



Identification of Main Phenolic Compounds of *Nitraria Retusa* Leaf and Stem Extracts and Evaluation of Their Anti-Adipogenic Activity in 3T3-L1 Cells

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Received Date: June 04, 2022; **Accepted Date:** June 13, 2022; **Published Date:** June 21, 2022

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Abstract

The edible halophyte *Nitraria retusa* known in traditional medicine purposes was used as a support in this study. The present study investigated the chemical analyses of the general composition of leaf and stem extracts using RP-HPLC. Results showed the richness of these extracts in phenolics especially flavonoids as luteolin-7-O-glucoside, isorhamnetin-3-O-rutinoside, isorhamnetin, quercetin and others. In total, nine compounds were identified for each organ extract. Luteolin-7-O-glucoside, isorhamnetin-3-O-rutinoside and Isorhamnetin are three flavonoids which are common in leaves and stems. Adipogenesis assay was performed to investigate the effect of organs, as well as the three last compounds on the adipocyte differentiation and on the lipid droplets accumulation in 3T3-L1 cells. Based on Oil-Red-O content quantification in 3T3-L1 cells, results showed that each extract of *N. retusa* at 25, 50, 100, 200 and 400 µg/mL could not inhibit the lipid droplet accumulation compared to untreated cells, in dose dependent manner. However, compounds showed better effect especially with isorhamnetin even with 5µM. Moreover, the effect of

extracts and single bioactive components on cell proliferation of 3T3-L1 cells showed that stem extract was more efficient than leaf one. Besides, according to morphological observation, isorhamnetin strongly and significantly affected cell proliferation.

Keywords: *Nitraria retusa*; Phenolic compounds; adipogenesis; Phytochemical

Introduction

The plant kingdom has become a target for the search by multinational drug companies and research institutes for new drugs and biologically active compounds [1]. The world health organization (WHO) reported in 2002 that the herbal medicine serves the health need of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries [2]. As known actually, phenolic compounds are receiving increasing attention because of their health promoting effects, attributed to their various biological activities and strong capacity to improve food quality and stability [3]. Phenolic compounds represent the most studied

phytochemicals and have been widely exploited as model systems in different areas of plant research such as pharmaceutical, food, cosmetic and chemical industries [4]. Improving knowledge on the use of anti-obesity medicinal preparations, and encouraging obese patients to consume them along with a healthy diet should be continued [5]. In order to use safely the medicinal plants in controlling and treating obesity, further chemical, biological, and clinical studies are needed on the efficiency of selected plants. Plant extracts may be the whole plant or plant organs (stems, leaves, flowers, and roots). Several investigations should focus on the difference between the combination and the single effect of such compounds. In view of the diversity and the structural complexity of these bioactive molecules, their extraction is influenced by several parameters and technology including the nature, polarity, extracting power of the solvent and extraction method [6, 7]. Other factors influence the extraction and activities of compounds are genotype of plants as well as their physiological state, organs and environmental conditions such as temperature, salinity, drought, light intensity and bacterial infection [8-12].

High performance liquid chromatography (HPLC) is the method of choice for the analysis of phenolic compounds, because of its versatility, precision and relatively low cost [13]. Most frequently, reversed-phase (RP) C18 columns, a binary solvent system containing acidified water and polar organic solvent (acetonitrile or methanol), and UV-Vis diode array detection (DAD) are used and so far to constitute a crucial and reliable tool in the routine analysis of plant phenolic compounds [14].

In this present work we propose to study *Nitraria retusa* plant, a North African halophyte of the Nitrariaceae family (formerly belonging to the Zygophyllaceae) well adapted to the arid climate thanks in particular to its fleshy leaves and also known in Tunisia under the name of "Ghardak". *N. retusa* is known for their biological effects. A previous study was carried out on this plant by some authors of this present work in order to enhance the role in the control of cellular accumulation of fat [15]. In addition, research in Morocco has shown that fresh leaves of *N. retusa* serve as a remedy for poisoning and stomach and abdominal pain [16]. *N. retusa* is also known for its antioxidant, antiproliferative [17] and antimicrobial [18] activities. In addition, the fruits of *N. retusa* are also used as a treatment for hypertension and difficult menstruation [17]. On the other hand, older studies have shown that *N. retusa* is also known for its effect against swelling, its ability to remove tumors and as a traditional remedy for infected wounds [19]. Regarding the phytochemical profile, the genus *Nitraria* has long been known for its richness in polyphenols, mainly the group of flavonoids such as isorhamnetin with strong biological activities, particularly antioxidant and antiproliferative [17, 20].

The aim of this work is to analyze the phenolic composition of *Nitraria retusa* extracts (stem and leaf extracts). In a further step, we aim to test the anti-adipogenic effect of extracts on 3T3-L1 cells as well as some of their identified phenolic compounds. At the end and to better

understand the treatment effects on cells, the morphological observations as well as their proliferative analysis were done.

Materials and Methods

Plant sampling and extracts preparation

Plant samples of different parts of *Nitraria retusa* were collected from a salt flat "sebkha of El Kelbia" located in the region of Kairouan, Tunisia with the GPS following coordinates N 35 48 44, E 10 09 06. After rinsing samples with distilled water and dried in shadow. The organs of *N. retusa* (stems and leaves) were ground separately using a ball mill type "Dangoumeau" and extracted with 70% of ethanol with a ratio of 1 g of plant powder in 10 ml of extraction solvent. Then the solution was kept in the dark for 2 weeks. During this period, the extract solution was manually shaken for 5-10 minutes every day. Finally, the extract was filtered through 0.22 μm filter (MILLIPORE, U.S.A.), and stored for further experiments [21].

