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RESEARCH ARTICLE

Anti-inflammatory and antioxidant activities of *Inula viscosa* and *Senecio anteuphorbium*

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Abstract

The present study is focused on the evaluation of anti-inflammatory and antioxidant properties of aqueous extract from the leaves of *I. viscosa* and the stems of *S. anteuphorbium.* The anti-inflammatory activity of these plants was studied both in vitro on lipopolysaccharide (LPS)-stimulated J774A.1 mouse macrophages and in vivo using carrageenan-induced paw edema in mice. The antioxidant activitrty of extracts was studied using two in vitro model systems ($DPPH^{\cdot}$ radical-scavenging assay and $ABTS^{+\cdot}$ assay) and their chemical profiles were investigated. The pre-treatment of J774A.1 cells with *S. anteuphorbium* aqueous extract (SAAE) and *I. viscosa* aqueous extract (IVAE) decrease nitric oxide (NO) production and inhibited the expression of inducible nitric oxide synthase (iNOS) in a dose-dependent manner, while the expression of toll-like receptor 4, myeloid differentiation primary response gene 88 and tumor necrosis factor alpha were not affected. The protective anti-inflammatory effect of SAAE and IVAE at an oral dose of 200 mg/kg was also confirmed in vivo. They significantly reduced paw edema after 3 and 6 h of carrageenan stimulation, resperctively. IVAE exhibited a high antioxidant activity and contained high total levels of polyphenols and flavonoids. The major compounds identified in the extract derived from IVAE were tementosin, 3 α-hydroxycostic acid and hydroxycoumarins, and might involve in these effects. This study is the first to report the anti-inflammatory potential of SAAE and IVAE both in vivo and in vitro. The effect demonstrated in vitro may be explained by the inhibition of the LPS-induced NO production through the inhibition of iNOS expression.

Keywords Inflammatory · NO · Sesquiterpene · Carrageenan · Flavonoid

Abbreviations

IVAE	*Inula viscosa* aqueous extract
SAAE	*Senecio anteuphorbium* aqueous extract
DMEM	Dulbecco's modified Eagle's medium
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MyD88	Myeloid differentiation primary response gene 88
TLR-4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
NO	Nitric oxide
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
ABTS	Azino-bis(ethylbenzothiazoline 6-sulfonic acid

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Introduction

In the inflammatory process, one of the primary mechanisms for increased production of inflammatory mediators is the activation of macrophages (Laskin and Pendino 1995; Tsujimoto et al. 2012). LPS-activated macrophages can produce large amounts of these inflammatory mediators by several pathways including toll-like receptors 4 (TLR-4) (Kawai and Akira 2007). MyD88 is an essential adaptor molecule of TLR-4, and the canonical MyD88-dependent pathway

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activates nuclear factor-kappa B (NF-kB), which regulate the expression of numerous inflammatory cytokines including nitric oxide (NO) through inducible NO-synthase (iNOS) activity and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin IL-6 (Pålsson-McDermott and O'Neill 2004). Several reports have provided evidence that NO is involved in the pathogenesis of several chronic diseases including rheumatoid arthritis, atherosclerosis, and hepatitis (Napoli et al. 2006; Gonzalez-Gay et al. 2009; Spruss et al. 2010), it is associated with regulation of the cytokines TNF-α, IL-1, and IL-10, which is believed as one of the most important inflammatory mediators (Wu et al. 2003; Pokharel et al. 2007). The inflammatory responses may be controlled by modulating these inflammatory factors and inhibition of TLR-4/MyD88 signaling.

A large number of plant species contain various bioactive compounds exhibiting health beneficial properties as antioxidative and anti-inflammatory effects. The discovery of herbal anti-inflammatory compounds and understanding their mode of action would contribute to the development of new therapies against chronic inflammation and its associated disorders (Pan et al. 2011). Many previous studies have shown that various phytochemicals exert anti-inflammatory effects by suppression of TLR-4 mediated proinflammatory signaling cascades (Patra et al. 2016) and possess antioxidative and radical scavenging activities, therefore can play an important role in the neutralization of free radicals (Birasuren et al. 2013). Consequentively, the suppressive effects of the antioxidant compounds on the production of inflammatory mediators are associated with their antioxidant activities.

Inula viscosa is a widespread plant in the Mediterranean region. The leaves of this plant have been traditionally used in Algeria as a decoction in the treatment of diabetes (Bellakhdar 1997) and for its anti-inflammatory and antiseptic activities (Lauro and Rolih 1990; Al-Dissi et al. 2001). Recent studies have reported that extracts obtained from *I. viscosa* have anti-inflammatory activity when assayed against different in vivo and in vitro experimental models (Schinella et al. 2002; Zeggwagh et al. 2006). Many biological active compounds have been identified from the leaves such as flavonoids and sesquiterpenes and are characterized by various pharmacological functions, including antioxidant and anti-inflammatory properties (Bohlmann et al. 1978; Wollenweber et al. 1991; Grande et al. 1992; Abu Zarga et al. 1998; Máñez et al. 1999; Hernández et al. 2001).

Senecio anteuphorbium is an endemic plant of South-Western Morocco and Canary Islands. This medicinal plant is used in Morocco for the treatment of rheumatism (cataplasm) (Bellakhdar 1997) and the maceration of the areal part is claimed by the population to be useful in the treatment of diabetes. Indeed, the *Senecio* genus is a source of pyrrolizidine alkaloids (PAs), which are the natural toxins because they are known to induce hepatotoxicity and/or tumorigenicity in humans (Cook et al. 1950; Schoental 1968; Fu et al. 2002). For this reason the safety of using *Senecio* genus raised particular concerns. Lin et al. (2009) have reported that hepatotoxicity depended on the total content dosage of toxic PAs in the herb. Further studies on the isolation and characterization of PAs are necessary to determine the safe dose level of the extract for therapeutic use in traditional medicine. However, to date there were no reports on the toxicity and the systematic research components of *S. anteuphorbium*, except the identification of two sesquiterpenes (Bohlmann et al. 1982).

