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EFFECTS OF MEGESTROL ACETATE ON GROWTH AND ENDOCRINE DISRUPTING OF ROTIFER *BRACHIONUS CALYCIFLORUS*

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SUMMARY

In aquatic ecosystems, invertebrates are the basis of the food chain and play a growing role in assessing the impact of environmental pollutants. The substances that affect organisms at concentrations lower than the normal levels of toxicity are endocrine disruptors. Endocrine disorders are a phenomenon that alters function(s) of the endocrine system and causes harmful effects on the health of the organism. Rotifer is the group of model aquatic organism with the ecological significance in the freshwater ecosystems, they are capable of constitute a high proportion of the biomass and has the widely distributed, reproduce quickly with the short time, short life cycle, small size and sensitive to toxic substances. The aim of this study was to evaluate the effect of megestrol acetate on the endocrine disorders of *Brachionus calyciflorus* rotifer. The results showed that megestrol acetate was endocrine disruptors, when the rotifers were exposed to these substances at a concentration of 0.05, 0.1, 0.5 and 1 $mg.l^{-1}$, the cysts growth was recorded and reached the highest value at concentration of 0.1 $mg.l^{-1}$. The researching results of Rotifer's genomic study on the impact mechanism of megestrol acetate and cyproterone acetate demonstrated that the endocrine system of Rotifer can be interrupted by environmental pollutant substances.

Keywords: *Brachionus calyciflorus, endocrine disorders, growth, invertebrates*

INTRODUCTION

According to the definition of the World Health Organization (WHO) and the European Commission, an endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations (Dănulescu *et al.*, 2011; Bruni *et al.*, 2002; Parent *et al.*, 2011; Robu *et al.*, 2007). In Vietnam, there is almost no study on endocrine disrupting chemicals (EDCs). Institute for Environment and Resources of the Vietnam National University Ho Chi Minh City initially studied the effects of these compounds. However, the research process is just beginning and there are no concrete results. Besides, the Law on Protection of the Environment in Vietnam also has no term for EDCs. The invertebrates are a main group that are very important in ecosystems, especially aquatic ecosystems. Invertebrates play an increasing role in assessing the impacts of environmental contaminants. In aquatic ecosystems, planktonic organisms play a major role as they constitute a high proportion of the biomass and are at the basis of pelagic food chains.

Until now, the phenomena of endocrine disruption in this group are still poorly understood, partly because the endocrine system of invertebrates has not been documented comprehensively (Highnam, Hill, 1977). In addition, many aquatic and terrestrial invertebrates have complicated life histories, display various forms of hermaphroditism and poorly resolved sexual dimorphism in some cases (Highnam, Hill, 1977). Almost of invertebrate taxa makes use of hormones to control biochemical, physiological and behavioural processes as well as

development, growth, and reproduction. Invertebrates use steroids, terpenoids, and peptide hormones, but the latter are by far the most common among these phyla (Gorbman, Davey, 1991; Lafont, 2000). As a function of hormone agonists, EDCs inhibit the activity of natural hormones by binding to estrogen or androgen receptors. Studies on the effects of EDCs have received a number of findings such as: estradiol-17b (E2) or ethinylestradiol which cause abnormalities in sexual development (Honer *et al.*, 2003). Lehmann *et al.*, 1998); Biutide tributyltin (TBT) causes a number of malformations in aquatic mammals, mollusks (Bryan, Gibbs, 1991; Matthiessen, Gibbs, 1998). The first adverse effects of TBT on mollusks were observed in *Crassostrea gigas* at the Bay of Arcachon and showedthat TBT was the causative agent with trace concentrations as low as 10 ng TBT/L in ambient water being effective (Bryan, Gibbs, 1991). Some substances induce effects on organisms at concentrations lower than those of the conventional toxicity (Effective Dose - ED50, lowest Observed Effect Concentration - LOEC) is considered the endocrine disruptors. Recently, pesticides and other compounds such as alkylphenols, phthalates, ethinylestradiol… also have been exhibited the same type of effect in vertebrates and ethinylestradiol may increasing of feminization in fishs which is living near effluent treatment plants in Great Britain (Tyler,Jobling, 2008).

