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Research Article

Phytochemical analysis with biological activities of *Calendula officinalis (Asteraceae),* growing wild in Mount Athos.

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ABSTRACT

This is the first study on the phytochemical content, antioxidant and anti-inflammatory activity of Calendula officinalis (Asteraceae) growing in the close and protective ecological system of Mount Athos. The purpose of this study is to determine the antioxidant and anti-inflammatory activities of C. officinalis extracts and to specify the involved compounds. The aerial parts of Calendula officinalis (Asteraceae) were extracted exhaustively with petroleum ether, dichloromethane and methanol. The concentrated methanol extract was reextracted, successively, with diethyl ether, ethyl acetate and n-butanol. The antioxidant and anti-inflammatory activities of all these extracts were evaluated through two in vitro model systems: the inhibition of lipid peroxidation induced by 1,1-diphenyl-picryl-hydrazyl stable free radical and the inhibition of soybean lipoxygenase. The last experiment is carried out for the first time in the genus Calendula. The extracts with the most significant antioxidant capacity with the first model system, was the diethyl ether and ethyl acetate and were equivalent. With the second model system, the extracts of the ethyl acetate as well as of the butanol and their residues, proved to be the strongest inhibitors of lipoxygenase. Phytochemical analysis of the plant lead to isolation of pseudotaraxasterol, quercetin, chlorogenic acid, diglucoside of oleanolic acid and a triglycoside of oleanolic acid. The triglycoside of oleanic acid was isolated for the first time in the genus Calendula. The results of biological experiments as well as the secondary metabolites presented in the extracts of this plant, which are known as antioxidant and anti-inflammatory factors, provide support to usage of Calendula officinalis, growing wild in Mount Athos, as a protecting factor from body cells damages caused by oxidation and as a reducing inflammation factor.

Key words: *Calendula officinalis, Antioxidant activity, anti-inflammatory activity, lipoxygenase, oleanic acid triglycoside.*

INTRODUCTION

Calendula officinalis is a genus of about 15–20 species of annual and perennial herbaceous plants in the daisy family Asteraceae. The common name "marigold" refers to the Virgin Mary. The most commonly cultivated and used member of the genus is the pot marigold (*Calendula officinalis*). The flower petals of the calendula plant (*Calendula officinalis*), have been used for medicinal purposes since at least the 12th century. *Calendula* is native to Mediterranean countries but is now grown as an ornamental plant throughout the world. Traditionally, *Calendula* has been used to notably speed the healing of burns, bruises, cuts and the minor infections that they cause[1] , but there is no scientific evidence that *Calendula* works for these problems. Triterpenoid-containing extracts of *Calendula* have been investigated in chemical-induced inflammation in mice[2,3]. Calendula extracts alleviated signs of chronic conjunctivitis and other chronic ocular inflammatory conditions in rats and also had a systemic anti-inflammatory effect.[4] The chemical composition of *Calendula officinalis* flowers comprises various classes of active biological compounds: glycosides of triterpenique saponins, (named

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calendulosides), flavonoids, sterols (cholestanol, campestanol, stigmasterol , sitosterol, s.o.), neutral lipids (which contain C12-C22) fatty acids chain, especially lauric, myristic, palmitic acid, as well as polyunsaturated fatty acids), carotenoids saccharides (water soluble polysaccharides, pectic substances, hemicelulose)[5]. Mount Athos is well known as a close and protective ecological system, for its spirituality and religious interests but also for the rich flora growing there. Because of the economic value of *C. officinalis* as a herbal medicine and its wide use in cosmetics, perfumes and pharmaceutical preparations we decided to study *C. officinalis* growing in Mount Athos. Although there are several reports on the chemical composition of this plant no such information is available on the plant growing in Mount Athos, which is designed to be used for galenic and cosmetic preparations. This is the focus of an extensive study in our laboratory in combination to an *in vitro* antioxidant and anti-inflammatory study.

MATERIAL AND METHODS

Plant material

Fresh flowers of *C.officinalis (*Asteracae) were collected from Mount Athos in June 2011 and were air dried. A voucher specimen (EK[44]) was kept in the laboratory of Pharmacognosy and Phytochemistry, School of Pharmacy, Faculty of Health Siences, Aristotle University of Thessaloniki.

