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EFFECT OF DIFFERENT TYPES OF SESQUITERPENE LACTONES ON THE MATURATION OF *Rhinella arenarum* OOCYTES

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Running Title**: Effect of sesquiterpene lactones on anphibian oocytes maturation**

Keywords: **Oocyte maturation; Sesquiterpene lactones; Anphibian; meiosis**

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Abstract

Sesquiterpene lactones (STLs) constitute a great class of plant secondary metabolites generally found in Asteraceae family, with high diversity with respect to chemical structure as well as biological activity. STLs have been classified into different groups such as guaianolides, germacranolides, melampolides, etc, according to their carboxylic skeleton.

In amphibians, fully grown ovarian oocytes are arrested at the beginning of meiosis I. Under stimulus of progesterone, this meiotic arrest is released and meiosis progresses to metaphase II, a process known as oocyte maturation.

The purpose of this work was to determine whether sesquiterpene lactones from the germacranolide and melampolide groups might act as inhibitor agents on the meiosis of amphibian oocytes *in vitro*.

Results indicated that the germacranolides: deoxyelephantopins caused high percentages of inhibition, minimolide showed a moderate inhibitory effect and glaucolide A is inactive.

Results with melampolides (uvedalin, enhydrin, polymatin A, polymatin B) show inhibitory effects. In the case of enhydrin and uvedalin, the results showed inhibitory effect at the higher concentrations assayed.

The results of this study suggest that the inhibitory activity on meiosis of *Rhinella arenarum* oocytes of sesquiterpene lactones tested, does not depend on which group they belong, ie the carboxylic skeleton, but probably to the arrangement and type of function groups present in the molecules.

All the lactones in the germacranolide group assayed showed low toxicity. In contrast, in the lactones of the melampolide group, important differences in toxicity were observed. While enhydrin and uvedalin showed low toxicity, polymatin A and B are highly toxic.

INTRODUCTION

The sesquiterpenic lactones (STLs) are a stable subfamily of terpenoids, a class of plant secondary metabolites of lipophilic character. They are almost exclusively derived from Asteraceae. STLs are 15-carbon (15-C) compounds consisting of three isoprene (5-C) units and a lactone group (cyclic ester). They can be categorized, relative to their carboxylic skeleton, into different groups: guaianolides, germacranolides, melampolides, etc.

Several biological activities of sesquiterpene lactones have been reported, including anti-tumor (Lee et al., 1977; Zhang, et al., 2005; Ghantous et al., 2010), anti-inflammation (Recio et al., 2000) and gastric cytoprotector effects (Giordano *et al*., 1992; Penissi *et al.*, 1998).

It has been previously shown that a sesquiterpenic lactone of guaianolide group, the Dehydroleucodine (DhL) isolated and purified from the aerial parts of *Artemisia douglasiana* Besser, selectively induces a dose-dependent transient arrest in G2 of both meristematic cells (López *et al.*, 2002) and vascular smooth muscle cells (Cruzado *et al.*, 2005). Treatment with DhL of *Rhinella arenarum* fully grown oocytes arrested at G2, at the beginning of meiosis I, induces an inhibition of spontaneous and progesterone induced maturation in a dose-dependent manner (Sánchez Toranzo *et al.*, 2007).

In amphibians, fully grown ovarian oocytes are arrested at the beginning of meiosis I, in the G2 / M of the cell cycle. Under hormonal stimulus, this meiotic arrest is released and meiosis progresses to metaphase II, a process termed oocyte maturation (Fortune *et al.,* 1975; Schuetz, 1985)**.** Meiotic maturation, which represents the transition from G2 to the M phase of the cell cycle, is induced by progesterone (Zelarayán *et al.,* 1996).

In amphibian oocytes meiosis, the transition from G2 to M phase is regulated by the maturation promoting factor (MPF), a complex of the cyclin dependent kinase p34/cdc2 and cyclin B. (Lohka *et al*., 1988; Masui, 1992). In immature oocytes there is an inactive complex (pre-MPF), where cdc2 is phosphorylated on both Thr-161 and Thr-14/Tyr-15 residues. The dephosphorylation of Thr-14/Tyr-15 is necessary for the start of MPF activation and it is induced by the activation of Cdc25 phosphatase (Perdiguero and Nebreda, 2004; Dekel, 2005),

