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YERLİ PAKİSTAN TIBBİ BİTKİLERİNİN ÖZELLİKLERİ ÜZERİNDE ÇALIŞMALAR

STUDIES ON THE CONTACT DERMATITIC PROPERTIES OF PAKISTANI MEDICINAL PLANTS. PART VI. DERMAL IRRİTATİNG PROPERTİES OF *LAVANDULA STOECHAS* L.

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SUMMARY

The irritant potentials of ***Lavandula stoechas*** were investigated on albino mice. These effects were mainly due to the monoterpenes present in its esential oil, obtained by distillation from the flowering stalks. Six irritant compounds, cineole, fenchone, camphor, borneol, α- terpineol and caryophyllene from the oil were isolated and purified by chromatographic methods. They were identified by comparing their physico - chemical characteristics and chromatographic behaviour with the authentic samples. The irritant behaviour of these compounds were evaluated by open mouse ear assay, while their potencies were compared by ID_{50} (Irritant dose in 50 % individuals) after the acute peak effects, which were calculated by probit analysis and by IU (Irritant units after chronic time). Fenchone and borneol were stronger and persistent irritant than camphor and α - terpineol with least ID_{50} i. e. 0.415 µg / 5 µl and 0.667 µg / 5 µl after 1.5 and 2 hours; while their reactions were lasted for 24 hours, with IU = 0.625 and 1.25 µg / 5 µl respectively. Cineole and caryophyllene were the least irritating and least persistent compounds with higher values of ID_{50} i. e. 1.867 µg / 5 µl and 3.141 µg / 5 µl after 3.5 and 5.25 hours respectively. The reactions of latter compounds did not last more than 12 hours under the concentrations used.

ÖZET

Albino sıçanlarında ***Lavandula stoechas***'in irite gücü incelendi. Bu etkiler başlıca; bitkinin çiçekli saplarından distilasyon ile edle edilen ve bitkinin esansiyel yağlarında mevcut monoterpenlerden dolayıdır. Yağdan sineol, fenkon, kafur, borneol, m - terpinol ve karyofelen adlı 6 tahriş edici bileşik izole edilerek kromatografik yöntemlerle saflaştırıldı.

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Bunların teşhisleri, fizikokimyasal özellikleri ve kromatografik özellikleri standard örneklerle kıyaslanarak yapıldı. Bu maddelen iritan hareketleri " açık sıçan kulak tayini yöntemi ile değerlendirildi. İrite etme güçleri " Probit analizi" ve IU (Kronik zaman sonrası iritan ünite) ile hesaplanan ID50 (Bireylerin % 50'sindeki irite edici doz) ile kıyaslandı. Fenkon ve borneol'un 1.5 ve 2 saat sonra 0.415 µg / µl ve 0.667 µg / µL lik ID50 değerleri ile kafur ve α - terpinol' den daha güçlü ve kalıcı iritan karakterde oldukları gözlendi. Bu maddelerin reaksiyonu, sırası ile 0.625 ve 1.25 µg / µL' lik IU değerleri ile 24 saat sürdü. Sineol ve karyofelen'in de sırası ile 3.5 ve 5.25 saat sonra, 1.867 µg / µL ve 3.141 µg / µL gibi ID50 nin en yüksek değerlerine sahip, en az irite edici ve en az kalıcı bileşikler olduğu gözlendi. Bu son iki bileşiğin reaksiyonu, kullanılan konsantrasyonlarda 12 saatten fazla sürmedi.

INTRODUCTION

Many plant derived irritant substances are well known, which frequently produce inflammatory changes in the skin of human beings and animals as a result of direct cytotoxic effects (1) and are capable of producing damage, without mediating any antibody system (2). The response is often characterised by the presence of inflammation, vesiculation and necrotic changes at the contact sites often called as irritant contact dermatitis or irritant contact sensitization (3,4). The incidence of contact sensitization by ***Lavandula stoechas*** L. are uncommon, but few reports indicated that the essential oil obtained from its flowering stalks has caused pigmentation of the face when used in 1 - 2 % (v /w) concentration in petrolatum (5).

