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ABSTRACT

Previous work has shown that roots of *Arbutus unedo* induce endothelium-dependent relaxation of rat thoracic aorta. In this study, we examined the vascular effect of Arbutus leaves and describe the isolation of several fractions responsible for their vasorelaxant activity. Aqueous extract (AE) of leaves was tested on rat aortic rings pre-contracted with 0.1 µM noradrenaline. At 10^{-2} g/L, AE produced an endothelium dependent relaxation of 66±5%, (n=8). Leaves of Arbutus were then extracted successively with different solvents (hexane, dichloromethane, ethyl acetate, methanol and water) and the extracts obtained were tested on pre-contracted aorta. The methanolic and the ethyl acetate extracts were the most active. When tannins (primarily condensed tannins) were precipitated from the methanolic extract, they showed a strong vasorelaxant activity (87±4%, n=5) whereas the elimination of tannins in the methanolic extract reduced significantly its vasorelaxant activity (42±8%, n=8, $p<0.005$). Methanolic extract was further separated semi-preparatively by reversed-phase HPLC and fractions were collected and tested for their vasorelaxant activities. Four fractions (Fr2, Fr3, Fr4 and Fr6) were most active and produced 88±2% (n=5), 75±6% (n=5), 76±3% (n=7) and 76±3% (n=9) relaxation, respectively. These four fractions mainly correspond to polyphenols compounds. The analysis of Fr6 chromatogram indicates that this fraction contains catechin gallate. Dose-response curves for the vasorelaxant effects of Fr6 and commercial catechin gallate were superimposable confirming the likely contribution of catechin gallate to the effect of Fr6. In conclusion, the vasorelaxant activity of Arbutus is present both in leaves and roots. This activity is likely due to

polyphenol compounds, primarily condensed tannins and catechin gallate enhanced by quercetol heterosides.

Key words: *Arbutus unedo*; Ericaceae; Tannins; Vasorelaxation; Rat thoracic aorta

INTRODUCTION

Arbutus unedo L. (Ericaceae) is a species widely spread in Mediterranean basin and used in traditional medicine as astringent, diuretic and urinary antiseptic (Grieve, 1967). In Morocco, the roots of Arbutus are employed in decoction form for several uses (Bellakhdar, 1997) specially in the therapy of hypertension and diabetes (Ziyyat *et al.*, 1997). In the spontaneously hypertensive rat (SHR), oral administration of the root aqueous extract of Arbutus produces vascular, diuretic and natriuretic effects (Ziyyat & Boussairi, 1998). Recently, we have shown that root-aqueous extract also induces an endothelium-dependent vasorelaxation on rat isolated aorta (Ziyyat *et al*., 2002), giving a rationale for its use in folk medicine against hypertension. This effect could be attributed to polyphenolic compounds likely to be present in the extract. Indeed, phytochemical studies on Arbutus have led to the isolation of several polyphenolic compounds, mainly tannins in the roots (Garnier *et al.*, 1961) and flavonoids such as afzelin, quercitrin and hyperoside in the aerial part (Dauguet & Foucher, 1982). The present study was thus undertaken to isolate some of the fractions present in Arbutus extract which might be responsible for its endothelium-dependent vasorelaxant activity and to examine the implication of some polyphenolic compounds, such as tannins, in this effect.

MATERIALS AND METHODS

Vegetal material

Leaves of *Arbutus unedo* were collected from Jbel Tazekka in oriental Morocco in October 2000. Taxonomic identification was performed by Pr B. Haloui from the Biology Department of Oujda Sciences Faculty (Morocco) where voucher specimen has been deposited (collection N° ZL 14).

Plant extract preparation

Aqueous extract (AE): 20 g of dried and powdered leaves of *Arbutus unedo* were extracted by infusion with 1 L of water for 30 min. The aqueous extract was obtained after filtration and evaporation to dryness *in vacuo*.

Soxhlet extraction: 79 g of dried and powered leaves of *Arbutus unedo* were extracted successively with different solvents (hexane, dichloromethane, ethyl acetate, methanol and water). The extracts were evaporated to dryness *in vacuo* to afford respectively 5.0 g, 1.15 g, 3.75 g, 19.0 g and 2.53 g of dry extract. (Rendement : 6.4%, 1.5%, 5.2%, 24.1%, 3.2%)

Semi-preparative HPLC separation of the methanolic extract: a final purification of the methanolic fraction was performed using repeated reversed-phase semi-preparative HPLC on Whatman (Maidstone; UK) Partisil 10 ODS-3 column (25×0.94 cm *i.d.*, 10 µm), flow rate 5 mL/min, UV detection 280 nm, 30 mg injection and step gradient elution as follows: 0-16 min (methanol/water/formic acid 25/75/0.02 v/v), 16-96 min (methanol/water/formic acid 35/65/0.02 v/v).

Equipment: semi-preparative separations were carried out with a Waters HPLC preparative system (St Quentin en Yvelines, France) equipped with a 590 pump coupled to a solvent select valve, a 484 detector and a ABB SE120 recorder.

