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Antioxidant, Antimicrobial, Cytotoxic, Anticholinesterase, Antityrosinase Activities and Characterisation of Volatile Compounds of *Verbascum oocarpum* by SPME and GC-FID/MS

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

The objective of present study was to specify the antioxidant, antimicrobial, cytotoxic, anticholinesterase, and antityrosinase activities of *Verbascum oocarpum* Murb., together with its volatile components. The methanolic and aqueous extracts of aerial parts of *V. oocarpum* indicated substantial antioxidant activity derived from benzoic acid, sinapic acid, and quercetin compounds. The methanolic extract displayed moderate antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa,* and *Mycobacterium smegmatis.* It also exhibited a higher IC_{50} value on tyrosinase than kojic acid, and lower acetylcholinesterase and butyrylcholinesterase inhibitor activities than galantamin. MTT analysis revealed that high concentrations of *V. oocarpum* extract can result in cytotoxicity, with an IC_{50} of 0.444 mg/mL. Common volatile components included pentadecane, hex-2(E)-enal, limonene, phenylacetaldehyde, isophorone, 1-methoxy-4-(2-propenyl)-benzene, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4h)-benzofuranone and hexadecane. *V. oocarpum*may be of potential benefit to the food, cosmetical, and pharmaceutical sectors owing to its potent anticholinesterase, antioxidant, and antimicrobial activities, volatile components, and limited cytotoxicity.

Keywords: Antioxidant; antimicrobial; volatile components; Verbascum oocarpum.

1. INTRODUCTION

Oxidative stress refers to the homeostasis between reactive oxygen species (ROS) and antioxidant defense mechanisms being impaired in favor of ROS, and can lead to significant cellular damage. DNA is a particular target of ROS, and ROS-induced DNA injury has been linked to a range of pathological circumstances, with the inclusion of Alzheimer's disease, Parkinson's disease, hypertension, and asthma. Plants contain various antioxidant components, including polyphenolic and volatile compounds, which serve to preserve the cells against the harmful impacts of ROS. There has recently been considerable scientific research into potential sources of novel and natural antioxidants.

Tyrosinase is a mono-oxygenase responsible for catalysing the production in melanocytes of melanin, the main dermatological pigment. Various factors can give rise to excessive melanin production, including overexposure to sunlight, melisma and other hyper-pigmentation diseases. Various depigmenting agents have been developed to treat cosmetically unattractive skin discoloration. This has also furthered investigation into novel and potent tyrosinase inhibitors aimed at preventing discoloration in foodstuffs and for use in skin whitening cosmetics. Several inhibitors have been already been identified and described [1-3]. This study investigated the tyrosinase-, acetylcholinesterase-, and butryrylcholinesterase-inhibiting potential of *V. oocarpum* from Turkey as part of the ongoing search for novel enzyme inhibitors with potential applications in skin-whitening products and cosmetics.

The phenolic profiles and biological activities of a number of *Verbascum* species have already been described. The ethanol extract of *V. sinaiticum,* for instance, has been declared to indicated substantial antiproliferative effect against a range of different cell lines. Greater activity has been determined in *V. sinaiticum* flowers than extracts obtained from the aerial parts [4]. Another study reported that ethanol extracts derived from the flowers may also exhibit antimicrobial activity against methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Salmonella typhimurium* [5].

Despite the known importance of *Verbascum* species deriving from their biological activities, our review of the literature revealed no previous studies of the phenolic profile, biological effects or cytotoxicity of *V. oocarpum*. The goal of our research was therefore to investigate volatile composition, antioxidant, antimicrobial, anticholinesterase inhibitory and cytotoxic activities of methanolic extract of this species for the first time in the literature for the phenolic profile.

2. MATERIALS AND METHODS

2.1 Chemicals and Instrumentation

The DPPH (2,2-diphenyl-1-picrylhydrazyl) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide) reagents employed were procured from Sigma-Aldrich (St. Louis, MO, USA). Merck (Darmstadt, Germany) supplied the methanol, ethanol, acetic acid, dimethyl sulfoxide and acetonitrile. Trolox (6-hydroxy–2,5,7,8-tetramethylchroman–2-carboxylic acid), TPTZ (2,4,6-tripyridyl-s-triazine) and Folin-Ciocalteu's phenol reagent were procured from Fluka Chemie GmbH (Buchs, Switzerland), while polytetrafluoroethylene membranes (porosity 0.45 µm) for use during extract filtration procedures were obtained from Sartorius (Goettingen, Germany). AR42J (ATCC® CRL-1492™) was obtained from American Type Culture Collection (ATCC, Manassas, VA). All cell culture materials were purchased from MulticellWisent Inc. (Montreal, Canada).Vanillic acid, p-hydroxy benzoic acid, syring aldehyde, p-coumaric acid, sinapic acid, benzoic acid and quercetin standards were procured from Sigma-Aldrich.