In this study, anti-inflammatory and antioxidant activities were selected to prove and validate the traditional use of *S. anteuphorbium* and *I. viscosa*. We report for the first time the anti-inflammatory activity of aqueous extract from the stem of *S. anteuphorbium* and the aqueous extract from leaves of *I. viscosa* both in vivo, experimenting on carrageenan-induced inflammation in the mice paw and in vitro, based on the expression of TNF-α, TLR-4, MyD88 and iNOS, as well as the release of NO, from LPS-stimulated J774A.1 macrophages. The antioxidant activity of *S. anteuphorbium* and *I. viscosa* extracts was studied using two in vitro model systems. The results have been correlated with total phenolic and total flavonoid contents and major compouds characterized by UHPLC-HRMS.

Materials and methods

Plant material

The stems of *S. anteuphorbium* were collected in Taghazout (Agadir, Morocco) in October 2013 and the leaves of *I. viscosa* in April 2013 in the location of Laazib, Department of Bejaia (Algeria). The stems and leaves were air-dried at room temperature and ground to a fine powder.

Preparation of the aqueous extracts

Ten gram of the leaves and stems dried powders of plants were boiled in 100 ml of distilled water for 15 min, it was then filtered through a Whatman filter (Millipore 0.25 mm, Germany) and centrifuged. The extracts were concentrated at 40 °C for 3 days. They were then lyophilized (14.2% yield for *I. viscosa* and 15.5% yield for *Senecio anteuphorbium*).

Anti-inflammatory activities

J774A.1 stimulated with LPS and treated with extract

Cultured J774A.1 macrophage cells (mouse ascites macrophages; American Type Culture Collection) are plated

in 6-well plate at 25×10^4 cells/well with 2 ml of DMEM media (PAN) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin until 70% confluency. Cells were pre-incubated with different concentrations of aqueous extract of *S. anteuphorbium* and *I. viscosa* (0.1, 1, 10, 100 µg/ml) at 37 °C for 2 h. Then they were treated with 25 ng/ml of lipopolysaccharide (LPS; Escherichia coli O111:B4) for 18 h. Several LPS concentrations (6.25; 12.5; 25; 50; 100 and 200 ng/ml) were examined in order to find significant concentration which produce high concentration of NO and the dose 25 ng/ml was selected (data not shown). After incubation, 100 µl of media were collected immediately for the detection of nitrite level as determined by Greiss reagent kit (Promega). Cells were lysed with Trizol (In vitro gen) for RNA extraction. Plant extracts in concentrations used in the cell culture experiments did not affect cell viability as assessed by trypan blue exclusion test. In brief, the cells were treated with different concentrations of extracts and after 18 h incubation they were rinsed with PBS, trypan blue (0.1%) was added and leaved 2 min. Then, the washed monolayers were fixed with 4% formaldehyde and the percentage of cell viability was determined by counting the dead cells at least 5 microscopic fields with a minimum of 50 cells per field. The some positions of fields should be maintained for all wells.

Measurement of NO production

The nitrite concentration in the culture medium was measured as an indicator of NO production by the Griess reaction system (Promega). The cell supernatant was mixed with the same volume of Griess reagent and incubated at room temperature for 5 min. The concentration of nitrite was determined by measuring the absorbance at 540 nm using an ELISA microplate reader. The amount of nitrite present in the samples was calculated by means of a standard curve generated using serial dilutions of $NaNO_2$ in fresh culture medium.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

RNA isolation and RT-PCR has been previously detailed by Kanuri et al. (2009). SYBR Green® Supermix (Agilent Technologies, Böblingen, Germany) was used to prepare the PCR mix. The expression levels of TNF-α, iNOS, TLR-4 and MyD88 were normalized using S18 as an internal control.

Carrageenan-induced mice paw edema

Adult male and femelle albino swiss mice (25–35 g each) were obtained from "Office National de Securité Sanitaire des Aliments" (ONSSA), Agadir-Morocco, and were allowed to acclimatize for 1 week in animal house of the Faculty of Sciences (Agadir-Morocco) before experiment started. Animals were kept on a 12-h light/12-h dark cycle at 22 ± 2 °C, and had free access to pallet chow and tap water ad libitum, however they were deprived of water only during the experiment. All the procedures were in strict accordance with "Guidelines for the care and use of laboratory animals" (Ministry of Agriculture, Law No. 28-7, Decree No. 2-10-473). Anti-inflammatory activity of aqueous extracts of *S. anteuphorbium* and *I. viscosa* on carrageenan-induced hind paw edema was determined according to the method described by Winter et al. (1962), using diclofenac as a standard drug. The mice were divided into four groups (n = 6). The control group received through gavage normal saline [0.9% NaCl, 10 ml/kg body weight (b.w.)], treated groups received some volume IVAE and SAAE at a dose of 200 mg/kg b.w. or diclofenac (Sigma-Aldrich) at a dose of 50 mg/kg b.w. (extracts and drug were dissolved in water and administrated by gavage in 10 ml/kg). An edema was induced on the right hind paw of each mouse by sub-plantar injection of 0.1 ml of 1% freshly prepared suspension of carrageenan (Sigma-Aldrich) in normal saline. The administration of extracts and drug was 1 h prior carrageenan injection. The paw diameter was measured before and at 1, 30, 3 and 6 h after the induction of inflammation using sliding caliper. Anti-inflammatory activity of aqueous extracts was measured as the percentage reduction in edema level when drug was present, relative to control and was calculated by the following equation: $\text{Edema}\% = \left(1 - \frac{D}{C}\right) \times 100$, where D represents the difference of paw diameter after the administration of drug to the mice and C represents the difference of paw diameter in the control groups.