Phytochemical study

General procedures, chemicals and standards

All solvents and chemicals used were of analytical or HPLC grade and obtained from Merck (Darmstadt, Germany). Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden).

Extraction process

Extraction and isolation

The air-dried aerial parts of *C.officinalis* (33 g) were exhaustively extracted (Soxhlet) with petroleum ether (bp 50–70 ^{o}C), dichloromethane and methanol. The extracts were then concentrated until dry on a rotary evaporator under vacuum distillation (Buchi 461) and the following solid residues were received: petroleum ether 3.5 g, CH_2Cl_2 3g, MeOH 16.5g

Part of the residue of the methanolic extract (12g) was redissolved in boiling water and filtered. The filtrate was extracted with diethyl ether (5 x 200 ml), ethyl acetate (5 x 200 ml) and n-butanol (5 x 200) successively. The extracts were concentrated to dryness and gave 1.50 g and 3.5g and 4.25 g of residue respectively.

The solid residue of dichloromethane was chromatographed on a Silica gel 60 (21x 1.8 cm) column using as mobile phase solvent mixtures of hexane, dichloromethane and methanol with increasing polarity. Fractions 26-31(corresponding to 25:75 hexane: dichloromethane) were combined and chromatographed again on a preparative TLC Silica gel 60 (20x20) plate with dichloromethane. From the zone with Rf=0,9 after elution with dichloromethane was isolated **compound 1 (14 mg)**.

The solid residue of diethyl ether was chromatographed on a preparative TLC (cellulose, CAW (CH_3Cl:CH_3COOH:H_2O 50:45:5). Two zones with different Rf were observed. From the zone with Rf=0,375 after elution with methanol was isolated **compound 2 (5 mg)**, while from the zone with Rf=0,77 after elution with methanol was isolated **compound 3 (1 mg)**

Half of the solid residue of the ethyl acetate extract was chromatographed on a Sephadex LH-20 (70X1.4) cm column with methanol and **compound 4 (3mg)** was isolated. The rest of the residue was chromatographed on a preparative TLC (Cellulose 20% CH_3COOH). **Compound 5 (4 mg)** was isolated from the zone with Rf=0,5.

Antioxidant and anti-inflammatory activity

Biological assays

1,1-diphenyl-picrylhydrazyl **(**DPPH), nordihydroguaeretic acid (NDGA) and trolox were purchased from the Aldrich Chemical Co. Milwaukee, WI, (USA), soybean LOX, and linoleic acid sodium salt were obtained from Sigma Chemical, Co. (St. Louis, MO, USA). For the *in vitro* tests a Lambda 20 (Perkin–Elmer) UV–Vis double beam spectrophotometer was used. Each in vitro experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

The stock solution of the tested extracts contained 5 mg/ml in DMSO.

Evaluation of antioxidant activity

Determination of the reducing activity of the stable radical 1,1-diphenyl-picrylhydrazyl (DPPH)

To a solution of DPPH (final concentration 0.05mM) in absolute ethanol an equal volume of the extracts dissolved in DMSO was added. As control solution ethanol was used. 20μl of the solutions of the extracts were used in the determination. After 20 and 60 min at room temperature the absorbance was recorded at 517 nm and compared with the appropriate standard NDGA

Evaluation of anti-inflammatory activity

Soybean lipoxygenase inhibition study in vitro

The bioassay was evaluated as reported previously[6]. The tested extracts 10 μl (5 mg/ml stock solution in DMSO) were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 ml of enzyme solution (1/9 x 10^4 w/v in saline) at a final volume of 1ml. The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor (nordihydroguaiaretic acid 0.1 mM 83.7 % inhibition).

