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PROPERTIES AND EFFECTS ON ISOLATED GUINEA-PIG ILEUM OF *ZYGOPHYLLUM GAETULUM* SPECIES ENDEMIC IN MOROCCAN SAHARA

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ABSTRACT

This paper examines the effects of extracts, chromatographic fractions, and pure compounds from Zygophyllum gaetulum *(Zygophyllaceae) on electrically-induced contractions of isolated guinea-pig ileum and morphine withdrawal* in vitro. *The results indicated that all the extracts, partially purified fractions from the MeOH extract and two pure triterpenene saponins, significantly and dose-dependently reduced both the electrical contractions of the ileum and morphine withdrawal. The two active compounds are triterpenene acid bisdesmosides with different sugar residues at C-3 and C-28 of the aglycones.*

INTRODUCTION

In the course of our research program on African medicinal plants, we investigated *Zygophyllum gaetulum* Emb. Maire (Zygophyllaceae) species endemic in Moroccan Sahara and used in the indigenous system of medicine as an antispasmodic, antieczema, hypoglycemic drug and as a remedy against stomach and liver pain with the vernacular name "aggaya" (Bellakhadar et al., 1991). In a previous paper we reported on the isolation and structural determination of three new triterpenic saponins, zygophylosides I (**1**), L (**2**), M (**3**) and three known quinovic acid glycosides **4–6** (Safir et al., 1997). There are no reports in the literature on the pharmacological properties of *Z. gaetulum* whereas a similar species, *Zygophyllum propinquum*, is known for its antihistaminic activity and to cause stimulation and then depression of isolated amphibian heart, relaxation of isolated intestine, and contraction of the uterus (Ahmad et al., 1990). Therefore, the present research was undertaken to evaluate a possible antispasmodic activity induced by the extracts, partially purified fractions, and pure compounds of *Zygophyllum gaetulum*, using electrically-induced contractions of guinea-pig ileum (E.C.I.), and their influence on morphine withdrawal *in vitro*.

Keywords: Zygophyllum gaetulum, Zygophyllaceae, triterpenene acid bisdesmosides, antispasmodic activity, morphine dependence *in vitro*.

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MATERIALS AND METHODS

Plant Material

Zygophyllum gaetulum Emb. Maire was collected in September 1992, near Zreouila, 27 km from Goulmine, South of Morocco. The plant was identified by Dr. A. Khaoudji of the Department de Biologie, Faculté des Sciences, Rabat, Maroc. A voucher specimen is deposited at the herbarium of Laboratoire de Chimie des Plantes et de Synthèse Organique et Bioorganique, Faculté des Sciences, Rabat, Maroc.

Extraction and Isolation

Air-dried and powdered aerial parts of *Z. gaetulum* (1 kg) were sequentially extracted at room temperature with $CH_3COOC_2H_5$, $CHCl_3$/MeOH (9:1), MeOH and