Antioxidant activities

DPPH scavenging activity

Radical scavenging activity of SAAE and IVAE against the stable synthetic radical, diphenylpicrylhydrazil (DPPH, Sigma-Aldrich), were determined spectrophotometrically using the method of Masuda et al. (1999). Fifty microliter of test sample or reference compound were mixed with 950 µl of $DPPH^{\cdot}$ methanolic solution (0.1 mM). After 30 min of incubation at room temperature, the absorbance was recorded at 517 nm.

ABTS scavenging activity

The free radical scavenging capacity of plant extracts was also studied using azino-bis(ethylbenzothiazoline 6-sulfonic acid azino-bis(ethylbenzothiazoline 6-sulfonic acid (ABTS, Sigma-Aldrich) radical cation decolorization

assay (Re et al. 1999), which is based on the reduction of $ABTS^{+\cdot}$ radicals by antioxidants of the plant extracts tested. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{+\cdot}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulphate (final concentration) and allowing the mixture to stand in the dark, at room temperature for 12–16 h before use. The kept solution was diluted in deionized water to an absorbance of 0.7 ± 0.02 at 734 nm. After the addition of 100 µl of aqueous plant extracts solutions to 3 ml of $ABTS^{+\cdot}$ solution, the absorbance reading was taken 10 min after initial mixing.

Phytochemical analysis

Determination of total phenolic content

The amount of total soluble phenolics was determined according to the Folin-Ciocalteu method (Singleton and Rossi 1965) using gallic acid as the standard. The extract samples (25 µl) were mixed with Folin Ciocalteu reagent (110 µl) for 3 min and aqueous sodium carbonate (Na_2CO_3, 200 µl) and distilled water (1.9 ml) were then added. The mixture was allowed to stand for 30 min and the phenols were determined by colorimetry at 750 nm. The total phenolic content was expressed as mg gallic acid equivalent (GAE)/g of extract.

Determination of total flavonoids content

Colorimetric aluminum chloride method was used for flavonoid determination (Jay et al. 1975). Briefly, 0.6 ml of each plant extracts were mixed with 0.3 ml of 10% aluminum chloride and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 430 nm. Total flavonoid content was expressed as mg rutin equivalent (RE)/g of extract.

Thin layer chromatogtaphy (TLC) analysis

The screening of bioactives components of the aqueous extracts of *S. anteuphorbium* and *I. viscosa* were performed by Thin-layer chromatography (TLC), on silica gel N-HR/UV_{254} (MACHEREY–NAGEL, Ref. 804023) plate using Ethyl Acetate/Methanol/Formic Acid/Water (100: 13.5: 2.5: 10) as a developing system. Chromatographed plate was sprayed with (NEU-reagent) and visualised under UV light at 365 nm. Various phenolic acids (gallic and chlorogenic acids) and flavonoids (quercetin, quercitrin, myricetin, myricetrin and luteolin) were used as standards.

Ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) analysis

Chromatographic separation was carried out using a DIONEX Ultimate 3000 UHPLC system and a Phenomenex Synergy 4u Hydro-RP 80 column (250×3 mm, 4 µm particle size). The injection volume was 10 µl. The chromatographic conditions were as follows: Flow rate: 600 µl/min, Column temperature: 45 °C. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). A gradient elution program was performed as follows: 0 min, 100:0; 40 min, 0:100; 40.1 min 100:0; 45 min, 100:0. High resolution accurate mass (HRAM) data was acquired in positive ion mode Electrospray using a Thermo Scientific Q Exactive Plus Orbitrap mass spectrometer operated at 140,000 resolution (FWHM) in MS mode and 35,000 resolution (FWHM) in MS2 mode. The data was analyzed using METLIN. The METLIN metabolite database is a repository of metabolite information and tandem mass spectrometry data designed to facilitate metabolite identification in metabolomics. For each compounds of interest we have compared experimental results with the bank to obtain more precise identification based on the high resolution of the mass spectrometer in the two modes MS and MS–MS.

Statistical analysis

GraphPad Prism Software version 5 was used for statistical analysis and one-way ANOVA with Tukey's post-hoc test was used for the determination of statistical significance among treatment groups and p value < 0.05 was defined as significant.

Results

Effect of IVAE and SAAE on NO production

The stimulation of J774A.1 cells with LPS caused a significant increase in cellular nitrite levels compared to the unstimulated control. Pre-treatment with the extracts resulted in a significant reduction of NO in media. Specifically, as shown in Fig. 1a, in the presence of SAAE at concentrations of 10 and 100 µg/ml a dose-dependent and significant decrease in nitrite accumulation was observed. Although IVAE also had an inhibitory effect on NO production at 100 µg/ml (Fig. 1c), the effect was not as strong as that exerted by SAAE.

