural Science, Sulaimani University according to the morphological description presented in flora of Iraq [16]. Plant samples were dried at room temperature 25 ± 2°C for one week to a constant moisture content of 10%. Plant leaves were used as a source of essential oils determination.

Sterilization of explants

Mature leaves were excised, rinsed with tap water for 10 minutes then submerged in 10% diluted commercial bleach for 15 minutes, rinsed with sterilization DH_2O, then submerged in 70% ethanol for 30 seconds. Explants then rinsed with sterilized DH_2O three times inside a laminar air flow cabinet. The ends of each leaf were cut to remove tissues affected by sterilization solution. Finally, leaf discs 1 cm in diameter were transferred to the culture medium [16].

Initiation of Callus culture

Leaf explants were placed on MS medium containing Thidiazuron (TDZ) at the concentration 2.0 mg/L and indole-3-acetic acid (IAA) at 0.5 mg/L to determine the most appropriate plant growth regulator for callus induction. The cultures were incubated at 25^{o}C for 16/8 hrs (light/dark) photoperiod at a light intensity of 1000 lux. The response of these explants to callus initiation was evaluated after 4 weeks of culture [17].

Cell suspension cultures

Cell suspension cultures were initiated and maintained according to the previously described methods [18 & 24].

Analysis by ITEX / GC-MS

The extraction of volatile compounds was performed using the in-tube extraction technique (ITEX). Samples (2, 10 and 20 g of dry, fresh and cell suspension calli) initiated from leaf explants were incubated respectively at 60^{o}C for 20 min. and 30 extraction strokes were performed from the headspace phase of the vial and the volatile compounds were adsorbed onto the ITEX fiber by thermal desorption, an aliquot of the extracted volatiles was directly introduced in the GC injector. The analysis of volatile compounds was carried out using a GC-MS QP-2010 (Shimadzu Scientific Instruments, Kyoto, Japan) model gas chromatograph-mass spectrometer. The volatile compounds were separated on a Zebron ZB-5ms capillary column of 50 m x 0.32 mm i.d and 0.25 mm film thickness. The carrier gas was helium, 1.3 ml/min and the split ratio 1:20. The temperature program used for the column oven was: from 40˚C rising to 2800˚C at 5˚C/min and held for 5 min. The injector, ion-source and interface temperatures were set at 250˚C. The MS mode was electron impact (EI). The mass range scanned was 50-550 m/z. The volatile compounds were tentatively identified using the spectra of reference compounds from NIST27 and NIST147 mass spectra libraries. The results were expressed as area % from the total area of peaks (100%) [19].

Results and Discussion

The major compounds identified in the essential oils of the plant leaves of *H. triquetrifolium* are listed in Table (1.A) total of 33 types of oils were determined, representing 92.95% of the total oil recording; Hexenal, (E) (12.63%), Octane, 2,3,3-trimethyl (11.36%), Pentadecane, 7-methyl (9.7%), Naphthalene derivatives (8.82%), Undecane (6.15%), alpha.-Pinene (5.75%), and Cycloheptane, 4-methylene-1-methyl-2-(2-1-yl)-1-vinyl-methyl-1-propen (4.5%) are the most predominant of the 33 compounds. In a previous work on *H. triquetrifolium* the essential oils were obtained by hydrodistillation from the aerial parts of plants, they were 1-hexanal (18.8%), 3-methylnonane (12.5%) and - pinene (12.3%) were shown to be the major compounds [20], but a total of 109 compounds included 92.2% of total oils that determined were identified as essential oils were obtained from the aerial parts of Tunisian endemic *H. triquetrifolium* Turra (Clusiaceae) [21]. A total of 22 types of essential oils were identified from fresh calli initiated from leaves, representing 98.26% of the total oil. The most abundant constituents were Dodecane (23.78%), Nonane, 3-methyl-(10.45%), Limonene (9.68%), Furan, 2-pentyl-(9.11%), Toluen (8.18%), Undecane (7.45%), 2,5-Cyclohexadiene-1,4-dione, 2,5-diphenyl (5.7%) and Eucalyptol (4.07%) Table (2).