Rotifers are ecologically important in freshwater ecosystems because they convert primary production with remarkable efficiency and have very short reproduction time; consequently they are widely distributed, very abundant and sometimes dominate freshwater plankton (Snell, Janssen, 1991). Rotifers are microscopic aquatic animals of the phylum Rotifera. It is a standard animal in 24 hour acute and 48 hour asexual reproduction tests (ASTM, 1998; APHA, 1998). Phylum Rotifera is divided into three classes including: *Monogononta, Bdelloidea* and *Seisonidea*. The largest group is the *Monogononta*, with about 1500 species, followed by the *Bdelloidea*, with about 350 species (Barnes *et al*., 2001). There are only two known species of *Seisonidea* (Aisha*et al*., 2008). Otherwise, most planktonic rotifers reproduce via cyclical parthenogenesis, which includes both asexual and sexual phases and the sexual reproduction in *Brachionus calyciflorus* (*B. calyciflorus*) is more sensitive to androgens than estrogens (Snell, Joaquim-Justo, 2007). Although little is known about rotifer endocrine systems, endocrine signals are likely to be important regulators of rotifer biology, development and life history of this species showed that their sexual reproduction is regulated by a system as in the case of most organisms (Gallardo *et al*., 1997, 1999, 2000). Therefore, the study of the effects of pollutants and endocrine disruptors on invertebrates and rotifers is necessary. The aim of this study to determine whether megestrol acetate is acting via an interference with a steroid receptor. PCRs with degenerate primers were carried out to determine whether *B. calyciflorus* has a genomic sequence that could correspond to an androgen or a progesterone receptor.

MATERIALS AND METHODS

Rotifer *B. calyciflorus*

All experiments carried out in the framework of this study were conducted on the *Monogononta* rotifer, *B. calyciflorus* at Liege University (Belgium). The *B. Calyciflorus* (Pallas) strain used was originally collected in 1983 in Gainesville, Florida by Professor T. W. Snell of Georgia Institute of Technology (GA, USA) (Snell *et al.*, 1991).

Test animals consisted of cyst hatchlings which were females 4 - 6 hours old, before each experiment, cysts were kept at -20^{o}C and set to hatch in continuous light at 25^{o}C. After 18 to 24 hours, a density of 3.10^{6} algae/ml *Nannochloris atomus* (CCAP 251/6 strain obtained from the Culture Collection of Algae and Protozo (Scotland, UK)) was added into experimental medium. The experimental and culture medium is Volvic water (Maule P, 2007). *N. atomus* was cultured in a Bold nutrient medium (BBM) with continuous illumination at 25^{o}C (Brown, 1964; Nichols, 1965).

Preparation of megestrol acetate

Megestrol acetate was obtained from Sigma-Aldrich (Germany). Stock solutions were prepared by dissolving test compounds in acetone. Stock solutions of both compounds were further diluted in test media so that final acetone concentration never exceeded 0.4%. Dilution water consisted of mineral water (Volvic).

Effects of megestrol acetate on demographic parameters of *B. calyciflorus*

Effects of megestrol acetate on asexual and

sexual reproduction of rotifers were assessed following a protocol based on Preston *et al* (2000). Neonates were incubated in test medium containing 3.0 x 10^6 cells.mL^{-1} of the green algae *Nannochloris oculata*. Six rotifers were placed in 16×150 mm glass test tubes containing 12 ml of acetone as control and contaminated medium (0.4% V:V). All tubes were then incubated at 25°C for 96 hours in darkness on a wheel rotating at 10-15 rph to reduce the sedimentation of algae but without disturbing rotifers. Ten replicates were performed for each concentration of megestrol acetate and for acetone controls. An acetone concentration of 0.4% has no effect on either asexual or sexual reproduction as demonstrated in preliminary tests. Four concentrations were tested 0.05, 0.1 0.5 and 1 mg.l^{-1} for megestrol acetate.

After 48, 72 and 96 hours, test tubes were emptied into a petri dish and the number of rotifers in each individual tube were counted including non-ovigerous females, amictic ovigerous females, mictic ovigerous females, fertilized females, fertilized eggs on female, detached fertilized eggs and males. Ovigerous females were classified according to the shape of their eggs which differ between female, male and resting eggs (Wallace, Snell, 1991). At 48 hours, rotifers were transferred to freshly prepared test media. On the basis of these observations, we can calculate the intrinsic rate of growth, the mixis rate and the number of resting eggs produced per females of rotifer.