H_2O to afford 10.85, 7.03, 19.46 and 11.03 g of residue, respectively. Part of the MeOH residue (4 g) was chromatographed on a Sephadex LH-20 column (100 × 5 cm) eluting with MeOH. Fractions of 8 ml were collected and combined by TLC based on similarity (Si gel plates in *n*-BuOH-HOAc-H_2O [60:15:25] and $CHCl_3$-MeOH-H_2O [80:18:2]) to give nine main fractions A-I. Fractions B (161 mg), E (520 mg), and I (329 mg) exhibited greatest biological activity and were separated by RP HPLC (μ-Bondapak C-18 column, 30 cm × 7.8 mm id, flow rate 1.5 ml/min) with MeOH-H_2O (54:46) as the solvent system. This procedure gave pure compounds **1** (30 mg), **2** (12 mg), and **3** (9 mg) from fraction B, **3** (6 mg), **4** (15 mg), **5** (9 mg) and **6** (10 mg) from fractions E and I, which were identified by their NMR spectra as compared with literature data (Safir et al., 1997; Aquino et al., 1988; Ahmad et al., 1993; Pollmann et al., 1997). Saponin **1** was identified as 3β-*O*-[α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→2)-β-D-glucopyranosyl]-urs-20(21)-en-28-oic acid 28-*O*-[β-D-2–*O*-sulphonylglucopyranosyl) ester (zygophyloside I), saponin **2** as 3β-*O*-[α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→2)-β-D glucop yranosyl]-urs-20(21)-en-28-oic acid-28-*O*-[β-D-gluco pyranosyl) ester (zygophyloside L), saponin **3** as 3β-*O*-β-D-quinovopyranosyl-27-nor-oleanolic acid-28-*O*-β-D-glucopyranosyl ester (zygophyloside M) previously reported in *Z. gaetulum* (Safir et al., 1997). Glycoside **4** was identified as 3β-*O*-β-D-glucopyranosyl-quinovic acid-28-*O*-β-D-glucopyranosyl ester previously isolated for the first time from *Guettarda platypoda* (Aquino et al., 1988); glycoside **5** as 3β-*O*-[β-D-2–*O*-sulphonylglucopyranosyl]-quinovic acid-28-*O*-[β-D-glucopyranosyl) ester (zygophyloside G) and glycoside **6** as 3β-*O*-[β-D-2–*O*-sulphonylquinovopyranosyl]-quinovic acid-28-*O*-[β-D-glucopyranosyl) ester (zygophyloside E) previously isolated from *Z. propinquum* (Pollmann et al., 1997; Ahmad et al., 1993). Figure 1 shows glycosides **1**–**6** from *Zygophyllum gaetulum*.

Pharmacological Screening

Animals. Male Charles River guinea-pigs (180–200 g) were used for all the experiments. The animals were housed in colony cages (4 guinea-pigs each) under conditions of standard light (light on from 7.00 a.m. to 7.00 p.m.), temperature (22 ± 1°C) and room humidity (60 ± 10%) conditions for at least 1 week before the experimental sessions. Food and water were available *ad libitum*.

Transmurally Stimulated Guinea-pig Ileum Test

The animals were sacrificed with CO_2 and bled. Guinea-pig ileum was prepared as described previously (Capasso et al., 1996a).

Experimental procedure. The samples of the extracts [$CH_3COOC_2H_5$, $CHCl_3$/MeOH (9:1), MeOH and H_2O] were dissolved in dimethylsulfoxide (DMSO, Merck) whereas the partially purified fractions (A-I) and pure compounds **1–6** were dissolved in distilled water.

In preliminary experiments, the administration of DMSO up to 100 μl was added to the organ bath to determine the baseline contraction. The effects of the extracts, partially purified fractions and pure compounds on the electrically-induced contractions of guinea-pig ileum (E.C.I.) were investigated according to the following experimental schedule:

(a) $CH_3COOC_2H_5$, $CHCl_3$/MeOH (9:1), MeOH and H_2O extracts at concentrations of 400, 200, 100 μg/ml organ bath: 15 min contact period;
(b) Partially purified fractions A-I at concentrations of 200, 100, 50 μg/ml organ bath: 15 min contact period;
(c) Pure compounds [**1**–**6**] at concentrations of 1×10^{-4}, 5×10^{-5}, 1×10^{-5} M: 15 min contact period.

Morphine Withdrawal on Guinea-pig Ileum

The experimental procedure was that described previously (Capasso et al., 1996b). Figure 2 shows a typical tracing of morphine withdrawal *in vitro*.

Experimental procedure. The administration of the extracts, partially purified fractions and pure compounds **1–6** was performed according to the following schedule:

(a) three Ach responses
(b) electrical stimulation (10–20 min)
(c) morphine injection (10^{-5} M) in absence of electrical stimulation (4 min) and the addition of naloxone (10^{-5} M) with subsequent contraction (first opioid withdrawal)
(d) washout and Ach response
(e) electrical stimulation (30 min)
(f) extracts (100, 200, 400 μg/ml organ bath) or partially purified fractions (50, 100, 200 μg/ml) or pure compounds **1–6** (1×10^{-4}, 5×10^{-5}, 1×10^{-5} M) from *Z. gaetulum* without electrical stimulation injected 10 min before morphine followed by naloxone (second opioid withdrawal)
g) washout Ach response