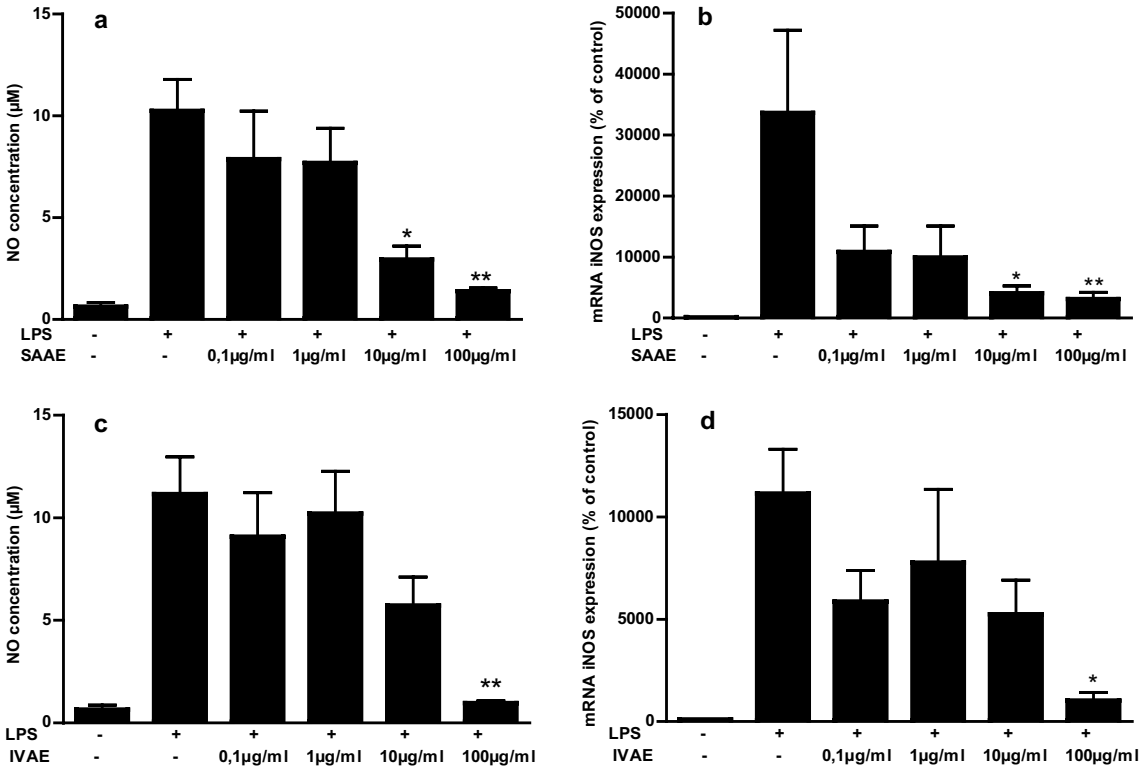

Fig. 1 Effects of IVAE and SAAE on LPS-induced NO production (**a**, **c**) and iNOS mRNA expression (**b**, **d**) in J774A.1 cells. Cells were pre-treated with the indicated concentrations of IVAE and SAAE for 1 h before LPS (25 ng/ml) stimulation and incubated for 18 h. The levels of nitrite were measured in the culture media by Griess reagents (**a**, **c**). The mRNA expression levels of iNOS was determined by RT-PCR analysis (**b**, **d**). The data shown are mean ± S.E.M. of different samples. $*p < 0.05$ and $**p < 0.01$ were compared to LPS-stimulated cells. *IVAE Inula viscosa* aqueous extract, *SAAE Senecio anteuphorbium* aqueous extract

Effect of IVAE and SAAE on mRNA expression of iNOS, TNF-α, TLR-4 and MyD88

To investigate the mechanism responsible for the observed attenuation of NO release in response to LPS, the expression of iNOS is investigated at the transcriptional level by quantifying mRNA levels. As shown in Fig. 1b, LPS-dependent induction of iNOS expression was significantly attenuated by SAAE treated cells in a dose-dependent manner, with inhibition being more pronounced in response at 10 and 100 µg/ml. LPS-induced iNOS expression was also significantly attenuated in cells pre-treated with IVAE (Fig. 1d); however, a significant effect was only found when cells were treated with 100 µg/ml of IVAE. In the same set of experiments, SAAE and IVAE showed no effect on TLR-4, MyD88 and TNF-α expression in response to LPS stimulation for 18 h (Table 1).

Carrageenan-induced mice paw edema

The carrageenan-induced mouse paw edema model was used to evaluate the in vivo anti-inflammatory effect of the extracts at the dose of 200 mg/kg and the results are summarized in Fig. 2. The results showed that injection of carrageenan stimulated local inflammation and then induce edema of the paw tissues which was most pronounced after 6 h. After 3 h, the induction of edema treatment with diclofenac, a standard anti-inflammatory drug, significantly attenuated signs of inflammation ($p < 0.1$) and was more pronounced after 6 h ($p < 0.001$). Pretreatment of SAAE attenuated markedly paw edema after 3 h of carrageenan stimulation, in a similar manner to the positive control group ($p < 0.01$). IVAE moderately inhibited the carrageenan-induced mice paw edema after 6 h.