Chemical analysis presented in Table (3) showed that 21 types of essential oils were found in dry calli of *H. triquetrifolium* that initiated from leaf explants grown at 16 hrs light and 8 hrs dark are characterized by a high content of Benzaldehyde derivatives (26.7%), Limonene (17.18%), Cyclohexane derivatives (14.67%), Undecane (12.21%), Beta.-Myrcene (5.51%) and Toluene (4.93%).

Results obtained from this study are in agreement with those of [22], who reported that essential oils isolated from *in vitro* cultures were less complex than from *in vivo* plants, while the main essential oil contents in cell suspension culture are; Limonene (6.69%), Toluene (2.98%), Eucalyptol (2.53%), o-Cymene (0.77%) and alpha-Pinene (0.53%) as described in Table (4). Results are in accordance with [17, 23], who mentioned that the accumulation of some secondary metabolites in *in vitro* cultures (callus and cell suspensions) were higher than those in wild type plants after optimization of cultural conditions. This may due to the presence of plant growth regulators (PGRs) supplemented to the medium for callus initiation. The effects of different PGRs on accumulation of some active compounds have previously been studied and confirmed in various plants and their tissue cultures in some medicinal plants that produce secondary metabolites more than intact plant [24].

Table (1)
Essential oils Composition in H. triquetrifolium leaves analyzed by Head space.

Essential oil	Ret. time	Conc. %	Area	Height	SI
1-Penten-3-ol	4.056	2.03	680924	300847	97
Pentane, 2,2,4-trimethyl-	4.106	2.35	788545	311034	97
Furan, 2-ethyl-	4.245	1.03	347387	176424	95
2-Pentanone, 3-methyl-	5.104	0.37	122989	58877	95
2-Butenal, 3-methyl-	5.801	0.98	327951	124524	95
Cyclohexane, (1,1-dimethylethyl)-	6.061	3.29	1105612	392010	99
2-Hexenal, (E)-	7.37	12.63	4238895	1630041	99
Octane, 2,3,3-trimethyl-	7.671	11.36	3814119	1652218	99
p-Xylene	7.848	1.08	363192	164253	94
Hexane, 2,2,3,3-tetramethyl-	8.661	6.42	2156533	848513	99
.alpha.-Pinene	9.737	5.75	1929745	596385	99
Nonane, 3-methyl-	10.833	0.41	138437	68805	99
Octane, 3,5-dimethyl-	11.763	0.48	161330	94975	81
Benzene, 1-ethyl-2,4-dimethyl-	12.61	1.04	349893	126768	93
Cyclohexanol,1-methyl-4-(1-methylethenyl)-, acetate	12.766	3.01	1011443	388712	96
Benzeneacetaldehyde	13.223	1.08	361428	127717	98
Pentadecane, 7-methyl-	13.837	9.79	3284678	1371280	99
2-Furanmethanol,5-ethenyltetrahydro-alpha.,.alpha.,5-trimethyl-	14.098	0.82	275328	102378	87
Undecane	14.995	6.15	2063901	816827	98
Nonanal	15.141	0.85	286722	103367	96
Undecane, 2,3-dimethyl-	17.237	0.52	175015	70977	96
Hexadecane	18.168	1.15	386947	145230	96
Undecane, 3-methyl-	20.114	0.02	8132	15463	68
Dodecane	21.205	0.29	97392	54539	81
Ylangene	23.401	0.84	280950	89826	96
Copaene	23.585	1.94	652656	225799	91
Cycloheptane,4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	24.853	4.5	1509083	427415	98
1R,3Z,9S-2,6,10,10 Tetramethylbicyclo [7.2.0]undeca-2,6-diene	25.093	0.9	303667	104592	99
Naphthalene,1,2,3,4,4a,5,6,8aoctahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)-	26.294	3.85	1292126	465111	98
Naphthalene,1,2,3,4,4a,5,6,8aoctahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)-	27.325	1.92	643098	213535	98
Naphthalene1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	27.423	1.62	544998	182986	89
Naphthalene,1,2,3,4-tetrahydro-1,6 dimethyl-4-(1-methylethyl)-, (1S-cis)-	27.524	1.43	478556	163600	96
2,5-Cyclohexadiene-1,4-dione,2,5 diphenyl-	45.681	3.05	1022131	265322	96

Ret. time= Retention time SI= Similarity

Table (2)
Composition of essential oils in H. triquetrifolium fresh calli initiated from leaves analyzed by Head space.

