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DPPH Scavenging, PRAP Activities and Essential Oil Composition of Edible *Lathyrus ochrus* L. (Cyprus Vetch, Luvana) from Cyprus

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Abstract: The essential oil of the aerial parts of edible *Lathyrus ochrus* L. was investigated by simultaneous GC, GC/MS analyses under the same conditions. Trace amount of oil (0.01> mL) obtained by hydro distillation of 200 g fresh plants was trapped in 1 mL *n*-hexane. Twenty components were detected representing 91.55 ± 0.56 % of the oil. The main components were phytol 49.39 ± 0.44 %, hexadecanoic acid 20.64 ± 0.89 % and pentacosane 4.20 ± 0.09 %. Essential oil solution (1% oil: *n*-hexane) afforded similar DPPH scavenging activity (9.28 ± 1.30 %) when compared with positive controls α-tocopherol (9.74 ± 0.21 %) and BHT (7.79 ± 0.26 %) at the same concentrations. Antioxidant activity of the oil was determined using a new HPTLC-PRAP assay. The oil afforded two fold higher reducing activity of phosphomolybdenum complex (594.85 ± 5.14 AU) when compared with positive controls α- tocopherol (271.10 ± 2.86 AU) and BHT (210.53 ± 1.81 AU) at the same concentration.

Key words: *Lathyrus ochrus*, essential Oil, HPTLC-DPPH assay, HPTLC-PRAP assay, phytol

1 INTRODUCTION

Lathyrus species (Fabaceae) are well known edible plants which are cultivated or gathered from wild. Several *Lathyrus* species have been reported as food products. Edible *Lathyrus* species include *L. ochrus*, *L. aphaca* L., *L. cicera* L. and *L. sativus* L.[1-4]. However, only *L. ochrus* is cultivated in Cyprus as food product which is known with the local name "Luvana". Usually fresh leaves of *L. ochrus* are consumed in salads and the seeds are used as a soup ingredient[4]. Other *Lathyrus* species that are edible could also be found on the island however their use as food products is not known. *L. sativus* is also cultivated in Cyprus but not used as food instead it is used as a livestock feed material.

Previously phytochemistry of many *Lathyrus* species have been reported. However there are only a couple of reports indicating the volatile and essential oil composition of this genus. Previously new flavonoids, flavonols, chalcones, pterocarpans, saponins as well as known sterols were reported from the extracts of *L. aphaca*, *L. pratensis* L., *L. chrysanthus* Boiss., *L. cloranthus* Boiss., *L. nissolia* (L.) Döll., *L. odoratus* L., *L. sativus*, *L. palustris* L. var. *pilosus* LEDEB. and *L. japonicus* Willd.[5-12]. Essential oil composition of *L. rotundifolius* Willd. and *L. vernus* L. were also reported. The essential oil of the aerial parts of *L. rotundifolius* was reported to have germacrene and elemene type sesquiterpenes (Germacrene D 50.4%; germacrene B 18.7%; γ-elemene 9.5%) in high amounts[13]. The flower and stem oils of *L. vernus* reportedly contained high amounts of palmitic acid (34.2 - 35.3%) and 1-octen-3-ol (49.8%)[14]. Floral volatiles of three different cultivars of *L. odoratus* were also investigated. Acyclic monoterpenes were reported as the main components with varying amounts for each cultivar ((*E*)-β-ocimene 22.9-46.5%, linalool 16.6-26.2%, nerol 3.3-10.1%, (*Z*)-β-ocimene 2.3-7.3%, geraniol 4.5-6.5%)[15]. *Lathyrus* species also afforded new primary metabolites including a new carbohydrate L-bornesitol[16] and amino acids α-*N*-oxalyl-L-α,β-diaminopropionic acid (α-ODAP), β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP)[17]. The beta isomer of ODAP is the known cause of the neurodegenerative disease called "*lathyrism*". The disease is initiated with over-consumption of *Lathyrus* species that contain this amino acid[18].