All absorbance values were determined using a Spectro UV-Vis Double PC–8 auto cell spectrophotometer (Labomed Inc.). An IKA® RV 05 Basic (IKA®, Werke, USA) rotary evaporator system was employed for evaporation processes, and a Heidolph Promax 2020 shaker for extraction procedures. pH values were calculated with a Hanna Instruments microprocessor pH meter.

2.2 Plant Material and Preparation of Extracts

V. oocarpum specimens were collected from Erzincan, Turkey. All samples were deposited in the Erzincan University Science Faculty herbarium (herbarium number: 10866). Fifty grams of dried plant powder was first weighed and mixed with 500 mL methanol. The mixture was then filtered following 24-h continuous stirring at room temperature. The resulting filtrate was evaporated by means of a rotary evaporator (IKA-Werke RV05 Basic, Staufen, Germany). The resulting extract was employed to investigate antioxidant, antimicrobial, cytotoxicity, anticholinesterase, and antityrosinase activities. The extract intended for HPLC analysis was further dissolved in HPLC-grade methanol and passed through 0.45-µm membranes for additional filtration.

2.3 HPLC Conditions

Vanillic acid, *p*-hydroxy benzoic acid, syring aldehyde, *p*-coumaric acid, sinapic acid, benzoic acid and quercetin standards were used for HPLC analysis [6]. Stock solutions of the prepared standards were diluted at concentrations of 5-100 µg mL^{-1} to produce calibration curves. HPLC analysis of phenolic compounds was carried out with a reverse phase column (150 × 4.6 mm i.d, 5 µm) (Waters Spherisorb, Milfort, MA, USA), on a gradient program employing a two-solvents system [A: 100% methanol; B: 2% acetic acid in water (pH 2.8)] and a constant solvent flow rate of 1.5 mL min^{-1} on a HPLC system (Shimadzu Corporation, LC 20 AT, Kyoto, Japan) (Table 1). The injection volume was set at 20 µL. Signals were determined at 232, 246, 260, 270, 280, 290, 308, and 328 nm using a diode array detector (DAD) detection with a column temperature of 25ºC.

Table 1. Phenolic composition of the methanolic extract of *Verbascum oocarpum*

Phenolic compounds	Retention time (min)	Amount (mg g^{-1})
p-hydroxy benzoic acid	4.411	-
Vanillic acid	5.102	-
Syringaldehyde	6.383	-
p-coumaric acid	7.437	-
Sinapic acid	7.947	28.48
Benzoic acid	9.588	6.08
Quercetin	14.720	0.43

Table 2. The antioxidant activities of *Verbascum oocarpum* extracts

Test Compounds	TPC[†]	FRAP[‡]	CUPRAC[§]	DPPH[¶‖]
Aqueous extract	13.9 ± 0.602	577 ± 3.245	1133 ± 14.089	0.0132 ± 0.0035
Methanolic extract	12.2 ± 0.300	607 ± 5.006	1228 ± 9.739	0.2998 ± 0.0021
BHT				0.0031 ± 0.0002

[†] Total phenolic content expressed in mg of gallic acid equivalent (GAE) per gram of dry plant weight.
[‡] FRAP value expressed as µM trolox equivalents (TE) per gram of dry plant weight.
[§] CUPRAC value expressed in µM trolox equivalents (TE) per gram of dry plant weight.
[¶‖] Concentration of test sample (mg/mL) required to produce 50% inhibition (SC_{50}) of the DPPH radical.

2.4 Determination of Antioxidant Capacity

The Folin-Ciocalteu procedure (Singleton & Rossi, 1965) was used to calculate total phenolic contents in the extracts, expressed as mg of gallic acid equivalents per g of 100 g sample [7].

The ferric reducing antioxidant power (FRAP) assay is used to calculate the iron-reducing capacities of an extract [8]. The Fe^{2+}-TPTZ complex exhibits a blue colour in the presence of 2,4,6-tripyridyl-S-triazine (TPTZ), which is read at 593 nm. The spectrophotometric method described by Apak et al. (2004) was used to determine CUPRAC levels [9].CUPRAC and FRAP values were expressed as µM Trolox equivalent per gram of sample. DPPH radical-scavenging activity derives from an antioxidant's DPPH cation radical scavenging capacity [10].The results were expressed as SC_{50} (mg sample per mL).

2.5 Antimicrobial Activity Assessment

All micro-organisms employed in this study, *Escherichia coli* ATCC 25922, *Yersinia pseudotuberculosis* ATCC 911, *P. auroginosa* ATCC 43288, *S. aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 43251, *Bacillus cereus* 709 ROMA, *Mycobacterium smegmatis* ATCC607, *Candida albicans* ATCC 60193, and *Saccharomyces cerevisiae* RSKK 251, were procured from the Hifzissihha Institute of Refik Saydam (Ankara, Turkey). Materials were prepared by dissolving extracts in dimethylsulfoxide (DMSO) in a stock solution of 178–256 mg mL^{-1}.

