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Quantitative analysis of the essential oil of *Origanum vulgare* (L) growing in the Kashmir valley

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Abstract
Aromatic and medicinal plants are widespread and Kashmir valley possesses their rich repository. The agro climatic conditions prevailing in this region provide an ideal habitat for the natural growth of variety of plant species which are of prime importance in pharmaceutical, food and cosmetic industries. The present study was conducted to analyse the essential oil obtained from *Origanum vulgare* (L.) family Lamiaceae and the variability in oil composition of the same species growing at different geographical locales. Essential oils obtained from aerial parts of three *Origanum vulgare* L. samples, growing wild in different locations viz. Srinagar, Pulwama and Yaarikhah regions of Kashmir were obtained by hydro-distillation of the aerial parts and analysed by Gas Chromatography coupled with Mass Spectrometry (GC-MS) for their chemical composition. Overall sixteen to thirty four numbers of constituents were isolated and identified in its oil. The major compounds were gamma terpinene, para cymene, carvacrol, cymene, sabinene and cis sabinene hydrate acetate. Three chemotypes were found: the first growing at Srinagar locale with a prevalence of γ-terpinene/ cymene / sabinene; the second growing at Pulwama characterised with a prevalence of γ-terpinene/p- cymene / sabinene /carvacrol and the third growing at Tangmarg featuring a prevalence of p-cymene / carvacrol /cis-sabinene hydrate acetate /γ-terpinene. The results of essential oil analysis show rich composition of oil compounds. Moreover, the percentage composition of the main components of its essential oil showed marked differences as a result of changing geographical regions. One can expect that the germplasm of *O. vulgare* is a potentially important source of genetic variation.

Keywords: *Origanum vulgare,* phytochemistry, GC-MS, essential oil.

Introduction
Origanum is an important multipurpose perennial medicinal plant which belongs to the family Lamiaceae, tribe Mentheae and comprises of 42 species and 18 hybrids widely distributed in Eurasia and North Africa (Ietswaart, 1980; Duman *et al*., 1988, Kokkini, 1997) [13, 9, 19]. It is native to the mountainous parts of Mediterranean region of Europe and Asia. *Origanum vulgare L.* (Oregano) is one of the important ethno medicinal plant having Ayurvedic importance and trade values as well. *Origanum vulgare (L.)* is locally known as Jungali Tulsi or Oregano or Himalayan marjoram. This is the only species of genus *Origanum* which is found in India. It is found in temperate Himalayas from Kashmir to Sikkim at an altitude of 1500 – 3600m. It is particularly grown in Shimla Hills, Giligit, Nilgris and in the Kashmir valley.

Oregano is the commercial name of those *Origanum* species that are rich in the phenolic monoterpenoids, mainly carvacrol and occasionally thymol (D'antuono *et al*., 2000) [6]. A number of chemically related compounds i.e. *p*-cymene, γ-terpinene, carvacrol methyl ethers, thymol methyl ethers, carvacrol acetates and thymol acetates, as well as *p*-cymenene, *p*-cymen-8-ol, *p*-cymen-7-ol, thymoquinone, and thymo hydroquinone are present in the oil. The other chemical compounds, usually less significant quantitatively, are the acyclic monoterpenoids such as geraniol, geranyl acetate, linalool, linalyl acetate and β-myrecene. Some sesquiterpenoids such as β- caryophyllene, β-bisabolene, β-bourbonene, germacrene-D, bicyclogermacrene, α-humulene, α- muurolene, γ-muurolene, γ-cadinene, allo-aromadendrene, α-cubebene, α-copaene, α-cadinol, β-caryophyllene oxide and germacrene-D-4-ol could also be present. In some *Origanum* plants sabinyl compounds such as *cis*- and/or *trans*-sabinene hydrate, α-thujene, sabinene, *cis*- and *trans*-3 sabinene hydrate acetates, *cis*- and *trans*-sabinol and sabina ketone can also be found (Skoula and Harborne 2002) [34]. Despite its economic importance the genetic resources and variability, its potential for utilization have not yet been fully explored (Putievsky *et al*., 1997) [29]. This is due to its high heterogeneity and complex taxonomy. A number of studies have shown that variation within species may occur in its morphological and chemical features (Kokkini *et al*., 1994, 1997a, 1997b; Chalchat and Pasquier, 1998; Skoula *et al*., 1999; D. Antuono *et al*., 2000) [16, 18, 19, 4, 33, 6].