- The intrinsic rate of growth

$$r = \frac{\ln N_t - \ln N_0}{T}$$

Where $\ln N_t$ = natural logarithm of the number of female rotifers in test tube after 96 hours, $\ln N_0$ = natural logarithm of initial number of female rotifers in each tube N_0 = 6, and T = 4 days. Tests were considered invalid if *r* at 96 hours was lower than 0.7 (Preston *et al* (2000; Wallace, Snell, 1991). The parameter *r* is representative of asexual reproduction as it is directly related to the increase in female numbers that occurs exclusively via asexual reproduction. A significant effect of test compounds on *r* was considered to be indicative of general toxicity and not endocrine disruption.

Genomic study of potential androgen and progesterone receptors in *B. calyciflorus*

PCRs with degenerate primers were run to determine whether *B. calyciflorus* has genomic sequences that could correspond to an androgen and progesterone receptor. The first step consisted of design of degenerate primers. For that purpose, the amino-acid sequences of the ligand binding domain (LBD) of the androgen receptor in the vertebrates *Homo sapiens, Heterocephalus glaber, Polypterus senegalus, Pagrus major, Gambusia affinis* were compared and aligned to seek conserved regions in these sequences, after that one of the conserved sequences were reverse-translated in all the possible nucleotide sequences. These sequences are degenerated by inserting altered codons at appropriate sites. The primers patterns obtained were as follows:

Degenerate primer prog1:

(prog1 Forward: 5'- CTA AAT GAG AAA ATG ATC TGC CAA ARA TGH SNA A - 3'; pdgprog 1 Reverse: 5'- GGT CTC AGC AGA TTT CCA ACA ATA KSR TAY TTN TC -3')

Degenerate primer progman1:

(progman1 Forward: 5'- CCG ACG GTT CTT TRA YGA YGT - 3'; pdg progman1 Reverse: 5'- ACA GTG GGC TCY TCN CCN GG -3')

Degerate primer progman2:

(progman2 Forward: 5'- TCA TCTC CGG TCA ATA TTT TTC TAG TAT HTG YTA YTT -3'; pdgprogman 2 Reverse: 5'- CCT TAG CAA ATT CAA GTG GRT AYT TYT CYT T -3')

Primer ProgSnell:

(ProgSnellForward: 5'- CGA CGG TTC TTT GAC GAT GT -3'; ProgSnell Reverse: 5'- TTC GGC TGA CTC TTC TTC GT -3')

These sequences were sent to Integrated DNA techniques Inc (Leuven, Belgium) for production of the primers to be used in the degenerated PCRs. DNA extraction was performed on 1000 live rotifers according to QIAamp DNA Micro Kit. The amplified DNA strands were subjected to an electrophoresis on agarose gel in order to separate DNA fragments according to their length and to estimate their size. Promega ladders were used to estimate the size of the fragments isolated and the rotifer mitochondrial gene CO1 was used as positive control. After extraction we run PCR according to following Table 1 such as:

Table 1. The heating mechanism in PCR technique

Temperature (°C)	Time
94	4 min
94	45 sec
52	45 sec
72	45 sec
72	10 min
4	Pause
	Total time: 16 min 25 sec

Statistical analysis

Results for experiments were compared to solvent controls and the data were calculated as mean ± SD (standard deviation, one-way ANOVA) by Dunnett's multiple comparison when appropriate. Results were considered significantly different for $p < 0.05$.

All tests were performed using the program SigmaStat 3.5.

RESULTS AND DISCUSSION

Effects of megestrol acetate on demographic parameters of B. calyciflorus

According to the results of Cruciani *et al* (2009) showed that cyproterone acetate, a synthetic anti-androgen which is also a strong progestagen, increased the production of resting eggs per female at concentrations between 0.05 and 0.5 $mg.l^{-1}$. Megestrol acetate is a progestagen that shared a similar structure at the end of the molecule with cyproterone acetate (Figure 1). This is why it is interesting to test effects of megestrol acetate on demographic parameters in *B. calyciflorus*. The range of concentrations tested was from 0.05 to 1 $mg.l^{-1}$.