Antioxidant activities

Antioxidant activity of extracts was investigated by using two different methods for stable $DPPH^{\cdot}$ and $ABTS^{\cdot+}$. The results (Table 2) showed that IVAE exhibited strong scavenging potential against $DPPH^{\cdot}$ ($IC_{50} = 11.4 \pm 1.28$ µg/ml) and $ABTS^{\cdot+}$ ($IC_{50} = 11.25 \pm 2.03$ µg/ml) compared with SAAE (IC_{50}

Table 1 Effects of IVAE and SAAE on LPS-induced TLR-4, MyD88 and TNF-α mRNA expression in J774A.1 cells

	Plant extract	Control	LPS	LPS+	LPS+	LPS+	LPS+
			0 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml
TLR-4,% of control	IVAE	100	74.65 ± 4.30	44.94 ± 4.90	50.13 ± 3.87	46.10 ± 17.77	88.95 ± 24.94
	SAAE	100	292.12 ± 139.69	148.57 ± 36.85	88.83 ± 31.10	120.83 ± 27.71	100.09 ± 26.44
MyD88,% of control	IVAE	100	113.68 ± 11.13	56.70 ± 11.96	46.26 ± 14.61	44.66 ± 19.55	81.05 ± 9.56
	SAAE	100	333.79 ± 142.48	140.90 ± 40.42	80.52 ± 1.83	152.83 ± 26.04	103.85 ± 20.80
TNF-α,% of control	IVAE	100	189.93 ± 23.19	128.08 ± 12.83	93.18 ± 27.78	152.45 ± 48.79	242.95 ± 20.34
	SAAE	100	517.84 ± 218.15	201.35 ± 57.84	144.03 ± 24.97	265.28 ± 27.04	281.82 ± 56.84

All values expressed as mean ± SEM, n = 4

IVAE Inula viscosa aqueous extract, *SAAE Senecio anteuphorbium* aqueous extract, *TLR-4* toll-like receptor 4, *MyD88* myeloid differentiation primary response gene 88, *iNOS* inducible nitric oxide synthase, *TNF-α* tumor necrosis factor alpha

of 444 ± 0.011 µg/ml and 50.03 ± 3.33 µg/ml. resp.). SAAE present highest activity against $ABTS^{\cdot+}$.

Quantification of total phenolic and flavonoid in IVAE and SAAE

Total phenolic and flavonoid content of the extracts are shown in Table 2. IVAE present a higher phenolic and flavonoid contents with 239.75 ± 0.49 mg GAE/g ext. and 102.22 ± 1.92 mg RE/g ext., respectively. Total phenolic and flavonoid contents in the stem of SAAE were 25.92 ± 2.73 mg GAE/g ext. and 27.11 ± 2.02 mg RE/g ext., respectively.

TLC analysis

The TLC analysis of SAAE and IVAE show the presence of several phenolic acids and flavonoids which appeared as blue and yellow spots, respectively (Fig. 3). The result showed the presence of luteolin and chlorogenic acid in IVAE. The flavonoid and phenolic controls produced orange-yellow and blue spots, respectively with NEU-reagent.

Characterization of major compounds in IVAE and SAAE

The UHPLC-HRAM/MS technique was performed for the identification of some compounds and the results are presented in Table 3 and Fig. 4, the peaks were selected on the basis of their abundance (strongest peaks were selected), in order to further investigate the relation of major compounds and the potential anti-inflammatory effects of the plant extracts. The SAAE depicted main unsaturated toxic PAs, namely, seneciphylline N-oxide (most abundant compounds) and four no identified compounds. Moreover, three compounds 3 α-hydroxycostic acid, tomentosin and hydroxycoumarine were found in IVAE. Sesquiterpenes are the major compounds identified in IVAE including tomentosin.

Discussion

In marcophages cells, binding of LPS to TLR-4 activates MyD88-dependent TLR-4 signaling pathway that leads to the production of pro-inflammatory cytokines including TNF-α, IL-12 and ROS (Nagy 2003). Overproduction of these mediators is present in macrophage in many inflammatory diseases, including rheumatoid arthritis, diabetes (Choy and Panayi 2001; Pickup 2004; Tilg and Moschen 2010). NO produced by iNOS is believed as one of the most important inflammatory reactions in activated macrophages (Pokharel et al. 2007; Tilg and Moschen 2010; Spruss et al. 2011). Recently, many researchers have

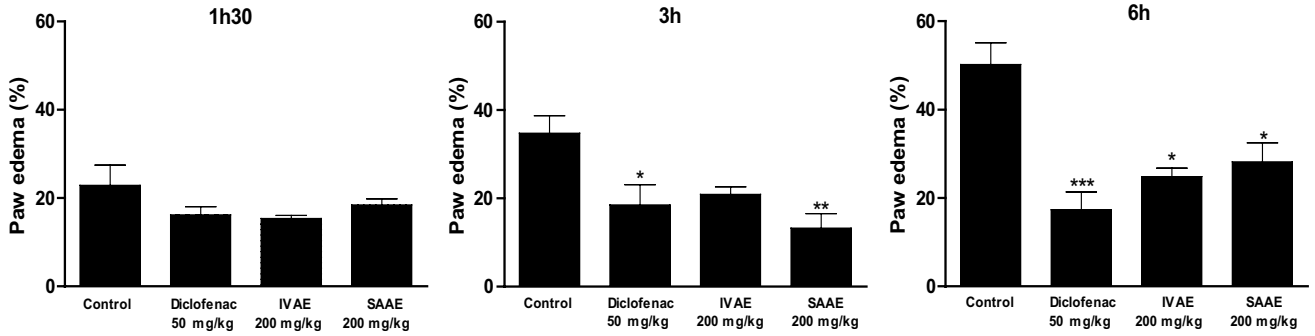

Fig. 2 Effect of IVAE and SAAE on paw circumference in control and experimental mice. Values are expressed as mean ± SEM of 6 mice. The drugs were administered by gavage 1 h before the subplantar injection of 0.1 ml of carrageenan (1% w/v in 0.9% saline); $*p<0.05$. $**p<0.01$ and $***p<0.01$ were compared to control. *IVAE Inula viscosa* aqueous extract, *SAAE Senecio anteuphorbium* aqueous extract

Table 2 Content of phytochemicals extracted from IVAE and SAAE and their radical scavenging activity determined by DPPH and ABTS assay[a]