There have been previous investigations on the chemical composition and content of the essential oil of *O. vulgare* from the Kumaon Himalayas (Verma *et al*., 2010) [39], Campania, Southern Italy (De Martino *et al*., 2009), Bosnia (Stoilova *et al*., 2008) [36], Bulgaria (Kula *et al*., 2007) [21], Lithuania (Mockute *et al*., 2004) [25]. However, there is no report on the comparative study of the essential oil of *O. vulgare* growing at different geographic locales of the Kashmir Himalaya. In this study chemical analysis and the comparative assessment of essential oils of *O. vulgare* grown at different locales of Kashmir Himalayas was done, because the geographic characteristic of the particular growing region is an important factor determining its aroma composition and other specific characteristics.

Materials and methods

Essential oil isolation: The plant material of *Origanum vulgare* (L.) collected from three different geographical locales of Kashmir Himalaya (viz. Srinagar, Pulwama and Yaarikhah) were identified at KASH herbarium, University of Kashmir, under voucher specimen Nos. 1822, 1823 and 1900 respectively. The aerial parts like stem, branches and leaves weighing 100 grams each, were separately shade dried, crushed and subjected to hydro distillation using a Clevenger apparatus until oil distillation ceased after 3 hours, according to the protocol described in the European Pharmacopeia (Council of Europe, 2011) [10]. The volume of essential oils was determined from a calibrated trap. The essential oils in the distillate were dried over anhydrous sodium sulphate (Na_2SO_4) so as to remove water after extraction. The essential oils were separately stored in sealed vials at 4^0C until analysed. The oil yield was calculated on fresh weight basis percentage of volume by weight.

Analysis of Essential Oils

Analysis of Essential Oil was done using Gas Chromatography coupled with mass spectrometry (GC/MS) to know the composition of oil and quantity of each composition.

Gas Chromatography-Mass Spectrometry (GC/MS) analysis

GC-MS analysis were carried on a Varian Gas Chromatograph series 3800 fitted with a VF-MS fused silica capillary column (60m x 0.25mm, film thickness 0.25 µm) coupled with a 4000 series mass detector under the following conditions: injection volume 0.5µl with split ratio 1: 60, Helium as a carrier gas 1.0 ml / min constant flow mode, injector temperature 230 ^{0}C, oven temperature 60 to 280^{0}C at 3 ^{0}C / min. Mass spectra: electron impact (EI^+) mode 70 ev and ion source temperature 250 ^{O}C. Mass spectra were recorded over 50 - 500 amu range.

Identification of essential oil constituents was done on the basis of retention Index (RI), determined with respect to homologous series of n- alkanes (C_5-C_{28}, Polyscience Corp., Niles IL) under the same experimental conditions, co - injection with standards (Sigma Aldrich and standard isolates), MS Library search (NIST 05 and Wiley), by comparing with the MS literature data (Jennings and Shibamoto, 1980; Adams, 2007; NIST 05) [14, 1]. The relative percentages of individual components were calculated based on GC peak area (FID response) without using correction factors.

Results and discussion

Chemical composition of the essential oil

Chemical composition of essential oils of *Origanum vulgare* (L.) growing at different geographical locales of Kashmir Himalaya, their yield and the retention time of the compounds are presented in Table - 1 and in total 16-34 constituents were identified in the three oils.