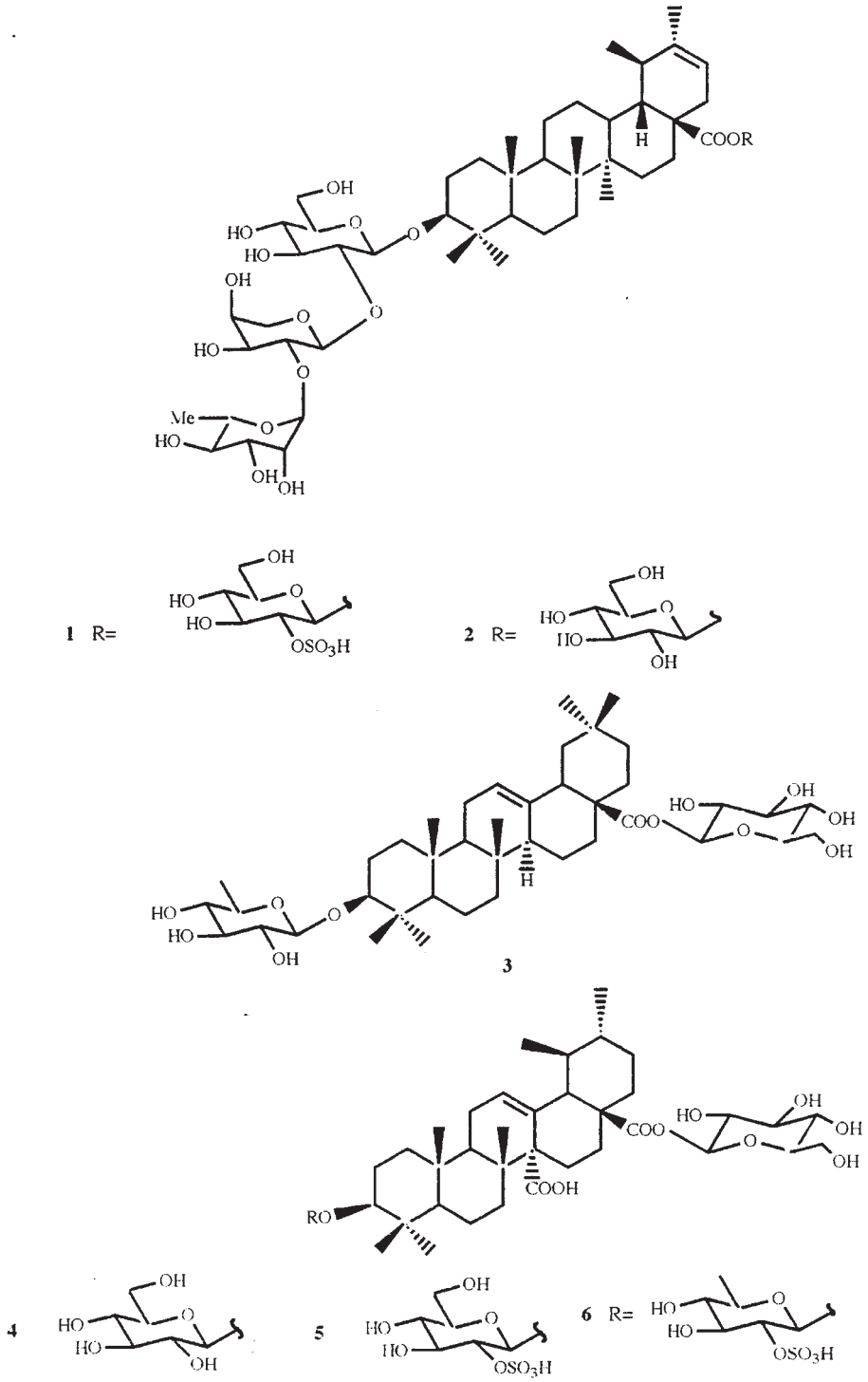

Fig. 1. Glycosides **1–6** from *Zygophyllum gaetulum*.

h) electrical stimulation (30 min)
i) final control opiate withdrawal (third opioid withdrawal)

Each experiment was performed on at least 6 to 9 isolated preparations from different animals.

