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Biological activities and luteolin derivatives of *Verbascum eskisehirensis* Karavel., Ocak & Ekici

Gözde ÖZTÜRK [1]*, Hale Gamze AĞALAR [1], Gülsüm YILDIZ [1], Fatih GÖGER [1,2], Neşe KIRIMER [1]

[1] Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, 26470, Eskişehir, Turkey.
[2] Department of Pharmacy, Yunus Emre Vocational School, Anadolu University, 26470, Eskişehir, Turkey.
* Corresponding Author. E-mail: gozde_ozturk@anadolu.edu.tr (G.Ö.); Tel. +90-222-335 05 80.

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ABSTRACT: In this present study, the aerial parts of *Verbascum eskisehirensis* were dried in the shade and air-flowed area. The dried samples were macerated with 70% methanol in water for 24 h by three times. The biological activities of the extract was determined by *in vitro* methods. The antioxidant activity was evaluated in 2,2′-diphenyl-1-picrylhydrazyl (DPPH˙) radical scavenging assay and the trolox equivalent antioxidant capacity (TEAC) was measured the ability of the extract to scavenge the stable radical cation $ABTS^{+}$ (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)). The antibacterial effects against some clinical pathogens were tested by broth microdilution assay. The *in vitro* antiinflammatory activity was also examined by 5-Lipoxygenase (5-LOX) enzyme inhibitory test. In the same time, the phenolic composition of the extract was determined by LC-ESI-MS/MS system. According to results, the extract was found rich-in luteolin derivatives. The major compound was identified as luteolin-pentosyl-hexoside. The antioxidant activities of the extract were moderate. The extract showed weak antibacterial activity against tested bacteria strains. *Salmonella typhimurium* ATCC 13311 was more sensitive to the extract than the others. The extract showed weak antiinflammatory activity.

KEYWORDS: *Verbascum eskisehirensis*; luteolin; antioxidant; antimicrobial; antiinflammatory.

1. INTRODUCTION

The genus *Verbascum* Linnaeus commonly known as 'mullein' in English and as 'sığır kuyruğu' in Turkish, is the one of the largest genus of the Scrophulariaceae, comprises more than 2500 species in the world [1]. Members of this genus have been known as potent medicinal plants according to the folk medicine for centuries. The leaves and flowers are known as expectorant, mucolytic and demulcent. For this reason, *Verbascum* species are used to treat respiratory disorders such as bronchitis, dry coughs, tuberculosis and asthma in traditional Turkish medicine. In Europe, Asia and North America, various *Verbascum* species are used to for antiseptic, antimalarial, astringent, demulcent, emollient, sedative, narcotic effects and also, they are used for the treatment of tumors, inflammations, migraine, asthma and spasmodic coughs. The species are recorded to treat haemorrhoids, rheumatic pain, superficial fungal infections, wounds and diarrhoea, and have inhibitory activities against some influenza viruses [2-5].

Phytochemical studies on *Verbascum* species indicated the presence of several secondary metabolites such as iridoids and its glycosides, flavonoids, phenylethanoid and its glycosides, neolignan glycosides and saponins [6-12].

As known, Turkey is very rich-in endemic plants which are used for medicinal and aromatic purposes. The genus *Verbascum* is represented in Turkey by 251 species in 13 groups. Among them 195 taxa are endemics to Turkey [13]. One of the endemic species of the *Verbascum* genus is *Verbascum eskisehirensis* Karavel., Ocak & Ekici. It is distributed in Eskişehir in Central Anatolia. It was firstly described by Karavelioğlu et al. [14].

In spite of the fact that most species of the *Verbascum* genus have been widely investigated for phytochemicals and potential biological activities, there was no report on the phytochemical profile and possible biological activities on *V. eskisehirensis* until now. In our study, it was aimed to determine the phenolic composition of the polar extract prepared from the aerial parts by LC-ESI-MS/MS system. The potential antioxidant, antimicrobial and antiinflammatory activities were evaluated by *in vitro* methods.

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2. RESULTS

2.1. Extraction yield

The extraction yield was calculated as 24% of the plant material.

2.2. LC-MS/MS results

According to spectral data and chromatogram, luteolin and its derivatives were identified in the extract. Table 1 summarizes HPLC-DAD and MS/MS data obtained for the extract.

Table 1. The phytochemical profile of *Verbascum eskisehirensis* extract.