L. stoechas is an important genus of family ***Labiatae***. The plant is an aromatic shrub that have strong ageeable camphoraceous odour, with a wide distribution in Arabian and Mediterranean coastal region particularly in Spain and Southern France to Asia minor. The medicinal importance of the plant is also well documented and considered to be cephalic, resolvent, deobstruent, a good stimulant, expectorant, antispasmodic and carminative (6). The essential oil obtained from its flowering twigs has been used in cosmetic preparations for centuries. It has also been used as a remedy againts colic and chest affections, to relieve nervous headache, biliousness and for cleansing wounds (6,7). The terpenoid chemistry has been studied by many eminent scientists and various investigations on the chemical constituents of terpenoid part of its essential oil reported the presence of camphor, fenchone, borneol, bornyl acetate, cineol, fenchyl alcohol, terpineol, lavanol, lavonyl acetate, Linolenic acids, phenolic acids, phenolic acids (8 - 13), amino acids (14) and various other types of terpenoid compounds (15 - 20). In the non terpenoid area,

Timmer ***et al.*** isolated and identified ten lactones in lavender oil; eight of which had been previously found (21). The medicinal importance of ***Lavendula*** species and their dermatitic behaviour have not received any attention in Pakistan. Since the local lavender oil is used in cosmetic preparations, its harmful effects, if any, particularly on the skin of human beings and animals have not been evaluated. The present communication describes the irritant effects of locally occurring ***L. stoechas*** on albino mice, followed by fractionation to isolate its active compounds, whose irritant potencies were evaluated by ID_{50}.

MATERIALS AND METHODS

Plant material

The plant material of L. stoechas was purchased from the local market of medicinal herbs. The material mainly consisted of the tops of the plant twigs containing dry inflorescence and minute seeds.

Extraction

10 kg of the plant material was pulverised and subjected to steam distillation for 4 hours in a clevenger hydrodistillation apparatus. The oily layer was separated from the distillate, dried over anhydrous sodium sulphate. Various physico-chemical properties of the oil were determined according to the standard procedure (22).

Column Chromatography

A portion of crude oil was fractionated into hydrocarbon and oxygenated part through the use of column chromatography. 5.8 g of the oil was adsorbed on 10 g silica gel and was applied at the top of a (5 cm x 110 cm) glass column, packed with 250 g of silica gel 60 (70 - 230 mesh ASTM No. 7734 of E. Merck, Darmstadt, Germany). The column was eluted first with hexane then with hexane / diethyl ether mixture. The polarity of the system was raised by incerasing the quantity of diethyl ether in hexane (each time the amount of diethyl ether was raised by the addition of 10 ml in 100 ml hexane (v / v). 20 ml fractions were collected and those having similar compounds were pooled, while monitoring with analytical TLC and detecting the isolated compounds by iodine. Hexane eluted fraction (500 ml) yielded terpene hydrocarbons, while oxygenated compounds were washed out by 50 to 80 % diethly ether in hexane.

The hydrocarbon part of the oil, after removing the solvent under reduced pressure (2.4 g) was further column chromatographed on silica gel 60 (125 g, 80 - 100 mesh in 4 cm x 80 cm sized column), eluting first with petroleum ether (40- 60°), then with petroleum ether / chloroform mixture. The polarity of the mobile phase was raised by incerasing the quantity of chloroform in petroleum ether (each time it was increased by 5 ml / 100 ml in petroleum ether v /v). 10 ml fractions were collected and similar compounds were pooled after monitoring with analytical TLC and detecting them by iodine.

The oxygenated part of the oil after removing the solvent under reduced pressure (3 g) was subjected to a third 5 cm x 64 cm sized glass column, packed with 150 g of active neutral alumina (with activity I, No. 1077 of E. Merck, Darmstadt, Germany), eluted first with chloroform then with 2 and 5 % methanol in chloroform, respectively. 10 ml fractions were also colected from this column. Similar compounds were bulked after monitoring with analytical TLC.

Thin Layer Chromatography

The analytical silica gel (PF 254 + 380) (25 mm) and preparative thin layer (75 mm) chromatographic plates were prepared with moving spreader, according to the method of E. Stahl (23). The following solutions were prepared in chloroform (1 % w / v) and applied to the chromatoplates using 5 µl Drummond microcaps. (i) Crude oily extract, (ii) Column fractinos (pooled), (iii) Isolated compounds and the standard known compounds such as (iv) Cineole, (v) Fenchone, (vi) Camphor, (vii) Borneol, (viii) α - Terpineol and (ix) Caryophyllene. The solvent systems used for the development of TLC plates were toluene / ethyl acetate (93 : 7) or petroleum ether / chloroform (95 : 5 or 90 : 10). Visualisation of the chromatograms were achieved by UV light at 254 and 360 nm (UV lamp TL 900 Camag Ltd), or by using vanillin / sulphuric acid spraying reagent and heating the plates at 110° C for 5 to 10 minutes (23). The compounds from the pooled (smaller silica gel and alumina columns) fractions, which correspond with the standard compounds were further isolated and purified by the preparative TLC. The isolated compounds were removed from the thick plates by eluting with dichloromethane. Pure compounds were recovered after complete elimination of dichloromethane under reduced pressure.