Chemical analyses of the general composition of *Nitraria retusa* extracts (stem extract and leaf extract) using reverse phase-high performance liquid chromatography (RP-HPLC)

Before injection into the HPLC system, *N. retusa* organ extracts were passed through a 0.45 μm nylon filter. The separation of selected phenolic compounds was carried out using HPLC system (consisting of a vacuum degasser, an autosampler, and a binary pump with a maximum pressure of 600 bar; Agilent 1260, Agilent technologies, Germany) equipped with a reversed phase C18 analytical column of 4.6 x100 mm and 3.5 μm particle size (Zorbax Eclipse XDB C18). Column temperature was maintained at 25°C. The injected sample volume was 2 μL and the flow-rate of mobile phase was 0.4 mL/min. Mobile phase B was milli-Q water consisted of 0.1% formic acid and mobile phase A was methanol. The optimized chromatographic condition was revealed as follows: 10% A, 90% B (0 min); 20% A, 80% B (5 min); 30% A, 70% B (10 min); 50% A, 50% B (15 min); 70% A, 30% B (20 min); 90% A, 10% B (25 min); 50% A, 50% B (30 min); 10% A, 90% B (35 min). UV-vis absorption spectra were recorded online during the HPLC analysis. The DAD detector was set to a scanning range of 200-400 nm. Peak identification was obtained comparing the retention time and the UV-vis spectra of *Nitraria retusa* phenolics chromatogram with those of available standards [22]. Quantification was performed by reporting the measured integration area in the calibration equation of the corresponding standard.

Preadipocytes differentiation and Oil-Red-O staining procedures

3T3-L1 preadipocytes were seeded into 96-well plates at 1.0×10^4 cells/well and cultured for additional two days until full confluence. Two days later (Day 0), cells were incubated with a differentiation cocktail (MDI) containing 1/10 insulin solution, 1/10 dexamethasone solution and 1/10 3-isobutyl-1-methylxanthine solution in standard culture medium for 3 days

followed by additional 48h with standard culture medium containing insulin alone. The differentiation-maintenance medium was changed every 2 days [21]. To investigate the effect *N. retusa* stem and leaf extracts on adipogenesis in 3T3-L1, different doses (25, 50, 100, 200 and 400 µg/mL) were added to the differentiation-induction and differentiation-maintenance media. The same procedure was conducted to investigate the effect of the determined phenolic compounds of *N. retusa* (luteolin-7-O-glucoside, isorhamnetin-3-O-rutinoside and isorhamnetin) using the following concentrations: 5, 25, 50, 100 and 200 µM. The staining procedure was conducted according to the adipogenesis assay kit (Cayman chemical company). The absorbance was read at 490 nm with a 96-well plate reader. The lipid droplet content was reported as percentage of control cells.

Cell culture

Murine 3T3-L1 preadipocytes (Riken Tsukuba Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (5000 µg/mL)-streptomycin (5000 IU/mL) in 75-cm² tissue culture flasks. Medium was changed every 3 days and cell passage was carried out at 80% confluence at one on two ratio using 0.25% trypsin (1 mM EDTA). 3T3-L1 cells were cultured in a humidified incubator at 37°C and 5% CO₂ [21].

Cell proliferation assay (MTT assay)

Cell proliferation was investigated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. 3T3-L1 cells were seeded in 96-well plates at 1 × 10⁵ cells/mL. After incubation for 7 days (adipocytes), leaf and stem extract samples diluted in medium were added at final concentrations of 25, 50, 100, 200, 400 µg/mL. The same procedure was conducted to investigate the effect of the determined phenolic compounds of *N. retusa* using 5, 25, 50, 100 and 200 µM concentrations for luteolin-7-O-glucoside,

isorhamnetin-3-O-rutinoside and isorhamnetin. MTT was added after treatment for 7 days and the resulting formazan was completely dissolved by 100 µL of 10% sodium dodecyl sulfate (SDS) for 24h. The absorbance was determined at 570 nm in a multi-detection microplate reader (Power-scan HT, Dainippon Pharmaceutical, NJ, USA). Absorbance caused by the ability of the sample to reduce MTT or by its color, was corrected using plates as blanks, prepared in the same conditions in the absence of cells.

Results

Chemical analyses of the general composition of *Nitraria retusa* extracts using reverse phase- high performance liquid chromatography (RP-HPLC)

After analysis, nine compounds were identified for each organ extract (Table 1). Peak identifications were obtained comparing the retention time and the UV spectra of *Nitraria retusa* phenolics chromatogram with those of available standards (Table 2). The chromatographic profiles of *N. retusa* extracts (stems and leaves), acquired at 254 nm, were presented in the (Figures 1 and 2) respectively. Quantification was performed by reporting the measured integration area in the calibration equation of the corresponding standard. Luteolin-7-O-glucoside, isorhamnetin-3-O-rutinoside and Isorhamnetin are three flavonoids which are common in leaves and stems. According to quantification measurements, the concentrations in leaf extract (µg/mL) of isorhamnetin, isorhamnetin-3-O-rutinoside and luteolin-7-O-glucoside were 0.539, 278.01, and 252.403, respectively (Table 1). In other side, the concentrations in stem extract (µg/mL) of isorhamnetin, isorhamnetin-3-O-rutinoside and luteolin-7-O-glucoside were 1.463, 130.417, and 7.902, respectively (Table 1). These flavonoids were chosen for further experiments to test the single effects of *N. retusa* components on cell viability, differentiation and lipid droplet formation in 3T3-L1 cells.

