



BIOLOGICAL STUDIES WITH PHENOLIC AND VOLATILE COMPOUND ANALYSIS OF *EVERNIA PRUNASTRI* EXTRACTS AND FRACTIONS

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ABSTRACT

Lichen *Evernia prunastri* of the Parmeliaceae family, also known as oakmoss, thrives on trunks of oak trees in mountainous temperate forests of Europe and North America. In the present study the evaluation of different extracts and fractions antioxidant, anti-inflammatory and inhibitory activity of aldose reductase enzyme, showed remarkable results. LOX and ALR2 inhibitory activities of *E.prunastri*, are presented for the first time in this study. The GC-MS analysis of the pentane extract by the use of two different solvents, pentane and acetone, led to the identification of a plurality of compounds found for the first time in this species. The major constituents found were Diacetone alcohol (1% and 38,27% in pentane and acetone respectively), the crown ether 1,4,7,10,13,16 Hexaoxacyclooctadecane (18,29% and 2.14%), and 3,5-Di-tert-butylphenol (9.12% and 1.51%). Furthermore, three known aromatic compounds and two depsides were isolated from the CH₂Cl₂ extract.

Keywords: *Evernia prunastri*, DPPH, lipoxygenase, aldose reductase, GC-MS analysis, chemical constituents.

INTRODUCTION

Evernia prunastri (L.) oakmoss was reported possessing antimicrobial [1], antioxidant and antiproliferative activity [2]. Large amounts are processed in the perfume industry and cosmetology as aromatic fixative agent [3], particularly the oakmoss absolute, a major contact sensitizer, is included in the fragrance mix used for diagnosing perfume allergy [4]. Moreover the lichen was applied as a biomarker in studies of natural or artificial environmental radioactive contamination [5]. Previous phytochemical studies on *E. prunastri*

have resulted in a variety of different compounds such as depsides, dibenzofurans, mono-aromatic compounds with part of them being chlorinated, as well as different terpenoids, triterpenes and steroids [6].

The scope of the present study, was the evaluation of oakmoss extracts and fractions for their antioxidant activity by DPPH• assay, their ability to inhibit the aldose reductase enzyme and their anti-inflammatory activity by inhibiting the lipoxygenase enzyme. The inhibition of the two enzymes, recorded for the first time, concluded in satisfactory results and therefore appeared the necessity for more efforts towards the isolation and identification of *E. prunastri* compounds with a potential of being ALR2 inhibitors and anti-inflammatory agents. GC-MS analysis [6-8] of *E. prunastri* along with the antimicrobial activity [9] has been previously mentioned. The essential oils were obtained by hydrodistillation method in a Clevenger-type

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apparatus [10] leading to the detection of monoterpenes, diterpenes and sesquiterpenes as major components. In this study the volatiles came directly from the pentane extract by partition with acetone. These two different polarity fractions, pentane and acetone, were analyzed with a polar column Alltech AT_{TM}WAX. There is no previous report though, on the GC-MS analysis in relation to the antioxidant, anti-inflammatory and aldose reductase inhibitory activity of the pentane extract which also led to the discovery of manifold volatile substances found for the first time in *E. prunastri*. Simultaneously a phytochemical study of the CH₂Cl₂ extract confirmed the presence of phenolic and depside compounds.

MATERIALS AND METHODS

Plant material

The lichen *E. prunastri* (L.), was collected from Lahana Kilkis, and the identification was carried out by Dr Theano Samara (National Agricultural Research Foundation, Forest Research Institute, Thessaloniki). A specimen (under voucher number E.K-54) has been deposited in the herbarium of the Laboratory of Pharmacognosy, School of Pharmacy, Aristotle University of Thessaloniki.

Reagents and solvents

Detection reagents, Naturstoffreagenz A and 1% vanillin/H₂SO₄ (Acros Organics). GC-MS solvents, Pentane (Pancreac Quimica San, Barcelona, Espana), Acetone (Pancreac, E.U.), EtOAc (Pancreac Quimica San, Barcelona, Espana). *In vitro* DDPH• assay, DPPH• (Aldrich Chemical Co. Milwaukee, WI, USA). *In vitro* LOX assay, Lipoxygenase, (Sigma Chemical Co. St. Louis, MI, USA) Tris.HCl (pH=9.00), NDGA (nor-dihydroguaretic acid) (Aldrich Chemical Co. Milwaukee, WI, USA) DMSO (Sigma Aldrich Corporation). Pentane (Carlo Erba, Pa, UN1208), Petroleum ether 40°-60 ° C (Panreac, PA 13.1315), CH₂Cl₂ (Panreac, PA 13.1315 and Carlo Erba PA, UN1593), Diethyl ether (Carlo Erba PA, No 447534). EtOAc (Merck Art. 9623), MeOH (Carlo Erba PA, UN1230).