Plant extract	Total phenolic mg GAE/g ext[b]	Flavonoid mg RE/g ext[b]	DPPH (IC_{50}) (µg/ml)	ABTS (IC_{50}) (µg/ml)
SAAE	25.92 ± 2.73	27.11 ± 2.02	444 ± 0.011	50.03 ± 3.33
IVAE	239.75 ± 0.49	102.22 ± 1.92	11.4 ± 1.28	11.25 ± 2.03

IVAE Inula viscosa aqueous extract, *SAAE Senecio anteuphorbium* aqueous extract

[a]All values expressed as mean ± SD of triplicate tests (n = 3)

[b]Data expressed in mg gallic acid and routine equivalent/g extract (mg GAE/mg. mg RE/mg)

Table 3 Major compounds identified in aqueous extracts of *I. viscosa* (IVAE) and *S. anteuphorbium* (SAAE)

	N	Tr (Min)	c	[M + H] + (m/z)	MS^2	ppm	Attempt identification of compounds
IVAE	1	8.88	187.07061	188.07077	146; 188; 144; 170	0.77820	$C_{11}H_9O_2N$ (ND)
	2	9.90	162.03897	163.03905	163; 145; 135; 117	0.47002	$C_9H_6O_3$ Hydroxycoumarine
	3	16.91	264.14344	265.14362	189; 207; 223; 201	0.67923	$C_{15}H_{20}O_4$ (ND)
	4	19.44	248.14852	249.14864	231; 203; 189; 185	0.46091	$C_{15}H_{20}O_3$ Tomentosin
	5	23.45	234.16926	235.16942	189; 217; 199; 235	0.68821	$C_{15}H_{22}O_2$ 3 α-hydroxycostic acid
SAAE	1	11.32	349.15981	350.15982	350; 120; 338; 322	0.01895	$C_{18}H_{23}O_6N$ (Seneciphylline N-oxide)
	2	12.27	337.196	338.19659	338; 320; 310; 292	0.85956	$C_{11}H_{27}O_3N_7S$ (ND)
	3	15.90	391.17038	392.17047	392; 332; 316; 228	0.23615	$C_{20}H_{25}O_7N$ (ND)
	4	17.14	–	485.23849	219; 237; 358; 385	–	–
	5	20.14	424.19184	425.19205	132; 150; 104; 122	0.48178	$C_{20}H_{28}O_8N_2$ (ND)

ND no determined, *IVAE Inula viscosa* aqueous extract, *SAAE Senecio anteuphorbium* aqueous extract

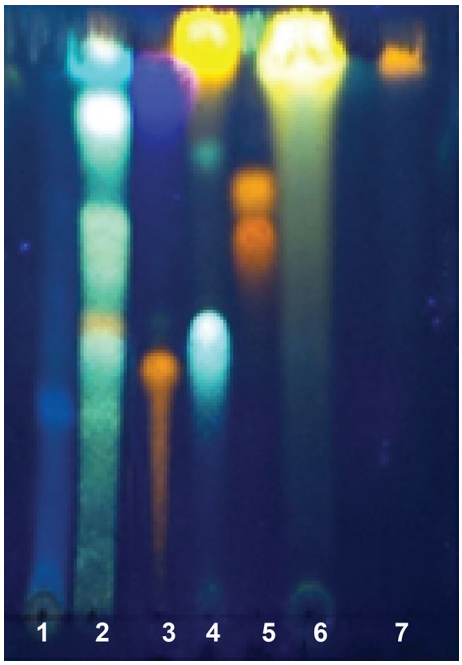

Fig. 3 Thin layer chromatogtaphy of SAAE and IVAE. *1*: SAAE (*Senecio anteuphorbium* aqueous extract); *2*: IVAE (*Inula viscosa* aqueous extract); *3*: Rutin + Gallic acid; *4*: Chlorogenic acid + Quercetin; *5*: Myricetrin + Quercetrin; *6*: Luteolin; *7*: Myricitin

attempted to investigate naturally occurring products to downregulate these inflammatory mediators, as this is thought to be a good strategy for curing LPS-induced inflammation.

In order to investigate the possible mode of the anti-inflammatory action of SAAE and IVAE, J774A.1 macrophage cells were induced with LPS. The results showed that non-toxic concentrations of SAAE high significantly suppressed the release of NO in LPS-stimulated J774A.1 cells being accompanied with a decrease in the expression of iNOS mRNA. These results suggest that the inhibition of NO release might be attributable to the suppression of iNOS expression at the transcriptional level further indicating an anti-inflammatory effect of the extract. While, IVAE present a high significant reduction of NO at activation J774A.1 cells and a significant suppression of iNOS expression. These results suggest that NO reduction can be attributing to suppression of iNOS mRNA expression and neutralization of NO by its antioxidant activity. Many researchers are developing agents to regulate the expression of NO via iNOS (Ortega et al. 2010; Park et al. 2010). Therefore, we also investigated the effect of extracts on the expression of pro-inflammatory cytokines TNF-α and TLR4 MyD88 LPS-stimulated J774A.1 cells. Both IVAE and SAAE did not influence their expression. Pro-inflammatory cytokines TNF-α that are usually released by macrophages play a critical role in initiating and sustaining the inflammatory response (Bradley 2008).