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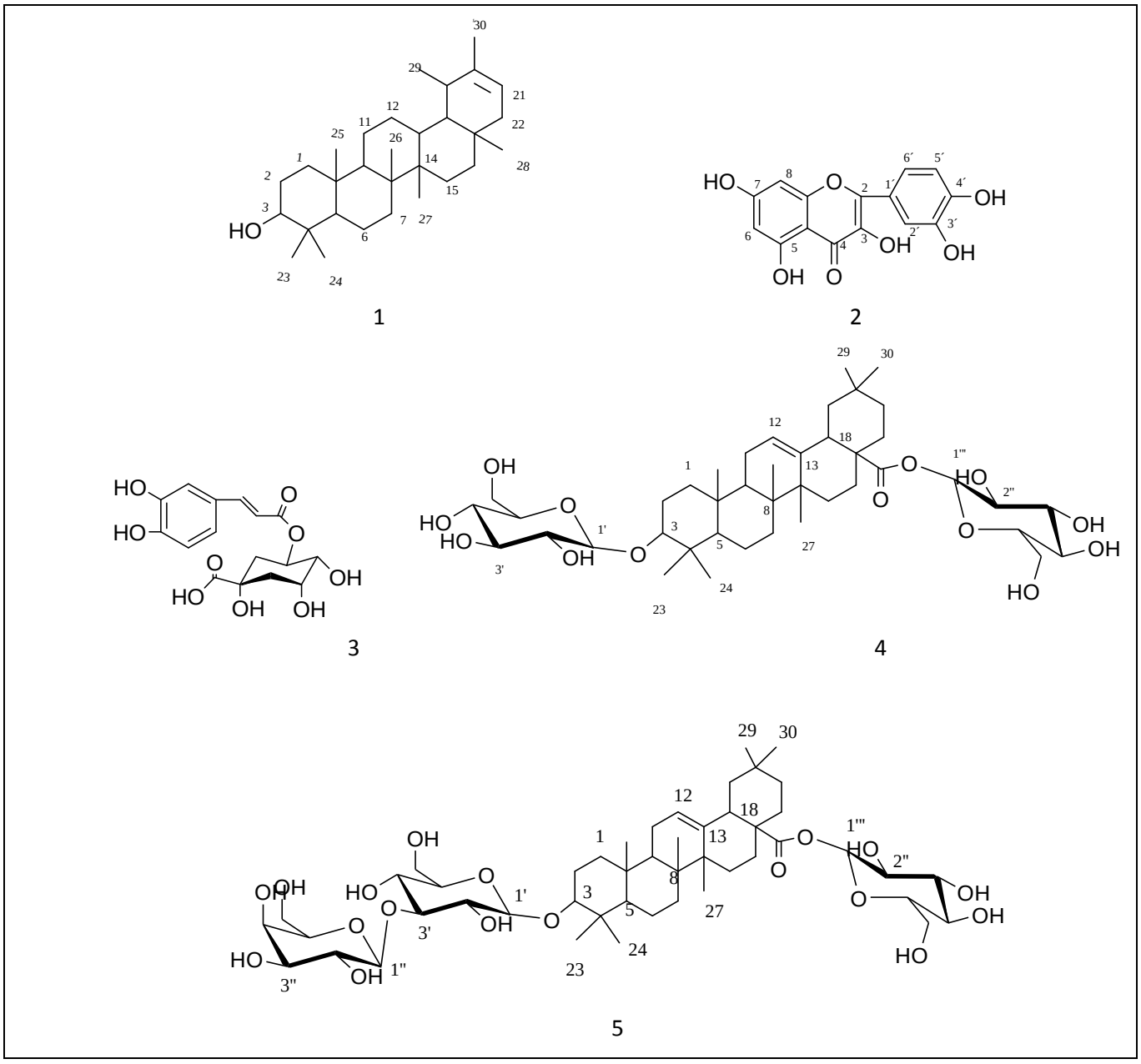
RESULTS AND DISCUSSION

Structure elucidation

The structure of **compound 1** (fig.1) was elucidated on the basis of spectral data (UV, ^{1}H NMR, ^{13}C NMR, HMBC, gCOSY and HSQC) as **pseudo-taraxasterol**. The results were also compared and are in total agreement with those of the data found in literature[7]. The structure of **compound 2** (fig.1) was elucidated on the basis of spectral data (UV, ^{1}H NMR) as **quercetin**[8]. The structure of **compound 3** (fig.1) was elucidated on the basis of spectral data (UV) as **chlorogenic acid**[9]. The U.V was taken with relation to a standard of chlorogenic acid and due to similarity of the U.V results, the Rf's taken and the fact that chlorogenic acid is a well known and studied compound, no further investigation was considered necessary.