Identifications	Peaks		Retention times (min)		Area		Quantifications (µg/mL)		Quantifications (mg/g extract)	
	Leaves	Stems	Leaves	Stems	Leaves	Stems	Leaves	Stems	Leaves	Stems
Resorcinol	1	1	10.813	10.882	27.2	30.5	69.152	77.542	2.305	2.584
Chlorogenic acid	2	2	15.685	15.882	150	43.2	20.690	25.958	0.689	0.865
<i>P</i> -coumaric acid	-	3	-	20.010	-	13.7	-	3.335	-	0.111
Sinapic acid	3	4	20.220	20.306	66.6	30.1	9.651	4.361	0.321	0.145
Luteolin 7- <i>O</i> -glucoside	4	5	21.191	21.179	2539.3	79.5	252.403	7.902	8.413	0.263
Isoquercitrin	5	-	21.756	-	23.5	-	16.708	-	0.556	-
Hyperoside	6	6	21.866	21.875	158.5	63	6.388	2.539	0.212	0.084
Isorhamnetin 3- <i>O</i> - rutinoside	7	7	22.839	22.804	1523.1	714.5	278.010	130.417	9.269	4.347
Quercetin	8	8	24.473	24.559	23.9	23.6	1.828	1.805	0.060	0.060
Isorhamnetin	9	9	25.840	25.840	11.9	32.3	0.539	1.463	0.017	0.048

Table 1: Identification and quantification of phenolic compounds of leaf and stem extracts of *Nitraria retusa*.

Standards	Retention time (min)
Resorcinol	10.835
Chlorogenic acid	15.918
<i>P</i> -coumaric acid	20.091
Sinapic acid	20.278
Luteolin-7- <i>O</i> -glucoside	21.145
Isoquercetin	21.745
Hyperoside	21.803
Isorhamnetin-3- <i>O</i> -rutinoside	22.986
Quercetin	24.285
Isorhamnetin	25.899

Table 2: List of standards and their retention times (min).

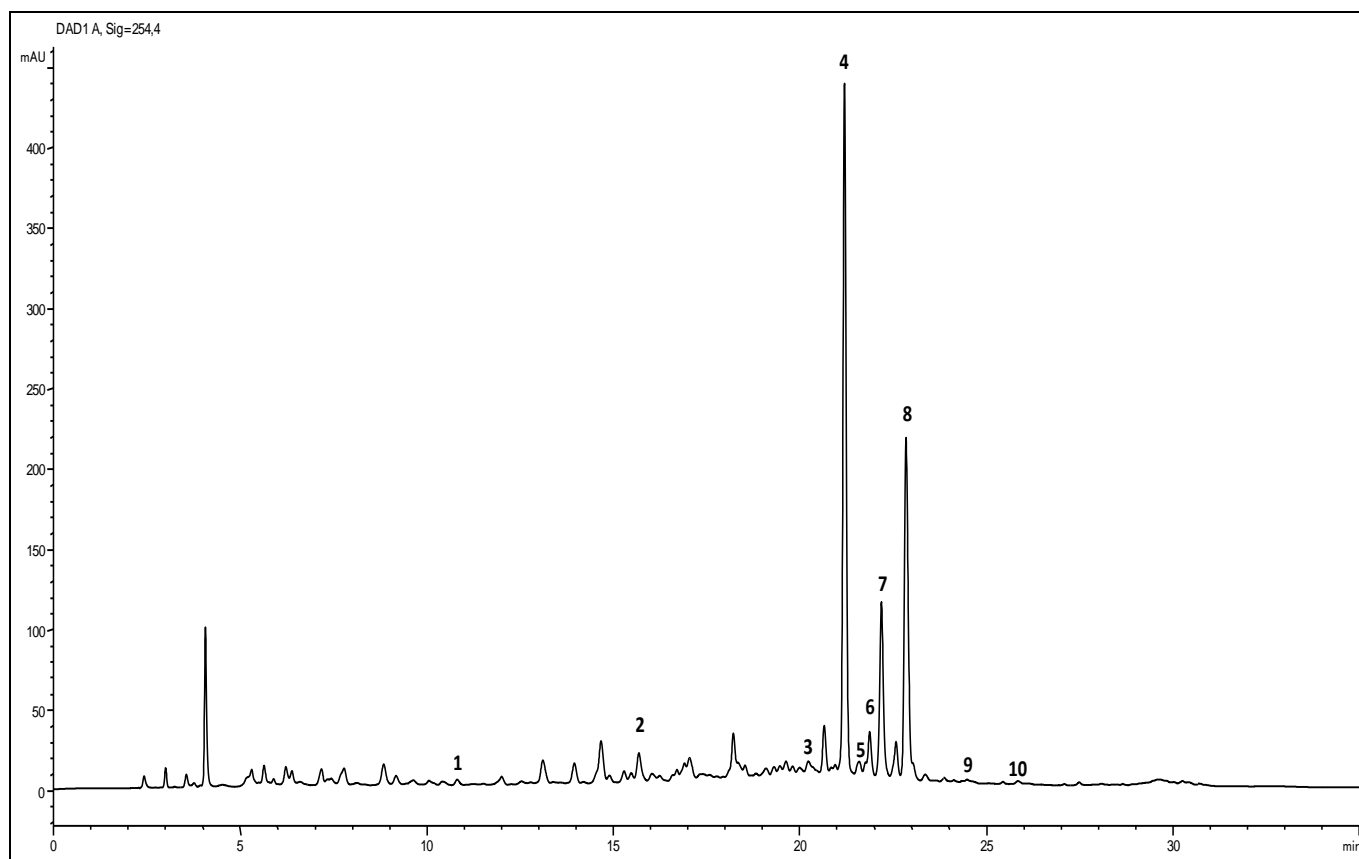


Figure 1: Chromatographic profile of leaf part extract of *Nitraria retusa* acquired at 245 nm. Peaks were identified based on the retention time of standards. 1: Resorcinol, 2: Chlorogenic acid, 3: Sinapic acid, 4: Luteolin 7-*O*-glucoside, 5: Isoquercitrin, 6: Hyperoside, 7: Isorhamnetin 3-*O*- rutinoside, 8: Quercetin, 9: Isorhamnetin.