Table 2. Impact of megestrol acetate on population intrinsic rate of growth of rotifer (r)

Concentration of megestrol acetate	Time (hours)		
	48	**72**	**96**
Solvent control	0.749 ± 0.142	0.686 ± 0.106	0.732 ± 0.051
0.05 mg/L	0.643 ± 0.11	0.59 ± 0.069	0.72 ± 0.051
0.1 mg/L	0.795 ± 0.104	0.601 ± 0.102	0.75 ± 0.037
1 mg/L	0.782 ± 0.063	0.667 ± 0.065	0.766 ± 0.059

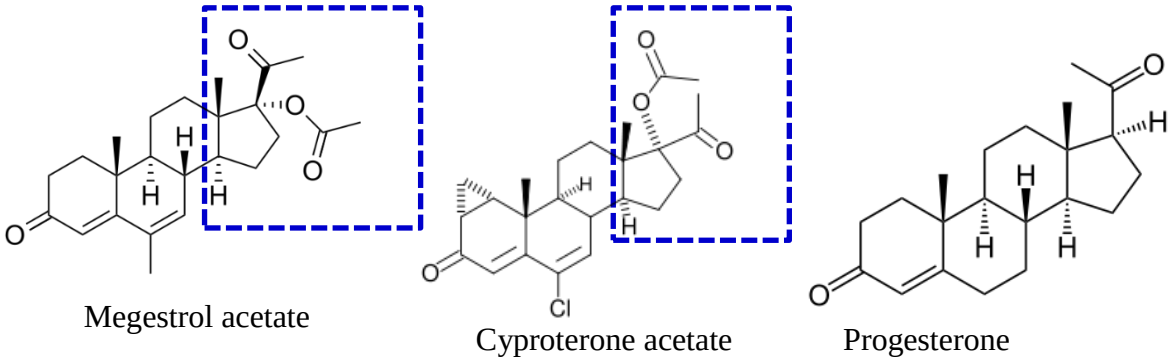


Figure 1. Structure of three progestative molecules. Dotted squares: acetate end.

Table 2 showed that megestrol acetate has no impact on the intrinsic growth rate (r) at the concentrations tested, indicating that this molecule has no effect on the asexual reproduction of rotifers until at least 1 $mg.l^{-1}$. At all concentrations, the r recorded in this experiment at 96 hours are higher than the threshold of 0.7 considered the value below which the reproduction is disrupted (Preston *et al.*, 2000). At 72 and 96 hours, we observed no statistical difference in mixis rate at all concentrations

compared to solvent control (Figure 2). Likewise, fertilization rate was not impacted by this molecule (Figure 3).

In contrast, Figure 4 showed that the production of cysts was significantly affected by the tested compound. Indeed, the values recorded at concentrations between 0.05 and 0.5 mg.l^{-1} are higher than control ones, however the difference is only statistically significant at 0.1 mg.l^{-1} ($p < 0.05$) due of high standard deviation. From these results, it could be seen that the inverted U-shaped dose-response relationship is one of the typical factors that disrupted endocrine (Jobling *et al.,* 2004). The similar observation has also been reported for a natural steroidal progestogen in vertebrates that induced an increase of the production of cysts in Brachionus manjavacas at concentrations of 5 - 10 mg.l^{-1} (Snell, Desrosiers, 2008). Stout et al (2010) showed that progesterone in rotifers, is a membrane associated protein bound receptor. However, the similar result of progesterone on *B. calyciflorus* was not recorded by other authors (Snell, Desrosiers, 2008; Stout *et al.,* 2010; Cruciani, 2009). It is thus possible that this species may contain receptors with a distinct structure than the rest of the rotifer species.