The structure of **compound 4** (fig.1) was elucidated on the basis of spectral data (UV, ^{1}H NMR, ^{13}C NMR, HMBC, gCOSY and HSQC) as diglucoside of oleanolic acid.[10] The structure of **compound 5** (fig.1) was elucidated on the basis of spectral data (UV, ^{1}H NMR, ^{13}C NMR, HMBC, gCOSY and HSQC) as **triglycoside of oleanolic acid**.[11]

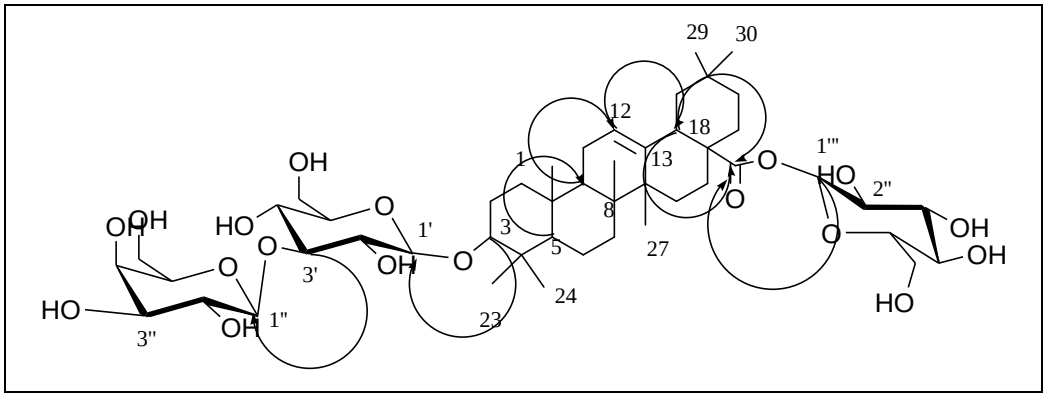
Fig.1: pseudotaraxasterol (1), quercetin (2), chlorogenic acid (3), diglucoside of oleanolic acid (4), triglycoside of oleanolic acid (5)



^{1}H NMR data of **compound 5** showed seven signals that correspond to seven –CH_3,(δ_H : 0.79, 0.83, 0.9, 0.92, 0.94, 1.04, 1.15) accounted correspondingly to C-23, C-24, C-25, C-26, C-27, C-29 and C-30 tertiary methyl protons. The appearance of all methyl signals between δ 1.15-0.79 supported the location of these methyl functionalities on saturated carbons. The ^{1}H NMR spectrum also displayed a one-proton downfield broad signal at δ 5.24 assigned to the vinylic C-12 proton. A one-proton multiple peak at δ 3.32 was ascribed to C-3 carbinol proton. A one-proton signal at δ 2.86 was associated with H-18. As seen on HMBC that peak was correlated with vinylic atoms of carbon and also with the carbon of carboxylic group. The remaining methylene and methine protons appeared in the range δ 2.86-1.04. The ^{13}C NMR spectrum of **compound 5** exhibited 36-carbon resonances which were compared with oleanen-type triterpenes.

Sugars: The ^{1}H NMR and ^{13}C NMR data showed three anomeric protons and carbons (δ_H 5.38, 4.38, 4.56 and δ_c 94.35, 104.96, 103.95) which indicates the presence of three sugar moieties. The sugar protons were unambiguously assigned using ^{1}H-^{1}H, COSY, HMBC and HSQC experiments. The attachments of glucose (Glc-1′) unit to C-3 of the aglycone, and of galactose (Gal 1′′) unit to C-3 of glucose (Glc-3′), were confirmed by HMBC. The main HMBC correlations are seen on fig 2 Also ^{1}H NMR showed a doublet of doublets at δ_H= 3.62 with J=5Hz and J=9Hz , attributed to H3′′, data that confirm that sugar 2 is galactose. All the data compared to literature confirm that sugar 1 is glycose and sugar 2 galactose .[12] The glycosilation site at C-28 was ascertained similarly. A Glc moiety attached to C-28 was confirmed from the presence of anomeric signal at δ_H 5.38 (J = 8Hz) and δ_C 94.35 The attachments of the glucose 1′′′ unit to C-28 of the aglycone, was confirmed by HMBC. Comparison of the ^{1}H and ^{13}C NMR data of compound 5 with those of compound 4 showed similar spectra with an additional sugar moiety in 5, which was deduced as galactose.

