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ARTICLE

Volatile Constituents of Leaves of *Trifolium alexandrinum*

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ABSTRACT

This study presents the volatile constituents from leaves of *Trifolium alexandrinum* from Pakistan. The dried leaves were subjected to hydro-distillation for extraction and extracted volatile oil was subsequently characterized by gas chromatography-mass spectrometry technique. A total of 22 compounds were identified where the most dominant constituents were: phytol (46.00%), palmitic acid (9.26%), phytol acetate (6.44%), and linolenic acid (3.47%). Most of these are well-known constituents to exhibit antibacterial, antioxidant, antitumor, and anti-inflammatory activities.

1. Introduction

In many developing countries (Pakistan, India, Bangladesh, Nepal, etc.) a vast majority living in rural areas not only utilize but also rely on traditional and herbal sources rather than synthetic medicines as they cannot afford expenses of the latter [1]. Additionally, natural remedies are preferred [2] and essential oils of aromatic plants have been employed as a useful source against various ailments because of their vast and diverse bioactive properties [3]. Because of fertile lands, these countries have diversity in plants of medicinal importance, and a variety of medicinal plants are grown and harvested. Although, many plants have been studied, however, there are numerous other plants either uninvestigated or have rare literature on them, and hence, there is a great need to explore their essential oil composition and medicinal properties especially antimicrobial activity [4-6].

Trifolium alexandrinum is an important winter fodder crop in Egypt that has been cultivated since ancient times [7]. It belongs to a family Fabaceae, commonly called berseem clover or Egyptian clover [8] that possesses

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antibacterial [9], hepatoprotective [10], phytoremediation [11], and antidiabetic [12,13] activities. It is distributed in Pakistan, Egypt, Syria, Iran, South Africa, South America, Italy, India, and Australia [7]. Most species of this genus have been utilized as folk medicine in many countries. In Turkish traditional medicine, they are used as analgesics, antiseptics, and expectorants [9]. The flowers of Egyptian clover are yellowish-white in color, stems are hollow with alternate leaves possessing oblong leaflets. It grows upright as tall as 18-30 inches and contains 18-28% crude protein [8]. Berseem clover has become one of the fastest spreading fodder species of recent time. According to the most recent findings of an ethnobotanical survey conducted in Pakistan, the seeds of *T. alexandrinum* are a source of antidiabetic treatments, and the dried flowers are used to cure asthma, congestion, and ulcer, among other ailments. Wound healing, on the other hand, is accomplished using the entire plant. It is standard procedure in Iraqi Kurdistan to make decoctions from this clover leave for the cure of diarrhea [14].

Berseem may only be grown by seed, which is often sowed in the early fall months. It can be planted in a normal breeding ground or directly injected into the ground. Seeds of berseem can be planted alone or in a mixture with other plant species. It is combined with grasses (ryegrass) or with a seasonal grain crop such as oats to yield sufficient silage that can be stored for several months. It can be incorporated into rice-wheat crop production as a winter and early spring nutrition crop, and it is planted before or immediately after rice production to provide nutrients to the rice yield. In Australia, it is cultivated with other pulses such as balansa clover (*Trifolium resupinatum*), arrow leaf clover (*Trifolium vesiculosum*), and Persian clover (*Trifolium resupinatum*) to produce a more diverse crop. It is sometimes mixed with vegetables such as turnips or sarson (*Brassica juncea*) in various regions [15].

Legume family is one of the largest plant families, and the clover belongs to the genus Trifolium of this family [16]. Approximately 240 different clovers species [17] are found in the *Trifolium*, which can be found in subtropical and temperate locations throughout both continents. It has been discovered that mountains of east Africa, Mediterranean basin, and northwestern America are all home to *Trifolium* species, whereas there are no *Trifolium* species found in the southeastern Asian and Australian continents [16]. Several plants of this genus are being used as herbage plants for generations (e.g., *T. pannonicum Jacq., T. repens* L.*, T. medium* L. etc.) apart from their use as traditional medicine in diverse cultures [16].