Peak	Rt	[M-H]-	MS^2	Compound	Reference
1	12.3	579	447, 285	Luteolin pentosyl-glucoside	[44]
2	13.6	449	285	Luteolin glucoside	[9]
3	14.2	461	285	Luteolin glucuronide	[18]
4	21.2	285	217,199, 151, 133	Luteolin	[18]

MS analysis was obtained in negative mode, because of the high sensitivity in the detection of the phenolic compounds. The chromatogram of the extract was given in Figure 1.

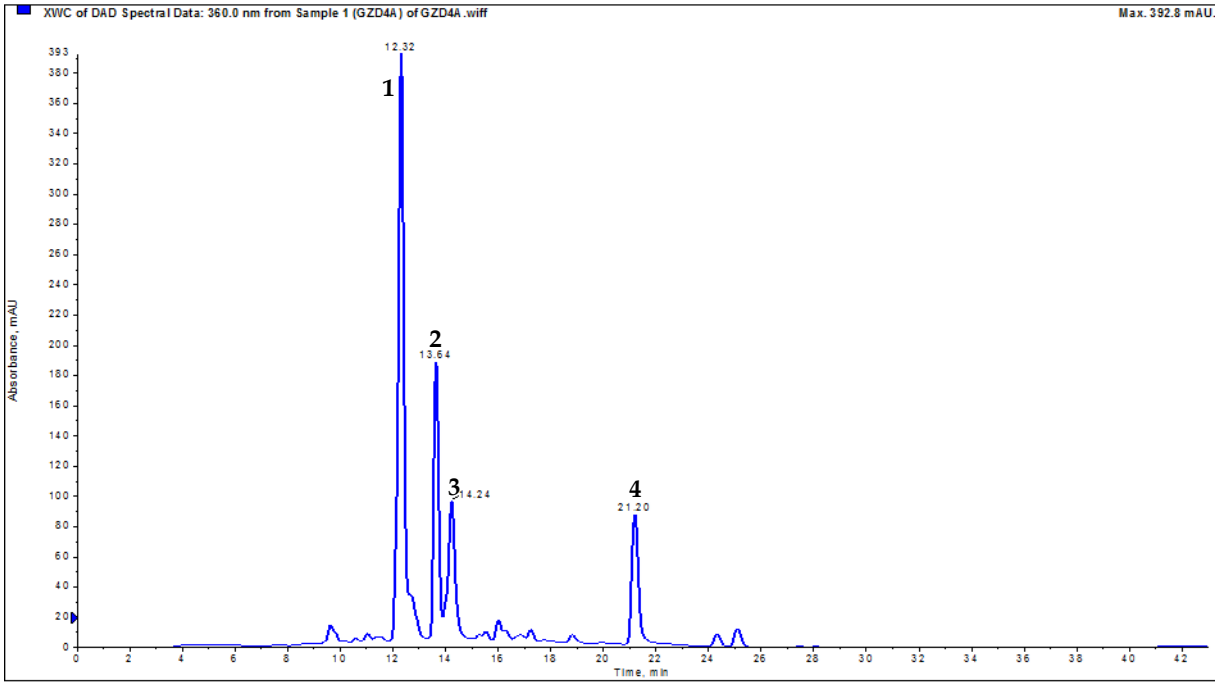

Figure 1. The HPLC chromatogram of the extract at 360 nm.

Compound 1 was identified as luteolin-pentosyl-glucoside. This compound showed a molecular ion at *m/z* 579 $[M-H]^-$ and fragmentations at *m/z* 447 $[M-H-132]^-$ and *m/z* 285 $[M-H-132-162]^-$. These fragments supported that compound was as an *O*-flavone. The fragment at *m/z* 447 occurred by the loss of *O*-pentosyl-moiety (-132 µ). The fragment at *m/z* 285 occurred by a sequential loss of pentosyl- (-132 µ) and glucosyl- (-162 µ) moieties. The fragment at *m/z* 285 was also characteristic for luteolin molecule. The spectrum was given in Figure 2.

Compound 2 was identified as luteolin-glucoside which produced a molecular ion at *m/z* 447 and yielded fragment ion at *m/z* 285. The fragment ion at *m/z* 285, corresponding to $[M-H-162]^-$, was produced by the loss of glucosyl- moiety.