Fig 2. **Most important H-C correlations as seen on HMBC for compound 5.**



Compound 1: Pseudo-taraxasterol. TLC (silica): Rf: 0.25 (75% Hexane-25% Dichloromethane), Rf: 0,9^{1}H NMR (500 MHz, CD_3OD)

δ: 5.26 (1H, d, J = 6.5 Hz, H = 21), 3.21 (1H, dd, H = 3), 1.78 (1H, m, H = 15), 1.7 (1H, d, H = 1), 1.63 (1H, d, H = 2), 1.63 (1H, m, H = 12), 1.62(1H, s, H = 30) 1.78 (1H, m, H = 15), 1.61 (1H, m, H = 13), 1.58 (1H, q, H = 2), 1.56 (1H, d, H =19), 1.53 (1H, m, H = 22), 1.53 (1H, d, H =6), 1.53 (1H, m, H =11),1.41 (1H, m, H = 7), 1.38 (1H, m, H =6), 1.38 (1H, m, H =22), 1.37 (1H, m, H =7), 1.31 (1H, d, H =16), 1.3 (1H, d, H =9), 1.27 (1H, m, H =11), 1.23 (1H, m, H =21), 1.21 (1H, t, H =16), 1.04 (3H, s, H =26, CH_3), 1.04 (1H,overlapped, H =18), 1.01 (1H, d, H =15), 0.98 (3H, s, H =29, CH_3), 0.97 (3H, s, H =23, CH_3), 0.96 (1H, t, H =1), 0.95 (3H, s, H =27, CH_3), 0.84 (3H, s, H =25, CH_3), 0.77 (3H, s, H =24, CH_3), 0.73 (3H, s, H =28, CH_3), 0.70 (1H, s, H =5). ^{13}C NMR: δ 139.8 (C-20), 119 (C-21), 79.62(C-3), 55.29 (C-5), 50.37 (C-9), 48.95 (C-18), 42.31 (C-14), 42.06 (C-22), 41.04 (C-8), 39.01 (C-1), 39.01 (C-13), 38.82 (C-4), 37.52 (C-10), 36.87 (C-16), 36.59 (C-19), 34.89 (C-17), 34.10 (C-7), 28.3 (C-23), 27.29 (C-15), 27.15 (C-2), 27.05 (C-12), 22.4 (C-29), 21.65 (C-30), 21.0 (C-11), 18.57 (C-6), 17.52 (C-28), 16.04 (C-25), 15.85 (C-26), 15.4 (C-24), 15 (C-27).

Compound 2: Quercetin. Spot appearance: yellow; TLC (cellulose) : Rf: 0.77 (CAW), UV (λmax , MeOH, nm) 253,368 .^{1}H NMR (500 MHz, CD_3OD) δ: 7.62(1H, d, J = 2.0 Hz, H-2') , 7.54 (1H, dd, J = 8.5, 2.0 Hz, H-6') , 6.88 (1H, d, J = 8,5 Hz, H-5'), 6.38 (1H, d, J = 2 Hz, H-8), 6.17 (1H, d, J=2 Hz, H-6). The results are in correspondence with literature data.

Compound 3: chlorogenic acid. Spot appearance: blue (UV), light blue F1 (UV/NH3); Rf = 0.74 (AcOH 15%); UV (kmax, MeOH, nm): 250 sh, 290, 329.

Compound 4: Diglucoside of oleanolic acid ^{1}H NMR (500 MHz, CD_3OD) δ: 0.78 (1H, m, H = 5), 0.79 (3H, s, H = 26, CH_3), 0.83 (3H,s, H = 24, CH_3), 0.90 (3H, s, H = 30, CH_3), 0.92 (1H, s, H = 29, CH_3), 0.94 (3H, s, H = 25, CH_3), 1.01 (1H,