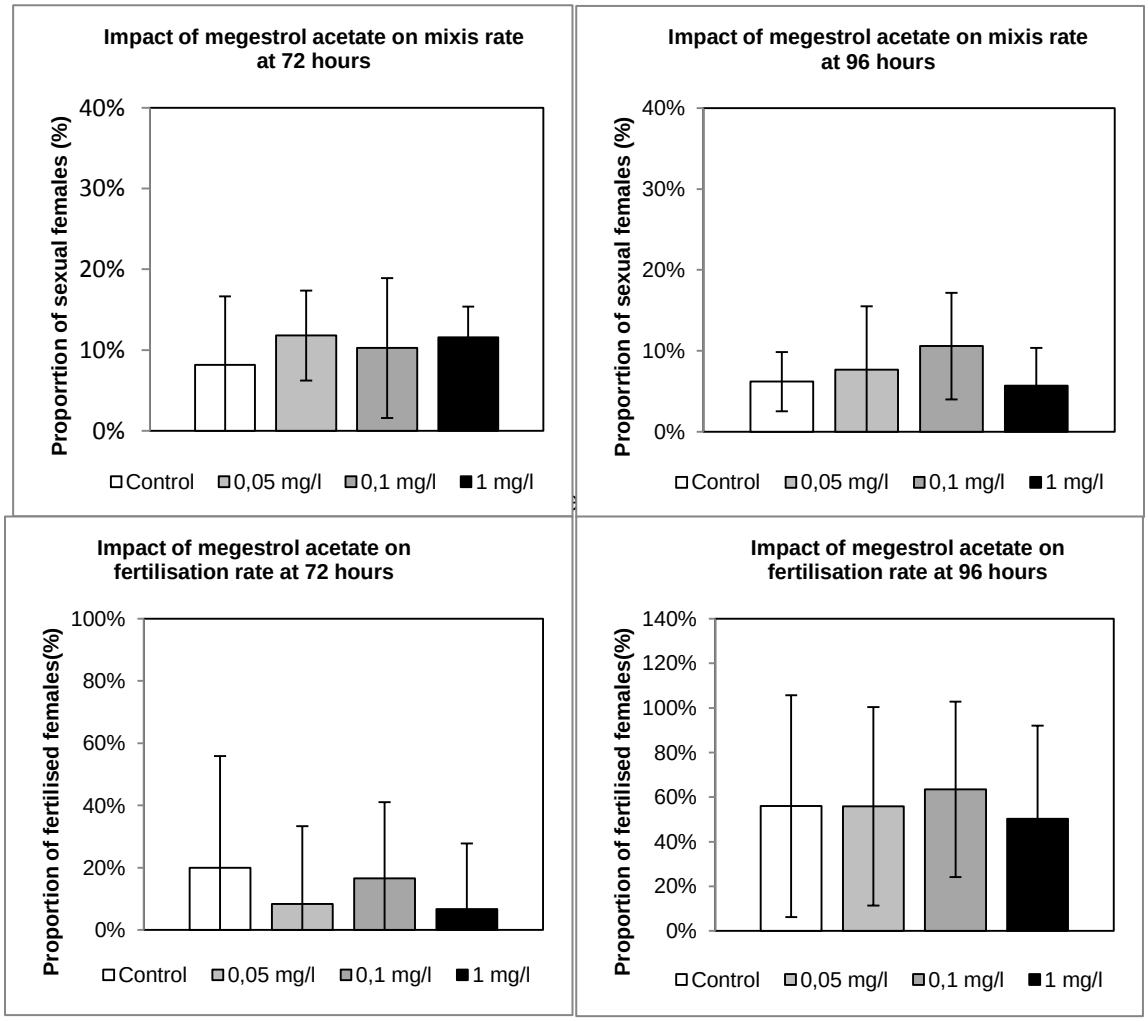


Figure 3. Impact of megestrol acetate (mg/l) on fertilization rate at 72 and 96 hours.

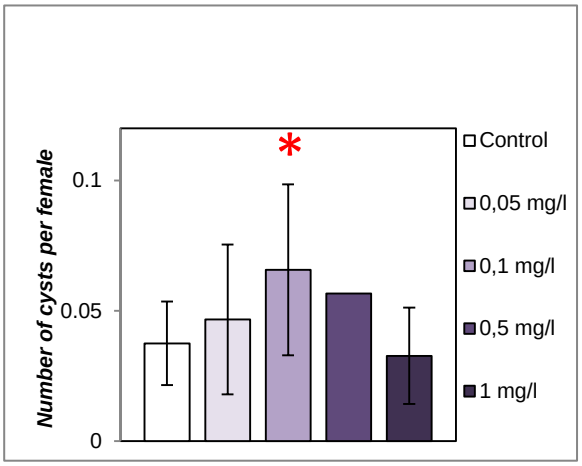


Figure 4. Impact of megestrol acetate on total number of cysts per total females at 96 hours (*: significantly different from the solvent controls, $p < 0.05$)