Tannins separation

Separation by caffeine: 1 g of methanolic extract was dissolved in 2 mL of water. Tannins were precipitated by 4 mL of a 2% caffeine aqueous solution. After filtration the caffeine tannate precipitate was washed by water and then dissolved in 50 mL of methanol. 50 mL of water was then added to the solution witch was extracted four times by 50 mL of chloroform and then evaporated under reduce pressure to give 288 mg of dry extract. In the same manner, to remove caffeine, the filtrate was extracted by chloroform and evaporated to dryness under reduce pressure to give 686 mg of dry extract.

Precipitation of tannins by skin powder: 100 mg of methanolic extract was dissolved in 20 mL of water. 200 mg of skin powder were added under stirring conditions during 1 hour. Tannins were removed by adsorption on skin powder after filtration. The filtrate led to 40 mg of dry extract.

Methanolic extract analysis

Analytical HPLC procedure: the methanolic extract and the different fractions isolated from methanolic extract were analyzed by using reversed-phase HPLC with a Waters μBondapak C_{18} (30×0.39 *i.d.* cm, 10 μm), 2 mL/min, UV detection 280 nm and a gradient elution with water (A) and methanol (B) both added with 0.05% (v/v) formic

acid as mobile phases with linear gradient from 5 to 60% B in 30 min, 60% B during 5 min.

Equipment: chromatographic analysis were performed using an HPLC system equipped with a Merck L-6200A pumpFontenay sous Bois, France), a Waters 717 autosampler, a Merck L-4000 detector and a Shimadzu C-R3A integrator(Croissy Beabourg, France).

Preparation of aorta and experimental device

The animal investigation conforms with the European Community guiding principles in the care and use of animals (86/609/CEE, *CE Off J* n°L358, 18 December 1986) and the French decree n°87/748 of October 19, 1987 (*J Off République Française*, 20 October 1987, pp. 12245-12248). Authorisations to perform animal experiments according to this decree were obtained from the French Ministère de l'Agriculture et de la Forêt (n°04226, April 12, 1991).

Male Wistar rats weighing 300-350 g were anaesthetised with sodium pentobarbital (50 mg/kg of body weight, i.p.), and the thoracic aorta was removed carefully in physiological salt solution (PSS). An aortic ring of about 3 mm in length was suspended between two stainless steel hooks in 10-mL water-jacketed bath containing PSS of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. The tissue bath solution was maintained at 37°C and gassed with 95% O_2 + 5% CO_2 (pH 7.4). The isometric contraction was recorded via a force-displacement transducer connected to an amplifier and a paper recorder. Data were also recorded on computer. A tension of 1 g was initially applied to the ring which was equilibrated in the medium for 30 min. Before

each experiment, vasoconstriction was initiated by 0.1 µM noradrenaline (NAdr) in normal PSS and when contraction reached steady-state, 10 µM carbachol (CCh, a cholinesterase-resistant analogue of acetylcholine) was added to induce endothelium-dependent relaxation. This step was necessary to verify the endothelium integrity. For relaxation studies, precontraction of the aortic rings was induced by 0.1 µM NAdr, the extract of *Arbutus unedo* was then added to the organ bath, and relaxation was evaluated as a percentage of the initial tension induced by NAdr alone. In denuded aorta, the endothelium was removed mechanically by rubbing the lumen of the artery with plastic tubing. In this case, relaxation was induced by 1 µM of sodium nitroprusside.

Chemicals. (-)-Norepinephrine hydrochloride (Noradrenaline, NAdr), carbamylcholine chloride (carbachol, CCh), catechin, gallic acid and catechin gallate were purchased from Sigma Chemicals Co. (St Quentin-Fallavier, France); Sodium nitroprusside (SNP) from Farco Chemicals (ville, pays); Arbutin, epicatechin, quercetin and hyperoside from Extrasynthèse(Genay, France); Formic acid from Prolabo (Fontenay sous Bois). Methanol was HPLC grade purchased from Carlo Erba (Val de Reuil, France). Water was purified by Milli-Q water purification system (Millipore,(St Quentin en Yvelines, France).

Statistics. Results are expressed as the means ± SEM for n separate experiments. Data were analysed by Student's *t*-test and a difference was considered as statistically significant when *p* value was less than 0.05.