During the present study sixteen compounds were identified in the essential oils of *Origanum vulgare* (L.) growing at Srinagar (I.I.I.M), which accounted for (98.38%) of total oil, while as twenty five each were found in the essential oils of *Origanum vulgare* (L.) growing at Pulwama (Bonnera) and Tangmarg (Yaarikhah) locales of Kashmir valley, accounting for 100.00% and 99.54% of total oil respectively. The essential oil composition of the three plant populations appeared quite different and allows us to identify three different chemotypes: the first growing at Srinagar locale with a prevalence of γ-terpinene/ cymene / sabinene; the second growing at Pulwama characterised with a prevalence of γ-terpinene/p- cymene / sabinene /carvacrol and the third growing at Tangmarg featuring a prevalence of p-cymene / carvacrol /cis-sabinene hydrate acetate /γ-terpinene. But the biosynthetic precursors of monoterpenoid phenols, (γ-terpinene and *p*-cymene), were almost constantly present in all cases. Sum of two phenols and their precursors constituted the bulk of oil of all the three locales: 39.281% at Srinagar; 61.692% at Pulwama and 69.732% at Yaarikhah. Generally, chemotypes form "biochemical varieties" or "physiological forms" in botanical species, each with a specific enzymatic equipment. These species are genetically codified and direct their biosynthesis to the preferential formation of a definite compound. In the case of phenolic compounds, the metabolic pathway is through the auto oxidative conversion of γ-terpinene to *p*-cymene followed by hydroxylation of *p*-cymene to thymol or carvacrol (Poulose *et al*., 1978) [28]. Data reported in this work should help to throw light in the apparent complex chemotaxonomy of the genus *Origanum*. Several chemo types of *O. vulgare* have also been reported from different places (Pande and Mathela, 2000; Kaul *et al.,* 1996) [26, 15].

The most abundant component in the essential oil of *Origanum vulgare* (L.) growing at Srinagar, which is at an altitude 1585 mts. absl was the precursor of thymol and carvacrol i.e γ terpinene (38.394%).But, surprisingly at this altitude, conversion of γ terpinene into carvacrol was least found (0.897%) and into thymol was totally absent .Other major components were identified as cymene (23.943%), sabinene (15.257%), cis- oscimene (5.114%), β trans ocimene (4.106%), methyl carvacrol (3.495%), alpha terpinene (2.250%) (Fig.1). It is to be noted that Cymene (23.943%), was found only at Srinagar (lower altitude) while it was totally absent at higher altitudes.

The phytochemistry of the essential oils of *Origanum vulgare* (L.) growing at Pulwama (Bonnera) locale of

Kashmir Himalaya which is at an altitude 1830mts. absl. revealed that the major component of its essential oils was γ - terpinene (25.725%), followed by para cymene (21.123%), sabinene (18.083%), carvacrol (14.782%), β trans ocimene (3.430%) and β caryophyllene (2.953%) (Fig. 1). It is worth mentioning that here even though γ-terpinene is present in comparatively minimum concentration among three geographic locales, but still its conversion into carvacrol is highest among three locales. Correspondingly, the percentage of terpene hydrocarbon- sabinene was markedly found at all the three locales, and was significantly higher for *Origanum vulgare* growing at Pulwama.

In examination of the essential oils of *Origanum vulgare* (L.) growing at Tangmarg (Yaarikhah) locale of Kashmir Himalaya which is at an altitude of 2180 mts. absl., the major component of its essential oil was found to be para cymene (33.290%), followed by carvacrol (27.198%), cis sabinene hydrate acetate (8.737%), γ terpinene (8.429%), sabinene (6.527%) and 4- terpineol (2.442%) (Fig. 1). Gounaris *et al*., 2002; Lawrence and Reynolds, 1984 reported p-cymene, carvacrol, γ-terpinene, sabinene, cis-ocimene, in the essential oil of *O vulgare ssp. vulgare*. Our *O. vulgare* oil composition at Yaarikhah, Tangmarg was found to be close to that reported by them except for some variations. Further, cis sabinene hydrate acetate was found only at Yaarikhah, which is a compound of intense spicy marjoramy aroma.

Table 1: Essential Oil composition (% total) identified by GC-MS of *Origanum vulgare,* collected from three different geographical locales of Kashmir Himalaya.

