

Antioxidant potential, biochemical activity and hypoglycemic effects of *R. obtusifolius* L. seed extracts used in Armenian traditional medicine

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Abstract. The diversity of plants in Armenia is due to the singularity of natural environment. However, biochemical activity of these plants has not been studied well. The goal was to investigate biological activities as well as antihyperglycemic properties of *Rumex obtusifolius* L. in rabbits with hyperglycemia. According to obtained data, *R. obtusifolius* extracts possessed high antioxidant activity in chemical-based tests. GS/MS analysis of its methanol extracts allowed identification of several compounds, which could have high contribution on biological activities. The sub-cytotoxic concentration of investigated extract was 10 mgmL⁻¹ on Murine microglial BV-2 WT cell lines. Oral administration of ethanol extract showed significant effect on hyperglycemia, reducing fasting glucose levels (57.3%, p<0.05) and improving glucose tolerance. These findings suggest that *R. obtusifolius* extracts could be source of new biologically active compounds and recommended for treatment of diabetes mellitus.

1 Introduction

Armenia is positioned at the junction of several biogeographical zones and appears to be rich in health promoting edible or medicinal plants. These zones are closely linked, resulting in relatively few endemic species of Armenian flora. Overall, 123 endemic plant species are described. These plants are commonly employed in traditional medicine for the prevention and treatment of various diseases from the 15th century and even earlier time. The privilege is the less cytotoxicity of plants to the humans. Armenian flora is rich with herb species which have been widely used in traditional medicine since ancient times [1]. However, this biodiversity was not studied properly for their biological activities, biochemical properties, including antioxidant properties [2], and hypoglycemic activities [3]. Therefore, there could be hidden a great potential for their antioxidant properties and antihyperglycemic effects which can have huge value for therapeutic uses, as well as food industry [4].

The aim of this research was to evaluate antioxidant potential of crude extracts of *R. obtusifolius*, identify biologically active compounds using GC-MS technique and to investigate biochemical properties and antihyperglycemic properties of *R. obtusifolius* alcohol extract in rabbits with hyperglycemia.

2 Materials and methods

2.1 Plant materials

Collection of plant material and preparation of plant crude extracts *R. obtusifolius* L. (seed) samples (voucher specimen number ERCB 13208) were collected from Tavush region (1300-1600 m above sea level). The collection, identification and preparation of plant material was done according to already established protocol [1]. *R. obtusifolius* L. samples were deposited to the Herbarium of Yerevan State University. Plant crude extracts were prepared by maceration technique using methanol (98%), ethanol (96%) and acetone (99.8%), according to method described previously [1]. Dry crude extracts stored in freezer (-18-20 °C). For determination of hypoglycemic properties, the dried seeds were extracted with 40% ethanol for 20 min at 60°C. Briefly, 270-280 mg of dry matter are in 6 ml of ethanol. The extract was filtered with Watman filter paper (N1, Unichem, China) and after cooling at room temperature was orally administrated.

2.2 Evaluation of antiradical activity by DPPH assay

Antiradical activity of tested plant crude extracts was evaluated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [5]. Test solutions contained 250 µL (1 mM) DPPH, 750 µL ethanol (96%) and 1000 µL plant crude extract with different concentrations. Catechin was used as positive control. IC₅₀ values for each plant extract were determined.

2.3 Hydrogen peroxide reducing activity

Hydrogen peroxide (H₂O₂) reducing ability of *R. obtusifolius* was evaluated by the method described by Ruch et al.[6]. Percent reduction of hydrogen peroxide

calculated by following formula: H_2O_2 reduction = $\frac{A_c - A_t}{A_c} \cdot 100\%$ where A_c - absorption of control solution, A_t - absorption of test solution. Ascorbic acid was used as positive control at $10 \mu\text{g mL}^{-1}$ concentration.*

2.4 Metal chelating activity

Metal chelating activity of *R. obtusifolius* extracts was assessed by color change due to the formation of ferrozine- Fe^{2+} complexes [7]. Percent metal chelating activity of plant extracts was calculated according the following formula:

$$\text{Chelating activity} = \frac{A_c - A_t}{A_c} * 100\% \quad (1)$$

where A_c - absorption of control solution, A_t - absorption of test solution. EDTA was used as a positive control at $22 \mu\text{g mL}^{-1}$ concentration.