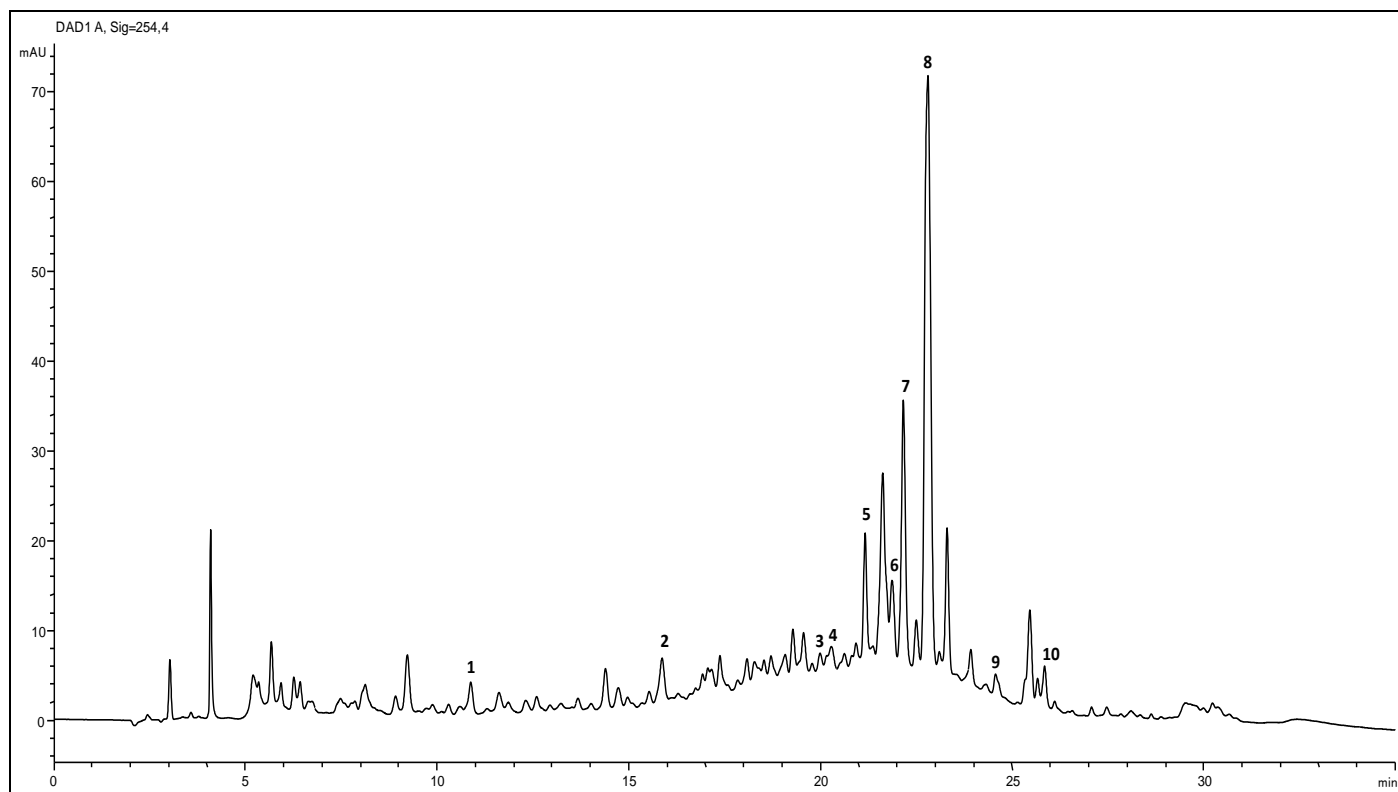


Figure 2: Chromatographic profile of stem part extract of *Nitraria retusa* acquired at 254 nm. Peaks were identified based on the retention time of standards. 1: Resorcinol, 2: Chlorogenic acid, 3: *P*-coumaric acid, 4: Sinapic acid, 5: Luteolin 7-*O*-glucoside, 6: Hyperoside, 7: Isorhamnetin 3-*O*-rutinoside, 8: Quercetin, 9: Isorhamnetin.

Effect of *Nitraria retusa* crude extracts and single bioactive components on cell differentiation and lipid droplet formation in 3T3-L1 cells

Adipogenesis assay was performed to investigate the effect of *Nitraria retusa* extracts (stem and leaf extracts), as well as the effect of selected bioactive components (Luteolin-7-*O*-glucoside, isorhamnetin-3-*O*-rutinoside and isorhamnetin) on the adipocyte differentiation and on the lipid droplets accumulation in 3T3-L1 cells. Differentiated 3T3-L1 cells were treated every two days with the different extracts described above and at various concentrations (25, 50, 100, 200 and 400 $\mu\text{g}/\text{mL}$) for 7 days. Moreover, in order to compare the effect of single compounds to the extract one, differentiated 3T3-L1 cells were treated every two days with various concentrations of selected chemical compounds (5, 25, 50, 100 and 200 μM). Based on Oil-Red-O content quantification in cells, results showed that each extract of *N. retusa* at 25, 50, 100, 200 and 400 $\mu\text{g}/\text{mL}$ could not inhibit the lipid droplet accumulation compared to untreated cells, in dose

dependent manner (**Figure 3**). Regarding the effect of compounds identified in *N. retusa* extracts on adipogenesis, results showed that dependent on the chemical structure and molecular weights of all compounds tested; there is a variability of their effect on 3T3-L1 differentiation and triglyceride accumulation within cells (**Figure 4**). Actually, isorhamnetin showed the strongest effect for all tested doses even for the lowest one (5 μM), compared to the other flavonoids glycosides (isorhamnetin-3-*O*-rutinoside and luteolin-7-*O*-glucoside). In fact, after treating cells with isorhamnetin in various doses (5, 25, 50, 100, 200 μM), the triglyceride accumulation significantly decreased to 24.45 %, 24.45 %, 28.33 %, 31.31 %, and 20.57 %, respectively (**Figure 4C**). However, high doses of luteolin-7-*O*-glucoside and isorhamnetin-3-*O*-rutinoside (50, 100, 200 μM) exhibited a significant reduction of lipid accumulation (**Figure 4A and B**), accompanied with cell morphological changes based on microscopic observation (**Figure 4D**). Therefore, it is necessary to correlate the obtained results with the MTT assay results in next part.