Compound 3 was identified as luteolin-glucuronide. The ions at *m/z* 461 $[M-H]^-$ and *m/z* 285 $[M-H-176]^-$ corresponding to loss of glucuronide unit supported the conclusion that this compound was luteolin glucuronide.

Compound 4 was identified as luteolin. The spectrum was characteristic to luteolin. As known, quasi-molecule ion of the flavone aglycones undergo a specific retro Diels-Alder reaction that contributed to the product ions. For luteolin, the molecular ion was *m/z* 285 $[M-H]^-$ and the characteristic product ion was *m/z* 133 $[M-H-C_7H_4O_4]^-$.

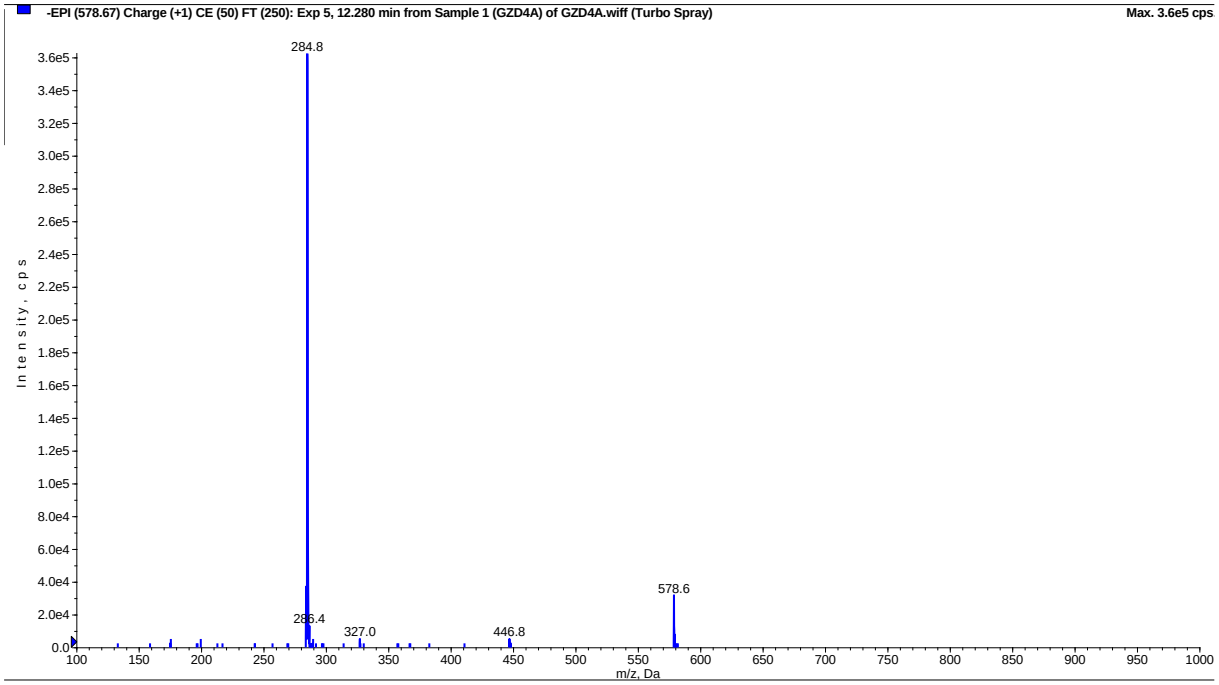

Figure 2. The mass spectrum of luteolin pentosyl-glucoside.

2.3. Biological activity results

2.3.1. Antioxidant activity results

The value of the Trolox Equivalent Antioxidant Capacity (TEAC) of the extract at 5.6 mg/ml concentration was calculated as 0.184 ± 0.08 mM while TEAC value of ascorbic was 0.869 ± 0.01 mM.

The extract showed very low 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity (IC_{50} value-176.7 µg/ml) when compared to ascorbic acid (IC_{50} value-6.3 µg/ml).

The extract was more effective against 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical than DPPH radical. But this effect was lower than ascorbic acid.

2.3.2. Antimicrobial activity results

The extract had antimicrobial effect against *Salmonella typhimurium* ATCC 13311 at 2.5 mg/ml (MIC). The MIC values of the extract were calculated as more than 5 mg/ml concentration against *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* ATCC 700699. Ciprofiloxacin used in bacterial infections in medical treatment had MIC at 0.04 mg/ml.