RESULTS

Figure 1A shows a typical experiment in which an intact thoracic aortic ring was exposed to 0.1 µM NAdr after equilibration in control solution. NAdr produced a dramatic increase in tension. As expected, application of CCh (10 µM) in the continuing presence of NAdr strongly (~80%) reduced the effect of NAdr. The tension obtained in the presence of 0.1 µM NAdr and the relaxation produced by 10 µM CCh were used as references in subsequent experiments evaluating the relaxant effects of plant extracts. On average, intact aortic rings developed a tension of 1.3±0.1 g in response to 0.1 µM NAdr (n=25), and 10 µM CCh induced a 83±2% relaxation (n=20). As shown in Fig. 1B, aqueous extract of Arbutus leaves (AE, 10^{-2} g/L) also induced a strong relaxation of pre-contracted aortic rings. The relaxation was preceded by a small rise in tension. On average, AE (10^{-2} g/L) produced a 66±5% (n=8) relaxation. As the relaxant effect of CCh requires the presence of an intact endothelium, we tested whether this was also the case for the relaxant effect of AE. Figure 1C shows an experiment performed in a denuded aortic ring. NAdr still produced a strong contraction of the aorta but, as expected, CCh had no effect. Interestingly, addition of AE (10^{-2} g/L) to the cocktail had no effect either, while application of the NO-donor SNP strongly relaxed the muscle. This result, which was obtained in all of 3 similar experiments, indicates that, similarly to root extracts (Ziyyat *et al*., 2002), leave extracts from Arbutus also require an intact endothelium to produce a relaxant effect.

(Figure 1 near here)

The subsequent experiments were aimed at identifying the active compounds present in AE which might be responsible for the relaxant effect. To do this, leaves of Arbutus were extracted successively with solvents of increasing polarity. Lipids were first removed with hexane, and four successive extracts were obtained with dichloromethane, ethyl acetate, methanol and water. These extracts were then tested on NAdr pre-contracted aortic rings at concentrations ranging from 10^{-3} to 10^{-1} g/L. As shown in Fig. 2, all four extracts caused a vasorelaxant effect. However, ethyl acetate and methanol extracts appeared most active, since at 10^{-2} g/L concentration they fully relaxed NAdr pre-contracted rings, while dichloromethane and water extracts required a 10-fold larger concentration to produce a similar effect (Fig. 2).

(Figure 2 near here)

In the following, we focused our attention to the methanolic extract, which caused a 79±4% relaxation (n=9) of NAdr pre-contracted aortic rings at 10^{-2} g/L and was selected for the purification of active compounds. Preliminary chemical research on this extract showed the abundant presence of condensed tannins (green coloration with $FeCl_3$ and precipitation with Stiasny reagent). Therefore, it was logical to question the implication of tannins in the vasorelaxant effects observed with this extract. We addressed this question by treating the methanolic extract with caffeine, a procedure used to precipitate most of the tannins (Okuda *et al.*, 1982). Methanolic extract was also treated with skin powder, which is commonly used to eliminate the tannins present in solution(European Pharmacopoeia 4^{th} edition, Council of Europe, Strasbourg, France). The fraction precipitated by caffeine and the filtrate of the fraction treated with skin

powder were tested on aortic rings pre-contracted by NAdr. Vasorelaxation was still robust (87±4%, n=5) when the fraction precipitated by caffeine was tested at 10^{-2}g/L. On the contrary, elimination of tannins by skin powder strongly reduced the vasorelaxant effect (42±8%, n=8, $p<0.005$). These experiments clearly indicate that tannins participate in the vasorelaxant effect of Arbutus leaves. However, the presence of a residual vasorelaxant activity in the solution treated by skin powder also demonstrates that other components besides tannins may also be involved.

(Figure 3 near here)

Methanolic extract was further separated semi-preparatively by reversed-phase HPLC. A complete chromatogram of the total methanolic extract is shown in Fig. 3A. This chromatogram was separated in thirteen fractions, named Fr1 to Fr13 (Fig. 3A). Each fraction was collected and tested at 10^{-2} g/L on pre-contracted aorta. Figure 3B shows that Fr2, Fr3, Fr4 and Fr6 were the most active, as they produced a relaxing effect of 88±2% (n=5), 75±6% (n=5), 76±3% (n=7) and 776±3% (n=10), respectively. The nine other fractions produced a 4 to 10 fold lower relaxation at the same concentration, and none of their effects was statistically significant. The four active fractions were further examined for their concentration-dependent effects. As shown in Fig. 4A, Fr2, Fr3, Fr4 and Fr6 produced a concentration-dependent relaxation. Fr2, Fr4 and Fr6 had a threshold value near 10^{-3} g/L and their effect was half maximal around $3x10^{-3}$ g/L. Fr3 was about 3-fold less active.

(Figure 4 near here)

Despite the complexity of the methanolic extract chromatogram (Fig. 3A), several components were identified by HPLC coupled with mass spectrometry and confirmed by retention time of standard. These include arbutoside, quercitin, epicatechin, catechin, catechin gallate, hyperoside and gallic acid (see Fig. 5 for chemical structures). Thus, we tested all these compounds at 10^{-2} g/L for their effects on NAdr pre-contracted aortic rings. As shown in Table 1, only catechin gallate induced a strong vasorelaxant activity. Arbutoside, quercitin and epicatechin had no significant effect whereas catechin and hyperoside induced a moderate vasorelaxant effect (Table 1).