Clovers are extensively used for the treatment of psoriasis and eczema for thousands of years, both in Asian and Europeans countries alike. As cough suppressant, antiseptic, painkiller, relaxant, and stimulant mixes*, T. repens* L.*, T. pratense* L.*,* and *T. arvense* L. have been used in ancient Turkish medicine [17]. In the Mediterranean region, few species of this genus are grown as fodder for animals [9]. *T. repens L. and T. pratense L.* are prominent medicinal herbs used in Pakistan, and are effective for the treatment of pneumonia, sinus infections, fevers, encephalitis, and a febrile sensation in the body [18]. Native Americans have traditionally employed these medicinal herbs to treat exterior skin issues, lung ailments, as well as various abnormalities of the mental and hormonal systems [8]. *T. pannonicum Jacq.* is a medicinal plant that grows wild, and is used by the locals for recovery of injury during the process of healing [19]. It is one of the most common natural pharmaceutical plants in the region. It was investigated that *T. angustifolium* L. extracts are used as the herbal remedy in Portugal to treat stomach cramps and diarrhea [20]. Egyptians have used the seeds of *T. alexandrinum L.* as an anti-diabetic medicine for thousands of years. *T. repens* L. is an antiparasitic cure used in herbal medicine by the Nagaland tribal communities to treat intestinal parasites. An in vivo investigation on animals has revealed that white clover has anti-inflammatory properties [16].

Over the last three years, more than 20 modern research works have been reported verifying the ethno - medicinal effectiveness of different *Trifolium* species. However, red clover is considered to be well-known among the *Trifolium* species, and it has been extensively studied in terms of therapeutic potential (particularly estrogenic effects) and agronomic relevance [14]. Red clover extracts are available for purchase as nutritional supplements in the marketplace. Although the antibacterial activity of isoflavones may have therapeutic efficacy for treating the disorders associated with hormones imbalancement, such as heart disease, menopausal problems, cancer, and osteoporosis, but there is currently no evidence to support this claim in the scientific community [21].

The production of phenolic and polyphenolic chemicals by *Trifolium* plants, in addition to isoflavones, is well documented. These compounds include phenolic acids, flavonoids, clovamides (caffeic acid esters), saponins, and a variety of other compounds. The apical parts of 57 *Trifolium* plants have been examined for their clovamide content, flavonoids (mostly isoflavones), phenolic acids, and then divided in 5 groups based on the results of the analysis [16]. There are several species in Cluster 1 that have the highest percentages of isoflavones (51-97 mg/g of dry mass), including *T. medium* L.*, T. lappaceum* L.*, T. phleoides* Willd. etc. where *T. bocconei* Savi. and *T. angustifolium* L. are the members of Cluster 2, that

contains species having very high flavonoid content of about 16- 32 mg /g of dry mass. Among plants of Cluster 3 are *T. isthmocarpum* Brot.*, T. resupinatum* var. *majus* Boiss.*,* etc. with low total phenolics. The species in cluster 4 have a high content of phenolic acids, ranging from 1-1.8 percent of dry matter [16]. Plants belong to Cluster 5 contain high levels of phenolic content. Each of these clovers exhibited substantial quantities of clovamides, flavanoids and polyphenolic compounds [16].

However, chemical studies on different parts of *Trifolium alexandrinum* showed the existence of proteins, isoflavonoids, flavonoids and their glycosides, steroids, terpenoids, amino acids and their derivatives, and fatty acids [8,9]. Considering the significant pharmacological properties and very rare information on its volatile composition, its aroma was studied.

2. Materials and Methods

2.1 Plant Material

Leaves of *Trifolium alexandrinum* were purchased from Akbari Market, Lahore, Pakistan. Drying, cleaning, and grinding of plant samples were carried out till a fine powder was obtained. 100 grams of the powdered sample was subjected to hydrodistillation for 5 hours and n-hexane was used as the collecting solvent through solvent extraction technique. The organic layer containing the volatile oil was then separated. The sample was stored in an air tight sample vial containing septum for the desired purpose and then kept at a low temperature (−10 ºC) for GC-MS analysis.

2.2 Gas Chromatography-Mass Spectrometry

Agilent 5977A series GC-MSD system was used to perform separation and characterization of volatile oil. A nonpolar capillary column, DB-5 MS (30 m × 0.25 mm ID × 0.25 µm film thickness) was used for separation of volatile compounds where operating conditions were: starting temperature was set at 100 °C and it was raised to 310 °C at a rate of 15 °C / min and then held for 2 mins. The flow rate of helium was 1 mL/min. An injection volume of 1 µL in split mode (split ratio 15:1) was used with a total runtime of 16 minutes. The conditions for the mass detector were: source, transfer line and MS Quad temperatures were 230 °C, 280 °C and 150 °C, respectively, where *m/z* was set in the range of 37-500.