Extraction and isolation of compounds

Air-dried material (198.59 g) was exhaustively extracted with pentane, petroleum ether, CH₂Cl₂ and MeOH under reflux. The evaporation of MeOH led to a residue that was dissolved in 1.5 l of boiling distilled water, filtered and extracted with EtOAc with the further collection of the aqueous part. Evaporation of the organic layers to dryness led to the corresponding weights: Pentane 1.52 g, petroleum ether 6.136 g, dichloromethane 6.562 g, methanol 17.635 g, ethyl acetate 0.467 g, water residue 13.56 g. The CH₂Cl₂ extract was subjected to CC (30x3.5cm) on silica gel 60, 0.040-0.063 (Merck, Art.9385) using C₆H₁₄-CH₂Cl₂-MeOH-water mixtures of increasing polarity as eluents and afforded 401 fractions.

The 51st fraction was isolated by silica gel using C₆H₁₄-CH₂Cl₂ (70–30%) to afford compound (1) (16.0 mg). The 69th fraction was separated by silica gel using C₆H₁₄-CH₂Cl₂ (70–30%) to afford compound (2) (4.2 mg). The 81st fraction was separated by silica gel using C₆H₁₄-CH₂Cl₂ (70–30%) to afford compound (3) (8.1 mg). The 107th fraction was isolated by silica gel using C₆H₁₄-CH₂Cl₂ (70–30%) to afford compound (4) (2.8 mg). Finally, the 272th was isolated by silica gel using C₆H₁₄-CH₂Cl₂ (40–60%) to afford compound (5) (67.2 mg).

Antioxidant activity with the DPPH assay

The experiment was carried out (according to the method described by Parejo et al [11] were 25 µl of various concentrations of all extracts were added to 975 µl DPPH• solution (2 x 10⁻⁵M) and each mixture was vortexed, while a blank in MeOH was utilized to zero the spectrophotometer. The mixtures were left to stand at room temperature for 30 min. When the reaction reached a plateau a decrease in absorbance was determined at 515 nm. The DPPH• concentration of the samples was calculated from the calibration curve. The percentage of the remaining DPPH• was calculated from the equation $DPPH\bullet = [DPPH\bullet]_{at\ t=T} / [DPPH\bullet]_{at\ t=0}$, where T is the time necessary to reach the steady state. Antioxidant capacity equals to the amount of sample required for the decrease of DPPH• initial concentration by 50% (EC₅₀) while AE=1/EC₅₀ represented the antiradical efficiency (AE).

Anti-inflammatory activity with the LOX• Assay

The experiment was carried out utilizing the method described in [12-15], where specific extract concentrations were dissolved in DMSO (20 µg in 1 ml DMSO). Samples and control solutions (10 µL were mixed with 100 µL of sodium linoleate (0.1 mM) and 0.2 mL of the enzyme solution (1/9) 10⁻⁴ % w/v salt solution, pH = 9). The incubation of the samples at room temperature for 3 min followed and the conversion of sodium linoleate to 13- hydroperoxylinoleic acid was recorded at 234 nm. NDGA was utilized as a standard inhibitor (NDGA % inhibition = 40 in 10µM).

ALR2 inhibitory activity

The method of the *in vitro* aldose reductase enzyme inhibition is being described in the literature [16]. Lenses from Male Wistar rats were quickly removed following euthanasia. The study was approved by the Ethics Committee of the Institute and performed in accordance with the Principles of Laboratory Animal Care (NIH publication 83-25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, Part 139, July 9th, 2003). Experiments were duplicated and compared to sorbinil used as an appropriate standard inhibitor. The % inhibition is calculated from the ratio $[(V_{control} - V_{(+inhibitor)}) / V_{control}] * 100$, where

V_{control} is the reaction rate of the control and V_(+inhibitor) is the reaction rate of the sample.