2.5 Determination of extent of lipid peroxidation

TBARs assay was used for evaluation of *R. obtusifolius* extracts on extent of lipid peroxidation[8]. Mice brain tissue homogenate (in 20 mM phosphate buffer (pH 7.4)) used during the test. Test solution contains 0.3 mL of plant extract (3 mg/mL concentration), 0.7 mL 250 mM HCl, 1 mL tissue homogenate and 2 mL TBA solution (0.375%). The absorbance of mixtures measured at 532 nm. Malondialdehyde concentration calculated using an extinction coefficient of $1.56 \cdot 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

2.6 BV-2 microglia cell culture

Murine microglial BV-2 WT cell lines were grown in a 5% CO_2 incubator at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) and 1% antibiotics (penicillin, streptomycin); culture medium was changed every 2 days. BV-2 cells were seeded on 96-well microplates at $25 \cdot 10^4$ cells per well for viability assay.

2.7 MTT assay

The cell proliferation and/or mitochondrial activity were measured using 3-(4,5-dimethyltrazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells, plated in 96-wells plates, were treated for 24-72 h with different concentrations of extracts ($50 - 5 \cdot 10^{-4} \mu\text{L mL}^{-1}$ in dimethyl sulfoxide). Cells were incubated for 2 h with MTT dye followed by the absorbance (Abs) measurement at the 570 nm with a microplate reader and the sub-cytotoxic concentration was selected for further investigations [9]. The MTT test was carried out in order to clarify the influence of *R. obtusifolius* crude methanol extract cell viability.

2.8 Identification of volatile compounds by GC-MS technique

For identification of volatile compounds contained in *R. obtusifolius* crude methanol extract, GC-MS technique was applied using a Hewlett-Packard 5890 Series II gas chromatograph, fitted with a fused silica HP - 5MS capillary column ($30 \text{ m} \times 0.25 \text{ mm}$, in thickness $0.25 \mu\text{m}$) [10]. The identification of peaks was tentatively carried out based on library search using National Institute of Standards and Technology (NIST)-2013.

2.9 Induction of hyperglycemia in experimental rabbits and blood sampling

The domestic rabbits (*Oryctolagus cuniculus domesticus*) (1800-1900g) were divided into three groups ($n = 9$) as follows: group 1: normoglycemic, group 2: hyperglycemic control, putting immobilization, and group 3: hyperglycemic experimental, in common with immobilization received *R. obtusifolius* seed extract (150 mg kg^{-1} body weight.). Hyperglycemia was induced by immobilization stress in the rabbits during 21 days (5 h daily) [11]. The animals were authorized by the "International Recommendation on Carrying out of Biomedical Researches with use of Animals," and the study plan has been approved by the National Center of Bioethics (Armenia).

2.10 Biochemical Analysis

Blood samples were taken from the aural vein and collected in serum separation tubes (Clot Activator & Gel, Turkey). Blood clot was removed by centrifugation at 3000 g for 10 min in a centrifuge at 4°C . The resulting supernatant was designated as a serum. The biochemical analysis was performed to measure the serum level of glucose, total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG). All parameters were assayed using enzymatic kit. Serum glucose level (mmol/L) was determined using glucose test kit based on the glucose oxidase method [12]. TC and TG were estimated by the method, as described [12] HDL and LDL were measured using the method, as described [13]. Analytical tests were conducted using automatic biochemical analyzers VITROS-5.1/FS (Germany) and MINDRAY B1-120 (China).

2.11 Oral Glucose Tolerance Test

On the 10th day of treatment an oral glucose tolerance test (OGTT) was carried out. Animals were fasted overnight before commencing of experiments. 20% glucose solution ($2 \text{ g kg}^{-1} \text{ b.w.}$) was administrated a signal oral dose to all groups of rabbits. The blood glucose level was measured by portable glucometer (Contour TS, Bayer, Switzerland). Blood samples were collected from aural vein at 0, 30, 60, 90, and 120 min after glucose loading.

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2.12 Data processing

All experiments were independently repeated at least 3 times. Obtained data were processed; mean values and standard deviations were calculated using GraphPad Prism 8.0.1 (GraphPad Software, Inc.; USA) software. The results obtained in the study are reliable ($p < 0.05$), unless another value is followed.

3 Results and Discussion

R. obtusifolius or broad-leaved dock is well known herb which have been widely used in traditional medicine from ancient times for treatment of infectious diseases, skin rash, mucosal inflammations, etc.[14]. In previous research works of our group, promising antimicrobial, antibiotic modulatory, and antiviral activity of its crude extracts was shown [1,15]. TLC-bioautographic analysis allowed separating active antimicrobial fractions from *R. obtusifolius* extracts against *Staphylococcus aureus* MDC 5233 and describing the chemical nature of those compounds [14]. It was hypothesized, that polar compounds could have considerable contribution to the antibacterial activity of acetone and methanol extracts of *R. obtusifolius*[14].