In *Rhinella arenarum* fully grown oocytes arrested in G2 / M, the treatment with DhL or the hydrogenated derivative of DhL, 11,13-dihydro-dehydroleucodine (2H-DhL), in which the alpha-methylenelactone function was inactivated, induced an

inhibition of progesterone-induced maturation in a dose-dependent manner (Sánchez Toranzo *et al.*, 2007). These results suggest that the inhibitory effect on meiosis progression of DhL does not depend only on the activity of the alpha-methylenelactone function, since its hydrogenated derivative, 2H-DhL, in which this function has been inactivated, causes similar effects on amphibian oocytes (Sánchez Toranzo *et al.*, 2009). In this sense, Schmidt et al.,(2006) has reported that the alpha-methylene-gamma-butyrolactone ring is not the only active group, but other groups such as the epoxide, aldheyde, alpha-beta-unsaturated carbonyl group, etc, also have reactivity.

The purpose of this work was to evaluate whether sesquiterpene lactones from the germacranolide and melampolide groups might act as G2 / M inhibitor agents on the meiosis of amphibian oocytes *in vitro.*

MATERIALS AND METHODS

Animals

Adult specimens of *Rhinella arenarum* were collected in the northwestern area of Argentina and kept at 15 °C until use.

Hormones and reagents

Plant Material: the sesquiterpene lactones were isolated from different plants of the Asteraceae family.

- Uvedalin, Enhydryn and Polimatin B were isolated from *Smallanthus sonchifollius* (Poepp. & Endl) H Robinson, according to Mercado et al., (2010).
- Minimolide from *Mikania minima* (Baker) BL Rob according to Cuenca et al., (1990) and Bach *et al*., (2011)
- Deohyelephantopin (and 2-epideoxyelephantopin) from *Gochnatia palosanto* (Cabrera) according to Ybarra *et al*. (1990)
- Glaucolide A from *Vernonia squamulosa* (Hook. et Arn) according to Catalán *et al*., (1986)
- Polymatin A from *Smallanthus macroscyphus* (Baker) A Grau according to Pedro *et al*. (2003).

The purity of all lactones was 98.0% as determined by HPLC analysis.

The aerial parts of Asteraceae specimens are deposited in the Herbarium of the Miguel Lillo Institute of the Universidad Nacional de Tucumán, Tucumán, Argentina.

STLs were dissolved in DMSO and various doses were added to the culture medium. Progesterone, purchased from Sigma Chemicals, was dissolved in ethanol and added directly to the culture medium to give a final concentration of 2.5 μM.

***In vitro* culture of denuded oocytes**

Experimental manipulation and culture were performed at room temperature (22-25 °C) in amphibian Ringer solution (AR) (6.6 g NaCl/l, 0.15 g $CaCl_2$/l and 0.15 g KCl/l) containing penicillin G-sodium (30 mg/l) and streptomycin sulphate (50 mg/l), pH 7.4.

Denuded fully grown oocytes were obtained according to Lin and Schuetz (1985). Follicle cells were removed by gentle shaking (100 oscillations/minute) (Zelarayán *et al*., 1995).

Randomized samples of 20 oocytes were distributed into separate wells containing 2 ml of AR; the reagents (5 μl) were added directly to the culture medium. Oocyte maturation was assessed 24h after hormone or reagent addition. Meiosis reinitiation was scored both by the presence of a transient white spot in the animal pole and by the absence of germinal vesicle (GVBD) after dissection of the oocytes fixed in trichloroacetic acid.

Toxicity

Impaired cell viability was measured using the reversibility assay based on the ability of viable cells to reinitiate meiosis. Briefly, oocytes were exposed for 1h to STLs (24 μM), washed twice in AR and then incubated with progesterone (2.5 μM) in AR for at least 24 h at 25 ºC.

Statistical analysis

Results are expressed as means ± SEM. Comparisons among different treatments were carried out using Student's test. A value of $P<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of lactones from the germacranolide group (deoxyelephantopins-mixture of analogs of deoxyelephantopin and 2-epi-deoxyelephantopin, glaucolide A, and minimolide).

To determine whether some lactones of the germacranolide group affect oocyte maturation, fully grown denuded oocytes of *Rhinella arenarum* were cultured in AR for 60 min with different doses of deoxyelephantopins, glaucolide A or minimolide (6, 12 or 24 μM respectively) before the addition of progesterone 2.5 μM. Oocyte maturation was evaluated by GVBD after 24h of culture**.**

The results indicated that, in our experimental conditions, deoxyelephantopins caused high percentages of GVBD inhibition (97%, 90% and 97% with doses of 6, 12 and 24 μM respectively) (Fig.1a).