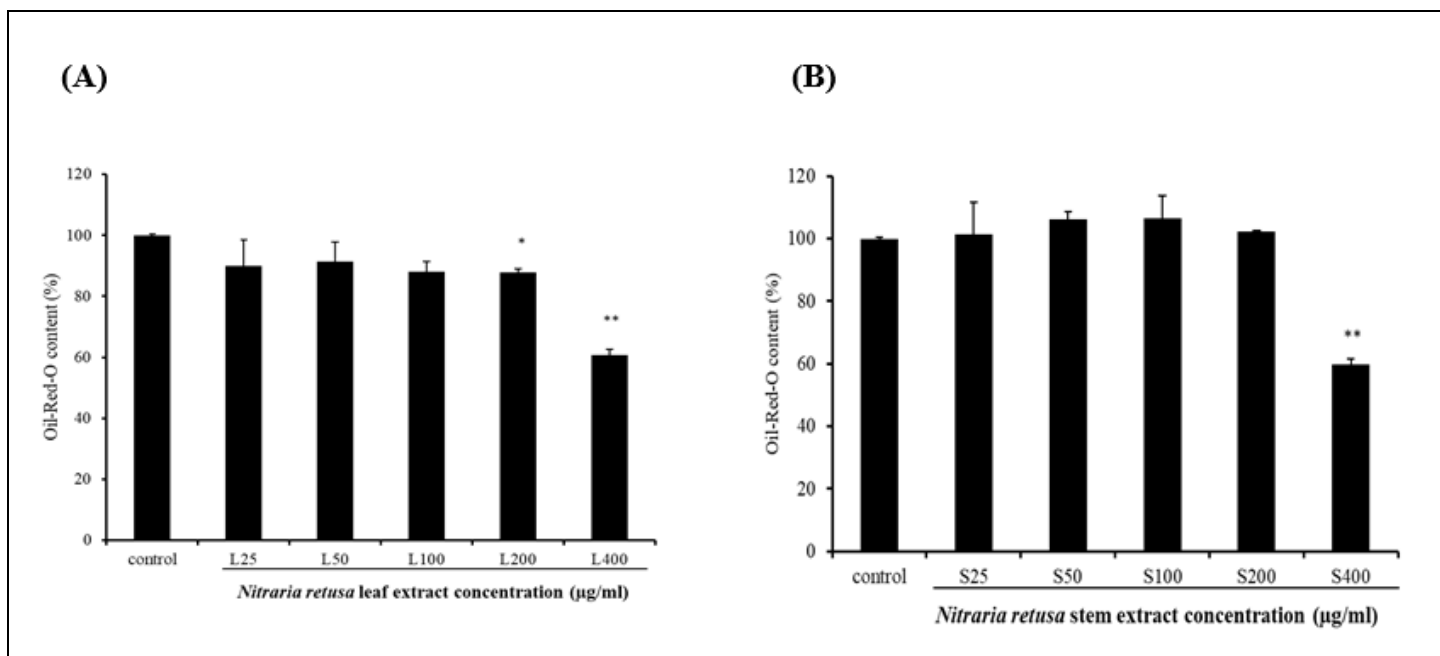


Figure 3: Effects of *Nitraria retusa* extracts using different concentrations (25, 50, 100, 200 and 400 µg/mL) of leaf (A) and Stem (B) on lipid droplet content in 3T3-L1 cells. Lipid droplet accumulation in treated cells was expressed as a percentage of control (untreated cells). Fat droplets in adipocytes differentiated for 7 days with or without treatments were stained with oil Red-O dye and relative Oil-Red-O absorbance was measured at 490 nm. Bars represent mean \pm SD, n=3, ** p<0.01 vs. Control.