Essential oil	Ret. time	Conc. %	Area	Height	SI
Oxirane, 2-(1,1-dimethylethyl)-3-methyl-	4.666	2.53	294818	123374	94
1,3,5-Cycloheptatriene	5.176	0.74	86664	54290	96
Toluene	5.273	8.18	954154	393178	98
Furan, 2,3-dihydro-4-methyl-	5.694	1.0	116384	50295	98
Cyclohexane, (1,1-dimethylethyl)-	5.963	1.93	225213	80843	98
Octane, 2,3,3-trimethyl-	7.61	1.61	188116	91343	94
Undecane	8.617	7.45	868926	290971	99
.alpha.-Pinene	9.701	1.07	125175	56556	98
Benzaldehyde	10.613	2.39	278944	92302	95
Nonane, 3-methyl-	10.815	10.45	1219198	418573	99
Phenol	10.964	1.35	157034	64554	96
Furan, 2-pentyl-	11.43	9.11	1062462	316604	99
o-Cymene	12.603	0.68	79048	35575	99
Limonene	12.761	9.68	1129006	401965	99
Eucalyptol	12.886	4.07	475269	168715	98
Benzeneacetaldehyde	13.228	1.09	127549	47386	100
Heptane, 5-ethyl-2-methyl-	13.847	1.05	121992	53788	99
Acetophenone	13.949	3.05	355683	121064	99
Dodecane	15.006	23.78	2773916	1094373	99
2-Nonenal, (E)	16.939	0.53	62415	30908	94
Propanoic acid, 2-methyl-, decyl ester	33.797	0.82	95712	39494	99
2,5-Cyclohexadiene-1,4-dione, 2,5-diphenyl-	45.716	5.7	665291	193871	98

Ret. time= Retention time SI= Similarity

In case of *H. perforatum* L. one hundred and thirty four compounds were identified mounting; 98.7% of the total oil, were obtained from fresh aerial parts. The main components of the oil were: germacrene D (18.6%), 2-methyloctane (9.5%), bicyclogermacrene (5.0%) and (*E*)-β-ocimene (4.6%) [25], some of these oil derivatives were not found in the current study, this may due to the variation of *Hypeicum* sp. Mainly because seasonal variation, geographic distribution, phenological cycle and type of the organ in which essential oils are produced and/or accumulated [26, 27], as well as conditions of biomass growth (e.g. in *vitro* or *in vivo*) [26]. These factors are known to influence the essential oil content and composition of this genus.

In the present study, the monoterpene hydrocarbon (limonene and cymene) recorded high percentage in dry callus cultures (17.18% and 2.48%) respectively, while suspension culture initiated from leaves recorded low limonene content (6.69%) but cymene concentration in fresh calli recorded minimum percentage (0.68%). On the other hand, these compounds were not detected in plant leaves. Fresh and dry calli initiated from leaves accumulated 4.07% and 1.79% of eucalyptol respectively while cell suspension cultures accumulated 2.53%. Dodecane showed the highest percentage (23.78%) in fresh callus culture, while dodecane recorded low amount in plant leaves reached 0.29%, but dodecane was not detected in dry calli and cell suspension cultures that initiated from leaf explants grown at 16 hrs photoperiod.

Table (3)
Essential oils composition of H. triquetrifolium dry calli initiated from leaves analyzed by Head space.