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The purpose of the present study is to identify the chemical composition of the essential oil of the popular food luvana (*L. ochrus*) for the first time. We believe the information obtained on the chemistry of this species will be used to evaluate general safety and beneficial properties of this edible species in Cyprus. Additionally free radical scavenging and antioxidant activities of the essential oil were also evaluated in order to provide information on the medicinal properties of this legume. This research was conducted as a part of our research project on the phytochemistry and biological activity of edible *Lathyrus* species of Cyprus.

2 EXPERIMENTAL

2.1 Plant Material

Plant materials were collected during the flowering period in 18 March 2012 from a cultivated field in the Güzelyurt region - Cyprus. Voucher specimens have been deposited in the Herbarium of the Near East University (Voucher no. 6772), Cyprus. Plant materials were identified by Dr. Kaan Polatoglu.

2.2 Isolation of the Essential Oil

Aerial parts of the fresh plant material (200 g) were chopped into small pieces (1 cm) and hydro-distilled (1000 mL DDH_2O) using a Clevenger apparatus for three hours. Plant material yielded an oil less than 0.01 mL which was trapped in *n*-hexane (1 mL) and dried over anhydrous Na_2SO_4.

2.3 Analysis of the Essential Oil

The essential oil analysis was done simultaneously by gas chromatography (GC) and gas chromatography–mass spectrometry (GC/MS) systems.

2.3.1 GC-MS analysis

The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m × 0.25 mm, 0.25 μm film thickness) was used with helium as a carrier gas (0.8 mL/min). The GC oven temperature was kept at 60℃ for 10 min and programmed to 220℃ at a rate of 4℃/min, and kept constant at 220℃ for 10 min and then programmed to 240℃ at a rate of 1℃/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250℃. Mass spectra were recorded at 70 eV. Mass range was from m/z 35 to 450.

2.3.2 GC analysis

The GC analysis was carried out using an Agilent 6890N GC system. The FID detector temperature was 300℃. To obtain the same elution order with GC-MS, the simultaneous auto-injection was done on a duplicate of the same column applying the same operating conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms. The results of the analysis was given in Table 1.

2.3.3 Identification of components

Identification of the essential oil and volatile components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder 3 Library)[19, 20], and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data[21, 22] was used for the identification.

2.4 HPTLC-DPPH Scavenging Activity Assay

DPPH scavenging activity of the essential oil was determined with our previous HPTLC-DPPH radical protocol[23]. Stock solutions (5 and 10 mg/mL) of the 1% essential oil (pure oil: *n*-hexane v/v), positive controls (±) α-tocopherol, BHT (Alfa aesar, Karlsruhe, Germany) (1, 5 and 10 mg/mL) and DPPH (0.1mM) (Fluka Chemie GmbH, Buchs, Switzerland) were prepared with CH_3OH. 200 μL of the sample solutions were mixed with 1000 μL of DPPH solution as well as positive controls and sample free blank controls in 1.5 mL Eppendorf tubes and vortexed for 2 minutes. Samples were incubated 1h in the dark at room temperature. Incubated samples (2 μL) were applied to known positions in x, y axis to an aluminum 60 F_{254} TLC Plate (Merck Darmstadt, Germany) with 5 mm band length using the TLC applicator system (CAMAG, Muttenz, Switzerland). After application of the samples and controls on the TLC; plates were scanned at 517 nm with a TLC Scanner (CAMAG, Muttenz, Switzerland) and absorbance of the bands were detected. The percentage of DPPH scavenging activity was calculated according to DPPH Scav. Act. % = $[(A_{Control} - A_{Sample})/A_{Control}] \times 100$ formula. The results of the DPPH scavenging activity test were given as mean ± standard deviation obtained from five parallel experiments. The results of DPPH scavenging activity were given in Table 2.