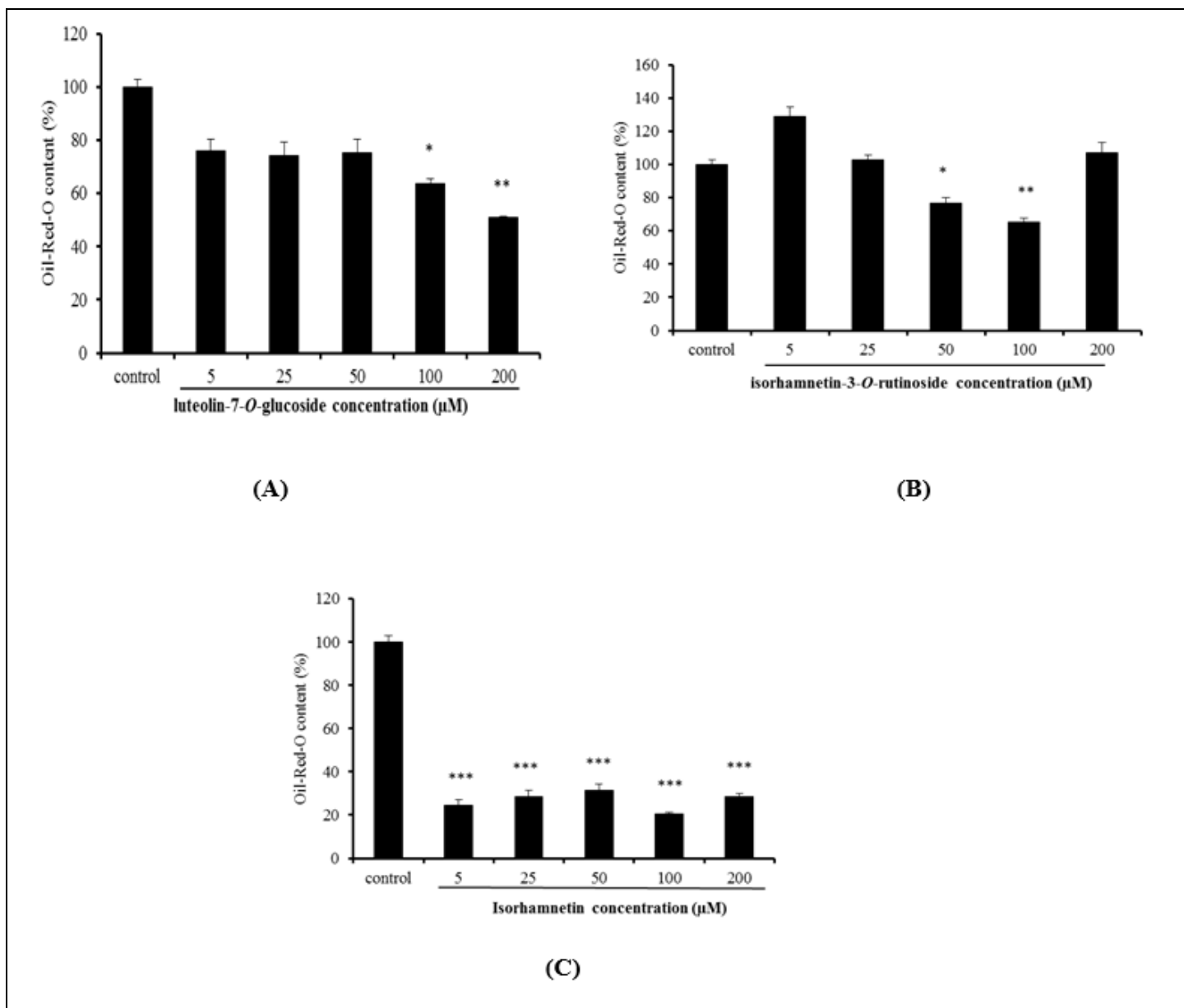


Figure 4: Effects of luteolin-7-O-glucoside (A), isorhamnetin-3-O-rutinoside (B) and isorhamnetin (C) using different concentrations (5, 25, 50, 100 and 200 μM) on lipid droplet content in 3T3-L1 cells. Lipid droplet accumulation in treated cells was expressed as a percentage of control (untreated cells). Fat droplets in adipocytes differentiated for 7 days with or without treatments, were stained with oil Red-O dye and relative Oil-Red-O absorbance was measured at 490nm. Bars represent mean ± SD, n=3, * p<0.05, ** p<0.01, *** p<0.001 vs. control.

Effect of *Nitraria retusa* extracts and single bioactive components on cell proliferation of 3T3-L1 cells

The effects of leaf and stem extract and compound ones (Luteolin-7-O-glucoside, isorhamnetin-3-O-rutinoside and isorhamnetin) on the morphology and the proliferation of 3T3-L1 adipocytes cells are presented in the (Figures 5 and 6). Results showed that treatments with the two organs of *N. retusa* revealed no significant decrease in cell proliferation as well as no changes in the morphology with the lower concentrations (25 and 50 μg/mL). However, at the concentrations of 100, 200 and 400 μg/mL, stem extract

significantly inhibited the growth of 3T3-L1 cells as compared to the control one by 20% for the two first doses and 30% for 400 μg/mL (Figure 5B). Concerning the tested single compounds, cell proliferation was slightly significant affected by isorhamnetin-3-O-rutinoside treatments (25, 50, 100, 200 μM) with just 5% maximum of cell growth reduction for the higher dose (Figure 6B). Luteolin-7-O-glucoside treatments affected more significantly the cell proliferation by decreasing it by a maximum around 40% using 100 μM (Figure 6A). Nonetheless, isorhamnetin strongly affected the 3T3-L1 growth by 30% and 60% reduction for 100 and 200 μM, respectively (Figure 6C).