(Figure 5 near here)

Analysis of Fr6 chromatogram indicated that catechin gallate was actually present in this fraction. Therefore, we compared the vasorelaxant effect of Fr6 and catechin gallate. As shown in Fig. 4B, Fr6 and commercially available catechin gallate produced a similar concentration-dependent vasorelaxant effect on NAdr pre-contracted aortic rings. In order to determine structure-activity relationships, this activity was compared that of catechin and gallic acid. As shown above, the vasorelaxant effect of catechin was weaker than that of catechin gallate (Table 1). Moreover, gallic acid exerted a strong vasoconstriction (51±13% at 10^{-2}g/L, n=5, $p<0.05$).

(Table 1 near here)

DISCUSSION

Arbutus unedo is one of the most popular medicinal plants used in oriental Morocco (Ziyyat *et al.*, 1997). Traditionally, roots of the plant are used in decoction to treat hypertension. In this study, we focused our interest on Arbutus leaves, because they can be collected more easily and do not require the destruction of the shrub, which is of ecological relevance. We found that the aqueous extract of Arbutus leaves produces a strong relaxation of pre-contracted aorta. This effect was endothelium-dependent and comparable, in potency and efficacy, to that of the root extract (Ziyyat *et al*., 2002). Thus, Arbutus leaves may be used instead of roots in folk medicine, provided that, like the roots, they do not present any toxic effect.

As we found that the vasorelaxant effect of Arbutus roots extract involves activation of endothelial NO-synthase and cGMP synthesis (Ziyyat *et al*., 2002), it is likely that Arbutus leaves also contain one or several active compounds which cause the endothelial cells to produce NO. Extraction of dried and powdered leaves by different successive solvents of increasing polarity led us to the observation that these compounds were likely present in the ethyl acetate and methanolic extracts. Indeed, these two extracts accounted for most of the vasorelaxant activity of Arbutus. Bioguided fractionation of these extracts, using Stiasny reagent and the green coloration with $FeCl_3$, led us to unmask the presence of polyphenolic compounds such as tannins.

Tannins and related polyphenols include more than 4000 identified compounds, and represent one of the largest groups of active phytochemicals (Waltner-Law *et al*., 2002). The participation of condensed tannins in the vasorelaxant effect of Arbutus leaves extract was suggested by the observation i) that tannins precipitated by caffeine

from the methanolic extract showed a strong vasorelaxant activity, whereas ii) the adsorption of tannins by skin powder in the methanolic extract reduced significantly its effect. Thus, the endothelium-dependent vasodilator effect of Arbutus leaves extract was mainly due to oligomeric condensed tannins. However, the presence of a residual vasorelaxant activity in the solution treated by skin powder also demonstrates that other components besides tannins may also be involved. These are likely to include flavonic heterosides (flavonoids), which are present in Arbutus leaves (Dauguet & Foucher, 1982). Both flavonoids and tannins have been shown earlier to produce endothelium-dependent relaxation of rat aortic rings (Fitzpatrick *et al.*, 1993; Chen *et al*., 1996; Chan *et al*., 2000), an effect which resembles the effect of Arbutus leaves extract. Moreover, tannins and related polyphenols were also shown to produce an endothelium-dependent, NO-derived vasorelaxation through an extracellular Ca^{2+}-dependent mechanism (Andriantsitohaina, 1999; Andrianbeloson *et al*., 1998; 1999). Oral administration of wine polyphenol compounds produces a decrease in blood pressure in normotensive rats associated with an endothelium-dependent relaxation and an induction of gene expression of inducible NO-synthase and cyclooxygenase 2 (Diebolt *et al*., 2001). Recently, provinol, a phenolic compound, was found to accelerate the regression of blood pressure and improve structural and functional cardiovascular changes produced by chronic inhibition of NO synthesis in L-NAME rats (Bernatova *et al*., 2002). This resembles the effect of an oral administration of the root aqueous extract of Arbutus in the SHR rat (Ziyyat & Boussairi, 1998). Thus, there is ample evidence to suggest that tannins or related polyphenols also mediate the vasorelaxant effect of Arbutus leaves extract.

Semipreparative separation of the methanolic extract by reversed-phase HPLC led to the identification of thirteen fractions. Out of these, four fractions (Fr2, Fr3, Fr4 and Fr6) were particularly active. These four fractions correspond to polyphenol compounds, primarily oligomeric condensed tannins. Interestingly, we found that Fr6 fraction contains catechin gallate, an oligomeric tannin. Test of its activity on aortic rings identified this compound as a potent vasorelaxant compound, similar to the complete Fr6 fraction. While this is the first report on the isolation of catechin gallate from Arbutus, we conclude that this compound represents one of the main compounds responsible for the vasorelaxant effect of the plant.

Catechins are generally known to produce vasorelaxant effects (Huang *et al.*, 1999). Yet, catechin derivatives were found to potentiate the contractile response to phenylephrine at low concentrations and to produce vasorelaxation only when used at high concentration (Sanae *et al.,* 2002). Although structurally related to catechin gallate, we found here that catechin exerted a much weaker relaxation than catechin gallate and gallic acid was even vasoconstrictive (see also: Sanae *et al.,* 2003).