In current research based on chemical tests it was shown that crude methanol and acetone extracts of seeds of *R. obtusifolius* possessed promising antioxidant activity. Particularly high DPPH, hydrogen peroxide reducing and metal chelating activity were shown (Table I). It was revealed that methanol extract of *R. obtusifolius* had 25.29 $\mu\text{g mL}^{-1}$ IC50 value in the presence of DPPH 0.05 mg mL⁻¹ concentration. It was shown that at the presence of 100 $\mu\text{g mL}^{-1}$ concentration *R. obtusifolius* acetone extracts have 99.30% hydrogen peroxide reducing activity when the concentration of H₂O₂ in the mixture was 36 mM. Its methanol extract exhibited moderate activity leading to 40.78% hydrogen peroxide reduction. On the other hand, *R. obtusifolius* methanol extracts exhibited expressed metal chelating activity as well at the concentration of 125 $\mu\text{g mL}^{-1}$ by reducing the number of Fe²⁺-ferrozine complexes by 73.02% at the presence of 0.125 mM FeCl₂. Its acetone extract had low metal chelating ability at tested concentrations. TBARs assay revealed that *R. obtusifolius* methanol and ethanol extracts have not possessed anti-peroxidative activity till 225 $\mu\text{g mL}^{-1}$ concentration.

3.1 GC-MS Analysis of *R. obtusifolius* methanol extract.

In order to identify biologically active volatile and semi-volatile constituents *R. obtusifolius* methanol extract GC-MS technique was used. Based on obtained data various biologically active compounds were identified, which could play important role in their antioxidant effect. In general, 21 compounds were identified in methanol extract of *R. obtusifolius*. The identified compounds and their characteristics are shown in Table II. Some of the identified compounds are considered to possess biological activities. These were linoleic acid (41.32%), cis-vaccenic

acid (24.43%), palmitic acid (12.25%), oleic acid (3.65%) 1,2,4-benzenetriol(0.46%), N-[4-bromo-n-butyl]-2-piperidinone (0.64%) which present in the extracts with relatively high concentrations and have different biological activities based on literature data [16,17]. These compounds could have a contribution to the antioxidant and antimicrobial activities of this plant extracts. Other bioactive compounds also identified in the extract but only at low concentrations (Table 2).

3.2 The effect of *R. obtusifolius* methanol extract on BV2 cell viability (MTT assay)

According to this test the sub-cytotoxic concentration of investigated extract was 10 mgmL⁻¹ (Fig. 1) ($p < 0.05$).

3.3 Effect of *R. obtusifolius* on Fasting Glucose Levels.

Antihyperglycemic activity of *R. obtusifolius* seeds ethanol extract was evaluated on hyperglycemic rabbit model. Fasting blood glucose levels in the hyperglycemic control (56.2%) and hyperglycemic + *R. obtusifolius* extract (48.0%) groups during the first day of immobilization were significantly increased, compared to the normoglycemic group, $p < 0.05$ (Fig. 2).

Therefore, it may be noted that disposable strong stressful pressure provokes of hyperglycemia. Treatment with *R. obtusifolius* (150mg/kg BW) single dose for 21 days showed a significant reduction in fasting glucose to hyperglycemic rabbits (57.3%, $p < 0.05$) in comparison with 1th day value.

3.4 Effect of *R. obtusifolius* on OGTT

The results of effect on OGTT were observed that the seeds extract showed a significant effect on hyperglycemia compared to the hyperglycemic group (Fig.3).

Blood glucose in all groups were increased at 30 min time point after glucose load, and then gradually decreased. following hours. At 120 min blood glucose levels were significantly reduced in treated group of rabbits (25.3%) and hyperglycemic control group (14.7%) compared to the values at 30min. Therefore, glucose tolerance was significantly improved in the *R. obtusifolius* treated animal groups, when compared to the hyperglycemic control group ($p < 0.05$).