2.3.3. Antiinflammatory activity results

The extract at 100 µg/ml concentration showed weak anti-inflammatory activity (17.9 ± 1.3 % inhibition of 5-lipoxygenase (5-LOX) while the positive control, Nordihydroguaiaretic acid (NDGA) showed strong antiinflamatory activity. The IC_{50} value of NDGA calculated as 5.9 ± 0.3 µg/ml. Unfortunately, the IC_{50} value the extract was not calculated because of the low activity.

3. DISCUSSION

3.1. Evaluation of LC-MS/MS results

The members of *Verbascum* genus are known as good sources of various iridoids, phenylethanoids, phenylpropanoids, saponins and flavonoids and their glycosylated forms [15-17].

As seen, the major compound of the extract was identified as luteolin-pentosyl-glucoside. Up to date, this compound has never been identified in *Verbascum* species before. Other luteolin derivatives identified in the extract were isolated in many species of *Verbascum* genus [18,19]. Based on the published data, flavanone, flavones and flavonols and their glycosides were identified in different *Verbascum* species. Also, isoflavonoids and bisflavonoid structures were isolated. Flavonoids identified in *Verbascum* species growing wild in Turkey were summarized in Table 2. According to Table 2, apigenin and luteolin and their 7-*O*-glucosides are common flavones in the species growing in Turkey.

To our knowledge, there is no report on phytochemical profile of *Verbascum eskisehirensis* samples. Furthermore, luteolin-pentosyl-glucoside was identified for the first time in *Verbascum* species.

Table 2. Flavonoids identified in Turkish *Verbascum* species.

Species	Collected area	Parts used	Flavonoids	References
V. dudleyanum (Hub.-Mor.) Hub.-Mor. (endemic)	Burdur	Aerial parts	Luteolin-7-*O*-*β*-glucopyranoside	[18]
V. salviifolium Boiss. (endemic)	Burdur	Aerial parts	Luteolin-7-*O*-*β*-glucoside Luteolin-3″-*O*-glucoside Apigenin-7-*O*-glucoside Chrysoeriol-7-*O*-glucoside	[18, 20]
V. mucronatum Lam.	Aksaray	Flowers	Luteolin-7-*O*-glucoside Luteolin-3-*O*-glucoside Apigenin-7-*O*-glucoside	[21]
V. pinetorum Boiss. O Kuntze (endemic)	Kahramanmaraş	Not defined	Myricetin Fisetin Quercetin Hesperetin Luteolin Kaempferol Apigenin Rhamnetin	[22]
	Hatay	Aerial parts	Rutin Naringin Hesperidin Eriodictiol Quercetin Morin	[23]
V. cheiranthifolium Boiss.	Bilecik	Aerial parts	Apigenin Luteolin Vitexin Swertisin	[24]
V. bugulifolium Lam.	İstanbul	Aerial parts	Apigenin-7-*O*-rutinoside Luteolin-7-*O*-*β*-*D*-glucopyranoside Luteolin-7-*O*-rutinoside	[25]
V. caesareum Boiss.	Hatay	Aerial parts	Luteolin 6-Hydroxy-luteolin Luteolin-7-glucoside Quercetin-3,7-diglucoside 6-Hydroxy-luteolin-7 glucoside Apigenin-7-glucoside	[26]
	Hatay	Aerial parts	Rutin Naringin Quercetin Vitexin Morin	[23]

Table 2. (*Continued*) Flavonoids identified in Turkish *Verbascum* species.

Species	Collected area	Parts used	Flavonoids	References
V. flavidum (Boiss.) Freyn & Bornm	Kahramanmaraş	Whole parts	Rutin	[27]
V. lagurus Fisch. & C. A. Mey	Kırklareli	Aerial parts	Luteolin Luteolin-7-glucoside Diosmetin-7-glucoside	[28]
V. antiochium Boiss.	Hatay	Aerial parts	Rutin Naringin Hesperidin Eriodictyol Quercetin	[23]
V. gaillardotii Boiss.	Hatay	Aerial parts	Naringin Hesperidin Quercetin Eriodictyol	[23]
V. galilaeum Boiss.	Hatay	Aerial parts	Naringin Quercetin Eriodictyol	[23]
V. sinuatum L.	Hatay	Aerial parts	Naringin Hesperidin Eriodictyol Quercetin Morin	[23]
V. tripolitanum Boiss.	Hatay	Aerial parts	Naringin Hesperidin Quercetin Vitexin	[23]
V. inulifolium HUB.-MOR. (endemic)	Mersin	Aerial parts	Myricetin Luteolol Kaempferol Isorhamnetin	[29]
V. obtusifolium Hub.-Mor. (endemic)	Mersin	Aerial parts & Calyx	Myricetin Luteolol Kaempferol Isorhamnetin	[30]