In addition to fraction Fr6, a strong vasorelaxant activity was also found in fractions Fr2, Fr3 and Fr4 of the methanolic extract. These fractions correspond to oligomeric condensed tannins. Oligomerization of tannins may be required for tannins to produce a vasorelaxant activity. Indeed, (+)-catechin, which is with (-)-epicatechin one of the basic units of oligomeric condensed tannins, produces no vasorelaxation while oligomeric tannins present in red wine (Andriambeloson *et al.*, 1998) or Arbutus (this study) relax pre-contracted aorta. Fraction Fr11, which contains heterosides of quercetin (hyperoside and quercitrin), had a much weaker activity. Genin alone (quercitin) showed a very weak activity, while the corresponding heteroside

(hyperoside) was more active. Finally, arbutoside (present in Fr1), which is responsible for the antiseptic activity of Arbutus leaves (Frohne, 1970), had no vasorelaxant activity.

In conclusion, like the root extract, Arbutus leaves extract possesses a strong endothelium-dependent, vasorelaxant activity which provides a rationale for the use of this plant in folk medicine to treat hypertension. A chromatographic fractionation of Arbutus leaves methanolic extract allowed to assign this vasorelaxant activity to polyphenolic compounds such as oligomeric condensed tannins and catechin gallate.

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Table 1. Mean values of the vasorelaxant effect (expressed as percent of relaxation on intact NAdr pre-contracted aorta) of the compounds identified in methanolic extract (n=4).

	10^{-2} g/L					
	Arbutoside	Quercitin	Epicatechin	Hyperoside	Catechin	Catechin gallate
% relaxation	1±1	5±1	3±1	23±6*	35±4*	81±6*

Values are means ± SEM. * $p<0.05$ vs. Controle (NAdr. Alone).

FIGURE LEGENDS

Figure 1. Vasorelaxant effect of Arbutus leaves aqueous extract

Original tracings showing the effect of carbachol (CCh, 10 µM, A, C), aqueous extract of Arbutus leaves (AE, 10^{-2} g/L, B, C) or SNP (1 µM, C) in intact (A, B) or denuded (C) rat aortic rings pre-contracted by 0.1 µM NAdr. The rings were first exposed to control solution and the solid lines indicate the period of drug perfusion.

Figure 2. Vasorelaxant effect of Arbutus leaves extracts

Original tracings showing the vascular effect of four extracts of Arbutus leaves obtained by soxhlet extraction with solvents of increasing polarity. Dichloromethane (A), water (B), ethyl acetate (C) and methanol extracts (D) of Arbutus leaves were tested at 10^{-3} - 10^{-1} g/L on noradrenaline (NAdr, 1 µM) pre-contracted intact rat aortic rings. The rings were first exposed to control solution and the solid lines indicate the period of drug perfusion.

Figure 3. Separation and test of different fractions from methanol extract of Arbutus leaves

A) complete chromatogram of the total methanolic extract of Arbutus leaves obtained by semi-preparative reversed-phase HPLC. This chromatogram was separated in thirteen fractions, named Fr1 to Fr13. B) Each fraction was collected and tested at 10^{-2} g/L on 0.1 µM NAdr pre-contracted rat aortic rings. The effect is expressed as percent relaxation. Each bar indicates the mean ± SEM of the number of experiments shown in parentheses.

Figure 4. Concentration-response curves for the effects of different fractions from methanol extract of Arbutus leaves

A) Concentration-response curves for the effects of fractions Fr2 (◆), Fr3 (■), Fr4 (triangles) and Fr6 (●) separated as shown in Fig. 3A. Aortic rings pre-contracted by 0.1 µM NAdr were exposed to 10^{-3}, $3x10^{-3}$ or 10^{-2} g/L of each fraction. B) Concentration-response curves for the effects of Fr6 (●, same data as in A) and catechin gallate (CG, ■). Each symbol indicates the mean ± SEM of the number of experiments indicatedin parentheses.

Figure 5. Chemical structures of compounds identified in methanolic extract.