Drugs. Naloxone HCl was purchased from Sigma Chemical Co (St. Louis, U.S.A.) and morphine HCl from Carlo Erba (Milan, Italy).

Parameter evaluation. Four parameters were evaluated as previously described by Capasso et al. (1996b).

Statistical Analysis

Regression methods were used for statistical analysis and critical significance was set at $P < 0.05$ for the transmurally stimulated guinea-pig ileum test. The results of morphine withdrawal in guinea-pig ileum were tested for statistical significance using the Student's *t*-test for paired data when results before and after treatments on the same preparation were compared. An IC_{50} was calculated according to the method reported by Tallarida and Murray (1987).

RESULTS

Effect of the Extracts, Partially Purified Fractions A-I, and Pure compounds 1–6 on Electrically-induced Contractions

$CH_3COOC_2H_5$, $CHCl_3$/MeOH (9:1), MeOH and H_2O extracts produced dose-dependent reduction in electrically-induced contractions. The IC_{50} values were 151 μg (C.L. (confidence limits) = 123–184), 145 μg (C.L. = 112–179), 129.7 μg (C.L.=110–142), and 159.4 μg

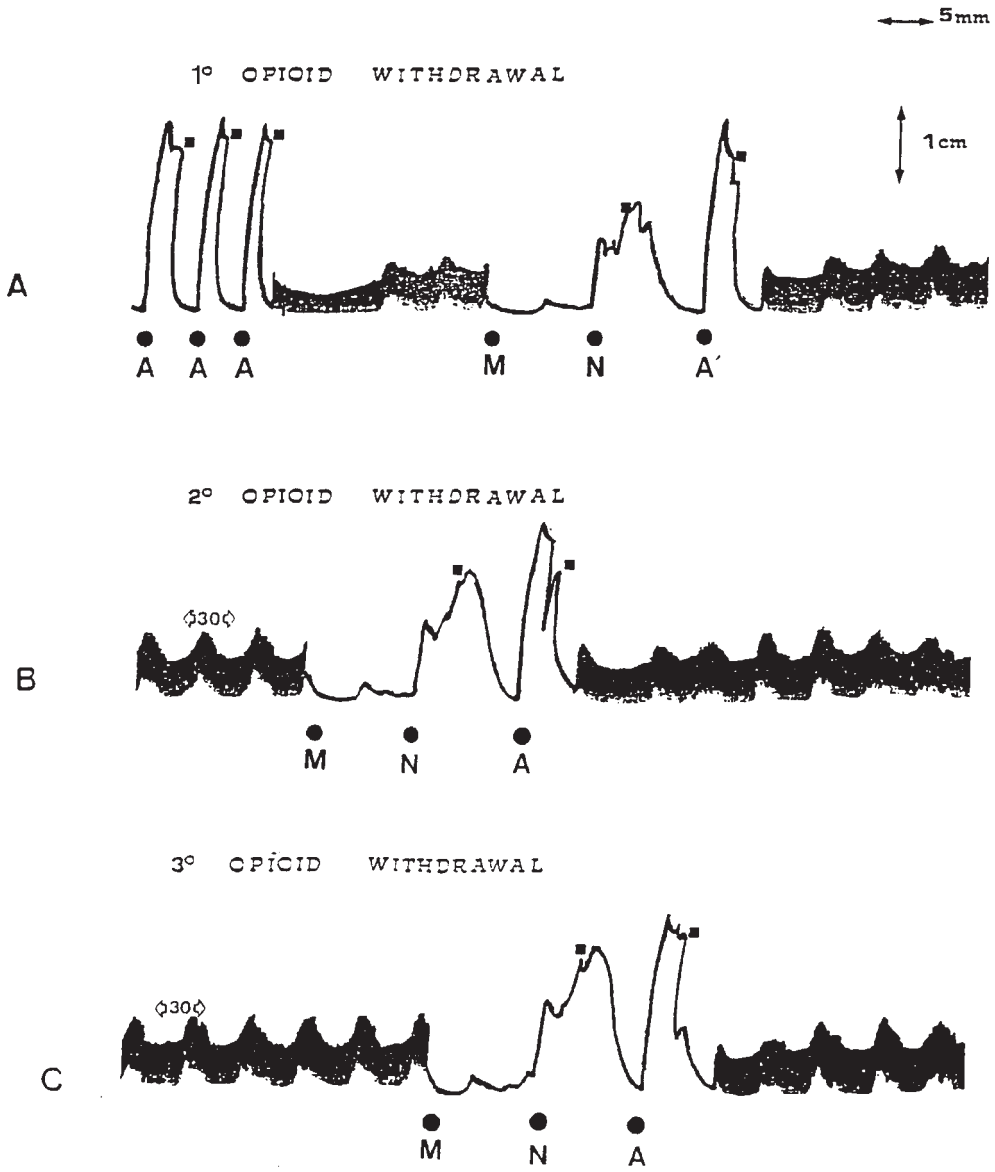

Fig. 2. Typical tracing of morphine withdrawal on guinea-pig ileum. (A) Three similar acetylcholine responses (A), electrical stimulation, injection of the opioid agonist (M.) followed after 4 min contact period by naloxone (N) which induces contraction (first opioid withdrawal). After washout (■), another A response was performed. (B) After a 30 min resting time under electrical stimulation, a further 4 min exposure of the ileum to M. and naloxone elicited a reproducible response (second opioid withdrawal). (C) After another 30 min resting period under electrical stimulation, the ileum responded again to M. and naloxone with the same intensity (third opioid withdrawal).

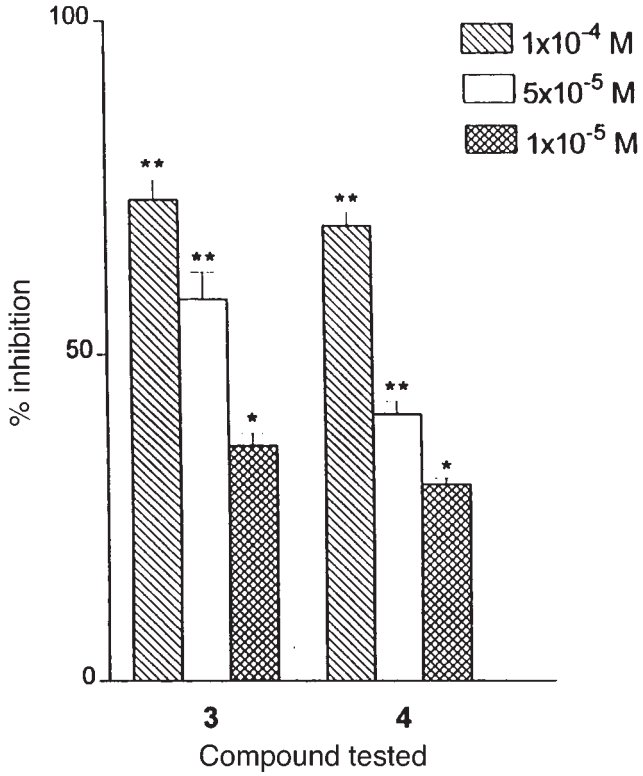

Fig. 3. Dose-related inhibition of pure compounds **3** and **4** (10^{-4}, 5×10^{-5}, and 1×10^{-5} M) from *Z. gaetulum* on the electrically-induced contractions of guinea-pig ileum. Results are expressed as mean ± s.e.m. $*P < 0.05$ ($n = 6$).