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m, H = 1), 1.04 (3H, s, H = 23, CH_3), 1.12 (1H, m, H = 19), 1.15 (3H, s, H = 27, CH_3), 1.27 (1H, m, H = 6), 1.3 (1H, overlapped, H = 15), 1.3 (1H, m, H = 2), 1.37 (1H, m, H = 6), 1.39 (1H, m, H = 21), 1.57 (1H, m, H = 9), 1.61 (1H, m, H = 7), 1.66 (1H, m, H = 2), 1.68 (1H, m, H = 22),1.71 (1H, m, H = 19), 1.72 (1H, m, H = 22), 1.73 (1H, overlapped, H = 21), 1.88 (1H, m, H = 11), 2.86 (1H, m, H = 18), 3.2 (1H, m, H = 3), 5.24 (1H, brs, H = 12), ^{13}C NMR: δ 14.59 (C-25), 15.66 (C-24), 16.21 (C-26), 17.62 (C-6), 22.11 (C-16), 22.39 (C-29), 22.6 (C-22), 22.80 (C-11), 24.75 (C-27), 26.95 (C-23), 27.38 (C-15), 28.6 (C-2), 30.19 (C-20), 32.01 (C-30), 32.50 (C-7), 33.49 (C-21), 36.43 (C-10), 38.65 (C-1), 38.96 (C-4), 39.20 (C-8), 41.10 (C-18), 41.45 (C-14), 45.94 (C-19), 46.48 (C-17), 47.61 (C-9), 55.23 (C-5), 89.56 (C-3), 122.36 (C-12), 144.43 (C-13), 176.13 (C-28) *Sugars glucose 1:* ^{1}H NMR (500 MHz, CD_3OD) δ : 4,54 (1H, d, J=7.5 Hz H-1’), 3.82 (1H, H-6΄), 3.67 (1H, H-6΄), 3.61 (1H, H-2΄). ^{13}C NMR: δ 104.35 (C-1΄), 71.31 (C-2΄), 61.3 (C-6΄). *glucose 2:* ^{1}H NMR (500 MHz, CD_3OD) δ : 5.37 (1H, d, J=8Hz, H-1΄΄), 3.82 (1H, H-6΄΄), 3.67 (1H, H-6΄΄), 3.31 (1H, H-2΄΄). ^{13}C NMR: δ 94.59 (C-1΄΄), 70.29 (C-2΄΄), 60.91 (C-6΄΄).

Compound 5: Triglycoside of oleanolic acid ^{1}H NMR (500 MHz, CD_3OD) δ: 0.78 (1H, m, H = 5), 0.79 (3H, s, H = 26, CH_3), 0.83 (3H, s, H = 24, CH_3), 0.90 (3H, s, H = 30, CH_3), 0.92 (3H, s, H = 29, CH_3), 0.94 (3H, s, H = 25, CH_3), 1.01 (1H, m, H = 1), 1.04 (3H, s, H = 23, CH_3), 1.12 (1H, m, H = 19), 1.15 (3H, s, H = 27, CH_3), 1.27 (1H, m, H = 6), 1.3 (1H, overlapped, H = 15), 1.3 (1H, m, H = 2), 1.37 (1H, m, H = 6), 1.39 (1H, m, H = 21), 1.57 (1H, m, H = 9), 1.61 (1H, m, H = 7), 1.66 (1H, m, H = 2), 1.68 (1H, m, H = 22),1.71 (1H, m, H = 19), 1.72 (1H, m, H = 22), 1.73 (1H, overlapped, H = 21), 1.88 (1H, m, H = 11), 2.86 (1H, m, H = 18), 3.2 (1H, m, H = 3), 5.24 (1H, brs, H = 12), ^{13}C NMR: δ 14.59 (C-25), 15.68 (C-24), 16.03 (C-26), 17.82 (C-6), 22.11 (C-16), 22.32 (C-29), 22.6 (C-22), 22.85 (C-11), 24.93 (C-27), 26.99 (C-23), 27.37 (C-15), 28.6 (C-2), 30.18 (C-20), 32.01 (C-30), 32.51 (C-7), 33.49 (C-21), 36.42 (C-10), 38.61 (C-1), 38.96 (C-4), 39.21 (C-8), 41.09 (C-18), 41.42 (C-14), 45.94 (C-19), 46.45 (C-17), 47.5 (C-9), 55.6 (C-5), 89.5 (C-3), 122.3 (C-12), 144.43 (C-13), 176.13 (C-28) *Sugars glucose 2:* ^{1}H NMR δ: 5,38 (1H, d, J=8Hz, H-1΄΄΄), 3.82 (1H, H-6΄΄΄), 3.67 (1H, H-6΄΄΄), 3.38 (1H, H-3΄΄΄), 3.34 (1H, H-4΄΄΄), 3.34(1H, H-5΄΄΄), 3.32 (1H, H-2΄΄΄). ^{13}C NMR: δ 94.35 (C-1΄΄΄), 77.19 (C-5΄΄΄), 76.84 (C-3΄΄΄), 72.50 (C-2΄΄΄), 69.69 (C-4΄΄΄), 60.91 (C-6΄΄΄). *glucose 1:* ^{1}H NMR δ : 4,38 (1H, H-1΄), 3.82 (1H, H-6΄), 3.67 (1H, H-6΄), 3.61 (1H, H-3΄), 3.60 (1H, H-4΄), 3.56(1H, H-5΄), 3.43 (1H, H-2΄). ^{13}C NMR: δ 104.96 (C-1΄), 84.57 (C-3΄), 75.66 (C-5΄), 73.55 (C-2΄), 70.64 (C-4΄), 61.3 (C-6΄). *galactose 1:* ^{1}H NMR: δ 4,56 (1H, H-1΄΄), 3.82 (1H, H-6΄΄), 3.67 (1H, H-6΄΄), 3.62 (1H, H-2΄΄), 3.61 (1H, H-5΄΄), 3.57 (1H, H-4΄΄), 3.49 (1H, H-3΄΄). ^{13}C NMR: δ 103.95 (C-1΄΄), 75.43 (C-5΄΄), 73.25 (C-3΄΄), 71.40 (C-2΄΄), 70.80 (C-4΄΄), 60.99 (C-6΄΄).