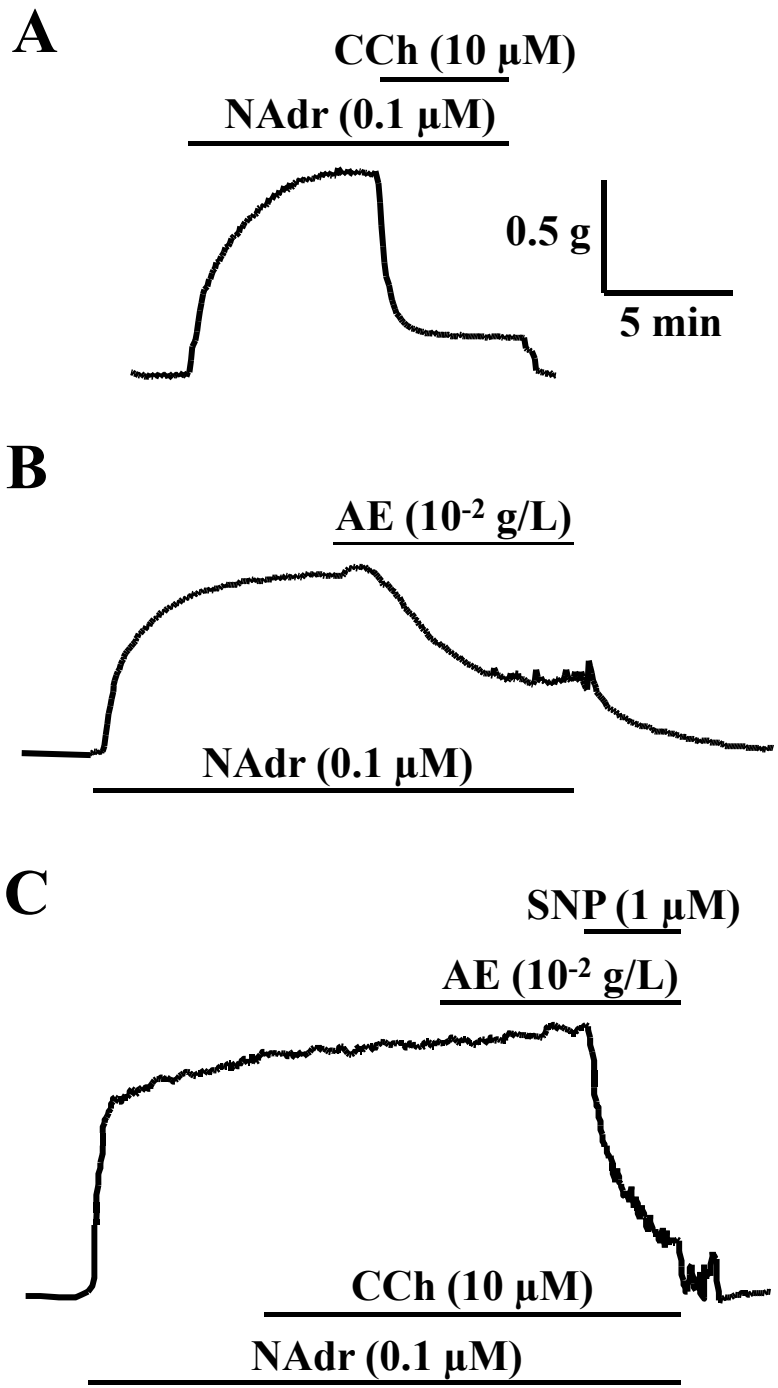
A
CCh (10 µM)
NAdr (0.1 µM)
0.5 g
5 min
B
AE (10-2 g/L)
NAdr (0.1 µM)
C
SNP (1 µM)
AE (10-2 g/L)
CCh (10 µM)
NAdr (0.1 µM)

