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The *Annona muricata* leaf ethanol extract affects mobility and reproduction in mutant strain NB327 *Caenorhabditis elegans*

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ABSTRACT

The *C. elegans* NB327 mutant strain is characterized for the knockdown of the *dic-1* gene. The *dic-1* gene is homologous to the *dice-1* gene in humans, encoding the protein DICE-1 as a tumor suppressor. Absence or under-regulation of the *dice-1* gene can be reflected in lung and prostate cancer [17,18]. This study evaluated the effect of EEAML on the *C. elegans* NB327 mutant strain. Phenotypic aspects such as morphology, body length, locomotion, and reproductive behaviour were analyzed. It is important to emphasize that the strain presents a phenotype characteristic with respect to egg laying and hatching. Reported studies showed that *Annona muricata* extract and its active components evidence anti-cancer and anti-tumor effects, through experimentation *in vivo* and *in vitro* models. However, neurotoxicity has been reported as a side effect. The results showed that the mutant strain NB327 was exposed to EEAML (5 mg/ml) concentration, it showed a significant decrease in average locomotion, resulting in 13 undulations in 30 s. This contrasts with the control strain's 17.5 undulations in 30 s. Similarly, the number of progenies was reduced from 188 progenies (control strain) to 114 and 92 progenies at the dose of (1 mg/ml and 5 mg/m) EEAML. The results of this study suggest that EEAML has a possible neurotoxic effect in concentrations equal to or greater than 5 mg/ml. Also, it does not have positive effects on the mutant strain of *Caenorhabditis elegans* NB327 phenotype.

1. Introduction

The leaves, seeds and fruit of *Annona muricata* (*A. muricata*), commonly known as Soursop-Graviola, have been recognized and used in ethnomedicine worldwide. These leaves have therapeutic properties, against oxidative stress, diabetes, neuralgia, arthritis, malaria, inflammation, tumors and cancer [1]. This has led to increasing attention on the use and impact of *A. muricata* in the population, including Latin American countries, where the fruit and leaves extract are marketed. Scientific literature has reported several phytoconstituents such as Acetogenins, whose biological activity is focused on the inhibition of mitochondrial complex I (NADH: ubiquinone oxidoreductase) [2]. Also, these Acetogenins have cytotoxic properties against different tumor cell lines such as lung, prostate, liver, and colon [3–6].

Cancer is a leading cause of death worldwide [7], and chemotherapy has been an invasive and degenerative treatment for it. In order to determine the effects of EEAML and bring to bioprospecting, the nematode *Caenorhabditis elegans* (*C.elegans*) was chosen in this study. This nematode was established in 1960 by the South African biologist Sydney Brenner as a model organism in the field of basic research. Its biological qualities can allow the improvement of the visibility and understanding of pathological diseases in humans and other multicellular beings. Some of the advantages of using *C. elegans* as a model for experimentation *in vivo* in this study are: Its perceptible phenotypes, the visibility of their internal organs, short life cycle (3 weeks), and the ability to adapt to different conditions in the laboratory [8,9].

Over the past decades, various mutant strains of *C. elegans* have been found to be ideal for the study of specific diseases, including cancer [10–13]. This worm model allows the analysis of signaling pathways, study of genes and proteins involved in embryogenesis, and its complete development. Moreover, this model could facilitate the understanding of the mechanisms of drug and other substance action [14]. In this study the *C. elegans* NB327 mutant strain was used, characterized for the knockdown of the *dic-1* gene, which can produce an alteration in the apoptotic process, and therefore a malformation in the gonads and eggs [15,16]. The *dic-1* gene is homologous to the *dice-1* gene in humans, encoding the protein DICE-1 as a tumor suppressor. Absence or under-regulation of *dice-1* gene can be reflected in lung and prostate cancer [17,18]. This study evaluated the EEAML *in vivo* effects on the *C. elegans* NB327 mutant strain. Phenotypic aspects such as

Abbreviations: EEAML, Ethanol extract of Annona muricata leaves; DMSO, Dimethylsulfoxide; LD50, Lethal Dose 50; MIC, Minimum Inhibitory Concentration; NGM, Nematode growth medium; MHA, Mueller Hinton Agar; MHB, Mueller Hinton Broth; CLSI, Clinical and Laboratory Standards Institute

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morphology, body length, locomotion, and reproductive behaviour were analyzed.