3. Results and Discussion

The light yellow oil was obtained and the ion-chromatogram for separation is shown in Figure 1. A total of 22 compounds were successfully identified that included numerous bioactive compounds and are provided in table 1 where most of the identified compounds showed matching quality above 90%. Major constituents identified were: phytol (46.00%), palmitic acid (9.26%), phytol acetate (6.44%), and linolenic acid (3.47%). Many of these are known to be aromatic and flavor imparting compounds such as phytol possesses a floral type odor, palmitic acid exhibits a faint oily fragrance and phytol acetate has a waxy odor.

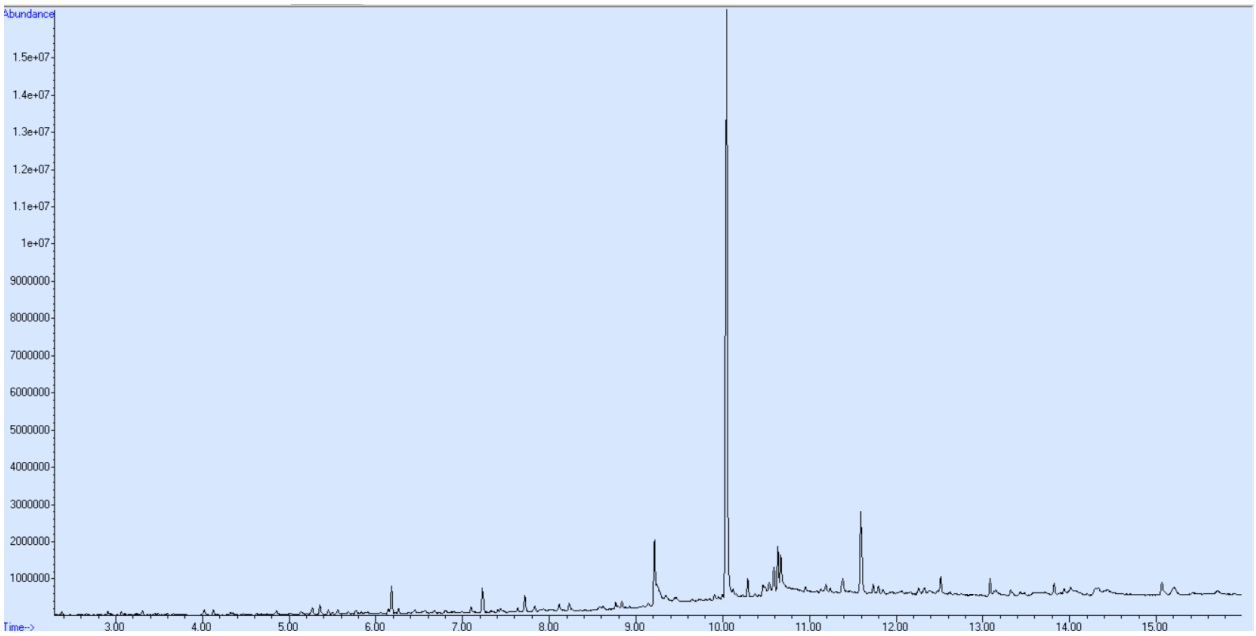

Figure 1. Total Ion Chromatogram of the volatile oil of leaves of *Trifolium alexandrinum*

Table 1. Volatile composition of leaves of *Trifolium alexandrinum*

Sr. No.	Retention Time (min)	Identified Compounds	*Abundance (%)*	*Retention Indices (RI_{Lit})*
1.	2.470	p-Cymene	T	1026
2.	3.162	Durene	0.11	1115
3.	4.021	Safranal	0.55	1173
4.	4.123	β-Cyclocitral	0.45	1196
5.	4.318	β-homocyclocitral	0.23	1235
6.	5.35	β-Damascenone	0.79	1354
7.	5.56	Damascone	0.42	1383
8.	6.18	trans-*β*-Ionone	2.02	1470
9.	6.26	β-Ionone epoxide	0.36	1488
10.	7.83	Ar-tumerone	0.52	1664
11.	8.23	Hexahydrofarnesyl acetone	0.67	1833
12.	8.76	Isophytol	0.43	1920
13.	8.83	Methyl palmitate	0.43	1930
14.	9.22	Palmitic acid	9.26	1960
15.	10.04	Phytol	46.00	2105
16.	10.28	Linolenic acid, methyl ester	1.21	2125
17.	10.46	Oleic acid	1.52	2141
18.	10.54	Linoleic acid	1.10	2145
19.	10.58	n-Nonadecanol-1	1.66	2156
20.	10.67	Linolenic acid	3.47	2143
21.	11.59	Phytol acetate	6.44	2218
22.	11.79	Tricosane	0.42	2300