Minimolide is an *exo*-methylene-γ-lactone with a -OH group in C-14 and acetyloxy groups on C-8 and C-14 that showed a moderate inhibitory effect (23%, 26% and 44%) at the concentration assayed (6, 12 and 24μM respectively)(Fig 1b).

Glaucolide A, which has the lactone double bond in position 7, 11 (within the γ-lactone ring), was inactive (Fig. 1c). This suggests that in order to observe activity the presence of the *exo*-methylene-γ-lactone group is necessary.

The reversibility assay showed that treatment with germacranolides at the assayed dose (24 μM) did no affect the viability of the oocytes, suggesting that these lactones are not toxic in our experimental conditions.

Is interesting to note that in nasopharynx carcinoma cells Kupchan et al., (1971) established that it is the exo- but not the endocyclic α methylene-γ-lactone that causes cytotoxicity. However, other studies showed that in some lactones as helenalin analogs, an endocyclic α,β-unsaturated ketone might cause more cytotoxicity than the exocyclic α-methylene-γ-lactone (Lee et al.,1971 and 1977).

Effect of lactones from the melampolide group (uvedalin, ehydrin, polymatin A, polymatin B)

Rhinella arenarum denuded oocytes were cultured in AR for 60 min with different doses (6,12, 24 μM) of uvedalin, enhydrin, polymatin A or polymatin B before the addition of progesterone (2.5 μM). GVBD was scored after 24h of culture.

Results indicated that uvedalin effectively inhibited meiosis resumption in a dose-dependent manner (74%, 85% and 92%) at concentrations of 6, 12, and 24μM respectively (Fig.2a).

Oocytes treated with polymatin A clearly showed a potent inhibition of the cell cycle (G2 / M arrest) independently of the dose (97%, 96% and 99%). Polymatin B showed a potent inhibitory effect similar to that of uvedalin (82%, 81% and 94%) at 6, 12 and 24 μM respectively (Fig.2b).

The reversibility assay indicated that polymatin A and B lactones are toxic because they cause about 40-50% death in the treated oocytes. This suggests that the inhibitory effect observed with these lactones is related to their high toxicity.

In the case of enhydrin (Fig.2c) and uvedalin, the results showed inhibitory effect at the higher concentrations assayed (89% with 12μM and 98% with 24μM). However, in the reversibility assay, enhydrin showed low toxicity with 7% of dead oocytes.

It is important to point out that the inhibitory effect of Polymatin A and B is counteracted by their toxic effects, while uvedalin and enhydrin present scarce toxicity, according to the reversibility assays performed.

In summary, our results indicate an inhibitory effect on meiosis progression of lactones from the melampolide and germacranolide groups. In this sense, they were able to inhibit progesterone induced maturation of *Rhinella* arenarum oocytes by blocking the G2 / M of the cellular meiotic cycle. In this inhibitory effect, does not depend on which group they belong, ie the carbon skeleton, but probably to the arrangement and type of group's functionality present in the molecules.With respect to toxicity, all the lactones in the germacranolide group assayed showed low toxicity. In contrast, in the lactones of the melampolide group, important differences in toxicity were observed. While enhidryn and uvedalin showed low toxicity, polymatin A and B are highly toxic.

ACKNOWLEDGMENTS

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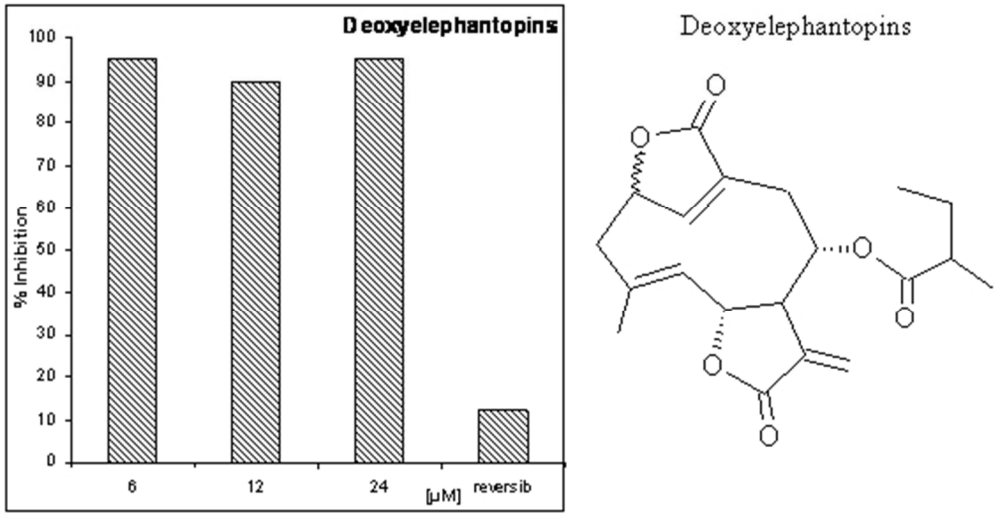