S. No	Compound name	% composition		
		Srinagar	Pulwama	Tangmarg
1	α-Thugene	0.888	1.163	0.0688
2	α-Pinene	0.959	0.713	0.451
3	Sabinine	15.257	18.083	6.527
4	β-Pinene	1.218	1.261	0.817
5	α-Terpine	2.250	1.449	1.758
6	Cymene	23.943	-	-
7	Limonene	0.163	0.367	0.378
8	Cis- ocimene	5.114	0.526	1.638
9	β-trans Ocimene	4.106	3.430	-
10	γ-Terepinene	38.394	25.725	8.429
11	Methyl carvacrol	3.495	-	-
12	Carvacrol	0.897	14.782	27.198
13	β-Caryophyllene	1.245	2.953	0.895
14	Germacerene-D	1.248	1.701	0.587
15	Cis-α- bisabolene	0.466	-	-
16	δ-cadinene	0.358	0.941	0.169
17	p-cymene	-	21.123	33.290
18	Cis sabinene hydrate acetate	-	-	8.737
19	4-Terpineol	-	0.216	2.442
20	Borneol	-	-	1.338
21	Thymol methyl ether	-	1.764	0.393
22	7-Tetracyclo undecane	-	1.436	0.440
23	β- borbonene	-	0.260	0.398
24	α-caryophylene	-	0.582	0.331
25	Thymol	-	0.062	0.815
26	Taugurgenene	-	0.734	-
27	Alloromadendrene	-	0.234	-
28	Cardreanol	-	0.215	-
29	α-cadinene	-	0.212	-
30	β-cubebene	-	0.069	-
31	3-carene	-	-	0.968
32	α-Himachalene	-	-	0.920
33	Terpinolene	-	-	0.398
34	Camphene	-	-	0.158
	Total identified compounds	98.34	100.00	99.54

As our results indicate, it could be noticed that there were variations in volatile oil percentages and composition according to the site samples. The total oil contents of plant and %age contributions of major oil constituents like carvacrol, sabinene, γ-terpinene and p-cymene varied markedly between localities. With increase in altitude, concentration of carvacrol increases i.e least in Srinagar and highest at Yaarikhah. The present study indicated that the oil of *O. vulgare* from the Kashmir region of India is mainly a carvacrol -rich chemotype which has great importance because of its high biological activity as well as antioxidant activity. It also indicated that the carvacrol biosynthetic pathway is favoured with altitude in Kashmir Himalayas and becomes more efficient than the thymol biosynthetic pathway. So, Kashmir Himalaya, conditions can be exploited for extraction of carvacrol. Also, in Kashmir Himalaya thymol biosynthesis is undermined by carvacrol biosynthesis, indicating correlation between the two compounds. This is in contrast to Russo *et al*., 1973, were thymol biosynthetic pathway is favoured over carvacrol biosynthesis but in conformity with studies on *O. vulgare* from the Mediterranean were carvacrol is a major constituent

(De Mastro, 1997; Kokkini *et al*., 1994) [7, 16]. Several samples rich in carvacrol were found also in Bulgaria (Konakchieve *et al*., 2004) [20].

The use of *O. vulgare* medicinal plant is believed to be due to biological properties of p-cymene and carvacrol. All our accessions showed large proportions of the same. The results obtained showed differences among the accessions of oregano in respect to chemical constituents of essential oils, indicating the existence of chemical polymorphism.