Essential oil	Ret. time	Conc. %	Area	Height	SI
Toluene	5.433	4.93	417521	166554	95
3-Penten-2-ol	5.61	1.76	149272	47052	91
Cyclobutanol, 2-ethyl- 2Ethylcyclobutanol	6.003	2.08	176277	38812	90
Cyclohexane, (1,1 dimethylethyl Cyclohexane, tert-butyl tert Butylcyclohexane 1-tert-Butylcyclohexane	6.088	14.67	1243806	530639	99
Acetic acid, butyl ester	6.406	0.93	78623	29848	99
Acetic acid, pentyl ester	8.083	0.73	61944	31363	97
Hexane, 2,4-dimethyl-	8.703	0.65	54782	32771	94
2,4-Heptadiene, 2,4-dimethyl- (4E)- 2,4-Dimethyl-2,4-heptadiene	9.054	2.39	202174	88894	99
alpha.-Pinene	9.783	1.79	151762	65873	100
Butane, 2,2-dimethyl-	10.875	0.62	52876	22688	100
.beta.-Myrcene	11.47	5.51	467126	143408	99
Acetic acid, hexyl ester	12.167	0.68	57662	29725	95
Octane, 3,3-dimethyl-	12.167	0.48	40632	23788	39
o-Cymene	12.647	2.48	210525	86904	99
Limonene	12.81	17.18	1456494	561348	99
Eucalyptol	12.936	1.79	152019	61452	94
3-Ethyl-3-methylheptane Heptane, 3-ethyl-3-methyl-	13.564	0.35	29953	21762	90
Undecane	15.042	12.21	1035042	423956	98
Benzaldehyde, 2,4-dimethyl- 2,4-Dimethylbenzaldehyde 2,4-Dimethylbenzenecarboxaldehyde	18.804	26.7	2263483	755977	99
Benzene, 1,3-bis(1,1-dimethylethyl)	19.74	0.77	65192	26861	90
Hexane, 3,3-dimethyl	20.5	0.54	45408	16352	85

Ret. time= Retention time SI= Similarity

High percentage of α-pinene (5.57%) was found in plant leaves while the less percentage was found in cell suspension cultures (0.53%). Undecane was found at high levels (42.92%) in cell suspension cultures initiated from leaf explants. Meantime percentage in plant leaves was found to be lower than cell suspension cultures (6.15%). Data also exhibit a high amount of toluene in leaves fresh calli (8.18%) compared with the dry calli and cell suspension cultures recording 4.93% and 2.98% respectively, while this was not detected in intact leaves**.**

To our knowledge, the analysis of essential oil in the present study using ITEX/ GC-MS method was reported for the first time in callus and cell suspension cultures.

Table (4)
Essential oil composition in H. triquetrifolium cell suspension cultures initiated from leaves analyzed by Head space.

Essential oil	Ret. time	Conc. %	Area	Height	SI
Oxirane, 2-(1,1-dimethylethyl)-3 methyl	4.763	9.84	2656847	838369	99
Toluene	5.375	2.98	805194	355684	99
2-Buten-1-ol, 3-methyl	5.533	2.21	595911	197306	99
2-Butenal, 3-methyl	5.739	2.87	775125	257735	99
3-Hexen-1-ol, (Z)-	7.456	0.38	102941	49632	95
1-Hexanol	7.804	0.37	100174	49340	100
1-Butanol, 3-methyl-, formate	8.035	1.83	494530	216136	98
Octane, 2,4,6-trimethyl-	8.664	13.71	3702148	1071360	99
2,4-Heptadiene, 2,4-dimethyl-	9.024	0.36	96929	41363	99
alpha.-Pinene	9.75	0.53	144451	65282	99
Benzaldehyde	10.644	0.8	215772	93393	96
Nonane, 3-methyl	10.846	2.38	644051	256334	99
Beta.-Myrcene	11.434	2.12	573587	229170	98
o-Cymene	12.629	0.77	207930	73352	94
Limonene	12.789	6.69	1807152	607698	98
Eucalyptol	12.906	2.53	682224	220450	98
Octane, 2,3,3-trimethyl-	13.861	0.44	119166	64685	98
Acetophenone	13.982	1.52	410591	129993	98
Undecane	15.018	42.92	11589833	4587385	99
Anisole, p-allyl-	18.199	1.68	452690	166713	98
n-Dodecyl acetate	29.538	0.85	228509	87757	97
Butyric acid, tetradecyl ester	33.806	1.7	460364	154026	100
2,5-Cyclohexadiene-1,4-dione, 2,5 diphenyl-	45.727	0.52	139639	65818	80

Ret. time= Retention time SI= Similarity

Conclusion

The comparative analysis of the essential oil of *H. triquetrifolium* obtained by ITEX technique showed variations in quantitative and qualitative oil content. Essential oil composition in *in vitro* cultures (callus and cell suspension) produce more compounds than intact plant. The essential oil extracted from plant leaves were more complex than that extracted from *in vitro* culture.

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