Biological Results

Antioxidants have been found to have some degree of preventive and therapeutic effects. Chemopreventive, antiviral and cytostatic properties are related to antioxidative activities. The antioxidant activity of the extracts was evaluated in different assays. The radical scavenging ability of the extracts was tested against the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) stable free radical. The interaction of the examined extracts with the stable free radical DPPH is shown in Table 1, in comparison to a well-known antioxidant agent, nordihydroguaiaretic. Both diethyl ether and ethyl acetate extracts presented the higher interaction % and they were almost equipotent. The methanolic as well as the butanolic extracts followed. No changes within the interaction values were observed after 60 min (Table 2). Literature data[13] suggest that after the use of ESR for examination of the antioxidant activity the fractions of ethyl acetate and total methanol fraction give relevant results. Water fraction appeared to have no antioxidant activity.

Table 1: Percent interaction of the extracts with the stable radical 1,1-diphenyl-picrylhydrazyl (DPPH) after 20 mins

MeOH	71,4%
D.ether	89.85%
E.acetate	86.61%
Butanol	33.47%
Water	8.92%
NDGA	81.0%

Table 2: Percent interaction of the extracts with the stable radical 1,1-diphenyl-picrylhydrazyl (DPPH) after 60 mins

MeOH	44.10%
D.ether	88.2%
E.acetate	87.08%
Butanol	35.85%
Water	1.78%
NDGA	82.6%

Antioxidants acting as lipoxygenase’s inhibitors could offer for health’s maintainance. Thus, our extracts were tested against soybean lipoxygenase LOX. Most of the LOX inhibitors present antioxidant activity or act as free radical scavengers[14]. Since lipoxygenation occurs via a carbon centered radical. It has been found that LOX inhibition is correlated to the reducing ability of the inhibitors of the Fe^{3+} at the active site to the catalytically inactive Fe^{2+}. Several LOX inhibitors proved to be excellent ligants for Fe^{3+}.

Table 3 : *In vitro* lipoxygenase (LOX) inhibitory activity of extracts

MeOH	16.666%
D.ether	5.970%

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E.acetate	44.138%
Butanol	38.820%
Water	34.195%
NDGA	83.700%

Herein, is for the first time that an anti-Lipoxygenase study has been conducted to these fractions using a LOX enzyme (of natural origin). The extracts of the ethyl acetate as well as of the butanol and their residues, proved to be the strongest inhibitors of lipoxygenase (table 3). This activity might be correlated to the presence of flavonoids in these extracts.

CONCLUSIONS

Phytochemical analysis of the plant lead to isolation of five compounds: pseudotaraxasterol, quercetin, chlorogenic acid, diglycoside of oleanolic acid and triglycoside of oleanolic acid. After investigation of literature data it seems that compound 5 has been isolated for the first time in this genus. Triglycosides of oleanolic acid have been isolated but not with this particular link between Gal and Glc 1. The ethyl acetate extracts exhibited the best biological profile, presenting simultaneously antioxidant and anti-lipoxygenase activities.

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