Essential oils of *O. vulgare* subsp. *vulgare* from Turkey were studied by Sezik *et al*., 1993 who found the essential oil rich in terpinen-4-ol,β-caryophyllene (21%) and germacrene D (17.8%) while Sahin *et al*., 2004 found that the main constituents of the essential oil of *O. vulgare* subsp. *vulgare* were β-caryophyllene and spathulenol, followed by germacrene D and α-terpineol. Melegari *et al*., 1995 investigated the essential oils of the inflorescences and found 4 chemotypes: p-cymene, terpinen-4-ol, thymol and β-caryophyllene. Mockute *et al*.2001 confirmed these results in a study of Lithuanian samples (β-caryophyllene, 10.8–15.4%; germacrene D, 10.0–16.9%; sabinene, 6.4–14.2%; (Z)-β-ocimene, 6.2–11.0%; and (E)-β-ocimene,7.0–11.5%).An Indian oil analyzed by Pande *et al*., 2000 unusually contained γ-muurolene (62.2%). Vokou *et al*., 1993 reported carvacrol, thymol, delta-terpinene and p-cimenene as major constituents of essential oil of *Origanum vulgare*. Also this composition is relatively similar to the composition of essential oil of leaves of *Origanum vulgare* growing in Italy (Russo *et al*., 1998) [30]. Previously, it was reported that *Origanum vulgare* growing in Brazil contained 4- terpineol (47.95%), carvacrol (9.42%), thymol (8.42%), and α-terpineol (7.57%) as major components (Cleff *et al*., 2010) [5]. The essential oils of 9 species and subspecies of oregano were studied by Figueredo *et al*., 2006 a. *O. vulgare* subsp. *vulgare* was particularly rich in the terpene hydrocarbons sabinene (16.3%), p-cymene (2.3%) and (Z)-β-ocimene (1.5%) and the sesquiterpene hydrocarbons germacrene D (13.3%), β-caryophyllene (10.7%) and β-bourbonene (1.9%). These hydrocarbons were accompanied by the functionalized terpenes linalool (4.0%) and terpinen-4-ol (4.8%).

It has been reported that the geographical region is one of the important factors affecting the composition of volatile oil in *Origanum* (Russo *et al*., 1998) [30]. There are many reports in the literature showing the variation in the yield and chemical composition of the essential oil with respect to geographical regions (Uribe-Hernandez *et al*., 1992; Souto- Bachiler *et al*., 1997; Celiktos *et al*., 2007; Van-Vauren *et al.*, 2007) [37, 35, 3, 38]. In examinations of the essential oil content, geographical variations have an impact on it because its oil mainly contains mono terepenes and sesquiterpenes which easily change by environmental conditions and geographic origins. Altitude seemed to be the most important environmental factor influencing the essential oil content. There are previous findings supporting this idea (Vokou *et al*., 1993) [40]. Our results are in conformity with them. So a number of studies have shown that variation in chemical features may occur within a single *Origanum* species.

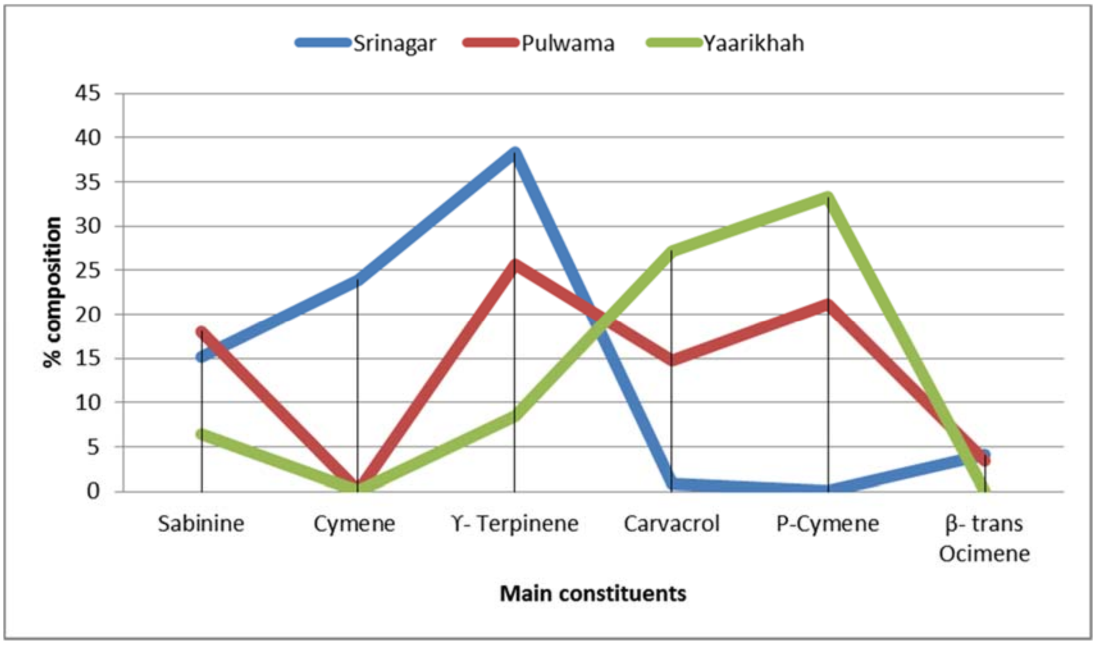

Fig 1: Comparative %age composition of major essential oil constituents of three different geographical locales.