Figure 1

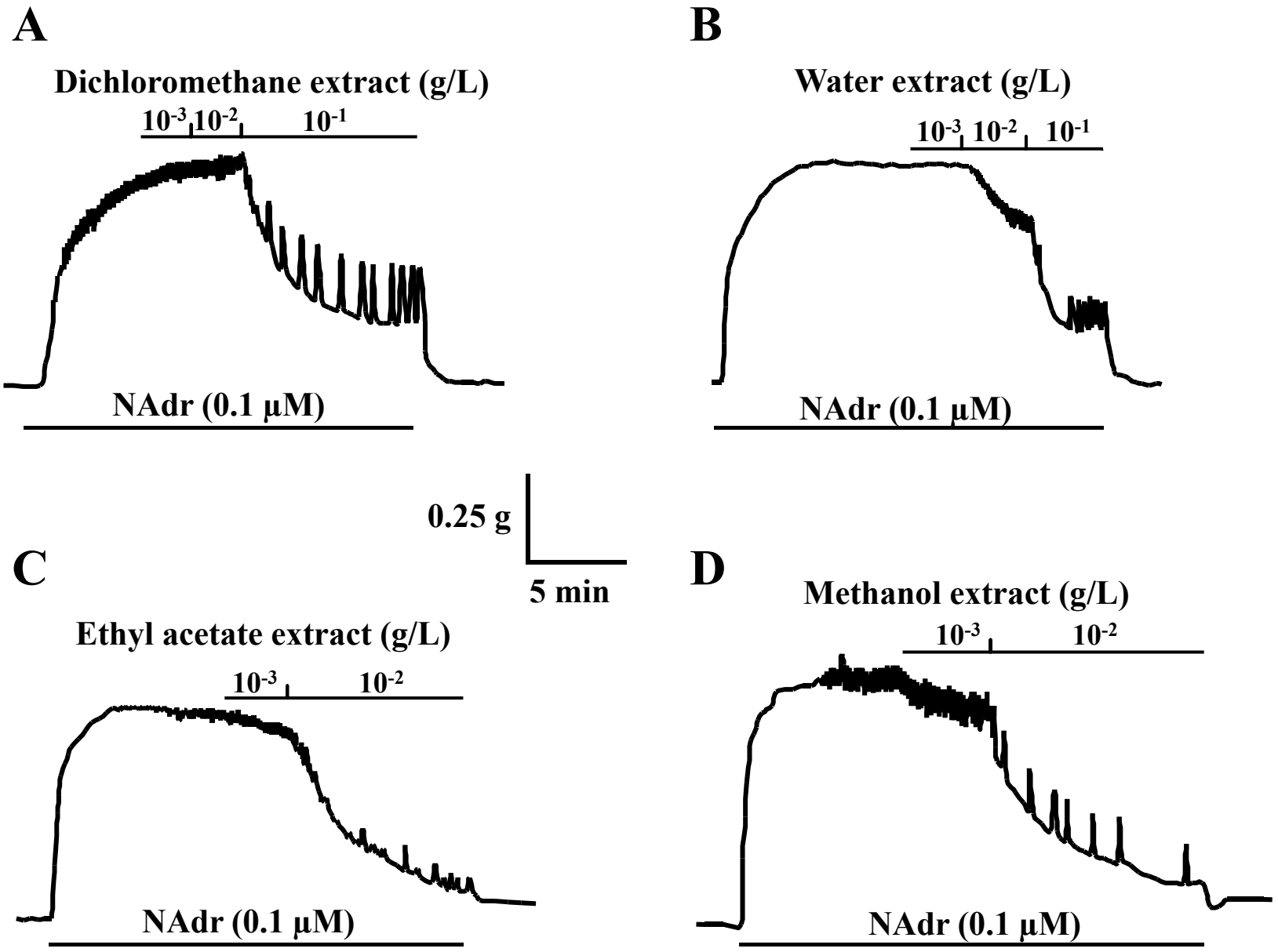
A
Dichloromethane extract (g/L)
10-3 10-2 10-1
NAdr (0.1 µM)
B
Water extract (g/L)
10-3 10-2 10-1
NAdr (0.1 µM)
0.25 g
5 min
C
Ethyl acetate extract (g/L)
10-3 10-2
NAdr (0.1 µM)
D
Methanol extract (g/L)
10-3 10-2
NAdr (0.1 µM)

Figure 2

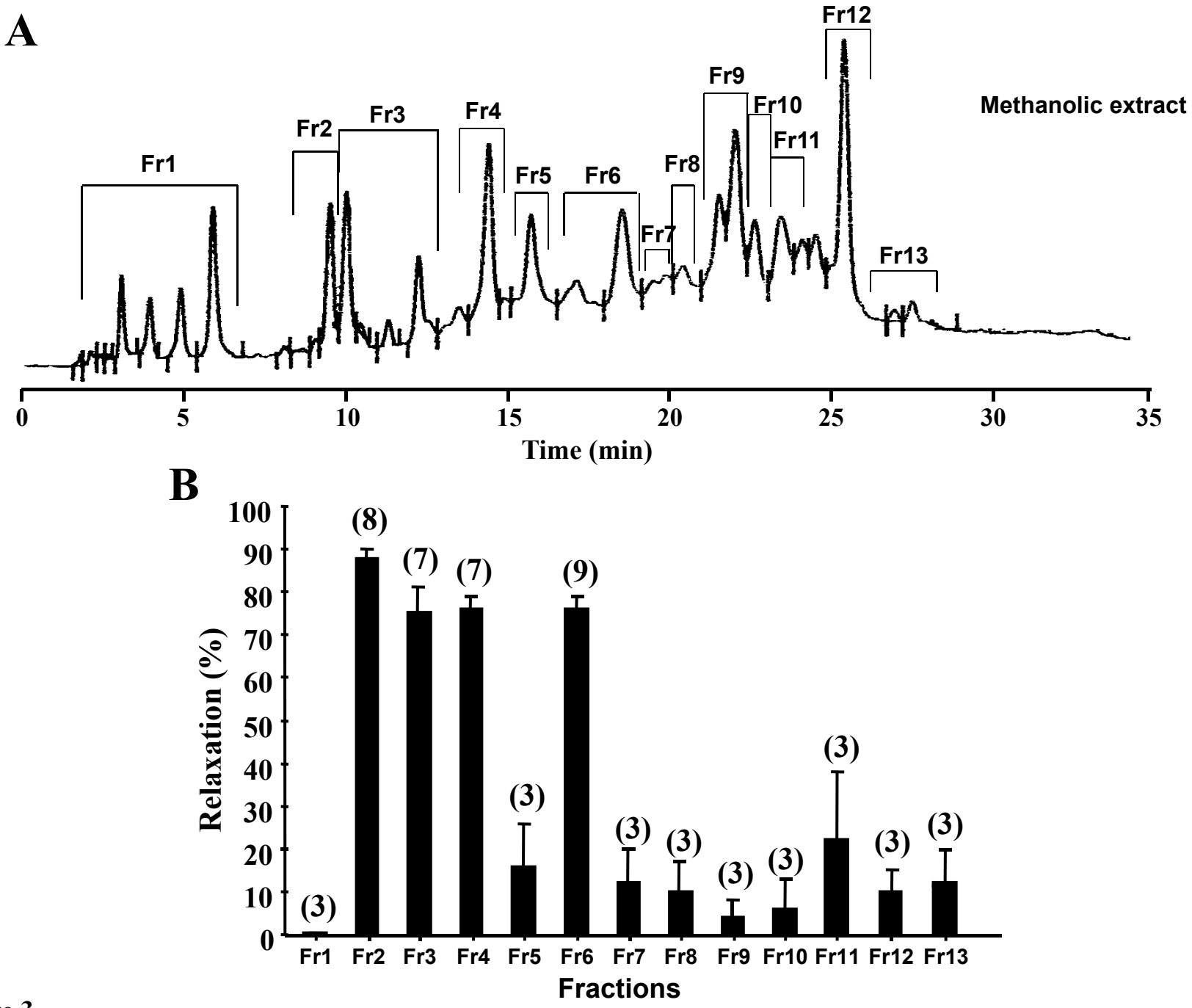
A
Fr1
Fr2
Fr3
Fr4
Fr5
Fr6
Fr7
Fr8
Fr9
Fr10
Fr11
Fr12
Fr13
Methanolic extract
0
5
10
15
20
25
30
35
Time (min)
B
Relaxation (%)
100
90
80
70
60
50
40
30
20
10
0
(8)
(7)
(7)
(9)
(3)
(3)
(3)
(3)
(3)
(3)
(3)
(3)
(3)
Fr1
Fr2
Fr3
Fr4
Fr5
Fr6
Fr7
Fr8
Fr9
Fr10
Fr11
Fr12
Fr13
Fractions