3.5 Effect of Ethanol Extract of *R. obtusifolius* on Serum Lipid Profiles

The data of effect of *R. obtusifolius* ethanol extract on serum lipid profiles showed that the TC and LDL-cholesterol levels in the hyperglycemic control group were significantly increased (68.8% and 61.9% respectively), TG level was increased (17.5%) compared to the normoglycemic group (Table III). After 21 days of oral treatment, physiological levels of blood lipids parameters demonstrated significantly decreased TC and LDL-cholesterol levels (53.3% and 38.4%, respectively),

and reduced TG level (17.5%) compared to the hyperglycemic control group. The HDL levels of the extract treated hyperglycemic group did not differ significantly from the hyperglycemic animals group.

Although the hyperglycemic control group of animals demonstrated a tendency towards increased LDL-cholesterol level compared to the other groups.

Table 1. Antioxidant activity of the tested plant methanol and acetone extracts using different chemical based methods

Plant name	Extract	DPPH* reduction IC ₅₀ value (µg mL ⁻¹)	H ₂ O ₂ %-reduction in the presence of 100 µg mL ⁻¹ plant crude extract	Fe ²⁺ % chelation in the presence of 100 µg mL ⁻¹ plant crude extract	MDA % reduction in the presence of 225 µg mL ⁻¹ plant crude extract
<i>R. obtusifolius</i>	Methanol	25.29±0.8	40.78±0.9	73.02±1.6	—*
	Acetone	ND**	99.30±2.1	5.56±1.0	—
Positive control		3 (Catechin)	11.23±0.9 (Ascorbic acid)	28.57±2.1 (EDTA)***	91.1±1.4 (Tocopherol)

*absence of activity, **ND – not determined, *** EDTA –Ethylene diamine tetraacetic acid. All experiments were independently repeated three times. Average means with standard deviations are represented, p<0.05.

Table 2. Compounds identified in the crude extract of *R. Obtusifolius*

Identified compound	RT*	% Peak area	Compound nature	Biological activities
1,2,4-Benzenetriol	15.540	0.46	phenolics	Antiseptic, fungicidal, insecticide, antioxidant
Tritetracontane	19.703	0.12	alkane	Insecticide
Hexadecanoic acid, methyl ester	21.619	0.30	fatty acid methyl esters	Antioxidant, pesticidal, antinematodal
1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester (Butyl isobutyl phthalate)	21.893	0.54	-	Not reported
Palmitic acid	22.572	12.25	fatty acid	Antibacterial, antioxidant, pesticidal, antinematodal
Hexadecanoic acid, ethyl ester	22.879	1.29	fatty acid ethyl esters	Antioxidant, anti-nematodal, pesticidal, hemolytic
Eicosane	23.251	0.19	alkane	Not reported
Oleic Acid	24.018	3.65	fatty acid	Antimicrobial
Methyl linoleate	24.686	0.45	fatty acid	Antifungal, antitumor
11-Octadecenoic acid, methyl ester	24.850	0.68	fatty acid methyl esters	Not reported
3,8-Dimethyldecane	25.168	0.16	alkane	Not reported
Linoleic acid	26.022	41.32	fatty acid	Antibacterial
cis-Vaccenic acid	26.209	24.43	fatty acid	Antimicrobial
4-Methyldocosane	29.11	0.15	alkane	Not reported
N-[4-bromo-n-butyl]- 2- Piperidinone	31.050	0.64	alkaloids	Antimicrobial, antioxidant, anti-inflammatory
1-Heptadecene	32.923	0.32	alkenes	Not reported
cis-9-Hexadecenoic acid	33.668	0.40	fatty acid	Antibacterial
Octadecane	34.204	0.27	alkane	Not reported
9-Octadecenal, (Z)-	34.872	0.53	fatty aldehydes	Antimicrobial
Heptacosane, 1-chloro-	35.190	0.31		Not reported
Supraene	36.351	0.21	triterpenoids	Antibacterial, antioxidant, pesticidal, antitumor
None pure ingredients		2.58		

*RT – retention time

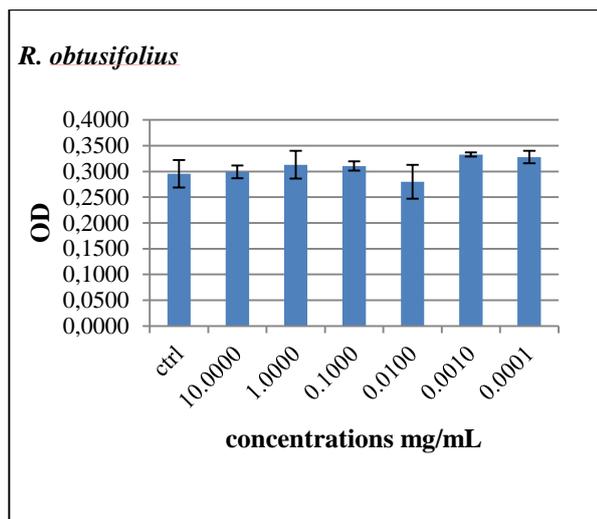


Fig. 1. Effects of *R. obtusifolius* extract on viability of BV-2 WT (MTT assay). Cells were treated for 24 h with extract at different concentrations (50 to 10^{-4} mg mL^{-1}). The results represent the mean \pm SD of the three repetitions. Values are given as average of the repetitions. The significance is presented with the Student-t test: $p < 0.05$.