3.2. Evaluation of biological activity

3.2.1. Evaluation of antimicrobial activity results

Antimicrobial activities against different microorganisms of various *Verbascum* species were investigated. In our study, *Salmonella typhimurium* ATCC 13311, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* ATCC 700699 were used. As known, *Staphylococcus* species are widespread in the world. These Gram-positive species cause various infections among people and animals. *S. aureus* causes superficial skin lesions and localized abscesses in other sites, deep-seated infections, such as osteomyelitis and endocarditis and more serious skin infections and also is a major cause of hospital acquired infection of surgical wounds and, with *S. epidermidis*, causes infections associated with indwelling medical devices. In the same time, it causes food poisoning by releasing enterotoxins into food [31]. *S. typhimurium* shows foodborne pathogenesis [32].

Among *Verbascum* species, the antibacterial activity against *S. aureus* was summarized at below. In a recent work, minimum inhibitory concentration of *V. sinuatum* L. methanolic extract was found 32 µg/ml against *S. aureus* ATCC 13709. Luteolin-7-glucoside, ajugol, aucubin, verbascoside, sinuatol from the methanolic extract of *V. sinuatum* L. were tested for their antibacterial activities. Verbascoside showed the highest antibacterial activity against all strains [33]. The methanol extract of *V. thapsus* aerial parts had growth inhibitory effects on *E. coli* and *S. pyrogenes* (MIC: 31.25 µg/ml) [34]. The methanolic extracts of the leaves, flowers, roots and seeds of *V. blattaria* L., *V. bombyciferum* Boiss., *V. chaixii* Vill., *V. dumulosum* P.H.Davis & Hub.-Mor., *V. nigrum* L., *V. olympicum* Boiss., *V. phlomoides* L., *V. phoeniceum* L. and *V. roripifolium* (Halacsy)

I.K.Ferguson had a strong antimicrobial activity against *S. aureus* ATCC 6538P [35]. It was indicated that *V. sinaiticum* Benth. extract (100, 50, 25 mg/ml) had strong antibacterial activity against *S. aureus* by agar well diffusion method [36].

In Turkey, *V. chionophyllum* Hub.-Mor., *V. cilicicum* Boiss., *V. pterocalycinum* var. *mutense* Hub.-Mor., *V. pycnostachyum* Boiss. & Heldr., *V. splendidum* Boiss., *V. prusianum* Boiss., *V. pseudoholotrichum* Hub.-Mor., *V. cymigerum* Hub.-Mor., *V. cholorostegium* Bormm. & Murb., *V. linguifolium* Hub.-Mor., *V. pellitum* Hub.-Mor. were evaluated for their antibacterial effect against *S. aureus* [37- 39]. Among these species, Dulger et al. [38] reported that the polar extracts of *V. pseudoholotrichum*, *V. cymigerum, V. cholorostegium, V. linguifolium, V. pellitum* had no significant activities against Gram (+) bacteria such as *S. aureus* ATCC 6538P. *V. prusianum* was found to have noticeable antibacterial activity against *S. aureus* ATCC 6538P [37]. Also, Dulger et al [37] recorded that the extracts of *Verbascum* L. species had antibacterial activity against *Staphylococcus aureus* ATCC 5538P while there were no significant activities against *Salmonella thyphi* ATCC 19430. The methanolic extracts of *V. chionophyllum, V. cilicicum, V. pterocalycinum* var. *mutense, V. pycnostachyum, V. splendidum* were evaluated for their *in vitro* antimicrobial activities against *S. aureus* and they were found % growth inhibition 19, 0, 9, 18, 55, respectively [39]. When compared to the published articles, our results indicated that this endemic plant was found to have a weak antibacterial activity.