Figure 3

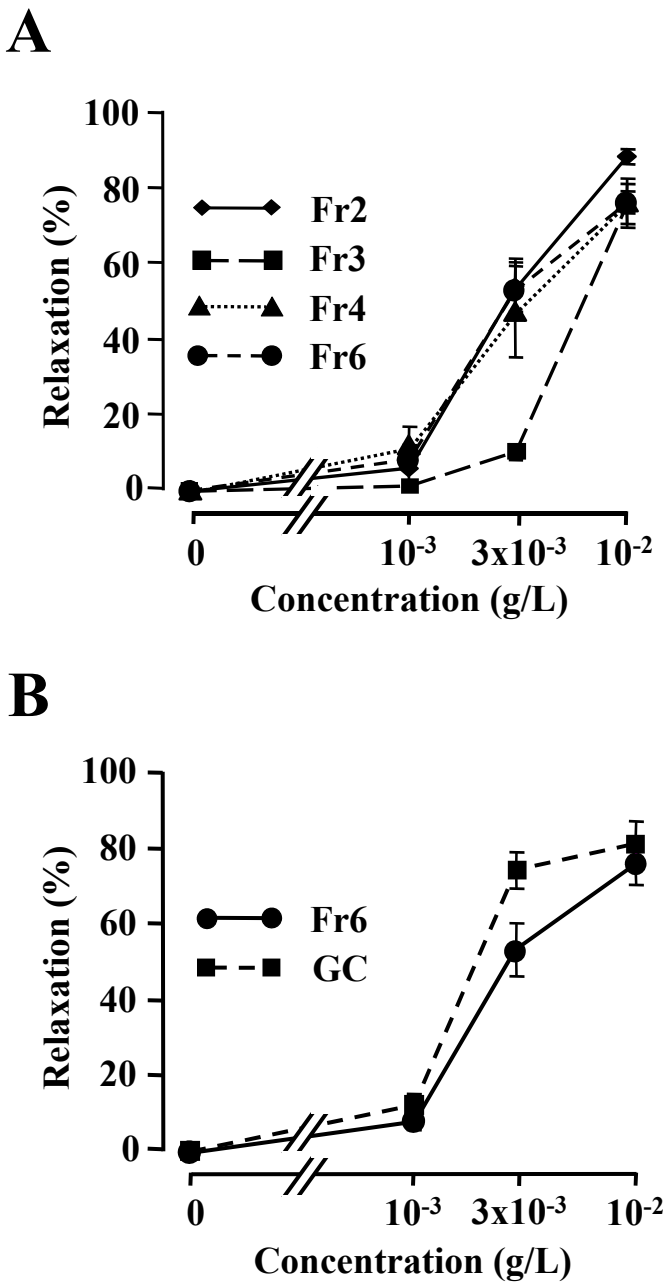
A
100
80
60
40
20
0
Relaxation (%)
Fr2
Fr3
Fr4
Fr6
0
10-3
3x10-3
10-2
Concentration (g/L)
B
100
80
60
40
20
0
Relaxation (%)
Fr6
GC
0
10-3
3x10-3
10-2
Concentration (g/L)

Figure 4