2. Materials and methods

2.1. Maintenance and cultivation of C. elegans NB327 mutant strain

The mutant strain of C. elegans NB327, *dic-1(tm1615) IV/nT1 [qIs51] (IV;V)*, was obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota (USA). Phenotypic characterization of the mutant strain NB327 was performed using a Leica DM IL inverted microscope, Olympus light microscope CX31, and an Olympus SZ51 stereoscope. The nematode was maintained on NGM Nematode growth medium [19], the Escherichia coli strain OP:50 used as a food source (donated by Calixto A, Chile Mayor University), and kept at 20 °C, according to Brenner standardization [19]. The synchronization protocol was standardized according to the characteristics of the mutant strain, adjusting the concentrations of NaOH 1 M/Cl_2 5%/H_2O solution (5: 3.8: 1.2) [20].

2.2. Extraction of EEAML

A. muricata leaves were collected in La Mesa, Cundinamarca (Colombia), wrapped in Kraff paper (226.43 g dry weight) under shade and dried at room temperature for two months [21]. Subsequently, the *A. muricata* leaves were ground and placed in 1590 ml of ethanol 95% for 3 weeks. After, the solution was filtered and placed in a rotary evaporator [22], and 11120 mg of ethanolic extract was obtained (yield 4.91%). After, 2820 mg was taken and suspended in 20 ml of dimethyl sulfoxide (DMSO). DMSO has a polarity property and a low percentage of reaction activity in bioassays, which allows the dilution of EEAML components [23,24]. A stock solution (141 mg/ml) was obtained and used in the preparation of NGM media for different tests.

The Minimum Inhibitory Concentration (MIC) identification was performed using *E. coli OP50* strain, by disk diffusion testing on Mueller Hinton Agar (MHA), under the Clinical and Laboratory Standards Institute (CLSI) protocols [25], where 20 µL doses of EEAML (0.01–100 mg/ml) were added to filter paper sensi-discs (0.5 mm), and DMSO solution was used as a negative control [26]. It was determined that there was no growth inhibition of the *E. coli OP50* strain. This data was confirmed with the Mueller Hinton broth dilution assay (MHB) [25]. The tests were performed in triplicate.

These bioassays were able to be performed using two concentrations: 1 mg/ml and 5 mg/ml reported in previous studies [27,28]. Initially, synchronized nematodes were placed in NGM media EEAML concentrations, as a controls were placed in the media without EEAML, and other with 0.2% DMSO. Finally the tests were performed, to able to find the phenotypic characteristics. The tests were performed in triplicate.

2.3. Body length and locomotion assay

Nematodes synchronized at the L1 stage were deposited in NGM media, each containing a dose of EEAML, and left for 6 days. Initially locomotion was determined by the number of undulations made by the head of the adult stage nematode in 30 s (Fig. 2B) [29]. Subsequently, the length of nematodes in the adult stage was measured with the Levamisole 100 mM treatment. This treatment was used in order to immobilize the worms and be able to measure them, using a microscope grid (Fig. 2A) [30]. These assays were determined with 10 larvae per replication.

2.4. Egg laying and reproduction assay

In order to count the number of eggs, the egg laying assay was first conducted. 5 L1 stage larvae were laid on the NGM agar, and when the larvae reached the L4 stage they were individually placed in a new NGM agar. Then, the eggs laid were counted and each larva was transferred to a new NGM agar for three days [31]. The reproduction assay was performed to determine the number of progenies, where the number of hatched progenies within the fertile period was evaluated. When the larvae reached the L4 stage, 5 larvae were placed individually in a new NGM medium. Each larva was transferred to a new NGM agar, maintaining initial conditions until the end of reproductive period. NGM agars with eggs laid were read after 48 h and the L1 –L2 stage larvae were counted [29].

2.5. Statistical analysis

All data were expressed as mean +/- standard deviation of the triplicate tests. Significant differences between control and treated groups were calculated by one-way ANOVA using Graph Pad Prims 6 program. Significant differences were determined as $P < 0,05$.

3. Results

3.1. Phenotypic comparison between the mutant strain (NB327) and the wild strain (N2)

Larval stages of *C. elegans* strain NB327 were initially characterized (Fig. 1A). The mutant strain NB327 is a knockdown model organism of the *dic-1* gene. *Dic-1* gene codes for the DIC-1 protein, which could be involved in the oogenesis, embryogenesis and nematode apoptotic processes [15]. The NB327 strain phenotype was recognized under a microscope and it was found that it differs from the wild-type strain N2 in two aspects: 1. The NB327 eggs were large, round and symmetrical, whereas the wild strain N2 eggs had an elongated shape and were smaller than NB327 eggs (Fig. 1B). 2. The NB327 distribution of eggs in the uterus were found to be disorganized, due to the large number of accumulated eggs; while the wild N2 eggs were considerably more organized, being similarly sized and having a specific amount of space between them (Fig. 1C).