3.2.2. Evaluation of antioxidant activity results

In a previous study, antioxidant properties of various fractions of the methanolic extract obtained from the aerial parts of *V. macrurum* Ten. were determined by monitoring their capacity to scavenge the free-radical diphenylpicrylhydrazyl (DPPH). Ten compounds were identified as the components of this methanolic extract. Their activities were compared to *a*-tocopherol and butylated hydroxytoluene (BHT), which were used as positive controls for the tests. Assessment of their antioxidant activities established that acteoside was the most potent free radical scavenger. Its activity was comparable to the synthetic antioxidant BHT and clearly superior to natural *a*-tocopherol [40]. The methanolic extract of *V. wiedemannianum* Fisch.&Mey., which is endemic to Turkey, and its phenylethanoid glycosides, wiedemannioside A-C, acteoside, martynoside, echinacoside and leucoseptoside B, were screened for possible *in vitro* antioxidant activity by two complementary test systems, namely DPPH free radical-scavenging (by bioautography and spectrophotometry) as well as *β*-carotene/linoleic acid test system. In the first case, *V. wiedemannianum* extract exerted an insignificant antioxidant activity with an IC_{50} value of 117 ± 0.56 µg/ml when compared to BHT (18.0 ± 0.40 µg/ml). In the *β*-carotene/linoleic acid test system, *V. wiedemannianum* exhibited antioxidant activity with 52.5 ± 3.11% inhibition rate [41-42].

According to recent work of Tatlı et al. [10], free radical scavenging of 36 secondary metabolites isolated from the methanolic extracts of *V. cilicicum* Boiss., *V. lasianthum* Boiss. ex Bentham, *V. pterocalycinum* var. *mutense* Hub. Mor., and *V. salviifolium* Boiss. were investigated. The isolated compounds, exhibited a dose dependent inhibition of bioautographic and spectrophotometric DPPH activities. Verbascoside was the most active with IC_{50} value of 4.0 µg/ml in comparison with vitamin C (IC_{50} 4.4 µg/ml).

3.2.3. Evaluation of antiinflammatory activity results

As known, several *Verbascum* species have been reported as a treatment for tumors, inflammations, migraine, asthma and spasmodic coughs in Europe, Asia and Nothern America [46]. In Turkish traditional medicine, *Verbascum* species are used to treat eczema, other types of inflammatory skin conditions and as a desiccant for wounds [49]. Studies on antiinflammatory activities of *Verbascum* species were reported by *in vitro* and *in vivo* models. The methanol extracts of the flowers of *V. chionophyllum* and *V. pycnostachyum*, and the aerial parts of *V. latisepalum* and *V. salviifolium*, displayed significant antinociceptive and antiinflammatory activity at 200 mg/kg oral dose in mice [48].

The methanolic extracts of *V. lasianthum* flowers and the aerial parts of *V. dudleyanum* were shown to possess significant inhibitory activity in the carrageenan-induced hind paw edema model and in *p*-benzoquinone-induced writhings in mice. Aucubin (an iridoid glucoside) and ilwensisaponin A (a triterpenic saponin) isolated from these plants were found to possess significant antinociceptive and antiinflammatory activities [9, 47].

The extract rich in iriodid glucosides of *V. mucronatum* was reported as antiinflammatory, antinociceptive and wound healing activities. Among isolated compounds, verbascoside was found to have significant activities [21].

Süntar et al [49] reported that the wound healing activities of the methanolic extracts of thirteen *Verbascum* species grown in Turkey by *in vivo* model. These species were *V. chionophyllum, V. cilicicum, V. dudleyanum, V. lasianthum, V. latisepalum, V. mucronatum, V. olympicum, V. pterocalycinum* var. *mutense, V. pycnostachyum, V. salviifolium, V. splendidum, V. stachydifolium* and *V. uschackense*. According to results, *V. olympicum, V. stachydifolium* and *V. uschackense* demonstrated the highest activities on tested wound models. Also, *V. latisepalum, V. mucronatum, V. pterocalycinum* var. *mutense* was reported as highly effective on tested models.

In published data on Turkish *Verbascum* species, the antiinflammatory effects were revealed by different models. In the present study, the polar extract of the endemic species, *V. eskisehirensis*, was evaluated for the possible antiinflammatory activity by *in vitro* 5-LOX inhibition. Our results showed that the endemic species had weak activity. In the same time, the extract was found to be rich in luteolin and its derivatives especially, luteolin-pentosy-glucoside.