The agar-well diffusion technique with various modifications was used for the purpose of susceptibility screening [11-14]. Each bacterium was suspended in Mueller Hinton (MH) broth (Difco, Detroit, MI), while yeast extracts broth was employed for yeast-like fungi. Micro-organisms were subsequently diluted to approximately 106 colony forming units (cfus) per mL. Sabouraud dextrose agar (SDA) (Difco, Detriot, MI) was employed for yeast-like fungi, and brain heart infusion agar (BHA) was used for *M. smegmatis* [15]. These were placed onto the surface of MH broth or SDA with the flood inoculation technique, and then dried. Next, a sterile cork-borer was used to open 5-mm diameter wells in the agar, with 8900-12,800 µg/50 µL of extract substances being placed into these. Incubation was then performed for 18 h at 35 ^{o}C. *M. smegmatis* was cultured over a period of 3 to 5 days on BHA plates at 35 ^{o}C. Ampicillin (10 µg), streptomycin (10 µg), and fluconazole (5 µg) were employed as standard drugs, with dimethylsulfoxide as a control. Minimal inhibition concentrations (µg mL^{-1}) of *V. oocarpum* were calculated in the final stage.

2.6 Evaluation of Cellular Viability

Dulbecco's Modified Eagles medium was used to culture rat acinar pancreatic cells (AR42J) at 37 °C and 5% CO_2 atmosphere with nutrient mixture F12 (DMEM/F12), including 10% FBS and 100 units mL^{-1} penicillin and 100 µg mL^{-1} streptomycin [16].

The safety of *V. oocarpum* extract was evaluated using the MTT test. This test is based on viable cells reducing yellow, water-soluble tetrazolium salt to insoluble purple-coloured formazan precipitate by succinate dehydrogenase. The cells were sown in a 96-well microplate, and then incubated using a range of concentrations of methanol (from 0.0625 to 2 µg mL^{-1}). We then calculated a sample concentration capable of inhibiting 50% of enzyme activity (the IC_{50} value) [16].

2.7 Tyrosinase Inhibitory Activity

Tyrosinase inhibitory activity (TIA) (EC 1.14.1.8.1, 30 U, mushroom tyrosinase, Sigma) was calculated with different concentrations of kojic acid solutions being used as standard [17]. Reagent Madley absorbance was read at 490 nm using the spectrophotometric method in a ELISA reader (VersaMax Molecular Devices, USA). The percentage of TIA was calculated using the formula

% inhibition = [[(A-B)-(C-D)] / (A-B)] x 100

2.8 Acetylcholinesterase (AChE)/ Butyrylcholinesterase (BChE) Inhibitory Activity

Acetylcholine esterase inhibitory (AChEI) and butyrylcholinesterase inhibitory (BChEI) inhibitory activities were investigated using Ellman's colorimetric assay with various modifications [18]. Acetylcholinesterase (AChE) and (BChE) served as enzymes during the experimental procedure. Acetylthiocholine iodide and butyrylthiocholine iodide were employed as substrates and 5,5'-dithio-bis 2-nitrobenzoic acid

(DTNB) as the colouring agent. Briefly, control and test compounds were dissolved in sodium phosphate buffer (pH=8) followed by dilution to a concentration of 25-200 µg/mL. In the next stage, 130 µL of sodium phosphate buffer, 10 µL of the tested compound and 20 µL of the enzyme were mixed in a 96-well plate, and incubated for 15 min at 25 ºC. Next, 20 µL of DTNB and 20 µL of substrate (acetylthiocholine iodide or butyrylthiocholine iodide for each assay) were placed onto all wells. The resulting substrate was hydrolysed, and thiocholine formed. Thiocholine reacts with 5,5-Dithiobis [2-nitrobenzoic acid] (DTNB) to elicit yellow 2-nitro-5-thiobenzoate. The absorbance was measured spectrophotometrically at 412 nm. AChE and BChE inhibition fractions in the samples were determined with the formula given below and were compared to galantamine used as standard.

% Inhibition = 100 - [(A1 / A2) x 100]

A1 = Absorbance of the sample solutions at 412 nm
A2 = Average absorbance of the control solutions at 412 nm.

2.9 Solid-phase Microextraction

A manual fıber SPME device was obtained from Supelco (USA). The fiber included for eliciting volatile components was polydimethylsiloxane/ divinyl-benzene (PDMS/DVB, 65 µm-blue hub plain). (precondition of the fiber at 250 °C in the injection port of a GC for 10 min) for 30 min (the highest concentration of aroma compounds without qualitative change of the composition; tested by 10 min extraction steps up to 30 min). After sampling, the SPME device was placed into the injector of the GC and the GC-MS instruments through the whole GC analysis time of 62 min using RTX-5M column. The SPME fibers were made ready in the GC injector for 30 min at 250 °C Extractions were performed at 50 °C at 30-min incubation and 10-min extraction times.