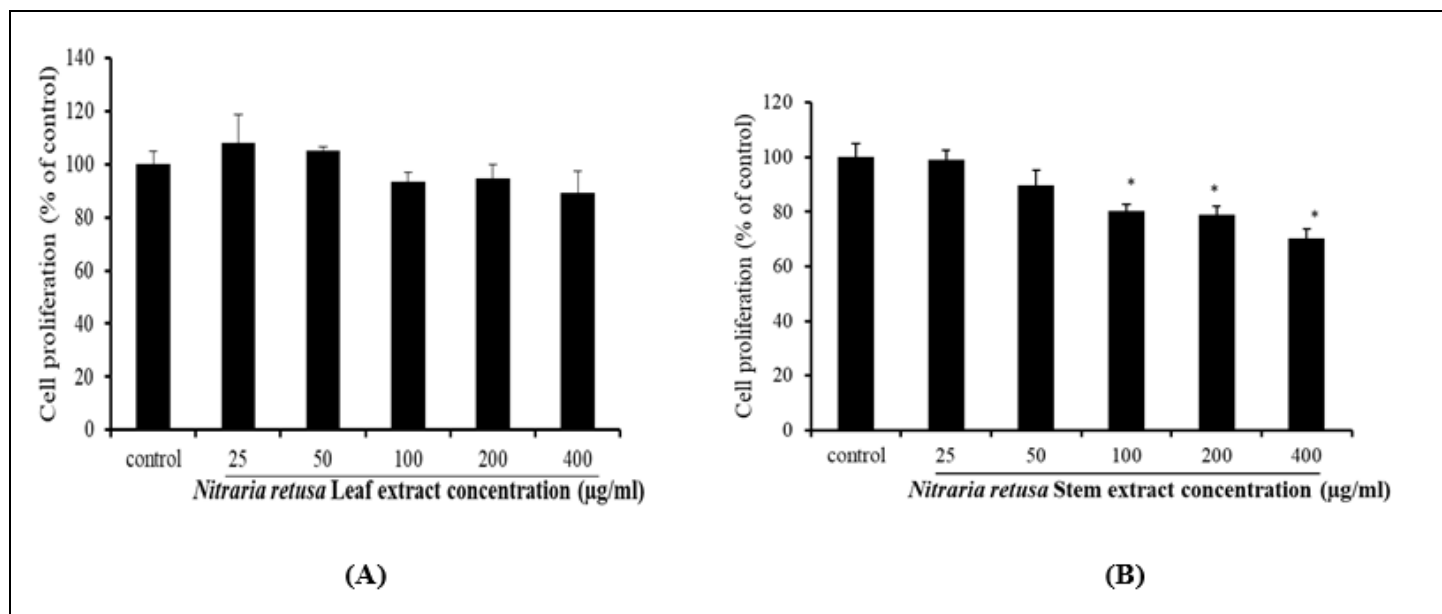


Figure 5: Effects of *Nitraria retusa* extracts using different concentrations (25, 50, 100, 200 and 400 µg/mL) of leaf (A) and Stem (B) on the proliferation of 3T3-L1 preadipocytes during differentiation (7 days of treatment). Bars represent mean ± SD, n=3, * p<0.05.

Discussion

The main idea of this study was to give an explanation to the anti-adipogenic activity of *Nitraria retusa* extracts of stems and leaves by the analysis of the phytochemical using the RP-HPLC system. Observation of results revealed the presence of many phenolics especially flavonoids as luteolin-7-O-glucoside, isorhamnetin-3-O-rutinoside, isorhamnetin, quercetin and others. In total, nine compounds were identified for each organ. Luteolin-7-O-glucoside, isorhamnetin-3-O-rutinoside and isorhamnetin are three flavonoids which are common in leaves and stems. For this work, 70% of ethanol was used for the extraction. It's important to mention that on the one hand, extraction is influenced by several parameters and technology including the nature, polarity, extracting power of the solvent and extraction method [6, 7]. On the other hand, genotype of plants as well as their physiological state, organs and environmental conditions such as temperature, salinity, drought, light intensity and bacterial infection influence extraction and activities of extracts and compounds [8-12]. Moreover, some of these compounds were already detected in other halophytes as the case of quercetin, isorhamnetin-3-O-rutinoside and isorhamnetin (176.33, 157 and 86.76 µg/g DW, respectively) in *Limoniastrum guyonianum* roots [23]. Isorhamnetin glycosides were also isolated from *Peucedanum aucheri* [24] and *Arthrocnemum glaucum* [25]. Resorcinol, hyperoside and quercetin were also detected in *Salsola kali* with different amounts in the leaves and/or stems respectively expressed as milligram per gram of residue (R): (0.09295 and 0.21138; 0.14912 and 0.18838; and 0.05178 mg g⁻¹R) [12].

All molecules in plants could work in synergy and structure of each compound could be the origin of the intensity

of the biological effects [14, 17, 20, 26]. In fact, the anti-adipogenic activity of *N. retusa* may be the result of its richness in phenolics, more probably in flavonoids as isorhamnetin and their combined activity or synergism. The lower anti-adipogenic activity of organ extracts compared to their single constituents could be explained by the concentrations of some compounds present in *N. retusa* [27]. In our study, isorhamnetin was more effective compared to the other compounds (isorhamnetin-3-O-rutinoside, luteolin-7-O-glucoside) even in the lowest dose (5 µM). For more understanding, the anti-adipogenic effect was previously showed with isorhamnetin in 3T3-L1 cells by reducing adipogenic gene expression as C/EBP-α and PPAR-γ [28] and the activation of the Wnt/β-catenin signaling [29]. Same tendency was observed with luteolin-7-O-glucoside from *Commelina communis*, an Asian dayflower, and the results showed that this molecule control lipogenesis by the reducing of the transcription level of glucose transporter 4 (GLUT4) and glucose uptake [30]. Moreover, the strong activity of isorhamnetin, as an O-methylated flavonol of quercetin, could be due to the contribution of its chemical structure since it is the only compound of *N. retusa* extracts possessing a methoxy group at a 3-position and glucose free. In this context, it was reported that flavonoids with methoxy groups showed stronger anti-diabetic effects particularly those with a methoxy group at the 3-position [31]. It has been also reported that several flavonoids with methoxyl substitutions exhibited higher anti-cancer activities which explain the antiproliferative effect of isorhamnetin compound [32]. As well, it was reported in comparison study between isorhamnetin free and isorhamnetin glycosides on their anti-oxidant activities *in vivo* system that isorhamnetin glycosides is metabolized *in vivo* by intestinal bacteria to isorhamnetin which plays important biological roles as among them antioxidant effect [33]. In fact, previous

studies confirmed that the biological activities depend on the presence or absence of the glycoside residue [17, 34]. However, in addition to the efficiency parameter, safety of tested doses of different compounds should be considered. Isorhamnetin in single effect showed a reduction on cell proliferation of 3T3-L1 cells compared to other glycosides compounds (Figure 6).