As known, inflammation is a complex biochemical reaction. To evaluate antiinflammatory activitiy, different models should be investigated. According to a study, luteolin 7-*O*-glucoside, luteolin 3'-*O*-glucoside, apigenin 7-*O*-glucoside and *β*-hydroxyacteoside from the aqueous extract of *V. salviifolium* aerial parts significantly inhibited carrageenan-induced paw edema at a 200 mg/kg dose, also luteolin 7-*O*-glucoside, luteolin 3'-*O*-glucoside and *β*-hydroxyacteoside also displayed antiinflammatory activity against the PGE1-induced hind paw edema model in mice. It was also reported that these compounds showed no effect in the TP-induced ear edema model [19]. The antiinflammatory activities of luteolin and its derivatives were critically reviewed by Aziz et al [50]. The review concluded that *in silico, in vitro, in vivo* and clinical studies strongly suggested that luteolin had antiinflammatory effect by regulting of transcription factors such as STAT3, NF-kB, and AP-1. In that review, luteolin was reported as a strong inhibitor of 5-LOX, a component of an important pathway leading to the production of leukotriene B4, a mediator of several inflammatory diseases. It can be concluded that the glucosylation of luteolin reduce the 5-LOX activity.

4. CONCLUSION

The results concluded that the polar extract of *Verbascum eskisehirensis* was rich in luteolin and its derivatives. Luteolin and other type of flavonoids and their derivatives were commonly found in vary *Verbascum* species. The extract was found to have weak antioxidant, weak antiinflammatory and moderate antibacterial activity.

5. MATERIALS AND METHODS

5.1. Chemicals and standards

Ultra-pure water was used throughout and was prepared using a Millipore Milli-RO 12 plus system (Millipore Corp., MA, USA). All remaining solvents were of the highest purity available: Methanol HPLC grade (Sigma-Aldrich), Acetonitrile anhydrous 99.8 % (Sigma-Aldrich), Ethanol absolute (Riedel), Dimethyl sulphoxide extra pure (Sial), Glycerol ≥ 99.5 % (Sigma-Aldrich), Formic acid ≥ 95 % (Sigma-Aldrich). The chemicals and standards were purchased from different companies: Ascorbic acid (Vitamin C) 99 % (Sigma-Aldrich), Ciprofloxacin (Sigma-Aldrich), Nordihydroguaiaretic acid (NDGA) ≥ 90 % (HPLC) (Sigma), 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) ≥ 98 % (HPLC) (Sigma), Trolox 97 % (Aldrich), Linoleic acid ≥ 99 % (Sigma), Resazurin (Sigma-Aldrich), Sodium persulfate (Sigma-Aldrich), Dipotassium hydrogen phosphate (Merck), Potassium dihydrogen phosphate (Merck). Microorganisms strains used for the evaluation of antimicrobial activity were obtained from the American Type Culture Collection (ATCC) in lyophilized form. 5-Lipoxygenase (5-LOX) from *Glycine max* was purchased from Sigma company.

5.2. Plant Material

Verbascum eskisehirensis Karavel., Ocak & Ekici. was collected from Sivrihisar, Eskisehir, Turkey. The plant was identified by Prof. Dr. Atila Ocak (Osmangazi University, Faculty of Science and Literature, Department of Biology, 26480, Odunpazarı, Eskişehir). The samples are kept in Anadolu University, the Herbarium of Pharmacy Faculty with archive number 38. The aerial parts of the plant separated from the roots were dried in the shade.

5.3. Extraction method

25 g of the dried and grounded aerial parts were macerated with 250 ml of 70% methanol in water for 24 h by three times. The extract was filtered and combined. The extract was freeze-dried after methanol removal. It was kept at -20°C until the assays.

5.4. LC-MS/MS analyzes

Separation and detection of phytochemicals of the extract were performed on a Shimadzu UPLC system consisting of a vacuum degasser, an autosampler (SIL20A Shimadzu Autosampler), a binary pump (LC20AD Shimadzu), an oven (CTO20A Shimadzu Column Oven) and DAD dedector (SPD M20A Shimadzu DAD Detector). The instrument was equipped with a GL Science Intersil ODS column (4.6 x 250 mm, 5 µm particle size). The mobile phase was acetonitril:water:formic acid (10:89:1, *v/v/v*) (solvent A) and acetonitril:water:formic acid (89:10:1, *v/v/v*) (solvent B) at a flow rate of 0.5 ml/min. The gradient elution started with 10 % solvent B at 0 min, 100 % solvent B reached at 40 min. The column temperature was kept at 40°C with an injection volume of 10 µl. The chromatogram was recorded at 360 nm.