2.10 Gas Chromatography-Mass Spectrometry/ Flame Ionisation Detector (SPME-GC-MS/FID)

The SPME procedure involved placing ~1.00 g of plant material into a 10-mL vial. Magnetic stirring was employed for all extractions. Fibers with extracted, aroma compounds were then inserted into the GC injector in split mode at a split ratio of 1:10 . Thermal desorption was carried out at 250 °C for 4 min. GC analysis was conducted using a Shimadzu 2010 Plus device attached to a Shimadzu QP2010 Ultra mass selective detector and flame ionisation detector simultaneously. A Restek Rxi-5MS capillary column (60 m length, 0.25 mm i.d. and a 0.25 µm phase thickness in split mode) was employed to assist with the separation procedures. The baseline oven temperature was 60^{o}C for 2 min, which was subsequently increased by 3^{o}C per min to 240^{o}C and finally held at 250^{o}C for another 4 min. Helium (99.999%) was employed as the carrier gas with a constant flow-rate of 1 mL min^{-1}. Electronic impact mode was used for detection once the ionisation voltage had been stabilised at 70 eV. Mass acquisition was performed in scan mode (40-450 *m/z*). Each volatile compound was compared to its respective RI (relative to C7-C30 alkane standards) for identification. Mass spectral data were subjected to comparisons against those held in the FFNSC1.2 and W9N11 library of mass spectra. All findings were finally compared against published data in previous studies on the subject [19].

3. RESULTS

3.1 HPLC Chromatograms

Phenolic standards and methanolic extract chromatograms are given in Figs. 1-2. The amounts of phenolic components identified in the specimens are shown in Table 1. Methanolic extracts of the plant were found to contain sinapic acid (28.48 mg/g), benzoic acid (6.08 mg/g) and quercetin (0.43 mg/g).

3.2 Antioxidant Activities of *V. oocarpum* Extracts

Methanolic and aqueous extract TPC values were 12.2 ± 0.300 and 13.9 ± 0.602 mg of GAE/g, respectively(Table 2).

Methanolic and aqueous extracts exhibited DPPH scavenging activity values of 0.2998 ± 0.0021 and 0.0132 ± 0.0035 mg/mL, respectively (Table 2). The radical scavenging capacity of extracts was lower than that of BHT (0.0031 ± 0.0002 mg/mL). Methanolic and aqueous extract FRAP values were607 ± 5.006, 577 ± 3.245 µM Trolox/g sample, respectively (Table 2). CUPRAC activity values for methanolic and aqueous were 1228 ± 9.739 and 1133 ±

14.089μM Trolox/g sample, respectively (Table 2).

3.3 Antimicrobial Activities of *V. oocarpum* Extracts

Minimum inhibition concentration values were used to evaluate the antimicrobial activity of *V. oocarpum* extract against the bacteria tested (Table 3). The methanolic extract of the plant exhibited antimicrobial effects against *E. coli, P. aeruginosa, S. aureus, E. faecalis and M. smegmatis*. However, we observed no effect on fungus.

3.4 Anticholinesterase Activities of *V. oocarpum* Extracts

We also investigated the effect of the methanolic and aqueous extracts of the plant on the tyrosinase, AChE, and BChE. Extracts' half-maximal inhibitory concentrations (IC_{50}) (>1000 μg/mL) were higher on tyrosinase thankojic acid (63,0957 μg mL^{-1}). AChE inhibitor activity values (% inhibition) of the aqueous extract were 8.2 ± 0.4, 15.5 ± 0.2, 18.5 ± 0.6 and 28.8± 0.5 μg mL^{-1} for 25, 50, 100 and 200 μg mL^{-1} , respectively. Methanolic extract AChE inhibitor activity (% inhibition) was 9.6 ± 0.3, 18.8 ± 0.5, 27.0 ± 0.3 and 44.9± 0.6 μg mL^{-1} for 25, 50, 100 and 200 μg mL^{-1} , respectively. AChE inhibitor activity (% inhibition) of galantamine was 68.6 ± 2.3, 74.3 ± 3.2, 79.4 ± 6.2 and 83.3 ± 4.3 μg mL^{-1} for 25, 50, 100 and 200 μg mL^{-1}, respectively (Table 4). BChE inhibitor activity (% inhibition) of the aqueous extract was 12.3 ± 0.3, 20.7 ± 0.2, 39.2 ± 0.7 and 55.3 ± 0.9 μg mL^{-1} for 25, 50, 100 and 200 μg mL^{-1}, respectively. BChE inhibitor activity (% inhibition) of the methanolic extract was 29.9 ± 0.5, 41.7 ± 0.7, 59.9 ± 0.6 and 66.7 ± 1.2 μg mL^{-1} for 25, 50, 100 and 200 μg mL^{-1}, respectively. BChE inhibitor activity (% inhibition) values of galantamine were 42.5 ± 1.1, 57.7 ± 2.1, 69.8± 2.4 and 79.5 ± 2.4 μg mL^{-1} for 25, 50, 100 and 200 μg mL^{-1}, respectively (Table 5).