(GC/MS) analysis

The analysis was performed using a Chromatograph GC-2010 Shimadzu; mass spectrophotometer GC/MS-QP 2010 Shimadzu MFG INC, USA; Autosampler Shimadzu AOC-20s; Autoinjector Shimadzu AOC-20i floated by special Shimadzu Lab Solutions software and Post Run Analysis (GCMS solution version 2.50) to proceed the analytical results. Separations were accomplished on a polar column from Alltech AT_{TM} WAX, Serial No. 707060483 (60m x 0.32mm, 0.25 μ m). Samples were swizzled in centrifuge Vortex Velp Scientifica S/N 170410 E.U. Operating conditions were as follows: carrier gas, helium 99.99%, with a flow rate of 3 mL/min; Detector gases were air, hydrogen and nitrogen at a flow of 60mL/min, 2.3mL/min, 10mL/min respectively. Temperature in injection port was stable at 230°C and in detector was 300°C; Split injection mode was used; Oven temperature was held at 60°C for 5 min, and then increased at a rate of 40°C up to 250°C, where it was held for 5 min, finally increased to 300°C at a rate of 20°C/min and held there for 7.5 min, to achieve column purification; injector temperature, 250°C; detector temperature, 270°C; volume injected was 2 μ L and total analysis time 57.5 min; The MS operating parameters were as follows: ionization energy, 70 eV; ion source temperature, 200°C; interface temperature, 250°C; mass detection, 40-500 m/z; solvent delay for pentane 8 min, for acetone 6 min; mass range 25-200 amu, Em voltage 3000 volts. The identification of the GC peaks corresponding to the components of the pentane extract was based on direct comparison of the retention time and mass spectral data with those of standard compounds, computer matching with the NIST 21, 27, 107, 147 and PMW Tox3 library and by comparison of fragmentation patterns of the mass spectra with those reported in the literature [17].

RESULTS AND DISCUSSION

Chemical composition

Compounds were identified on the bases of NMR spectra, as well as by comparison with data from the literature [18-22]. Our investigation of the dichloromethane extract resulted in the isolation of sparassol(1), 2'-O-methyl evernol(2), evernin(3), methyl β - orcinol carboxylate(4) and 3,5-dimethoxytoluene (5). (Figure 1).

Antioxidant activity

Results of radical scavenging activity with the DPPH• assay (Table 1), showed that the EtOAc extract presented the higher Antiradical efficiency AE=1/EC₅₀ of 2.17, with the Pentane and CH₂Cl₂ extracts following with Antiradical efficiency 1.40 and 1.18 each. MeOH,

petroleum ether and aqueous extracts presented the lowest Antiradical efficiency ranging from 0.62 to 0.21. All extracts exhibited low Antiradical efficiency in respect to the standard Quercetin. Compounds (2) and (3) are methylated derivatives of lecanoric acid, a depside exhibiting moderate activity in DPPH assays [23]. The presence of an electron-attracting group (-COOCH₃), the formation of two hydrogen bonds between 2-OH and 1'-COO- and between 2'-OH and 1'-COOCH₃ and the conjugation of the COO- group with an aromatic ring enhance the electron-attracting properties [23,24]. Compounds (1) and (4) being regarded as methylated derivatives of orsellinic acid are characterized by the formation of a hydrogen bond between the oxygen of the carbonyl group and the OH group in *orthoposition* contribute to a moderate antioxidant activity. The antioxidant potential of the CH₂Cl₂ extract could be attributed to a synergistic/cumulative effect of these and other yet unknown compounds [23,25].

Anti inflammatory activity

In regards to the lipoxygenase enzyme inhibition (Table 2), the CH₂Cl₂ extract exhibited the lowest inhibition. Petroleum ether extract inhibition reached up to 50% while MeOH fraction and EtOAc extract showed an inhibition slightly below the 50% with NDGA being considered as a standard. Usnic acid a constituent found in *E. prunastri*[6], manifested a dose-dependent anti-inflammatory activity when tested on rats [26] through inhibition of prostaglandin synthesis.

Aldose reductase inhibitory activity

Aldose reductase, the key enzyme of the polyol pathway, was found to be implicated in the secondary complications of diabetes. During an increase of intracellular glucose under diabetic conditions, retinopathy develops as a result of osmotic and oxidative stress. Attenuating the activity of aldose reductase has been gaining a lot of attention in recent years as a means to prevent the onset of retinopathy in chronic diabetic patients. Results of Aldose reductase inhibition (Table 3) appear to be remarkably interesting for all extracts tested, with the EtOAc fraction and CH₂Cl₂ extract presenting a remarkably high inhibition ranging from 93% to 92% at a final concentration of 50% mg/mL while the other extracts, petroleum ether, MeOH and pentane, showed an inhibition ranging from 84% to 70% at the same concentration. Results were compared to sorbinil used as a standard inhibitor. A recent study showed that lecanoric acid, atranorin and usnic acid, compounds found in *E. prunastri*[6], bind to different amino acid residues and the last two have a common amino acid Leu301 for binding, which is a non-polar residue, in the active site of aldose reductase [27].