Fig. 1a. Effect of Deoxielephantopins inhibitions on progesterone-induced maturation. Oocytes incompetent to mature spontaneously were preincubated in AR with Deoxielephantopins (6 – 24 µM) 60 min before progesterone addition (2.5 µM). The respective reversibility column is added to the graphic: for this test of reversibility, the oocytes were incubated with the lactone for 60 min and then were washed three times in AR. The GVBD was scored after 24 h of incubation. Values are the mean ± SEM of four experiments. Each experiment was performed on a different Animal
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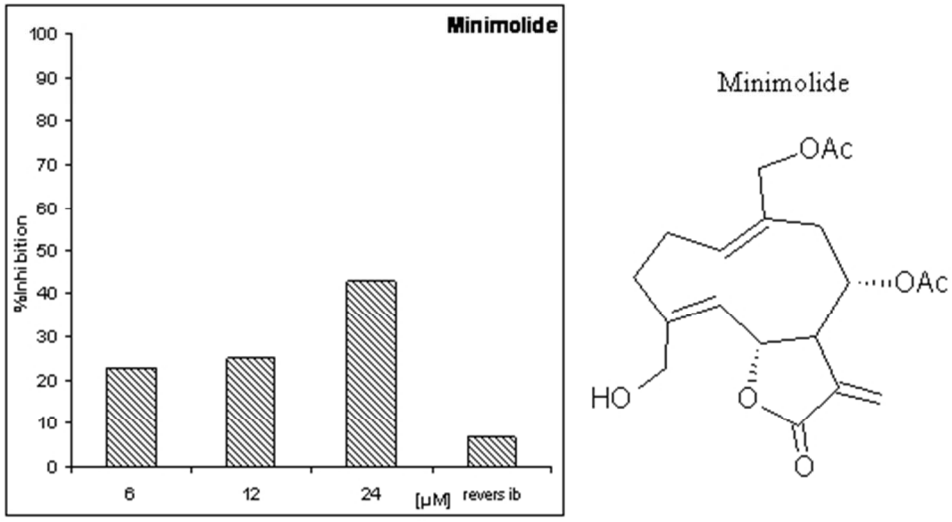

Fig 1b. Effect of Minimolide inhibition on progesterone induced maturation. Oocytes incompetent to mature spontaneously were cultured in AR with different doses of Minimolide (6 - 24 µM) 60 min before progesterone addition (2.5 µM). The respective reversibility column is added to the graphic The GVBD was scored after 24 h of incubation. Values are the mean ± SEM of four experiments. Each experiment was performed on a different animal.
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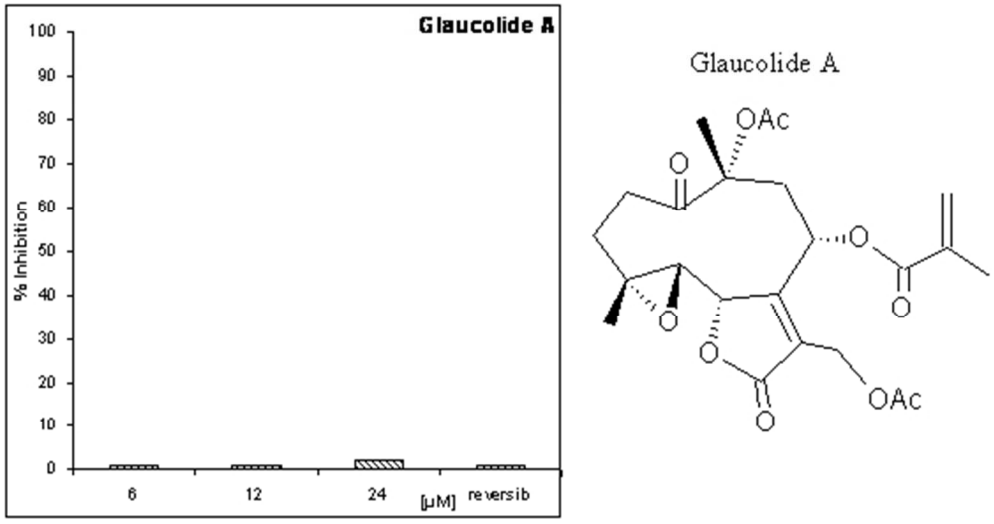