T = trace > 0.1; RI_{Lit} = Retention indices from literature

The spectra of major compounds are provided in Figure 2 and their fragmentation patterns and relative spectral data is provided as under:

Phytol: 297(<1, M+), 278(1), 123(28), 111(9), 95(16), 83(16), 71(100), 57(29). Peak match was 98%.

Palmitic acid: 258(1, M+), 257(4), 157(13), 129(41), 115(16), 73(100), 60(80), 57(71), 55(74). Peak match was 99% 71(100),

Phytol, acetate: 296(2, M+), 278(5), 151(4), 137(9), 123(58), 109(21), 95(50), 82(46), 68(64), 55(46). Peak match was 76%;

Linolenic acid: 204(1, M+), 189(1), 175(2), 161(1), 147(3), 133(4), 128(2), 121(16), 105(11), 93(49), 79(100), 67(79), 55(87); Peak match was 99%.

Numerous well-known and significantly bioactive constituents such as cymene, safranal, linolenic acid, linoleic acid, phytol, and palmitic acid were obtained from these oils. Phytol is a diterpene alcohol [22] and palmitic acid belongs to fatty acid class of compounds. Phytol being the major constituents of this oil where numerous studies have reported its pharmacological activities such as antibacterial [23], antioxidant, antinociceptive activities [24], antiallergic, anti-inflammatory [25,26], antiquorum [27], cytotoxic [28] and antitumor activities [29]. Besides, it has immunostimulant [30] and anticonvulsant properties as well [31]. It also acts as a precursor for the formation of vitamin E and K_1 [32]. Phytol acetate is a diterpenoid derivative of phytol and have been reported for its remarkable antifungal, antibacterial [33] anti-diuretic and anti-inflammatory [34] activities. On the other hand, palmitic acid is a well-known fatty acid that possesses antitumor [35], and anti-inflammatory activities and medicated oils rich in palmitic acid are used to treat rheumatic symptoms in the traditional medicine of India [36]. On the other hand, linoleic and linolenic acids are dietary essentials that also possess various medicinal properties. Linolenic acid is an omega-3 fatty acid whereas linoleic acid is an omega-6 fatty acid [37]. Linolenic acid, methyl ester has antioxidant and antimicrobial properties [38]. β-damascenone and damascenone are rose ketones and the former has been reported for it antispasmodic and anti-inflammatory properties [39]. Safranal is minor constituent of plant essential oil and it is reported for its antifungal [40] properties. Safranal also exhibits