Gas Chromatography

The oil was subjected to GC analysis for their chemical composition on a Pye Unicam 204 model gas chromatographic apparatus, using CBP1 (non polar methyl silicone) and CBP20 (highly polar polyethylene glycol) capilary column with 20 m length and 0.25 internal diameter, along with the flame ionisation detector (FID). The retention times of various peaks were compared with the standard compounds. Other conditions of GC operation were temperature programmed with initial column temperature at 75° C which was hold up for 10 min., then raised at a rate of 4° C / min. The final column temperature was kept at 190° C for 15 min. The injection port temperature of 250° C was maintained. Nitrogen was used as a carrier gas under split system at a flow rate of 25 ml / min.

Biological Assay

Animals

Albino mice weighing 20 to 25 g were housed in cages on wood shavings in animal house under 25 ± 3° C and relative humidity 35 ± 5.2 %. Pelleted food and deionised water was available ad libitum.

Procedure

10 mg of the substance under test was dissolved in 10 ml of acetone to prepare a 10 mg / 10 ml (w /v) solution. It was further diluted according to the method of Evans and Schmidt (24). Eight dilutions were prepared for the main assay. The pilot and main irritancy assay on mice ears were also adopted from Evans and Schmidt (24). For the main assay, group of 12 animals was used for each dilution. 5 µl of the solution under test was applied to the inner surface of one of the animal's ear using Drummond microcaps. The untreated ear was used as control. The ears were examined for redness after 30 minutes and then 15 minute interval until two observations indicated that further redness would not occur. The time of maximum erythema was noted. The number of ears eliciting the degree of redness coresponding to at least ++ intentensity on Hcker's scale at peak irritancy, (25) also mentioned by Evans and Schmidt (24) were noted and expressed in µg / 5 µl per ear. The animals were also number of red ears with at least ++ intensity after 24 and 48 hours were recorded and denoted by IU (Irritant units) (25). If no redness was obresved either after the acute or chronic stage, the procedure was repeated using more concentrated solution on the ears of other ani-

mal. The total number of red ears per dilution were tabulated. ID50 (Irritant dose in 50 % individuals) along with the upper and lower confidence limits were calculated by probit analysis (26).

RESULTS AND DISCUSSION

Irritation due to ***Lavandula stoechas*** was observed in the local people, who deal with teh extraction and trading of its oil. The skin of the dorsal side of hands and arms was often involved. It produced inflammatory patches after prolong handling the oil. The main irritant responsible for such behaviour seemed to be thir water insoluble essential oil.

L. stoechas plant material purchased from the local market was about two month old collection. It mainly consisted of the inflorescence portion of the plant containing 8.5 % tiny seeds. The physicochemical properties of the oil and its percentiage yield have been reported in Table -1.

Table - 1 : Physicochemical characteristics of Lavandula stoechas oil.

Characteristics	Values
Yield	0.82 %
Distillation time.	6.5 hours
Colour Yellowish red	
Specific gravity at 25° C	1.059
Refractive index at 20° C	1.508
Acid value	5.12 %

The gas chromatographic analysis of teh whole oil of L. stoechas revealed a number of components, out of which 12 terpenes could be identified, after comparison with teh standard samples (Table - 2). The standard samples were obtained from Mr. Abdus Sattar of Pakistan Council of Sicentific and Industrial Research (PCSIR) laboratories, Lahore.

For biological assay, the oil was fractionated into hydrocarbon and oxygenated terpenoid fractions through the column chromatogram on activated silica gel. The elusion with hexane, isolated hydrocarbon, while the oxygenated component remained adsorbed on silica gel and was eluted later by diethyl ether.

Table - 2 : Chemical constituents of Lavandula stoechas oil (As revealed by Ga Chromatograms)

R. Time (min)	Compounds	Percentage
8.71	α - Pinene	0.35
9.10	Camphene	0.40
11.21	β– Camphene	0.35
13.01	Cineole	3.62
15.12	Fenchone	0.23
16.15	Fenchol	1.29
18.86	Camphor	46.13
19.00	Borneol	6.27
19.72	α - Terpineol	2.95
20.51	Citronellol	0.58
22.84	Neral + Pulegone	0.76
23.50	Linalyl acetate	1.08
35.65	Caryophyllene	4.68

The hydrocarbon part of the oil was further resolved into various fractions after second time column chromatographed on silica gel, which were eluted with pertoleum ether. These fractions after pooling and subjecting to the preparative thin layer analysis, furnished cineol, fenchone and camphor as the predominant compounds. The oxygenated part of the oil on the other hand, when further subjected to an active neutral alumina column, eluting with chloroform and following TLC analysis, yielded borneol, α - terpineol and caryophyllene as major compounds.