GC MS analysis

In this study the volatiles of *E. prunastri* came directly from the pentane extract by partition with acetone. For the first time, these two different polarity fractions, pentane and acetone, were analyzed with a polar column Alltech AT_{TM}WAX. This study led to the identification and quantification of compounds already known in the literature and a plurality of compounds found for the first time in *E. prunastri*. The identification is accounting for the 79.83% of the extract in pentane in which 41 compounds were identified and for the 83.79% in acetone in which 42 compounds were identified. The compounds along with their retention indices, main fragment ions and percentages are summarized in Tables 4 and Table 5 while the compositional variation of the pentane extract is represented in Figure 2. The different types of constituents are listed in order to their elution time and in the tables are mentioned the compounds found previously in *E. prunastri*, along with any antioxidant^a, anti-inflammatory^b and antidiabetic activity^c.

The qualitative and quantitative determination of the pentane extract essential oil with pentane and acetone as a solvent showed that ketones (1% and 40.56%), hydrocarbons (9.91 and 29.08), ethers (19.24% and 2.49%), alcohols (16.37% and 3.66%) and acids (10.42% and 1.56%) were the major constituents in the two solvents, respectively. Oxygenated monoterpenes (7.59%), oxygenated sesquiterpenes (1.22%), terpene related compounds (1.18%) and sesquiterpene hydrocarbons (0.38%) were found only in pentane solvent. Esters (3.42% and 3%), others (3.99% and 2.87%) and aldehydes (5.11% and 0.07%) were the minor constituents in both solvents. The major compounds found were Diacetone alcohol (1% and 38.27%), the crown ether 1,4,7,10,13,16 Hexaoxacyclooctadecane

(18.29% and 2.14%), and 3,5-Di-tert-butylphenol (9.12% and 1.51%). Except from the crown ether, the other two compounds have been identified for the first time in *E. prunastri*. From the oxygenated monoterpenes 7.59%, α -Terpineol 0.25% and Eucalyptol 1.66%, were not mentioned before in the literature. From the sesquiterpene hydrocarbons 0.38%, Patchoulane was the only compound, while from the oxygenated sesquiterpenes 1.22% Patchouli alcohol was the only compound, both found for the first time in *E. prunastri*. In comparison with the literature, the differences are obvious and could be attributed to the usage in this study of a single polar column that allows the detection of more hydrophilic compounds as well as environmental factors that may influence the essential oil composition. The polar column allows the detection of alcohols, aldehydes, ketones, acids, esters and ethers plus the usage of two different solvents allows the detection of a variety of aromatic hydrocarbons. Considering the *in vitro* antioxidant activity, the pentane extract exhibited the second higher antiradical efficiency AE 1.40 due to the presence of many compounds acting as free radical scavengers such as phenolic compounds. As far as LOX inhibitory activity is concerned, studies revealed that a series of n-alcohols with carbon chains up to 8 or 10 carbons could act as competitive inhibitors by the binding of the hydrophobic alkyl tail to the catalytic site of the enzyme [28]. Additionally, phenolic compounds are known for their lipoxygenase inhibition activity [29]. A remarkable finding is the high ALR2 inhibitory activity 69.95% that could be attributed to long-chain fatty acids present in the essential oil that are known to act as aldose reductase inhibitors in lens homogenates [30,31,32] although other yet unknown non-volatile compounds could also act as ALR2 inhibitors.

Table 1. Antiradical efficiency of the six extracts/fractions of *E. prunastri*

Antiradical activity with the DPPH [•] method		
Extracts/fractions	EC ₅₀ ±SD ^a	AE ^b
Pentane	0.71±0.005	1.40
Petroleum ether	3.94±0.026	0.26
CH ₂ Cl ₂	0.85±0.004	1.18
MeOH	1.63±0.018	0.62
EtOAc	0.37±0.057	2.17
Aqueous	4.69±0.004	0.21
Quercetin	0.071±0.003	14.022

^aEfficient concentration (mg antioxidant/mg DPPH[•]): Amount of antioxidant required for the decrease of the initial concentration of DPPH[•] by 50 %. ^b (AE) Antiradical efficiency: 1/EC₅₀

Table 2. LOX inhibitory activity of the six extracts/fractions of *E. prunastri*

Extracts/fractions	10 µ(M)	%Inhibition ^a (LOX)
Pentane		27
Petroleum ether		50
CH ₂ Cl ₂		0.9

MeOH	44
EtOAc	41
Aqueous	34
NDGA	79

^aNumber of experiments: n=2, SD ≤ 10% Nordihydroguaiaretic acid (NDGA): 40% inhibition of soybean LOX in 3 min.