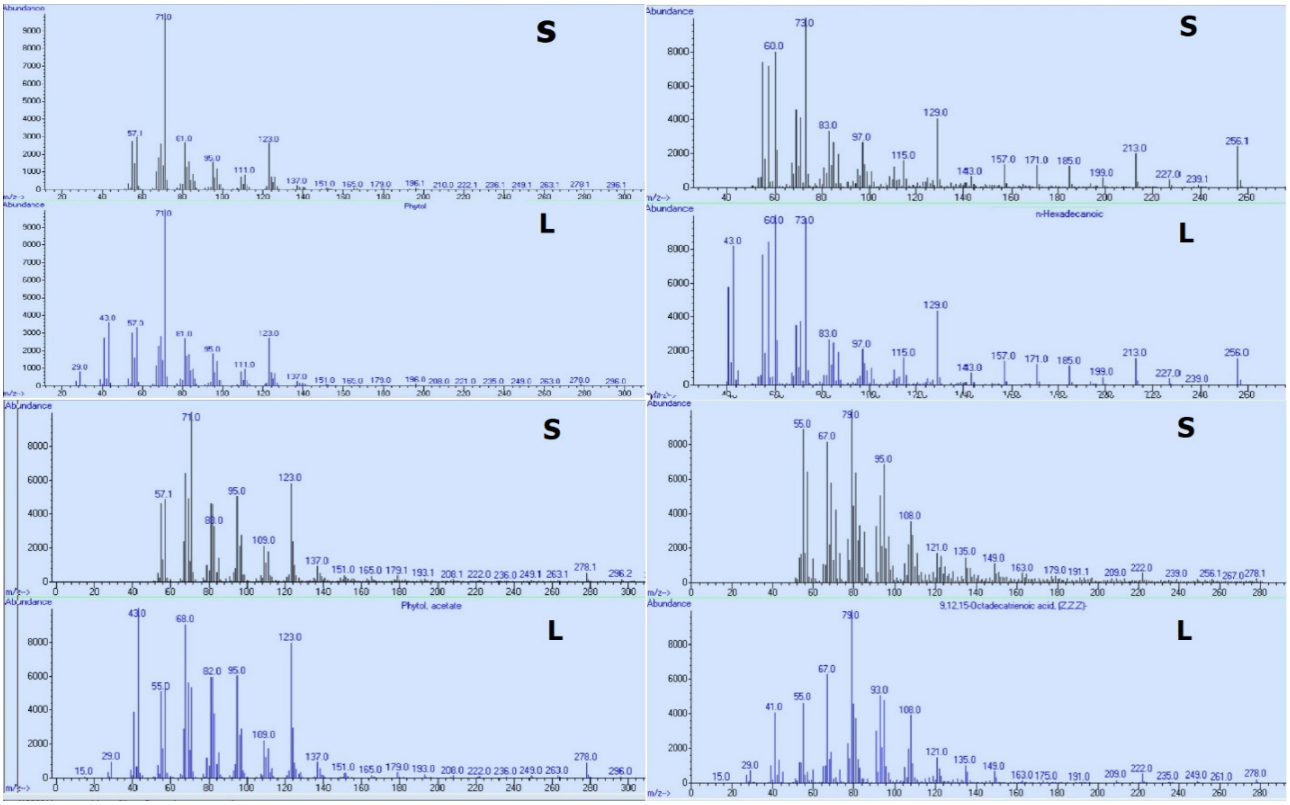

Figure 2. Matching mass spectra of major compounds.

S = sample spectra, L = Library match

anticonvulsant and antioxidant bioactivities. β-Ionone epoxide is a monoterpenoids derivative of β-Ionone which is a yellowish liquid. β-Ionone and its derivatives act like anticancer agents [41]. Immunological assay of ar-tumerone which is sesquiterpenoid that shows its strong potency against snake venom and thus used as a medicine for the treatment of snake bite [42]. p-Cymene is an important pharmaceutical component which is used to treat coughs and phlegm and is a monterpene having antioxidant properties which is used to cure ailments in which oxidative trauma shows pathophysiological role [43]. *β-Cyclocitral* is a monoterpene which possesses notable antimicrobial and antioxidant characteristics [44]. Besides, numerous other minor constituents are also present that exhibit aromatic and bioactive properties. The classification of the identified constituents is provided in Table 2. The major classes belong to terpenoids (including terpenes and terpenoid alcohols) and fatty acids where these classes also known to exhibit various pharmacological and aroma imparting properties [45-47].

Table 2. Classification of volatile oil constituents

Sr. No.	Class of volatile constituents	Serial numbers of compounds from Table 1	Percentage (%)
1.	Terpenes & Terpenoids	1, 4, 8, 9, 21	9.27
2.	Fatty acids	14, 17, 18, 20	15.35
3.	Ketones	6,7, 10, 11	2.4
4.	Terpenoid alcohols	12, 15	46.43
5.	Aldehyde	3, 5	0.78
6.	Esters	13,16	1.64
7.	Others	22, 2, 19	2.19

4. Conclusions

The volatile oil extracted from the leaves of *Trifolium alexandrinum* was found to contain a total of 22 constituents. In this study, it was discovered that phytol, a pharmacologically significant constituent, was present as one of the major constituents, and that it is associated

with a variety of bioactive properties. There were several major and minor active constituents present, which led us to propose that further bioactive studies should be conducted in order to evaluate the bioactive potential of this volatile oil.

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