Fig 1c. Effect of Glaucolide A on progesterone induced oocyte maturation. Denuded oocytes incompetent to mature spontaneously were cultured in AR with different doses of Glaucolide A (6 – 24 µM) 60 min before the addition of progesterone (2.5µM).). The respective reversibility column is added to the graphic. The GVBD was scored after 24 h of incubation. Values are the mean ± SEM of five experiments. Each experiment was performed on a different animal.
150x79mm (96 x 96 DPI)

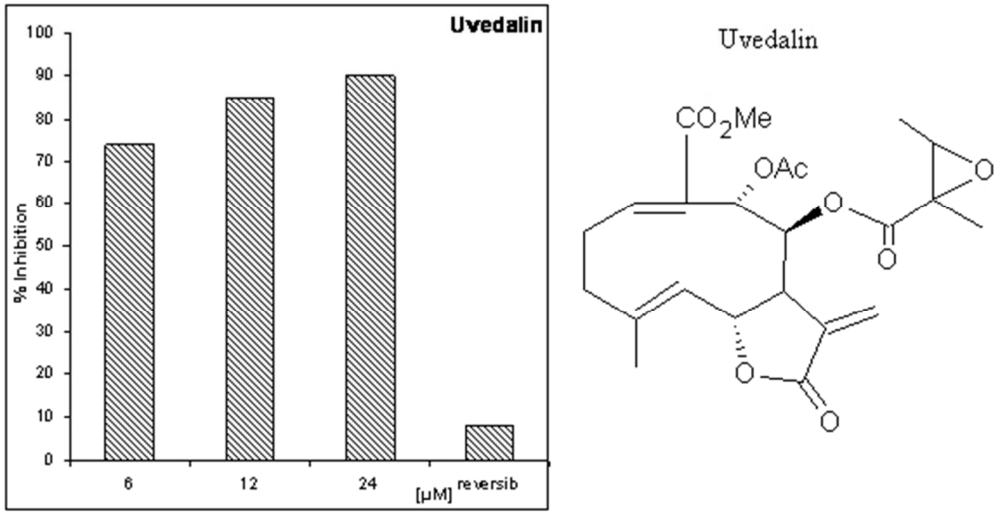
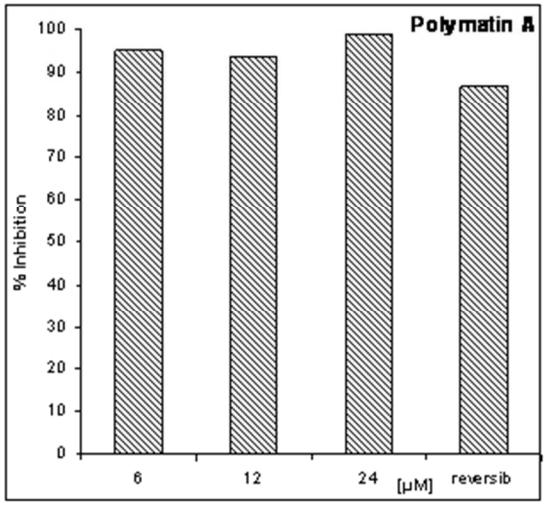
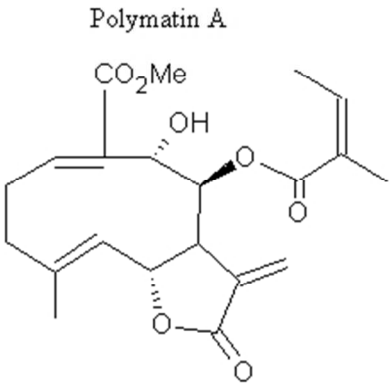
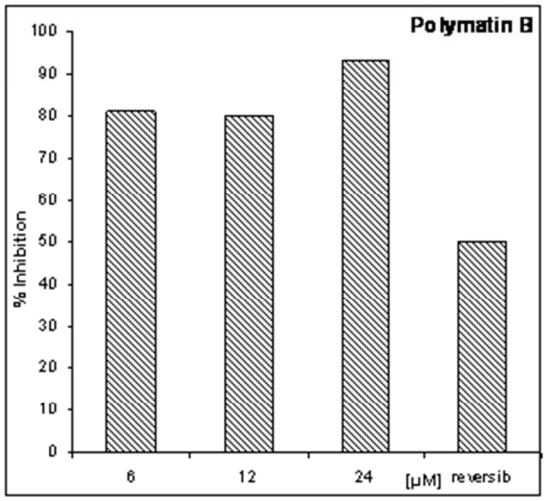