Table 3. Antimicrobial activities of extracts of *Verbascum oocarpum* (μg mL^{-1})

Tested Compounds	Microorganisms and Minimal Inhibition Concentration (μg/mL)										
	Quantity (μg/mL)	Gram negative			Gram positive				No gram	Yeast Like Fungi	
		Ec	Yp	Pa	Sa	Ef	Lm	Bc	Ms	Ca	Sc
Methanolic Extract	10000	125	-	62.25	350	350	-	-	62.5	-	-
Aqueous Extract	10000	-	-	-	-	-	-	-	-	-	-
Ampicillin	10	10	18	>128	35	10	10	15	-	-	-
Streptomycin	10								4		
Fluconazole	5									>8	>8

Ec: Escherichia coli ATCC 25922, Yp: Yersinia pseudotuberculosis ATCC 911, Pa: Pseudomonas aeruginosa ATCC 27853, Sa: Staphylococcus aureus ATCC 25923, Ef: Enterococcus faecalis ATCC 29212, Lm: Listeria monocytogenes ATCC 43251, Bc: Bacillus cereus 702 Roma, Ms: Mycobacterium smegmatis ATCC607, Ca: Candida albicans ATCC 60193, Sc: Saccharomyces cerevisiae RSKK 251, (-): no activity of test concentrations (10 000 μg/mL).

Table 4. Acetylcholinesterase inhibitor activities (% inhibition)

Samples	25 μg mL^{-1}	50 μg mL^{-1}	100 μg mL^{-1}	200 μg mL^{-1}
Aqueous extract	8.2 ± 0.4	15.5 ± 0.2	18.5 ± 0.6	28.8± 0.5
Methanolic extract	9.6 ± 0.3	18.8 ± 0.5	27.0 ± 0.3	44.9± 0.6
Galantamin	68.6 ± 2.3	74.3 ± 3.2	79.4 ± 6.2	83.3 ± 4.3

Table 5. Butyrylcholinesterase inhibitor activities (% inhibition)

Samples	25 μg mL^{-1}	50 μg mL^{-1}	100 μg mL^{-1}	200 μg mL^{-1}
Aqueous extract	12.3 ± 0.3	20.7 ± 0.2	39.2 ± 0.7	55.3 ± 0.9
Methanolic extract	29.9 ± 0.5	41.7 ± 0.7	59.9 ± 0.6	66.7 ± 1.2
Galantamin	42.5 ± 1.1	57.7 ± 2.1	69.8 ± 2.4	79.5 ± 2.4

3.4 Volatile Components of *V. oocarpum* Extract

To the best of our knowledge, this is the first study to report the volatile components of *V. oocarpum*. The volatile components of *V. oocarpum* extract were determined using GC-MS analysis, and as shown in Table 6, 13 components were identified, constituting 74.57%. The principal components of volatile components were identified as pentadecane (26.87%), and hex-2(E)-enal (12.55%). We also determined limonene, phenylacetaldehyde, nonanal, isophorone, 1-methoxy-4-(2-propenyl)-benzene, tetradecane, 5,6,7,7 a-tetrahydro-4,4,7a-trimethyl-2(4h)-benzofuranone and hexadecane at GC-MS analyses.

Table 6. Major volatile components of *Verbascum oocarpum* based on SPME-GC-FID/MS analysis

Compound classification	Compound name	Retention Time	% Area[a]	Retention Index[b]
alkyl aldehyde	Butanal, 2-methyl- (CAS)	6.069	2.19	713
alkyl aldehyde	Hexanal (CAS)	8.631	2.91	814
alkyl aldehyde	Hex-2(E)-enal	10.210	12.55	860
monoterpen	Limonene	16.940	2.59	1030
aldehyde	Phenylacetaldehyde	17.645	4.46	1046
alkyl aldehyde	Nonanal	20.026	3.08	1101
cyclic ketone	Isophorone	21.034	1.57	1124
steroid	1-methoxy-4-(2-propenyl)- benzene	27.926	6.06	1286
alkane hydrocarbon	Tetradecane	32.179	2.87	1392
alkane hydrocarbon	Pentadecane	36.000	26.87	1491
steroid	5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-Benzofuranone	37.748	5.72	1539
alkane hydrocarbon	Hexadecane	39.636	2.36	1591
alkane hydrocarbon	Heptadecane	43.129	1.34	1691
	Total		**74.57**	

a, % Area obtained by FID peak-area normalization; b, RI calculated from MS, retention times relative to that of n-alkanes (C6-C30) on the nonpolar Restek Rxi-5MS column.