The UPLC system was connected directly to a 3200 Q TRAP (AB Sciex, Toronto, Canada), supported with an electrospray ionization interface (ESI). For enhanced mass scan (EMS), the mass system was operated in negative polarity at a scan rate of 1000 Da/s within the mass range of 100-1200 amu. Mass scan (MS) and daughter (MS/MS) spectra were measured from *m/z* 100-1200. Collision-induced fragmentation experiments were performed in the ion trap using nitrogen. The software used for data acquisition and analysis is Analyst 1.6. The chromatographic conditions were used as described above.

5.5. The biological activity

5.5.1. DPPH• radical scavenging activity

Serial dilutions were carried out with the stock solutions (10 mg/ml) of the extracts to obtain the concentrations of 10, 5, 25×10^{-1}, 125×10^{-2}, 625×10^{-3}, 3125×10^{-4} mg/ml. Diluted solutions were mixed with DPPH (equal amounts) and allowed to stand for 30 min for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive control ascorbic acid. The percentage inhibition was calculated using Equation 1. The IC_{50} value, which is the concentration of the test material that inhibits 50 % of the free radical concentration, was calculated as mg/ml [43].

$$\text{Inhibition Percentage} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] x100 \qquad \text{(Eq. 1)}$$

5.5.2. TEAC assay (Trolox equivalent antioxidant capacity)

This assay assesses the capacity of a compound to scavenge the stable ABTS radical in comparison to the antioxidant activity of Trolox, a water-soluble form of vitamin E that is used as a standard. The blue-green ABTS was produced through the reaction of 7 mM ABTS with 2.5 mM sodium persulfate ($Na_2S_2O_8$) (final concentrations) in the dark at room temperature for 12-16 h before use. The concentrated ABTS solution was diluted with ethanol to a final absorbance of 0.8-0.7 at 734 nm. A 10 µl portion of extract was added to 990 µl of ABTS solution, and the reduction in absorbance was measured 1 min after addition of Trolox (final concentration 1-20 µM) and up to 40 min after addition of the extract. The stock solution of Trolox (2.5 mM) was prepared in ethanol. Absorbance was measured on a UV/spectrophotometer (Shimadzu PharmaSpec UV-1700) [43].

5.5.3. Antimicrobial activity

Microorganisms strains used for the evaluation of antimicrobial activity were stored at -85 °C in glycerol until inoculation. Antimicrobial activity of the extract was evaluated by broth microdilution assay. *Salmonella typhimurium* ATCC 13311, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* ATCC 700699 were used as test microorganisms. Standard antibiotic, ciprofloxacin was used as a positive control. The extract was dissolved in sterile dimethyl sulfoxide (DMSO). 100 µl of the extract solution was applied to 96-well microplates and two-fold serial dilutions were performed. After the dilution, 50 µl of microorganisms was inoculated (10^5-10^6 CFU/ml) on to the plates. After incubation at 37 °C for 24 h the first well was treated with

20 µl of resazurin, which insured on all microplates the Minimum Inhibitory Concentrations (MIC), where the lowest concentration of the samples prevented visible growth. Antibacterial assays were repeated three times.

5.5.4. Antiinflammatory activity

The antiinflammatory activity of the extract was determined by inhibition of 5-lipoxygenase (5-LOX) enzyme by *in vitro* method. The inhibition of 5-LOX activity was tested spectrophotometrically on a 96-well quartz plate according to method described by Baylac and Racine [45]. 1.94 ml of potassium phosphate buffer (100 mM, pH 9.0), 40 µl of the extract and 20 µl of the 5-LOX solution were mixed and incubated for 10 min at 25°C. After incubation, the reaction was then started by adding 50 µL of linoleic acid. The change of absorbance at 234 nm was followed for 10 min. The experiments were triplicate and the results calsculated from Equation 2. NDGA was used as a positive control.

Inhibition Percentage = (E – S) / E x 100 (Eq. 2)

E: the absorbance of the enzyme solution without the extract, S: the absorbance of the enzyme with the extract.

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