Another standard model to test drugs for acute inflammation and its effect as anti-inflammatory drugs includes carrageenan-induced hind paw edema study. Therefore, we have used this model for determining the acute phase of inflammation. Carrageenan is a potent inflammatory agent and the carrageenan-induced paw edema is a well-known acute model of inflammation that is widely used for screening novel anti-inflammatory compounds. Acute inflammatory response is characterized by an increase in vascular permeability and cellular infiltration leading to edema formation, as a result of extravasation of fluid and proteins and recruitment of different cell types, including monocytes that differentiate locally into macrophages and neutrophils (Medzhitov 2008). This leads to the production of various inflammatory mediators including prostaglandins, leukotriens, interleukins (IL-1 and IL-6) and reactive oxygen species (ROS) (White 1999; Nacife et al. 2004). Among these proinflammatory molecules, NO plays a vital role in carrageenan-induced paw edema, it is formed by a number of different cells, including leukocytes, endothelial cells and sensory nerve cells (Handy and Moore 1998). Di Rosa et al. (1971) reported that the carrageenan-induced inflammatory process in the rat involves three phases: the first phase (1.5 h), which is not inhibited by non-steroidal

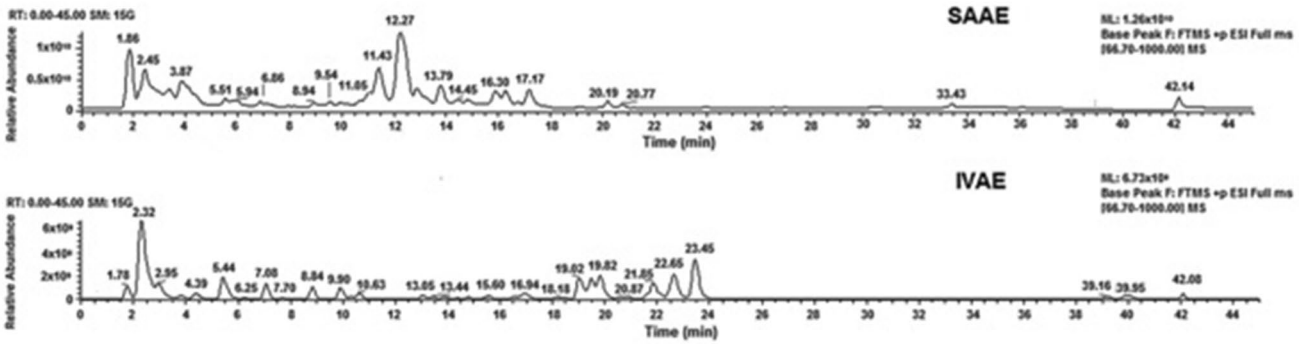

Fig. 4 Base peak chromatogram of SAAE and IVAE obtained from UHPLC-HRMS (positive ion). SAAE *Senecio anteuphorbium* aqueous extract, IVAE *Inula viscosa* aqueous extract

anti-inflammatory drugs such as diclofenac or aspirin, has been attributed to the release of histamine and serotonin and is characterized by increase in vascular permeability. The second phase (2 h) is mediated by release of bradykinin, an important chemical mediator of both pain and inflammation. In contrast, the third and final phase (3 and 6 h) has been correlated with the elevated production of prostaglandins and cyclooxygenases. Another key mediator in the carrageenan-induced inflammatory response is NO. It is generated by three distinct isoforms of nitric oxide synthase (NOS): neuronal (nNOS) and endothelial (eNOS) which are constitutively expressed, whereas inducible NOS (iNOS) which is mainly generated by stimulated macrophages and ist overproduction is associated with pathogenesis of several inflammaion-related diseases. NO produced by iNOS is involved in the maintenance of the inflammatory response at later time points (4–10 h) following carrageenan administration. In addition, iNOS mRNA was detected between 3 and 10 h after carrageenan administration and iNOS protein was detected at 6 h and was maximal at 10 h (Salvemini et al. 1996). NO is a potent vasodilatator its involvement during an inflammatory response may be related to its ability to increase vascular permeability and edema through changes in local blood flow and has been shown to increase the production of pro-inflammatory prostaglandins (Salvemini et al. 2003). In the present study, statistical analysis revealed that 50 mg/kg of diclofenac and 200 mg/kg of SAAE and IVAE significantly inhibited paw edema induced by carrageenan in the second phase. This finding suggests a possible inhibition of iNOS expression and NO production by extracts, as was shown in the macrophage cell model, with such an effect being a possible explanation for the reduction of edema. Therefore, this effect which produced by non-steroidal anti-inflammatory drugs such as diclofenac, is due to the inhibition of the cyclooxygenase enzyme, which catalyses the synthesis of cyclic endoperoxides important in the formation of prostaglandins (Al-Majed et al. 2003). It was observed that there was inhibition of edema formation from 3 to 6 h by SAAE, and which was the same as that of diclofenac. IVAE failed to inhibit inflammation during the first 3 h following carrageenan administration, but caused a significant inhibition at 6 h. The inhibition of inflammation at all the time points studied for SAAE and diclofenac than IVAE could be explained by the bioavailability and pharmacokinetic properties of the active secondary metabolites and diclofenac. Indeed, diclofenac presente a bioavailability of 65% with oral administration, therefore the digestion and absorption of secondary metabolites depend of their structure; they are slow processes especially for glycosylated forms because of the resistance of the osidicbonds resulting in decrease of bioavailability (Graefe et al. 2001). These results could explain the high anti-inflammatory potential of SAAE than IVAE.

These results do not preclude that plant extracts may also exert protection through antioxidant effect. Therefore, antioxidants occurring in plants may play a significant role in health protection and have shown some promise in reducing inflammation. The mechanism of antioxidant action can include free radical-scavenging activity and suppression of reactive oxygen species formation, either by inhibition of enzymes or by chelating trace elements (Van Acker et al. 1996; Singh et al. 2004).