(C.L.=143–194), respectively. The MeOH fraction was further purified by Sephadex LH-20 column chromatography and nine main fractions were collected. Only the partially purified fractions B, E, and I were able to reduce contractions significantly. The IC_{50} for fraction B was 75 μg (C.L. = 57–89); that for fraction E was 81 μg (C.L. = 65–94); and that for fraction I was 79 μg (C.L. = 68–110). Figure 3 shows that only pure compounds **3** and **4** from the above fractions, exert appreciable inhibitory activity, whereas saponins **1**, **2**, **5**, and **6** (data not shown) were inactive at 1×10^{-4}, 5×10^{-5}, 1×10^{-5} M. In all of the above experiments, inhibition appears 2–4 min after administration, it was enhanced with time, and lasted for the entire recording period (15 min).

Effects of Extracts, Partially Purified Fractions and Pure Compounds on Morphine Withdrawal

Both extracts (Fig. 4a), fractions B, E, and I (Fig. 4b) and pure compounds **3** and **4** (Fig. 4c) administered 10 min before the injection of morphine were able to reduce dose-dependently morphine withdrawal. After washout, Ach response, electrical stimulation and the final opiate withdrawal were still reduced. As during treatment, the treatment period of morphine was 10 min, when compared to the pre-drug period. To avoid a possible influence of the treatment period, we performed a series of experiments to verify whether a treatment period longer than 4 min may effect naloxone contraction. No differences were observed when the treatment period was 4 or 10 min (data not shown).

DISCUSSION

Although *Z. gaetulum* is empirically used in Moroccan folk medicine, there are no data in the literature as to the possible pharmacological effects of the plant. The results of the present study indicate that extracts, some partially purified fractions, and pure glycosides from *Z. gaetulum* are able to reduce dose-dependently the electrically-stimulated contractions of isolated guinea-pig ileum segments and morphine dependence *in vitro*. Between the tested extracts, the MeOH extract was the most active in inhibiting the contractions and in reducing morphine withdrawal. Only fractions B, E, and I, obtained via Sephadex LH-20 column separation of the MeOH extract, were active in both tests, showing the