2.5 HPTLC-PRAP (Phosphomolybdenum Reducing Antioxidant Power) Assay

This new HPTLC-PRAP assay involves use of "TLC plate" as a "microplate" and the "TLC Scanner" as a "microplate reader". The test substances were applied on x and y dimensions on the TLC plate and densitometric measurement was achieved using the TLC-Scanner, similar to our previous protocol[23]. The HPTLC-PRAP assay was based on a previously described spectrophotometric PRAP protocol[24]. The solutions of 10% (w/v) phosphomolybdic acid (Aldrich, St. Louis MO, USA), 10, 5, 1 mg/mL 1% essential oil (pure oil: *n*-hexane v/v), positive controls: α-topocherol (Alfa aesar, Karlsruhe, Germany) and BHT (Alfa aesar, Karlsruhe, Germany) was prepared. Methanol

Table 1 The Essential Oil Composition of *Lathyrus ochrus*[1].

RRI[2]	Compound	%[3]	Identification[4]
1400	Tetradecane	0.25 ± 0.01	t_R, MS
1700	Heptadecane	0.12 ± 0.03	t_R, MS
1779	(*E*,*Z*)-2,4-Decadienal	0.13 ± 0.01	MS
1800	Octadecane	0.10 ± 0.0	MS
1868	(*E*)-Geranyl acetone	0.21 ± 0.02	MS
1955	Neophytadiene isomer	3.53 ± 0.36	MS
1958	(*E*)-β-Ionone	0.85 ± 0.02	t_R, MS
1992	Neophytadiene	1.35 ± 0.13	MS
2041	Pentadecanal	1.56 ± 0.03	t_R, MS
2131	Hexahydrofarnesyl acetone	1.73 ± 0.04	MS
2296	Isophytol	0.68 ± 0.05	MS
2384	1-Hexadecanol	1.02 ± 0.03	MS
2500	Pentacosane	4.20 ± 0.09	t_R, MS
2509	Methyl linoleate	0.42 ± 0.01	t_R, MS
2583	Methyl linolenate	1.72 ± 0.42	t_R, MS
2622	Phytol	49.39 ± 0.44	MS
2670	Tetradecanoic acid	1.99 ± 0.06	t_R, MS
2700	Heptacosane	0.58 ± 0.0	t_R, MS
2822	Pentadecanoic acid	1.08 ± 0.12	t_R, MS
2931	Hexadecanoic acid	20.64 ± 0.89	t_R, MS
	Alkanes	5.25	
	Fatty acids + methyl esters	25.85	
	Diterpenes	54.95	
	Others	5.50	
	Total	91.55 ± 0.56	

[1] The analysis were carried out in triplicate
[2] RRI Relative retention indices calculated against *n*-alkanes
[3] % area ± SD, calculated from FID data
[4] Identification method: t_R, identification based on the retention times (t_R) of genuine compounds on the HP Innowax column; MS, tentatively identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.

was used as the negative control. Phosphomolybdic acid solution (200 μL) and samples (200 μL) were mixed then incubated at 80℃ for 30 minutes. After all of the samples and controls were cooled to room temperature they were applied (2 μL – 5 mm) on silica gel TLC plate (Precoated Silica gel 60 F254 0.2 mm aluminium TLC plate Merck, Darmstadt, Germany) with the help of TLC applicator (Linomat 5 – CAMAG, Muttenz, Switzerland). TLC plate was scanned at 600 nm with a TLC-Scanner (TLC-Scanner 3 – CAMAG, Muttenz, Switzerland). Absorbance of the each spot were detected with TLC densitometry as "Absorbance Unit" (AU) and increased absorbance of the reaction meant increased reducing power when compared to the negative control. The results of the PRAP activity test were given as AU mean ± standard deviation obtained from five parallel experiments. The results of the PRAP activity test were given in Table 2.

2.6 Statistical Analysis

Analysis of variance (ANOVA) was applied on the data obtained from the HPTLC-DPPH and HPTLC-PRAP assay followed by the post hoc "Tukey's test". The results of this analysis are given as the mean ± standard deviation followed by different letters which indicate compared results

Table 2 DPPH Scavenging & PRAP Activity of *L. ochrus* Essential Oil[1].