Table 3. ALR2 % inhibition of the six extracts/fractions of *E. prunastri* (at a final concentration of 50µg dry extract/ml solvent)

Extracts/fractions	Inhibition a	Inhibition b	MO	SD
Pentane	69.19	70.71	69.95	0.757576
Petroleum ether	84.85	83.84	84.34	0.505051
CH₂Cl₂	92.42	91.41	91.92	0.505051
MeOH	71.21	71.72	71.46	0.252525
EtOAc	91.41	93.94	92.68	1.262626
Aqueous	21.21	18.18	19.70	1.515152

Number of experiments n=2 sorbinil % inhibition = 44.67(±4.37)% at the same concentration.

Table 4. GC-MS analysis of the pentane extract of *E. prunastri* with pentane as a solvent in a polar column Alltech AT_{TM}WAX

Classification	Compounds	RI, min	Area, %	M ⁺ , m/z	Main fragment ions, m/z (rel. Int.)
Oxygenated monoterpenes	Eucalyptol*, b ³⁸	13.286	1.66	154	43(100), 55(50), 71(60), 81(70), 93(50)
	Camphor ^{6, 48, b 36,37}	24.086	2.51	152	95(100), 41(40), 55(40), 69(40), 81(60)
	Linalool ^{6, 48, b 33, a 34, c 35}	24.861	2.69	154	71(100), 41(60), 43(60), 55(80), 93(80)
	α-Terpineol*, b ³⁹	29.533	0.25	154	59(100), 45(20), 81(50), 93(60), 121(30)
	Borneol ^{6, 48, b40}	29.699	0.48	154	95(100), 41(10), 55(20), 67(10), 110(20)
Sesquiterpene hydrocarbons	Patchoulane*, b ⁴¹	33.411	0.38	206	105(100), 55(60), 67(70), 79(80), 91(90)
Oxyg. sesquiterpenes	Patchouli alcohol*, b ⁴²	43.305	1.22	222	43(100), 55(50), 67(40), 79(40), 98(90)
Terpene related compounds	Linalylisobutyrate*	25.234	1.18	224	93(100), 43(50), 69(20), 80(40), 121(20)
Hydrocarbons	3,8-Dimethylundecane *	9.249	0.41	184	57(100), 43(85), 70(40), 71(90), 85(50)
	n-Nonyl-cyclopropane*	14.415	1.74	168	55(100), 43(90), 69(80), 83(60), 97(50)
	1-Tridecene*	21.452	2.57	182	55(100), 43(80), 69(65), 83(70), 97(60)
	6-Methyl-1-octene*	21.926	0.25	126	55(100), 41(45), 43(45), 56(70), 69(50)
	3,7-Dimethylnonane ^{6, 48}	23.236	0.54	156	57(100), 41(20), 43(60), 71(60), 85(45)
	(E)-9-Octadecene*	27.887	0.78	252	55(100), 43(70), 57(90), 69(70), 83(80)
	Heptadecane ^{48, b 47}	29.441	0.88	240	57(100), 43(50), 71(60), 85(40), 99(20)
	9-Octadecyne*	31.225	2.19	250	67(100), 41(60), 55(60), 81(70), 95(60)
	TetramethylHeptadecane*	35.034	0.55	296	57(100), 43(60), 71(80), 85(50), 99(20)
	Isopentyl alcohol*	14.649	0.19	88	55(100), 41(40), 42(60), 57(60), 70(50)
Alcohols (Aliphatic)	Isooctanol*	14.940	0.48	130	55(100), 43(90), 56(60), 57(80), 69(55)
	3-Methyl-1-hexanol*	15.370	0.24	116	55(100), 41(40), 56(80), 57(50), 70(60)
	1-Hexanol*	18.298	0.61	102	56(100), 42(30), 43(40), 55(50), 69(30)
	4-methyl-2-propylpentan-1-ol *	19.876	0.45	144	57(100), 41(40), 43(55), 55(30), 56(40)

Table 4. GC-MS analysis of the pentane extract of *E. prunastri* with pentane as a solvent in a polar column Alltech AT_{TM}WAX