3.2. The EEAML affects the mutant strain NB327 locomotion, but not its length

The body length and locomotion of *C. elegans* are modulated by various signal pathways, which can be affected by specific substances and molecules [30,32]. Locomotion is one of the neuronal synaptic indicators, where the neuronal tissue constitutes the action targets for toxic molecules [29,33–36]. The mutant strain NB327 was exposed to EEAML (1 mg/ml and 5 mg/ml) concentrations for 6 days, where subsequently no significant alteration of the nematode body length was found, compared to the controls (Fig. 2A). However, when the mutant strain NB327 was exposed to EEAML (5 mg/ml) concentration, it showed a significant decrease in average locomotion, resulting in 13 undulations in 30 s, in contrast to the control strain's 17.5 undulations in 30 s (Fig. 2B). Meanwhile, the nematode exposed to EEAML (1 mg/ml) concentration did not show any locomotion changes (Fig. 2B). These data suggest that active compounds of EEAML can produce neurotoxic effects in concentrations greater than or equal to 5 mg/ml.

3.3. The EEAML affects the mutant strain NB327 reproductive behaviour

The nematode reproductive organs are a target for toxic substances [29,33,35,37,38]. The mutant strain NB327 reproductive behaviour could be deficient in the development of gonadogenesis, embryogenesis and oogenesis processes [15]. This behaviour was assessed by testing egg laying and reproduction. The NB327 exposed to EEAML, for 3 days at concentrations of 1 mg/ml and 5 mg/ml, showed a lower average of egg numbers (36 eggs per day and 31 eggs per day) than the control strains (Fig. 3A). Similar results were found when the NB327 was