Further, we studied comparatively the composition of *O. vulgare* oils distilled from the fresh drug and oils distilled from the plant after 1 year of storage at room temperature. In comparison with the essential oil distilled from the fresh drug, the essential oil distilled from the plant after 1 year of storage at room temperature was more stable. The phase of oil components of plants stored for 1 year showed a significant change (Table 2). As evident from the findings, there was increase in concentration of almost all the constituents of plants stored for 1 year, as compared to the oil from fresh plants. Remarkable increase in concentration was found in that of sabinene (28.447%), β caryophyllene (14.517%), β trans ocimene (12.453%) and germacrene D (12 .143%) Fig.2. It has been found that the pattern of variation of a single species follows its geographical distribution or depends on the season of plant collection as well as the conditions used for drying and storage (Kokkini *et al*., 1994, 1996,1997; Dorman *et al*., 2000) [16, 18, 19, 8].

Table 2: Essential Oil composition (% total) identified by GC-MS of *Origanum vulgare* stored for one year

S. No,	Compound name	% composition
1.	α - pinene	1.164
2.	sabinene	28.447
3.	β- pinene	2.072
4.	p- cymene	5.623
5.	limonene	0.256
6.	β- trans ocimene	12.453
7.	β- bourbonene	2.360
8.	β - caryophyllene	14.517
9.	α - caryophyllene	1.284
10.	Germacerene- D	12.743
11.	ϒ – gaurgenene	2.582
12.	δ – caudinene	5.156
13.	7-tetracyclo undecano	0.999
14.	Eicosapentaenoic acid	2.606
15.	Cedreneaol	0.921
16.	δ – maurolene	6.818
	Total identified compounds	100.00

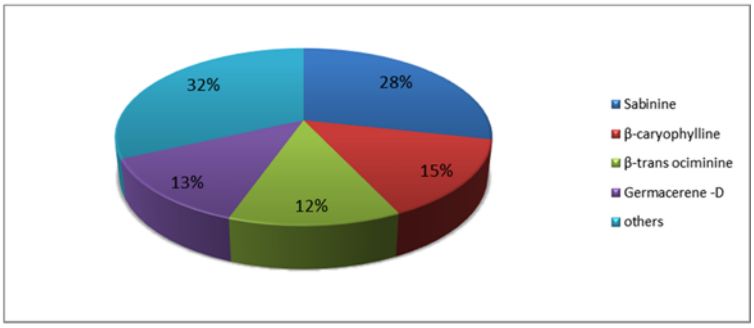

Fig 2: %age composition of main constituents of essential oil of *O.vulgare*, one year old sample

In general, our results discussed here concluded that it will be useful to study the conditions of the location where aromatic plants will be cultivated since the geographical regions, even in the same country, affected not only the volatile oil percentage but also its composition. More studies in this area of research are recommended in order to explain exactly how the environmental conditions as well as altitude can affect the oil content of aromatic plants. From the present study it is quite evident that the *Origanum vulgare* (L.) growing under Kashmir conditions is a new chemo type whose chemical profile is not completely matching either with the ones growing at different geographical locales of Kashmir nor with the *Origanum vulgare* (L.) growing elsewhere. The differences in the essential oil composition among the said plant growing at different geographical locales may be as a result of structural or physiological modifications of the plant caused by specific environmental factors (phenotypic plasticity). Thus composition differentiation requires a detailed analysis. Present studies suggested that the agro-climatic conditions of Kashmir are ideal for growing these crops of international standards and can be exploited by giving proper opportunities to the farmers of the region.

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