The mouse ear test is known to be useful for screening the extracts of higher plants for inflammatory reaction (3, 4,24,25). Since the plant extracts are compelx mixture of phytochemical compounds, they may act on the skin by different mechanisms, with different potencies and duration of action (1,3,4,24,25). In order to compare the irritant reactions due to the compounds isolated from lavender oil, the number of mice indicating inflammatory reaction were counted at the time of peak irritancy, which differ from compound to compound. The data was then analyzed by probit analysis (26), which enable us to compare the potencies by

Table - 3 : Irritant Responses of the Compounds Isolated from Lavandula stoechas oil on Albino Mice.

DOSE LEVELS (µg / 5 µl)		COMPOUNDS					
		Cineole	Fenchone	Camphor	Borneol	α-Terpineol	Caryophyllene
20		----	----	----	----	----	12/12
10		12/12	----	12/12	----	----	10/12
5		10/12	----	11/12	12/12	11/12	8/12
2.5		7/12	12/12	9/12	9/12	9/12	5/12
1.25		4/12	10/12	8/12	7/12	7/12	2/12
0.625		3/12	8/12	6/12	6/12	5/12	1/12
0.3125		1/12	4/12	3/12	5/12	3/12	---
0.15625		----	3/12	1/12	2/12	1/12	---
0.078125		----	----	----	----	----	---
ID 50	µg/5µl	1.867	0.415	0.793	0.667	0.853	3.141
	S.D.	0.112	0.130	0.103	0.127	0.128	0.124
	% 2	4.650	1.405	1.045	2.941	0.900	0.543
	t	3.5	1.5	2.5	2.0	3.25	5.25
	U.C.L.	2.755	0.603	1.208	1.064	1.369	4.649
	L.C.L.	1.342	0.285	0.520	0.421	0.552	2.133
IU(µg/5µl) after	24 h	2.5	0.625	5	1.25	0.625	>20
	48 h	>10	>5	>10	>20	>5	>20

Where : S. D. = Standard Deviation ; t = Time (hours) to peak reaction ; ID 50 = Irritant dose in 50 % animals, calculated by probit analysis ; U. C. L. = Upper confidence limit ; L. C. L. = Lower confidence limit ; IU = Irritant units after 24 and 48 hours.

means of ID50 (Irritant dose in 50 % individuals). The chronic effects of lavander oil compounds on the animals skin were also recorded after one and a two days to ascertain the chronic inflammatory dose in a similar way as Hecker performed for croton oil (25).

Previous reports on the dermatitic properties of lavander oil showed that the essential oil of ***L. stoechas*** has caused hyperpigmentation of the face in sensitized women, when used in 1-2 % (v / w) concentration in petrolatum (5). Our findings suggested that the irritant properties of lavender oil were probably due to the compounds present in its monoterpenes part (Table - 3, Fig - 1). Among the six isolated compounds, fenchone and borneol appeared to be the most potent and persis

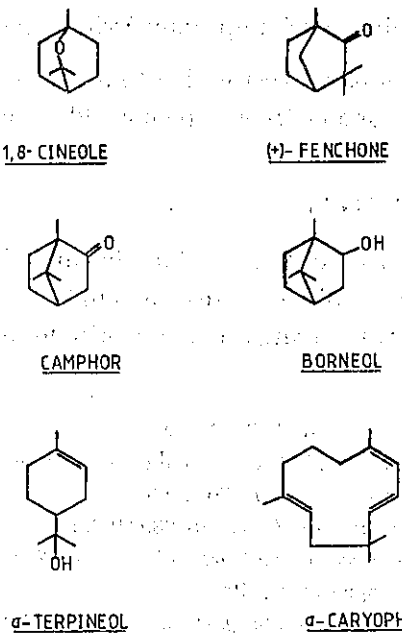

Figure - 1: Structures of the Compounds isolated from Lavender Oil

tent irritant than all other compounds with least ID50 (i. e. 0.415 µg / 5 µl and 0.667 µg / 5 µl) after 1.5 and 2 hours respectively. Their reactions were lasted for 24 hours, indicating IU = 0.625 and 1.25 µg / 5 µl after 24 hours (Table - 3). Cineole and caryophyllene were the least irritant and least persistent, with ID50= 1.867 µg / 5 µl and 3.141 µg / 5 µl after 3.5 and 5.25 hours respectively. Their reactions did not last more than 12 hours under the concentrations used (Table 3). The inflammatory reactions due to these compounds with at least ++ intensities appeared as red wheels that spread in 1.2 cm to 2 cm diameter areas of the skin of animal's ears. The potent and persistent inflammation induced by fenchone and borneol was probably due to the result of some tissue damage in the animal skin; while the least persistent inflammation of cineole and caryohyllene was possibly due to direct action at some skin receptor sites.

Further work is imperative to amplify this property through the preparation of their dervatives, that would possibility lead to the structure - activity relationship of such important irritant compounds.

We concluded from our investigation that the lavender oil contain closely related irritant monoterpenes, which could be harmful to the animal and human s skin.

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