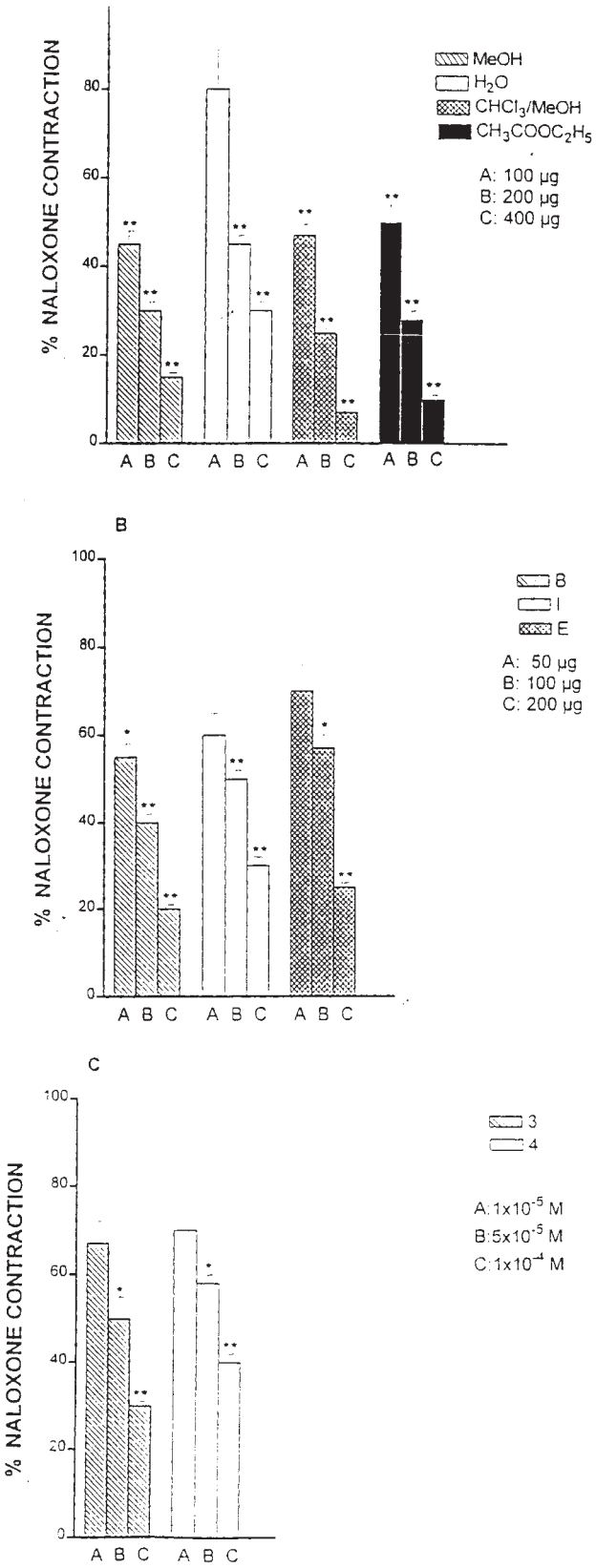

Fig. 4. The effects of the $CH_3COOC_2H_5$, $CHCl_3$/MeOH, MeOH and H_2O extracts (100, 200, and 400 mg/ml), partially purified fractions B, E, and I (50, 100, and 200 mg/ml) and pure compounds **3** and **4** (10^{-4}, 5×10^{-5}, and 1×10^{-5} M) from *Z. gaetulum* on morphine withdrawal. The drugs were injected 10 min before the opioid agonist. $^{**}P < 0.01$.

same relative order of potency. In comparison to the whole extract, the fractions were more potent, indicating that they should contain a mixture of the active principles. In order to identify the molecules responsible for the activity and which compounds induce the observed activities, the fractions were separated by HPLC to give compounds **1–6**.

Only compounds **3** and **4** were able to reduce both electrically-stimulated contractions and morphine withdrawal *in vitro* with the same order of potency. These data seem to validate the traditional uses of *Z. gaetulum* as an antispasmodic remedy (Bellakhadar et al., 1991). The active compounds isolated from *Z. gaetulum* are triterpenene glycosides of the ursane or oleane series. The saponins with an ursane or oleane skeleton possess a wide spectrum of biological activities ranging from cytotoxic, antitumor, antileukemic and antiviral effects, to hypolipidemic, antiatherosclerotic, antinflammatory, analgesic and antipyretic activities (Mahato & Nandy, 1991; Mahato et al., 1992). Previously, it was reported that some triterpenenes like alisol B and its monoacetate isolated from *Alisma orientale* inhibit contractions in rat isolated ileum induced by bradykinin, acetylcholine and 5-isoleucine-angiotensin. Also, a glycyrrhetinic acid derivative was able to suppress the acetylcholine-induced contraction of isolated colonic smooth muscle of guinea-pigs with a LD_{50} of 584 mg/kg (orally) (Mahato et al., 1992).

Our results confirm the antispasmodic activity of triterpenene saponins and show that it is influenced to a great extent by their molecular structure. All the tested compounds **1–6** (Fig. 1) are bisdesmosidic triterpenene glycosides possessing a 3-β-hydroxy-urs-28-oic acid (compounds **1**, **2**, **4**, **5**, **6**) or 27-nor-3-β-hydroxy-olean-12-en-28-oic acid (compound **3**) as the aglycone moiety, and sugar residues comprised of one (compounds **3–6**) or three (compounds **1–2**) units linked at C-3 as well as one monosaccharide bonded at C-28 (glucopyranose or 2-*O*-sulphonyl-glucopyranose) of the aglycones. It is interesting to note that the nature and the number of the sugar units linked at C-3 seem to modulate the activity of triterpenene saponins. In fact among the six glycosides tested, the active compounds **3** and **4** possessed only a sugar residue (quinovopyranose in **3** or glucopyranose in **4**) at C-3. Three sugar units at C-3 (as in compounds **1** and **2**) or the presence of a $-SO_3H$ group on the sugar unit at C-3 (as in compound **5** and **6**) of the triterpenene aglycones substantially reduces the activity. Further studies are necessary to verify this hypothesis.

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