	PRAP Activitiy (AU)				DPPH Scavenging Activity (%)		
	Oil[2]	α-tocopherol	BHT	Blank	Oil[2]	α-tocopherol	BHT
10 mg/mL[3]	593.87 ± 5.14e[3]	837.03 ± 1.38a	819.70 ± 2.10b	48.43 ± 0.34l	9.28 ± 1.30ef	89.13 ± 0.54b	90.81 ± 0.35a
1 mg/mL[4]	266.73 ± 5.08h	663.47 ± 2.60c	622.73 ± 1.64d		0.40 ± 0.14g	89.66 ± 0.30b	88.92 ± 0.17b
0.5 mg/mL[5]	187.27 ± 3.8 j	554.67 ± 7.17f	502.80 ± 4.46g		0.01»	60.15 ± 0.38c	45.36 ± 0.54d
0.1 mg/mL[6]	71.27 ± 1.14k	271.10 ± 2.86h	210.53 ± 1.81i		0.01»	9.74 ± 0.21e	7.79 ± 0.26f

[1] The results were given as the Absorbance mean ± standard deviation of five parallel experiments. (The given Absorbances were obtained as the absorbance unit (AU) of a TLC densitometric measurement.

[2] The stock solutions of the oil are prepared by dilluting 1% (oil/*n*-Hexane; v/v) oil solution in methanol.

[3] The essential oil stock solution contains 100 μg pure oil in 1 mL.

[4] The essential oil stock solution contains 10 μg pure oil in 1 mL.

[5] The essential oil stock solution contains 5 μg pure oil in 1 mL.

[6] The essential oil stock solution contains 1 μg pure oil in 1 mL.

have significant differences $p<0.05$. Statistical analysis was performed with XLSTAT-2012.6.02 trial version (Addinsoft, NewYork, USA).

3 RESULTS

The essential oil was obtained from the fresh aerial parts of *L. ochrus* (200 g) with a very low yield (<0.01 mL). We have identified twenty components of the essential oil representing 91.55 ± 0.56% of the oil. The largest portion of the oil was comprised diterpenes (54.95%) including phytol (49.39 ± 0.44%), isophytol (0.68 ± 0.05%) and neophytadiene derivatives (neophytadiene, 1.35 ± 0.13%; neophytadiene isomer, 3.53 ± 0.36%). Remaining portion of the oil was composed of alkanes (5.25%) and fatty acids and their methyl esters (25.85%) including hexadecanoic acid 20.64 ± 0.89%, pentacosane 4.20 ± 0.09%, tetradecanoic acid 1.99 ± 0.06% and methyl linolenate 1.72 ± 0.42.

In the present study DPPH scavenging and phosphomolybdenum reducing antioxidant power of the essential oil were also determined. The dilutions of the 1% essential oil (pure oil: *n*-hexane v/v), yielded significantly low DPPH radical scavenging activity when stock solutions with the same concentration were compared. Highest DPPH scavenging activity was observed at the highest concentration applied (10 mg/mL (corresponds to 100 μg oil/mL) – 9.28 ± 1.30%) which afforded tenfold lower activity than the positive controls. However, the exact concentration of the oil at the highest activity was 100 μg/mL which produced significantly similar results to α-tocopherol at 0.1 mg/mL (100 μg/mL) with higher activity than the BHT at the same concentration.

Similar to the DPPH scavenging activity, the oil presented lower reducing activity in PRAP assay when compared with the positive and negative controls at the same concentration. The dilutions of the essential oil solution (1% oil: *n*-hexane) showed significantly lower activity than the positive controls when the same concentration of the stock solutions were compared. Highest PRAP activity was observed at the application of the oil with the highest concentration (10 mg/mL (corresponds to 100 μg pure oil/mL) – 593.87 ± 5.14 AU obtained by densitometric measurement at 600 nm). The highest activity obtained from the oil solution corresponds to a higher activity than the activity obtained for BHT and α-tocopherol at 0.1 mg/mL (100 μg/mL) application (210.53 ± 1.81 and 271.10 ± 2.86 respectively).