Classification	Compounds	RI Time, min	Area, %	M ⁺ , m/z	Main fragment ions, m/z (rel. Int.)
	1-Heptanol*	21.813	0.16	116	56(100), 41(40), 43(40), 55(60), 70(80)
	1-Octanol* ^{b33}	25.151	0.46	130	56(100), 41(40), 43(40), 69(50), 84(40)
	1-Nonanol*	28.300	2.38	144	56(100), 41(50), 43(60), 55(90), 70(60)
	(Z)-9-Tetradecen-1-ol*	31.481	0.38	212	67(100), 41(60), 55(90), 81(80), 96(90)
	(E)-4-methyl-6-(2,6,6-trimethylcyclohexen-1-yl)hex-4-en-1-ol*	44.225	1.37	236	137(100), 41(40), 55(40), 81(70), 95(80)
(Aromatic)	Phenol ^{6, 48,}	38.075	0.53	94	94(100), 47(10), 55(10), 65(20), 66(30)
	3,5-Di-tert-butylphenol*, ^{a43}	45.173	9.12	206	191(100), 41(20), 57(80), 74(20), 206(20)
Aldehydes (Aliphatic)	7 Hexadecenal*	49.147	3.77	238	57(100), 43(80), 55(95), 67(90), 81(80)
(Aromatic)	2-Hydroxy-6-methylbenzaldehyde ^{6, 12}	34.286	1.34	136	135(100), 51(20), 63(10), 77(40), 90(40)
Ketones	Diacetone alcohol*	18.835	1.00	116	43(100), 58(20), 59(70), 98(20), 101(20)
Acids	Hexanoic acid*	33.999	3.25	116	60(100), 41(20), 43(20), 73(40), 87(10)
	Octanoic Acid ⁶	39.498	1.81	144	60(100), 43(40), 55(40), 73(50), 101(30)
	Nonanoic Acid ⁶	42.051	4.20	158	60(100), 41(40), 57(70), 73(60), 115(20)
	n-Decanoic acid*, ^{b 46}	44.493	1.16	172	60(100), 41(40), 55(40), 73(90), 129(30)
Esters	1-Octadecyl acetate*	49.313	3.42	312	43(100), 55(70), 61(60), 83(70), 97(80)
Ethers	1,4,7,10,13,16 Hexaoxacyclooctadecane ⁴⁸	8.230	18.29	264	45(100), 43(50), 44(40), 59(40), 89(40)
	Octyl ether *	44.400	0.95	242	71(100), 41(30), 43(30), 5(100), 82(40)
Others	Butanoic anhydride *	34.579	0.45	158	71(100), 43(50), 56(20), 72(10), 88(20)
	3-Methoxybenzyl alcohol*	49.852	3.54	138	138(100), 51(10), 77(40), 107(30), 109(60)

*Compounds detected for the first time in *E. prunastri*, **RI**, Retention Indices, **Area %**, percentage on the total extract, **M⁺**, molecular ion with main fragments, **a**: antioxidant activity, **b**: anti-inflammatory activity, **c**: antidiabetic activity.

Table 5. GC-MS analysis of the pentane extract of *E. prunastri* with acetone as a solvent in a polar column Alltech AT_{TM}WAX

Classification	Compounds	RI Time, min	Area, %	M ⁺ , m/z	Main fragment ions, m/z (rel. Int.)
Hydrocarbons	3,7-Dimethylnonane*	12.800	0.13	156	57(100), 43(70), 55(10), 71(60), 85(40)
(Aliphatic)	n-Nonyl-cyclopropane *	14.383	1.54	168	55(100), 43(80), 57(60), 69(70), 83(50)
	3-Methylundecane*	19.833	0.31	170	57(100), 41(30), 43(50), 71(50), 85(40)
	(2Z)-3-Methyl-2-undecene*	21.150	0.12	168	56(100), 43(60), 55(90), 69(55), 70(55)
	1-Tridecene*	21.400	5.53	182	55(100), 43(80), 69(70), 83(65), 97(60)
	2,2-Dimethylbutane*	26.367	0.27	86	43(100), 41(60), 57(90), 71(70), 85(10)
	1-isopropyl-2-nonyl-cyclopropane*	26.700	0.22	210	56(100), 41(40), 43(40), 55(40), 57(45)
	(E)-9-Octadecene *	27.833	5.01	252	55(100), 43(80), 69(70), 83(75), 97(80)
	(E)-3-Methyl-5-Undecene*	33.403	0.25	168	70(100), 41(50), 43(40), 55(80), 83(40)
	(E)-Icos-9-ene*	33.626	2.40	280	57(100), 43(90), 55(95), 69(70), 83(80)
	2-Butyl-1-decene*	37.912	0.18	196	56(100), 55(50), 70(40), 83(20), 98(10)