Fig. 2a. Effect of Uvedalin inhibitions on progesterone-induced maturation. Oocytes incompetent to mature spontaneously were preincubated in AR with Uvedalin (6 – 24 µM) 60 min before progesterone addition (2.5 µM). The respective reversibility column is added to the graphic: for this test of reversibility, the oocytes were incubated with the lactone for 60 min and then were washed three times in AR. The GVBD was scored after 24 h of incubation. Values are the mean ± SEM of four experiments. Each experiment was performed on a different Animal
152x79mm (96 x 96 DPI)







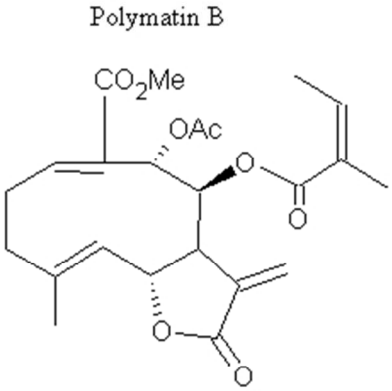


Fig 2b. Effect of Polymatin A and Polymatin B inhibition on progesterone induced maturation. Oocytes incompetent to mature spontaneously were cultured in AR with different doses of Polymatin A and Polymatin B (6 - 24 µM) 60 min before progesterone addition (2.5 µM). The respective reversibility column is added to the graphic The GVBD was scored after 24 h of incubation. Values are the mean ± SEM of four experiments. Each experiment was performed on a different animal
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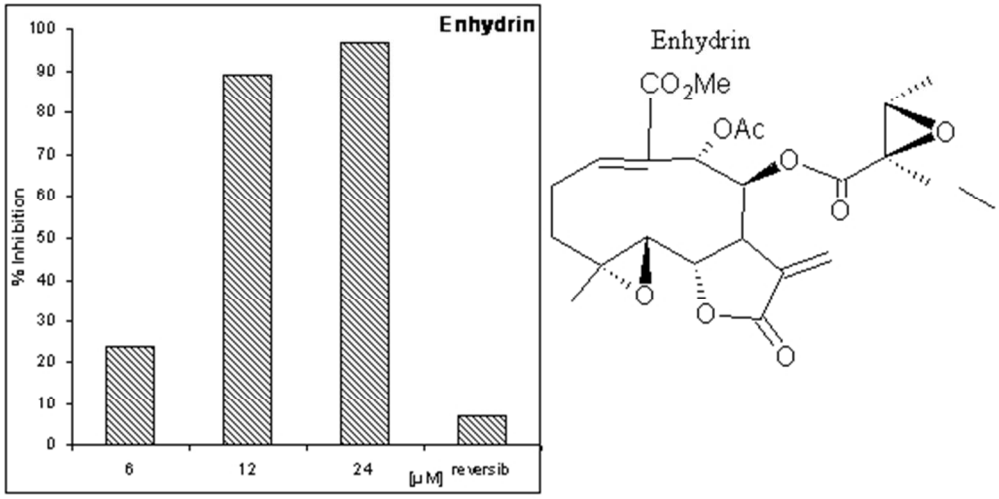

Fig 2c. Effect of Enhydrin on progesterone induced oocyte maturation. Denuded oocytes incompetent to mature spontaneously were cultured in AR with different doses of Enhydrin (6 – 24 µM) 60 min before the addition of progesterone (2.5µM).). The respective reversibility column is added to the graphic. The GVBD was scored after 24 h of incubation. Values are the mean ± SEM of four experiments. Each experiment was performed on a different animal.
155x78mm (96 x 96 DPI)