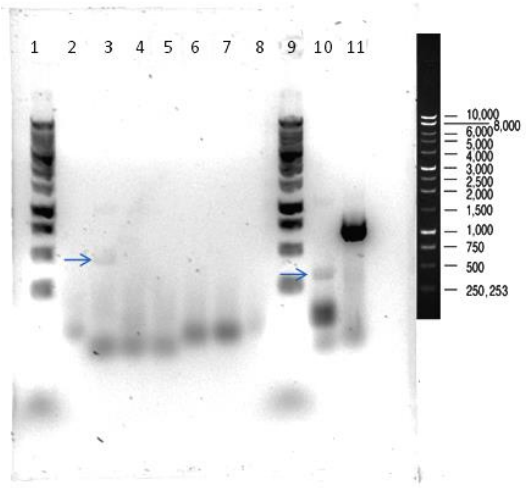
Genomic study of potential androgen and progesterone receptors in B. calyciflorus

Figure 5 showed that no DNA fragment was detected for both degenerated primers andro1 and andro2 *B. calyciflorus*. Promega ladder electrophoresis results of isolated gene segments and control samples (CO1) showed that the display ranges corresponding to the DNA fragments were detected and no bands were recorded on the electrophoresis columns 2, 6, 7 and 8. This indicates that no degenerate primer has been used to attach to the DNA of rotifers and no sequence in the genome of *B. calyciflorus* displays enough similarity to the sequence of the LBD of androgen and progesterone nuclear receptors of vertebrates for the degenerate primers to attach to the DNA extracts from the rotifers.

In contrast, columns 3 and 10 display bands of about 500 pairs of bases (pb), sizes that match the expected fragments corresponding to the attachment of these degenerate primers. These observations can regard as direct evidence to support the hypothesis that MAPR (membrance associated progesterone receptor) exists in *B. calyciflorus*. This new finding opens an explanation for the mechanisms of megestrol acetate action and agreement with the reported of Stout *et al.*, (2010). According to Stout *et al.*, (2010) progesterone binds to the MAPR in *B. manjavacas* has increased the production of resting eggs. However, effect of progesterone in *B. calyciflorus* was not found. This result supports the hypothesis that in this species, the MAPR is capable of binding to mo lecules with the same structure at the sites of acetate of megestrol acetate, therefore, when the progesterone is present, the resting eggs is increased (similar *B. manjavacas*).

The effects of progesterone on rotifers have been studied and reported by Snell and DesRosiers (2008). When *B. Manjavacas* females were exposed to progesterone with concentration up to 10 $mg.l^{-1}$ (32 µM) in the artificial seawater 15 ppt, the asexual reproduction was not affected but the production of resting eggs increased significantly. However, at 14 $mg.l^{-1}$ the asexual reproduction became strongly affected. Preston *et al.*, (2000) showed that exposure to nonylphenol (estrogen agonist), testosterone (androgen agonist), and flutamide (androgen antagonist), all had significant effects on reproduction in *B. calyciflorus*. In contrast, 17a-ethinylestradiol and nonylphenol, two estrogenic contaminants common in surface waters, had little effect on *B. calyciflorus* sexual reproduction until concentrations reached close to asexual no observed effect concentration (NOEC) values (Radix *et al.*, 2002).

This observation was also supported by Maule (2007), the results proved that the anti-androgens fenitrothion and cyproterone acetate had altered the number of resting eggs producing per *B. calyciflorus* female at concentrations as low as 50 $µg.l^{-1}$ and this value is lower 20-40 times than the asexual NOEC values for these chemicals. *B.calyciflorus* exposed to fenitrothion at concentrations of 0.1, 1,10 and 100 $µg.l^{-1}$ had longer reproductive periods and produced more offspring than controls (Linlan *et al.*, 2010). Exposure to concentration up to 1000 $µg.l^{-1}$ significantly reduced the number of resting eggs. Low concentrations of fenitrothion caused an increase in the duration of the juvenile and reproductive period, and lifetime reproduction of *B. Calyciflorus* (Linlan *et al.*, 2010).