	1-Octadecene*	38.885	0.93	252	57(100), 43(90), 55(90), 69(70), 83(80)
(Aromatic)	Benzene ⁴⁸	6.283	0.94	78	78(100), 52(10), 63(40), 73(10), 79(10)
	Toluene ⁴⁸	8.217	2.03	92	91(100), 51(10), 65(10), 74(10), 92(60)
	Ethylbenzene ⁴⁸	10.700	1.45	106	91(100), 51(20), 65(10), 77(10), 106(40)
	m-Xylene*	11.150	6.01	106	91(100), 51(10), 65(10), 77(10), 106(40)
	Isopropylbenzene ⁴⁸	12.200	0.74	120	105(100), 77(20), 79(20), 103(10), 120(30)
	o-Xylene*	12.583	0.66	106	91(100), 51(15), 65(10), 77(15), 106(45)
	Hemelit ⁴⁸	15.967	0.36	120	105(100), 51(10), 77(20), 91(10), 120(40)
Alcohols	Ethylhexanol*	22.950	0.47	130	57(100), 41(40), 43(40), 70(20), 83(20)
(Aliphatic)	1-Dodecanol*	36.801	0.43	186	55(100), 43(90), 56(70), 69(80), 83(70)
	2-Hexadecanol*	48.140	0.23	242	45(100), 43(40), 57(40), 69(30), 83(20)
(Aromatic)	Phenol ^{6, 48}	38.075	1.02	94	94(100), 55(10), 65(10), 66(40), 74(10)
	3,5-Di-tert-butylphenol	45.157	1.51	206	191(100), 41(20), 57(80), 74(20), 206(20)

Table 5. GC-MS analysis of the pentane extract of *E. prunastri* with acetone as a solvent in a polar column Alltech AT_{TM}WAX

Classification	Compounds	RI Time, min	Area, %	M ⁺ , m/z	Main fragment ions, m/z (rel. Int.)
Aldehydes	3,3-Dimethyl-hexanal*	24.617	0.07	128	84(100), 41(50), 43(80), 57(50), 69(80)
ketones	3-Penten-2-one*	10.850	0.52	98	83(100), 43(40), 53(15), 55(90), 95(50)
	Diacetone alcohol *	18.833	38.27	116	43(100), 41(10), 58(30), 59(70), 101(30)
	4-Hydroxy-2-pentanone*	22.167	0.52	102	43(100), 42(10), 58(20), 61(10), 84(20)
	1,2-Diacetylene*	27.383	1.25	112	43(100), 42(10), 69(30), 97(70), 112(20)
Acids	Acetic acid*	22.383	0.70	60	43(100), 42(10), 45(90), 57(10), 60(80)
	n-Hexadecanoic acid*, b ⁴⁵	49.055	0.86	256	43(100), 57(60), 60(80), 73(80), 129(25)
Esters	Sec-Butyl acetate*	24.733	1.87	116	43(100), 41(10), 83(10), 87(30), 101(10)
(Aliphatic)	2-methylbutan-2-yl pentanoate *	27.950	0.11	172	85(100), 43(60), 57(60), 70(60), 71(90)
	Tert-Pentylpentanoate *	34.412	0.27	130	43(100), 55(10), 70(10), 87(40), 89(10)
	Octadecanol acetate	49.266	0.49	312	43(100), 55(60), 69(50), 83(55), 97(55)
(Aromatic)	Diethyl phthalate*	46.627	0.26	222	149(100), 50(10), 65(20), 76(10), 93(10)
Ethers	1,4,7,10,13,16-Hexaoxacyclooctadecane ⁴⁸	8.433	2.14	264	45(100), 59(60), 72(20), 89(30), 133(10)
	Hexyl tert-butyl ether*	24.500	0.07	158	59(100), 43(40), 55(10), 57(60), 85(10)
	Octyl ether *	30.900	0.28	242	57(100), 41(20), 43(60), 69(40), 71(80)
Others	Chlorobenzene*	13.633	2.58	112	112(100), 51(40), 74(10), 77(80), 114(40)
	Dimethylformamide*	17.600	0.08	73	73(100), 44(80), 45(10), 56(10), 58(10)
	Dipropylene glycol*	34.829	0.21	134	59(100), 41(20), 45(30), 85(10),

*Compounds detected for the first time in *E. prunastri*, **RI**, Retention Indices, **Area %**, percentage on the total extract, **M⁺**, molecular ion with main fragments: **a**: antioxidant activity, **b**: anti-inflammatory activity, **c**: antidiabetic activity.