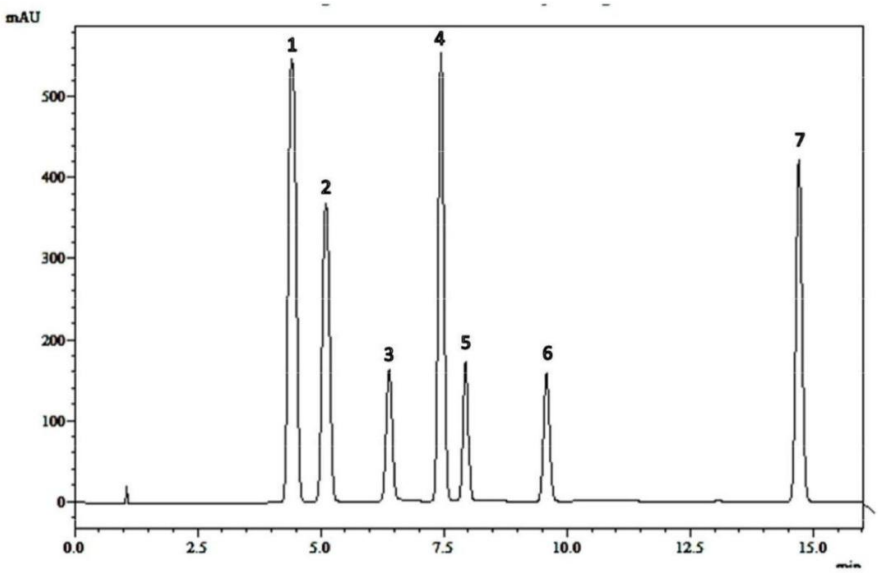

Fig. 1. RP-HPLC chromatogram of phenolic standards (50µM) searched in *Verbascum oocarpum* samples detected at 270 nm by DAD. Peak identification: (1) *p*-hydroxy benzoic acid, (2) vanillic acid, (3) syring aldehyde, (4) *p*-coumaric acid, (5) sinapic acid, (6) benzoic acid, (7) quercetin.

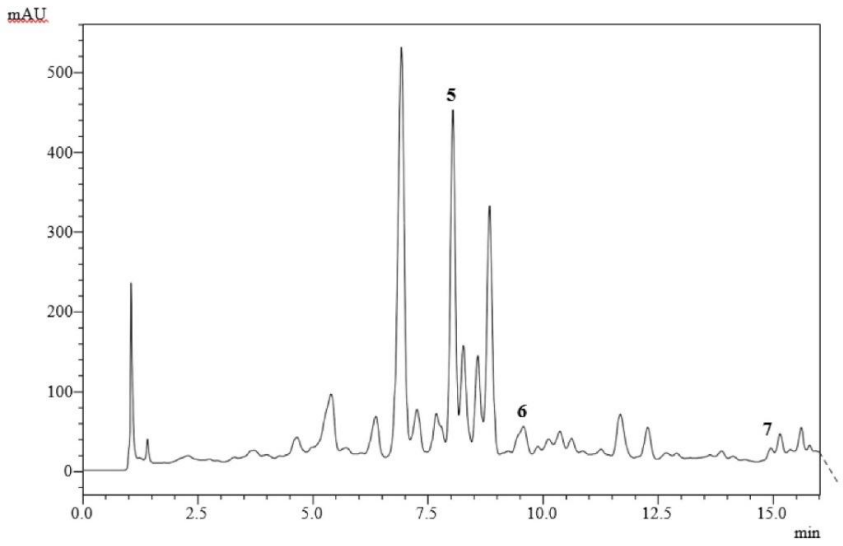

Fig. 2. RP-HPLC DAD chromatogram of *Verbascum oocarpum* methanol extract (50 mg/mL) at 270 nm. Peak identification: (5) sinapic acid, (6)benzoic acid, (7) quercetin.

3.4 MTT Assay

The MTT assay results indicated that *V. oocarpum* causes cell death in a concentration-dependent manner (Fig. 3). The IC_{50} value of the plant was measured at 0.444 mg/mL.