4 DISCUSSION

Previous reports indicate monoterpenes and sesquiterpenes are the main components of *Lathyrus* essential oils[13–15]. However, the oil of *L. ochrus* contained common acyclic diterpenes phytol and neophytadiene derivatives as main components. *L. ochrus* oil therefore can be defined as a good natural source of phytol. Phytol have many potential industrial uses it could be used as a precursor in the synthesis of α-tocopherol (Vitamin E)[25] and phylloquinone (Vitamin K1)[26] as well as its use in production of cosmetics, fragrances and household cleaners[27]. However when the phytol exist in food products a special care is required since the consumption of large doses of phytol by the patients with refrum's disease could manifest itself with ataxia, retinitis pigmentosa, anosmia, hearing loss and polyneuropathy. Refrum's disease patients cannot metabolize phytanic acid which is accumulated as a side product of the free phytol in the tissues[28]. The phytol is also known to be toxic and has growth inhibitory effect in test animals[29].

The beneficial properties of phytol have also been reported. Phytol has been identified as the anticonvulsant principle of *Lactuca sativus*. Phytol was shown to inhibit succinic semialdehyde dehydrogenase (SSADH) which acts in the degradation of γ-aminobutyric acid (GABA), a major

inhibitory neurotransmitter[30]. In vivo, anti-inflammatory[31] and antinociceptive[32] activities of pure phytol have also been reported. High phytol containing plant essential oils such as *Cleome* sp. were also evaluated for their toxicity against sweet potato weevil *Cylas formicarius elegantalus* and high insecticidal activity was reported[33, 34].

The PRAP activity method employed in the antioxidant evaluations is a new method for HPTLC. It involves densitometric measurement of the samples on TLC plates using a TLC scanner which enables quantitative evaluation of the PRAP activity of the extracts, essential oils and pure compounds. The method uses the TLC plate like a microplate and TLC scanner like a microplate reader. This method enables quantitative PRAP evaluation of a number of samples on a single TLC plate.

A previous research reported radical scavenging activities of the pure phytol against hydroxyl and nitric oxide radicals and its prevention of thiobarbituric acid reactive species (TBARS) *in vitro*. Pure phytol was reported to produce high radical scavenging and antioxidant activity in the applied tests[32]. The oil showed high DPPH and PRAP activities suggesting their relationship with phytol content. However, other components of the oil may also be responsible for the observed activity.

5 CONCLUSION

L. ochrus afforded an oil rich in phytol (49.4%). Phytol content in fresh plant material can then be estimated as ca. 2.5 mg/100 g. Therefore, patients with refrum's disease should consider avoiding over consumption of *L. ochrus* in their diets. The amount of the toxic amino acid β-ODAP which causes neurodegenerative disease "*Lathyrism*" is not reported for *L. ochrus*. In our literature survey, we did not find any lathyrism cases related to *L. ochrus* consumption. However the unknown β-ODAP content together with the reported toxicity and growth inhibition of phytol[29] long term frequent consumption of *L. ochrus* should be avoided for adults and especially for children.

Due to the high phytol content the oil could also have beneficial anti-inflammatory, anticonvulsant and antinociceptive as well as insecticidal activities. The essential oil produced high antioxidant and similar radical scavenging activities when compared to the positive controls at the same concentrations. The observed activities were concentration dependent. The PRAP activity of the oil was determined with a new HPTLC-PRAP assay. The new assay allows rapid determination of the PRAP activity of a number of samples quantitatively on a single TLC plate.

Overall, the *L. ochrus* plant could be regarded as a good source for phytol but the safety on the consumption of this plant as food product should be evaluated. Therefore, a more detailed phytochemical investigation of the non-volatile portion of this plant is deemed necessary, and is the subject of our ongoing research.

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