Fig 1. Isolated compounds from the CH₂Cl₂ extract of *E. prunastri*.

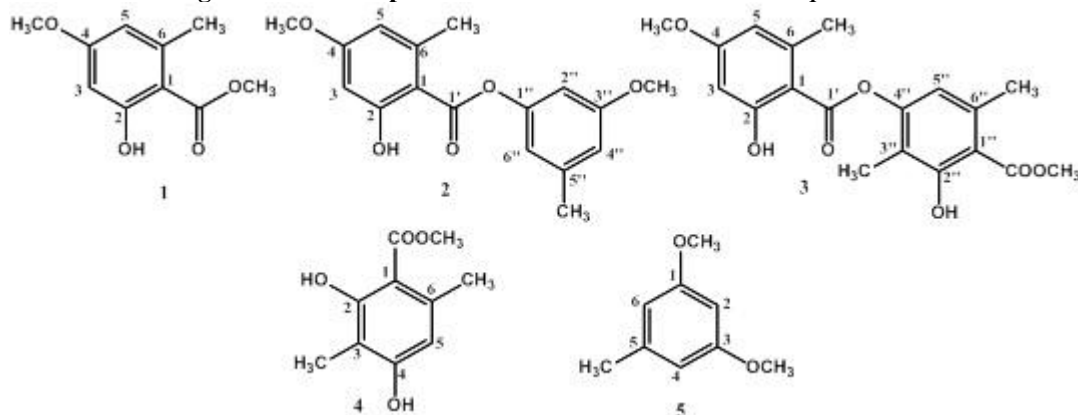
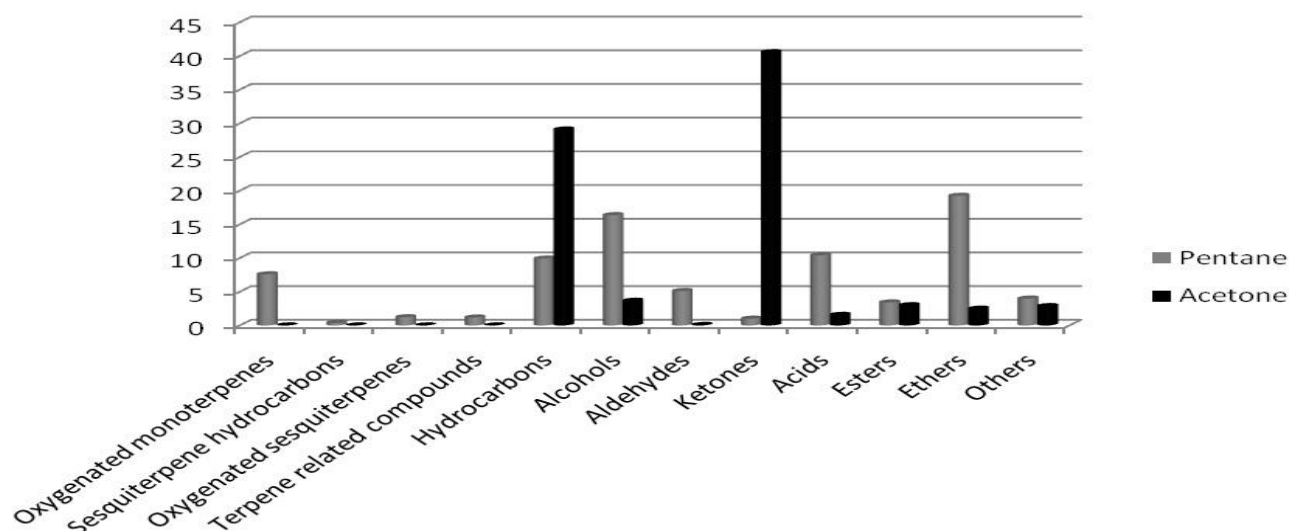


Fig 2. Compositional variation of the pentane extract essential oil in two different solvents (Pentane and Acetone)



CONCLUSION

The biological studies of various extracts and fractions provided evidence for the strong *in vitro* ALR2 inhibitory activity which reached the higher levels. With this in mind, our focus is to continue the isolation and structure elucidation of phytochemicals along with *in vivo* hypoglycemic studies in order to develop new chemical compounds for the treatment of diabetes type 2. Furthermore the GC-MS analysis led to the detection of compounds, identified for the first time in *E. prunastri*, with potential anti-inflammatory and ALR2 inhibitory activity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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