4. DISCUSSION

HPLC study of the extract showed the entity of benzoic acid, sinapic acid, and quercetin. Benzoic acid is a naturally occurring aromatic carboxylic acid found in both plant and animal tissues. Micro-organisms are also capable of producing it. Benzoic acid has a wide sphere of application as an antibacterial and antifungal preservative, as a flavoring additive in various foodstuffs, and by the cosmetic, hygiene, and pharmaceutical sectors [20]. Sinapic acid and its derivatives, particularly 4-vinylsyringol, are natural compounds with various anti-carcinogenic, antimutagenic, antiglycemic, neuroprotective and antibacterial properties, with proven health benefits [21]. The distribution of quercetin varies in different areas of the plant, in the form of aglycones, and also glycosides. It also possesses anti-histaminic, anti-cancer, and anti-inflammatory properties, which are mainly derived from its antioxidant properties. Quercetin is found in significant quantities in citrus fruits, apples, onions, parsley, sage, tea and red wine [22]. The principal components determined in *Verbascum phlomoides* by HPLC analysis in one previous study were rosmarinic acid (14.93 mg/g), caffeic acid (39.96 mg/g), ferulic acid (29.61 mg/g) and quercetin (17.29 mg/g) [23]. Saltan et al. (2011) identified caffeic acid, syringic acid, p-coumaric acid, rutin, ferulic acid, and quercetin as the principal components for *V. Pestalozzae;* protocatecuic acid, chlorogenic acid, vanillic acid, p-coumaric acid, and rutin for *V. Detersile;* protocatecuic acid, chlorogenic acid, p-coumaric acid, rutin, and quercetin for *V. Bellum* and protocatecuic acid, chlorogenic acid, caffeic acid, p-coumaric acid, rutin, and ferulic acid for *V. Myriocarpum* [24].

Plant polyphenols are known to be highly efficient scavengers of singlet oxygen, reducing agents and hydrogen atom donators [25]. Studies on the subject have usually employed the Folin-Ciocalteu method [26]. We determined high total phenolic contents in both the methanolic and aqueous extracts investigated in the present study. Karamian & Ghasemlou (2013) compared the plant against various species of the genus and determined total phenolic contents of 72.95 ± 0.033 mg GAE/g for *V. nudicaule,* 118.2 ± 2.46 mg GAE/g for *V. sinuatum,* and 95.83 ± 1.39 mg GAE/g for *V. Speciosum* [25].

The phenolic contents have also been reported to differ in plant species from this genus, being affected by a range of factors including temperature, soil content and altitude [27].

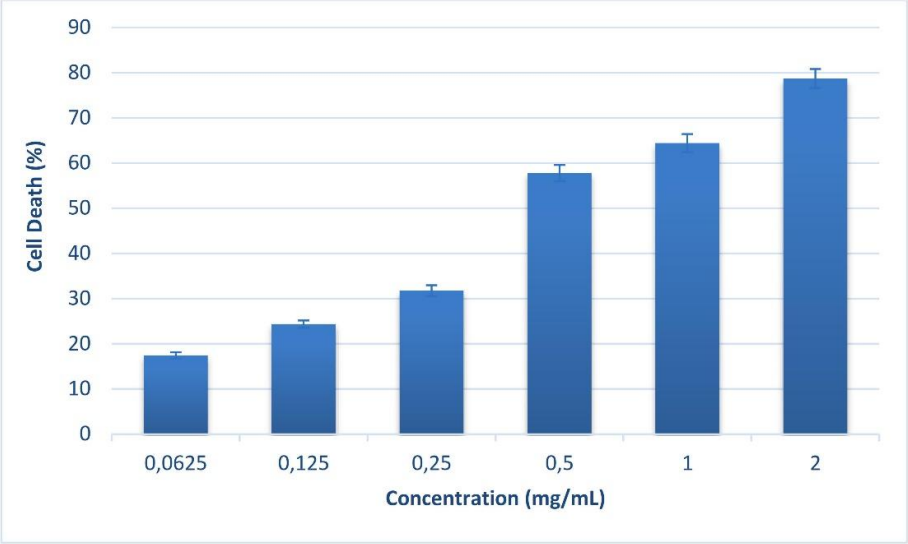

Fig. 3. Effect of *Verbascum oocarpum* (0.0625 – 2 mg/mL) on AR42 cell viability by MTT assay. All experiments were done in triplicates and each assay was repeated four times. The results were presented as mean with ± SD.

DPPH, FRAP, and CUPRAC assays have all been employed to measure antioxidant activity. When they interact with DPPH, antioxidants donate an electron or a hydrogen atom to it, so it is no longer free-radical in nature. DPPH's radical scavenging activity is expressed in the form of SC_{50}, lower values being indicative of higher antioxidant activity. DPPH scavenging activity values (expressed as SC_{50}) for the methanolic and aqueous extracts in this study were 0.2998 ± 0.0021 and 0.0132 ±0.0035 mg/mL, respectively. SC_{50} values determined in other studies include 15 µg/mL for *V. pestalozzae* Boiss., 27 µg/mL for *V. detersile* Boiss. & Heldr., 130 µg/mL for *V. bellum* Hub.-Mor., and 220 µg/mL for *V. myriocarpum* Boiss. & Heldr.[24]. Compared with other species, *V. oocarpum* appears to exhibit significant DPPH scavenging activity. We applied the FRAP assay to determine antioxidant activities involving the reduction of ferric tripyridyltriazine (Fe-(III)-TPTZ) complex to a blue-colored Fe (II)-TPTZ for different antioxidants. FRAP values for methanolic and aqueous extracts were 607 ± 5.006, and 577 ± 3.245 µM Trolox/g sample, respectively. The CUPRAC test also measures antioxidant capacities, with higher absorbance indicating higher reducing power. The technique is based on Cu (I)-neocuproin being reduced to Cu (II)-neocuproin by antioxidants. CUPRAC activities of the methanolic and aqueous extracts investigated in this study were 1228 ± 9.739, and 1133 ± 14.089µM Trolox/g sample, respectively. The antioxidant values determined in the extracts indicated that *V. oocarpum* possesses potent antioxidant activity. The sinapic acid and quercetin identified using HPLC analysis also possess powerful antioxidant properties. This activity may derive from the plant's phenolic compounds.