In the present work, two different methods were successfully used for evaluation of the antioxidant activity of the crude extracts: $DPPH^{\cdot}$ radical-scavenging assay, $ABTS^{+\cdot}$assay. Both $DPPH^{\cdot}$ and $ABTS^{+\cdot}$radicals have been used extensively to evaluate the antioxidant properties of natural products. Our results show that IVAE ixhibit a strong antioxidant effect than SAAE, suggesting that the bioactive constituents of *I. viscosa* can contribute in anti-inflammatory effect.

Bioactivities found in crude aqueous extract of *I. viscosa* and *S. anteuphorbium* could be attributed to secondary metabolites, so that the phyochemical of IVAE and SAAE were analyzed. The results obtained show that the antioxidant activity of IVAE is directely related to amount of total phenolic and flavonoid contents but not with anti-inflammatory effect. As reported, the high levels of phenolic compounds indicate high antioxidant capabilities (Dudonné et al. 2009).

Many compounds from medicinal plants have been known as antioxidant and inhibitors of expression of iNOS in LPS-activated macrophages (Kim et al. 1999; Raso et al. 2001) and iNOS may be a useful tool with therapeutic focus in many inflammatory processes. Concerning the bioactive compounds such as phenolic compounds from *S. anteuphorbium* responsible for the observed effects have not been properly characterized. To our knowledge no study of its composition has been published so fare. Accordingly, it is difficult to identify all the compounds except senecionphylline N-oxide (pyrrolizidine alkaloid). Thus, other data and analysis are necessary for their identification. On the other hand, biological activities of *I. viscosa* as antioxidant (Schinella et al. 2002; Danino et al. 2009; Mahmoudi et al. 2016) and anti-inflammatory effects (Máñez et al. 1999; Hernández et al. 2005, 2007; Mahmoudi et al. 2016) have been reported, and several bioactive compounds implicated in these effects are isolated and identified including phenolic compounds (cafeic acid, 1,3-dicaffeoylquinic acid, luteolin, chlorogenic acid, p-coumarin acid, rutin, quercetin) (Hernández et al. 2007; Gökbulut et al. 2013) and sesquiterpene acids (dehydrocostic acid, 3 α-hydroxycostic acid) and lactones (inuviscolide, tomentosin) (Grande et al. 1992; Abu Zarga et al. 1998; Hernández et al. 2001, 2005; Mamoci

et al. 2011). In our study, the presence of chlorogenic acid and luteolin in IVAE was revealed by TLC analysis. While, hydroxycoumarin, 3 α-ydroxycostic acid (major component) and tomentosin were identified by UHPLC-HRMS in IVAE. Mamoci et al. (2011) demonstrated similar profiles of tomentosin and 3 α-hydroxycostic acid in *I. viscosa.* Luteolin and chlorogenic acid have many beneficial effects such as anti-inflammatory property by inhibition of iNOS expression (Hwang et al. 2014; Pandurangan et al. 2014). Hydroxycoumarin is a new compound identified from *I. viscosa* which has not been reported in the literature, but it has been identified in *inula* genus (Jiang-Jiang et al. 2011). Indeed, it is a typical phenolic compound and, therefore can act as potent metal chelator and free radical scavenger (Kostova 2005) and iNOS inhibitor (Timonen et al. 2011; Adfa et al. 2012). Sesquiterpenoids were considered as the characteristic constituents from *Inula* genus (Guo and Yang 2005). Therapeutic applications of tomentosin, a sesquiterpene lactone, on inflammation have been reported (Park et al. 2014). Differently from simple sesquiterpenoids, the sesquiterpene lactones are a group with much higher biological activity. It has been established that the α-methylene-γ-lactone moiety is involved in their mechanism of action through alkylation of biological nucleophiles by a Michael-type addition. The covalent binding to free sulfhydryl groups in proteins can inhibit processes such as neutrophil migration and enzymatic activity (Hall et al. 1980). It has been demonstrated that tomentosin inhibits gene transcription factors such as NF-κB (Siedle et al. 2004; Abrham et al. 2010) and, in consequence, affect the expression of iNOS, TNF-α and COX-2 (Wong and Menendez 1999). In our study, IVAE did not affect the expression of TNF-α. However, it could well be that compounds found in IVAE act through different pathway. Further experiments are necessary to confirm this hypothesis and to verify a possible inhibition of other transcriptional factors. These results suggest a crucial contribution of these compounds in the modulation of NO-mediated inflammation, which act as scavengers of free radicals and/or inhibitors of the iNOS expression. These findings are in correlation with previous work cited previously on *Inula viscosa*, which found that anti-inflammatory and antioxidant potential were correlated to the presence of phenolic compounds and sesquiterpenes. *Inula viscosa* are generally used as decoction in water to treat inflammatory diseases. Our study confirmed that the high contents of phenolic compounds and sesquiterpenoids may be responsible for its therapeutic uses.

In conclusion, our study provides the first evidence that SAAE and IVAE exert anti-inflammatory effect by inhibiting NO production through suppression iNOS mRNA expression in vitro. In addition, We have demonstrated that they exhibit antioxidant activity. These activities may be due to their contents of phenolic coumpounds and sesquiterpenes. These results suggest that the *S. anteuphorbium* and *I. viscosa* can be a potential herbal component for the development of new anti-inflammatory drugs. Further studies are necessary to fully define the precise mechanisms involved.

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Compliance with ethical standards

Ethical statement All the experimental mice were treated following the Ethical Principles and Guidelines for Scientific Experiments on Animals (1995) formulated by The Swiss Academy of Medical Sciences and the Swiss Academy of Sciences.

Conflict of interest The authors declare that there are no conflicts of interests.

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