As mentioned previously in this discussion, the composition of crude extract will depend on the solvent used and also on the quality of the original material, and its composition. In order to obtain a high quality extract with antioxidant activity that is suitable for use in the food, cosmetic, and pharmaceutical industries, the extract must be purified to remove all inert and undesirable components. A

purification and fractionation processes that remove fractions with limited antioxidant activity might enable a good level of biological activity. Then, fractionation, purification, and/or the isolation of active principle(s) could increase bioactive component bioavailability in the extracts, and improve medicinal agent efficacy in weight loss [35, 36].

Because of their benefits to human health, natural antioxidants isolated from *N. retusa* could be an alternative used in food industries in order to avoid or at least reduce the use of synthetic products. Further chemical, biological, and clinical studies are needed on the effectiveness of selected medicinal plants in ameliorating and treating obesity in humans, in order to improve our knowledge on the use of anti-obesity medicinal herbal preparations.

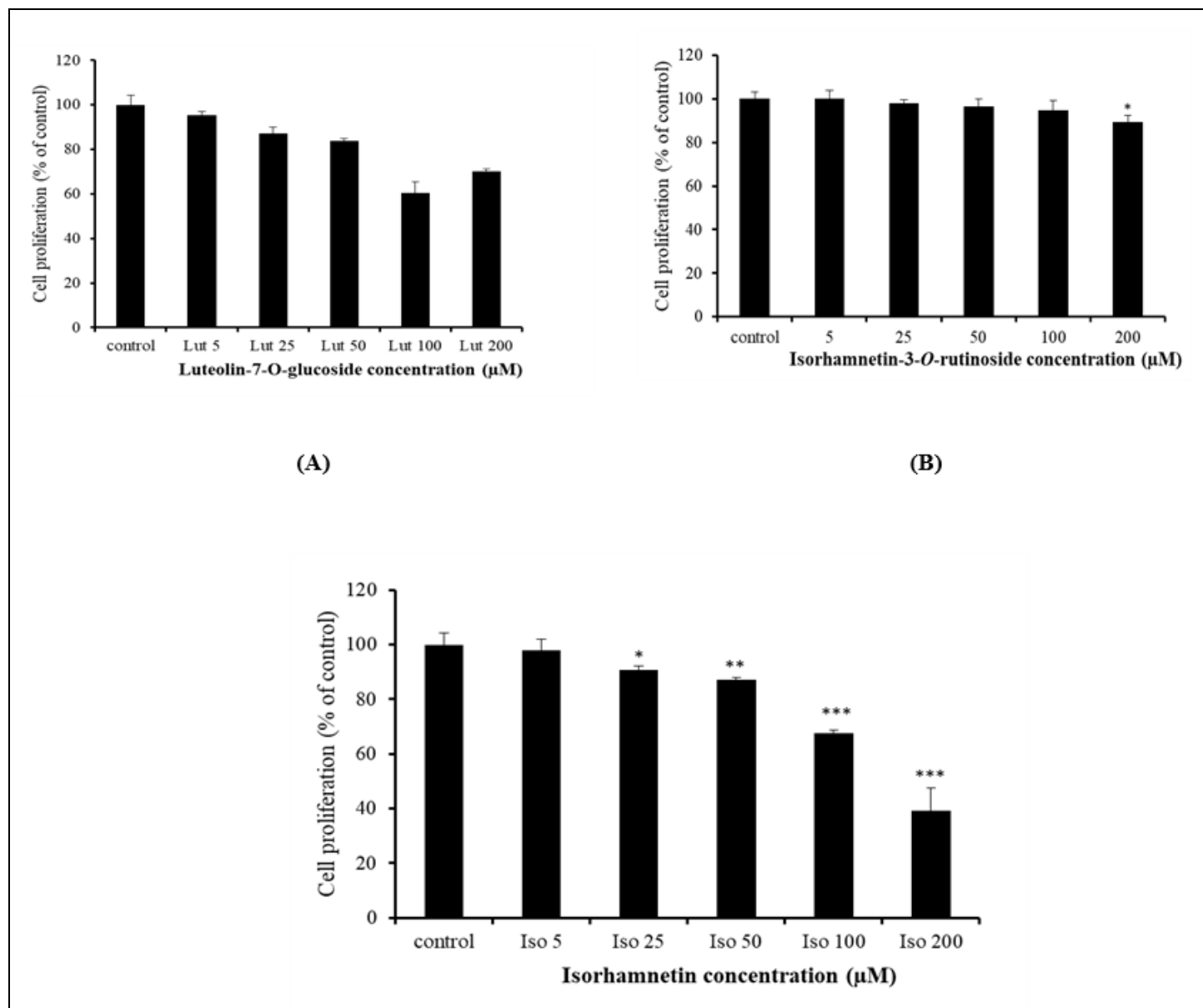


Figure 6: Effects of luteolin-7-O-glucoside (A), isorhamnetin-3-O-rutinoside (B) and isorhamnetin (C) using different concentrations (5, 25, 50, 100 and 200 μM) on 3T3-L1 preadipocytes proliferation during differentiation (7 days of treatment). Bars represent mean ± SD, n=3, * p<0.05, ** p<0.01, *** p<0.001.

Conclusion

The extracts of stems and leaves from *Nitraria retusa* tested in the current study were in crude form but contained many bioactive components which may well act in synergy. To determine which compounds are responsible for the observed effects further experiments of bioactivity-guided fractionation and isolation of bioactive compounds are required.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

This research was supported by the JICA/JST Science and Technology Research Partnership for sustainable Development (SATREPS) Project: “Valorization of Bio-resources in SemiArid and Arid Land for Regional Development.”

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Citation: Kalai FZ, Boulaaba M, ksouri R, Isoada H (2022) Identification of main phenolic compounds of *Nitraria retusa* leaf and stem extracts and evaluation of their anti-adipogenic activity in 3T3-L1 cells. *Adv in Nutri and Food Sci: ANAFS-233*