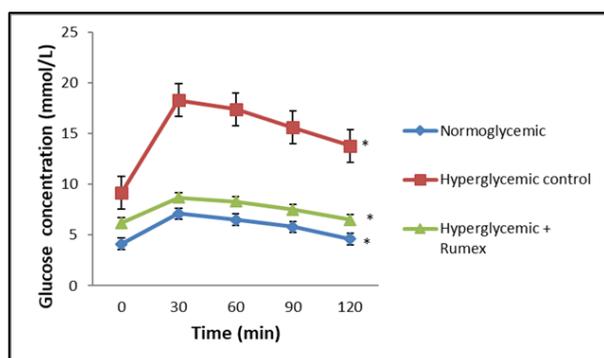


Fig. 2. Effect *R. obtusifolius* ethanol extract on OGTT in normoglycemic and hyperglycemic rabbits. Data are represented as mean \pm SD for 3 animals per group. *Significantly different levels compared to the normoglycemic group. The significance is presented with the Student-t test: $p < 0.05$.

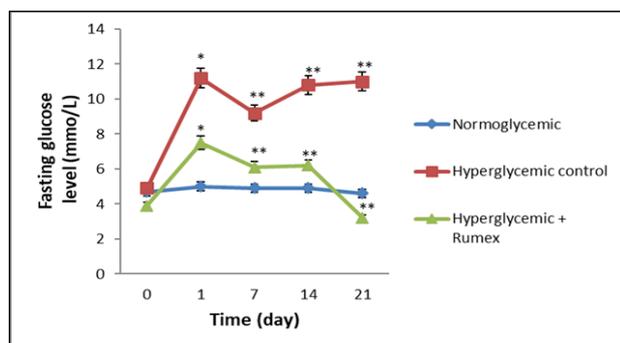


Fig.3 Effect of *R. obtusifolius* ethanol extract on fasting blood glucose levels in normoglycemic and hyperglycemic rabbits. Data are represented as mean \pm SD for 3 animals per group. *Significantly different levels compared to the nonhyperglycemic group. **Significantly different levels compared to the hyperglycemic control group. The significance is presented with the Student-t test: $p < 0.05$.

Table 3. The effects of SR aqueous extracts on Serum lipids in rabbits

Parameters (mmol L ⁻¹)	Experimental groups of animals		
	Normoglycemic	Hyperglycemic control	Hyperglycemic + <i>R. obtusifolius</i>
TC	1.4 \pm 0.08	4.5 \pm 0.16 *	2.1 \pm 0.09**
TG	0.85 \pm 0.12	1.03 \pm 0.4 *	0.85 \pm 0.05 **
HDL	0.78 \pm 0.04	1.02 \pm 0.15*	1.04 \pm 0.08**
LDL	1.0 \pm 0.15	2.63 \pm 0.09*	1.62 \pm 0.08**
VLDL	0.17 \pm 0.03	0.46 \pm 0.07*	0.38 \pm 0.02**

*Significantly different from normoglycemic group ($p < 0.05$). Data are represented as mean \pm SD for 3 animals per group.

4 Conclusion

Thus, high antioxidant activity of *R. obtusifolius* seed extracts was revealed. GS/MS analysis of *R. obtusifolius* seed methanol extracts allowed identification of several compounds, which could have high contribution on biological activities. On the other hand, it was shown that the sub-cytotoxic concentration of investigated extract was quite low (10 mg mL^{-1}) on Murine microglial BV-2 WT cells. The ethanol extract of *R. obtusifolius* seeds revealed hypoglycemic activity, improved lipid profile in hyperglycemia induced by immobilization stress in rabbits. Further investigations should be performed to clarify the mechanisms of action. These findings suggest that *R. obtusifolius* extracts could be source of new biologically active compounds and might be recommended for treatment of diabetes mellitus.

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