1. Ladder Promega
2. Degenerate primer prog1: (pdg prog1 Forward : 5'- CTA AAT GAG AAA ATG ATC TGC CAA ARA TGH SNA A – 3' ; pdgprog 1 Reverse : 5'- GGT CTC AGC AGA TTT CCA ACA ATA KSR TAY TTN TC -3')
3. Degenerate primer progman1 (pdg progman1 Forward : 5'- CCG ACG GTT CTT TRA YGA YGT – 3' ; pdg progman1 Reverse : 5'- ACA GTG GGC TCY TCN CCN GG -3')
4. Error
5. Error
6 . Degenerate primer andro1
(pdgandro 1 Forward : 5'-GAA CGA CAA CTG GTT CAT GTT GTN AAR TGG GC – 3' ; pdgandro 1 Reverse : 5'-TTT AGC CAT TCC AGC CAG AAT YTT NGG NAC -3')
7. Degenerate primer andro2
(pdgandro 2Forward : 5' TGA ACA TCG AAT GCA TAT ATC TAG TAT GTA YFV NCA-3' ; pdgandro 2Reverso : 5'-ACA GAA TCC AGC AGT TGT GTC ARY TGR TAR AA-3')
8. Degerate primer progman2 (pdgprogman2 Forward : 5'- TCA TCTC CGG TCA ATA TTT TTC TAG TAT HTG YTA YTT -3' ; pdgprogman 2 Reverse : 5'- CCT TAG CAA ATT CAA GTG GRT AYT TYT CYT T -3')
9. Ladder Promega
10. Primer ProgSnell (ProgSnell Forward : 5'- CGA CGG TTC TTT GAC GAT GT -3' ; ProgSnell Reverse : 5'- TTC GGC TGA

Figure 5. Electrophoresis on agarose gel after PCR as part of the research for possible receptors in *B. calyciflorus*

CONCLUSION

The study results showed that megestrol acetate, a steroidal antiandrogenic progestagen, induces a statistically significant increase in the production of cysts at 0.1 mg.l^{-1}. The inverted U-shape dose-response relationship indicates that this molecule is an endocrine disruptor. Genomic study of progesterone receptor (MAPR) in *B. calyciflorus* showed that this receptor had increased of production of resting eggs. Result study also have supplied information of rotifer *B. calyciflorus* to conduct other studies about invertebrates in ecology and ecotoxicology.

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ẢNH HƯỞNG CỦA MEGESTROL ACETATE ĐẾN SINH TRƯỞNG VÀ RỐI LOẠN NỘI TIẾT CỦA ROTIFER *BRACHIONUS CALYCIFLORUS*

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TÓM TẮT

Trong các hệ sinh thái thủy sinh, động vật không xương sống là cơ sở của chuỗi thức ăn và đóng một vai trò ngày càng tăng trong việc đánh giá tác động của các chất gây ô nhiễm môi trường. Những chất gây ra tác

động lên các sinh vật ở nồng độ thấp hơn so với mức độ độc tính thông thường là những chất phá vỡ nội tiết. Rối loạn nội tiết là hiện tượng làm thay đổi chức năng của hệ nội tiết và gây ra những ảnh hưởng bất lợi cho sức khỏe của sinh vật. Rotifers là nhóm sinh vật thủy sinh mô hình có ý nghĩa sinh thái trong các hệ sinh thái nước ngọt, chúng có khả năng tạo ra một lượng sinh khối lớn và có thời gian sinh sản nhanh, vòng đời ngắn, kích thước nhỏ, phân bố rộng rãi và nhạy cảm với các chất độc. Mục đích của nghiên cứu này là đánh giá tích ảnh hưởng của megestrol acetate đến sự thay đổi nội tiết của rotifer *Brachionus calyciflorus*. Kết quả cho thấy megestrol acetate là chất chất gây rối loạn nội tiết, khi rotifer tiếp xúc với các chất này ở nồng độ 0,05; 0,1; 0,5 và 1 mg/l thì sự gia tăng sản sinh khối u đã được ghi nhận và đạt giá trị cao nhất ở nồng độ 0,1 mg/l. Kết quả nghiên cứu hệ gen của Rotifer về cơ chế tác động của megestrol acetate chứng minh hệ thống nội tiết của Rotifer có thể bị gián đoạn bởi các chất gây ô nhiễm môi trường.

Từ khóa*:* *Brachionus calyciflorus, động vật không xương sống, rối loạn nội tiết, sinh sản*