The methanolic extract exhibited powerful antimicrobial activity against *E. coli, P. aeruginosa, S. aureus, E. faecalis,* and *M. smegmatis*, micro-organisms which are implicated in the etiology of numerous diseases. The antimicrobial effect exhibited by the plant against these micro-organisms suggests that an effective preparation might be produced from it. Significant research has already been conducted into the antimicrobial activities of a number of *Verbascum* species. Senatore et al. (2007) reported that the methanolic extract of *V. sinuatum* L. exhibited inhibitory activities against the totality of bacterial strains investigated (MIC 15.5-250 µg mL^{-1}) [28]. Another study examined the methanolic extracts of the leaves, flowers, roots and seeds of *V. blattaria* L., *V. bombyciferum* Heuff., *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* (Halácsy) I.K. Ferguson in the context of antimicrobial activity against *E. coli, P. aeruginosa, S. aureus* and *C. albicans* [29].

Analysis of the effect of the methanolic and aqueous extract of the plant on tyrosinase, AChE, and BChE enzymes revealed that the plant's tyrosinase activity was insignificant (IC_{50} >1000 µg/mL). The plant extracts were determined to possess moderate AChE and BChE inhibitor activities. Further studies are now needed to investigate whether the extracts could be beneficial in Alzheimer's disease. Kahraman's phytochemical studies identified four iridoid glucosides, ajugol, aucubin, lasianthoside I and catalpol, two saponins, ilwensisaponin A and C, and a phenylethanoid glycoside, verbascoside, in *V. mucronatum*. The authors observed a moderate anti-AchE effect. Analysis of the effect of the methanolic and aqueous extract of the plant on tyrosinase, AChE, and BChE enzymes revealed that the plant's tyrosinase activity was insignificant (IC_{50} >1000 µg/mL). The plant extracts were determined to possess moderate AChE and BChE inhibitor activities. Further studies are now needed to investigate whether the extracts could be beneficial in Alzheimer's disease. Kahraman's phytochemical studies identified four iridoid glucosides, ajugol, aucubin, lasianthoside I and catalpol, two saponins, ilwensisaponin A and C, and a phenylethanoid glycoside, verbascoside, in *V. mucronatum*. The authors observed a moderate anti-AchE effect [30]. The neurobiological activity of phenylethanoid glycosides has also been investigated in previous studies [31]. Kim et al. (2003) noticed that phenylpropanoids exhibit anti-amnesic properties against scopolamine-associated memory deficit in a mouse model. *E-p*-methoxycinnamic acid and other phenylpropanoids have also been observed to significantly suppress glutamate-related neurotoxicity in rat cortical cells [32].

Essential oils have a long history of use for therapeutic and other applications. Their antidepressant, stimulating, detoxifying, antibacterial, antiviral and sedative properties have resulted in essential oils becoming increasingly popular as natural, safe and inexpensive health products. *V. oocarpum* is rich in essential oils, suggesting a potential for use in the treatment of various health problems. Previous studies have investigated the volatile components of the flower, leaf and stem of *V. wiedemannianum* [33]. Hydrocarbons were identified as the principal constituents of oil in the flower and stem, at 183.3% and 32.1%, respectively. Aldehydes were the principal group identified in leaf oil, at 46.8%. Yılmaz Iskender et al. (2009) identified pentadecane (58.2%), (2E)-hexanal (33.2%) and hexadecanoic acid (24.6%) as the principal components of flower, leaf, and stem oils, respectively [28-33]. We think that the changes in the composition of volatile components isolated from *Verbascum* species may be attributed to the plant parts used, and to climatic, seasonal, and geographical factors.

The MTT assay results indicate that an IC_{50} value lower than 0.0625 mg/mL caused no decrease in cell viability compared with the controls, and that cell viability can only be significantly reduced by high concentrations. To the best of our knowledge, this is the first study to investigate the cytotoxicity of *V. oocarpum.* While our findings suggest that *V. oocarpum is safe*, this needs to be confirmed by further research.

5. CONCLUSION

In conclusion, our findings show that extracts of *V. oocarpum* are rich in phenolic compositions, with vigorous antioxidant and antimicrobial activities and essential oil contents. These extracts appear to be excellent sources of raw materials for the pharmaceutical, cosmetic, and food industries.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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