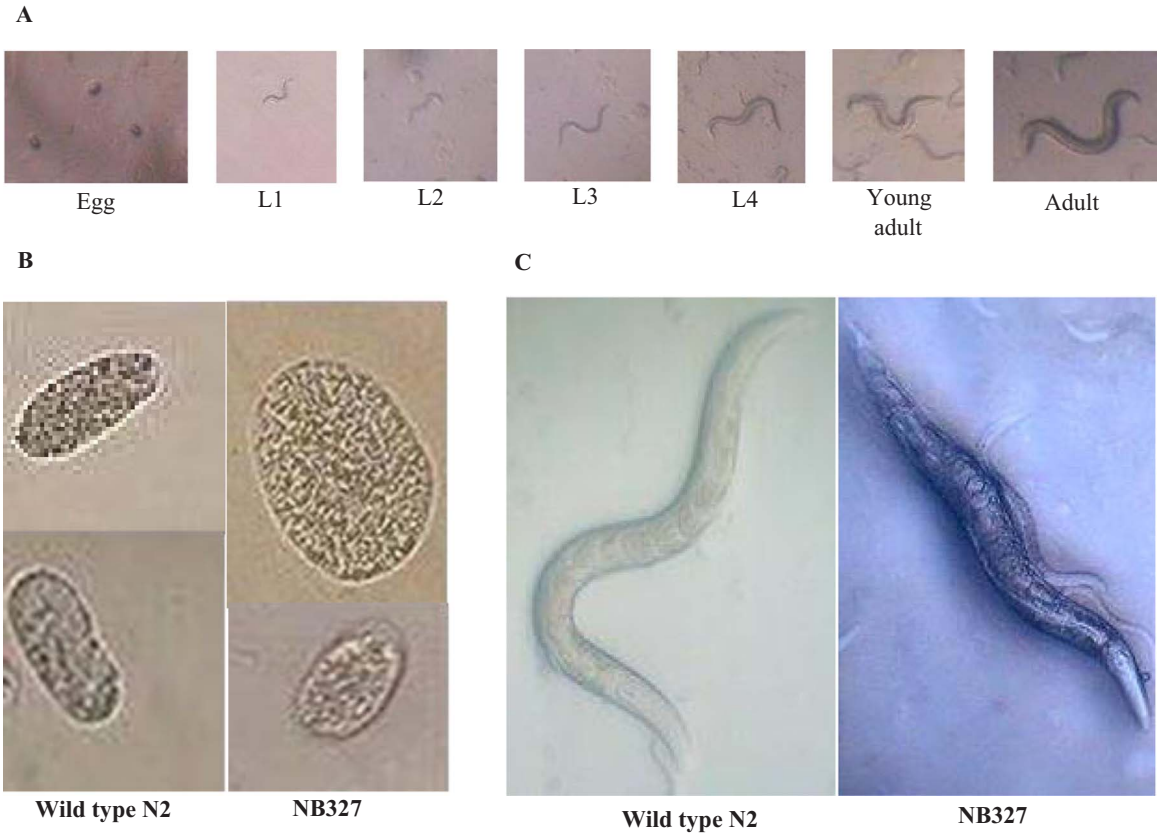

Fig. 1. Phenotypic characterization of the differences between the mutant strain NB327 and the wild strain N2. A. Strain NB327 larval stages, embryonic stage (egg) is observed, larval L1-L4 stage and adult larvae. **B.** NB327 eggs and N2 wild strain. The difference in size and shape **C.** The distribution of eggs in the uterus of the NB327 and N2 wild strain. Stereoscopic pictures taken from the Olympus SZ51 40X, Olympus optical microscope CX31, and Leica DM IL inverted microscope 40X.

exposed to EEAML (1 mg/ml and 5 mg/ml) concentrations for 3 days, presenting a significant decrease in the average number of progeny (114 and 92 progenies) compared to the control strains (Fig. 3B).

4. Discussion

A. muricata belongs to the *Annonaceae* family and has been a traditional plant remedy in America and Africa. Phytochemicals trials have shown that Acetogenins are the main constituent and active component for the developing of anti-cancer and anti-tumor effects, which have been demonstrated through experimentation *in vivo* and *in vitro*. However, neurotoxicity has been reported as a side effect [1]. In this study, *C. elegans* strain NB327 was exposed to two EEAML doses. Initially, the phenotypic strain characterization was performed by checking an abnormal eggs morphology and the reproductive organs, with similar results having been reported by Han et al. (2006) [15].

Locomotion is an indicator of the neuronal synaptic function in nematodes, specifically the type D GABAergic motor neurons [33]. The results of NB327 strain locomotion, showed undulations from 15 to 20 in 30 s, which were similar to those described by Calahorro Núñez F. (2011) [39]. In this study, the NB327 strain locomotion behaviour was increased with the addition of EEAML (1 mg/ml) concentration, compared to the control. However, the addition of EEAML (5 mg/ml) concentration presented a reduction of 22% compared to the presented control, could be indicating a possible neurotoxic effect on the NB327 strain. The neurotoxic effect was suggested by Moron Francisco Rodriguez J et al. (2010) [40]. This study argued that a possible neurotoxic effect of the *A. muricata* extract, with the appearance of Parkinson atypical forms, could be associated with the consumption of fruit (15 mg/fruit), and plant leaves (140 mg/cup of tea).

The Acetogenins mechanism is based on the inhibition of mitochondrial complex I (NADH: ubiquinone oxidoreductase), which is necessary in ATP synthesis, increasing oxidative stress, cytotoxic effect, and cell-proliferation in neoplastic cell lines. It has been considered that mitochondrial complex I inhibitors are precursors of neurodegenerative diseases such as Parkinson's, due to the increase of superoxide radicals in neuronal cells [41].

The reproductive behaviour of the NB327 strain was evaluated by egg laying assay and reproduction. The mutant strain without *A. muricata* extract, showed a decrease in the number of laid eggs and progenies, compared with those reported for the wild-type strain N2 [42]. This result agrees with the study described by Han et al. (2006), where it was reported that the NB327 strain could be a knockdown for DIC-1 protein, causing a deficit in the reproductive behaviour in terms of egg position and viability. The reduction of egg viability in this strain is due to the under-regulation of the DIC-1 protein, which can increase apoptosis and affects the gonadogenesis, oogenesis, and embryogenesis negatively, resulting in unviable progenies (infertile eggs) [15].

The egg positions showed a decrease in the number of eggs laid per day, using EEAML (1 mg/ml and 5 mg/ml) concentrations, where it can be suggested that the EEAML (1 mg/ml and 5 mg/ml) provides a hostile, toxic, and harmful environment, resulting in the nematode retaining the eggs to provide protection to their progeny. In order to normalize the egg laying, external conditions should be favorable [43,44]. Studies have shown that egg position and retention are regulated by neurotransmitters such as acetylcholine and serotonin, as well as specific motor action [43,45,46].

The evaluation of the nematode reproduction, using EEAML (1 mg/ml and 5 mg/ml) concentrations, showed a decrease in the number of progeny, suggesting that active compounds of EEAML can produce harmful effects on the hatching. The mechanisms with which the *A. muricata* extract prevents hatching are not known, but it has been

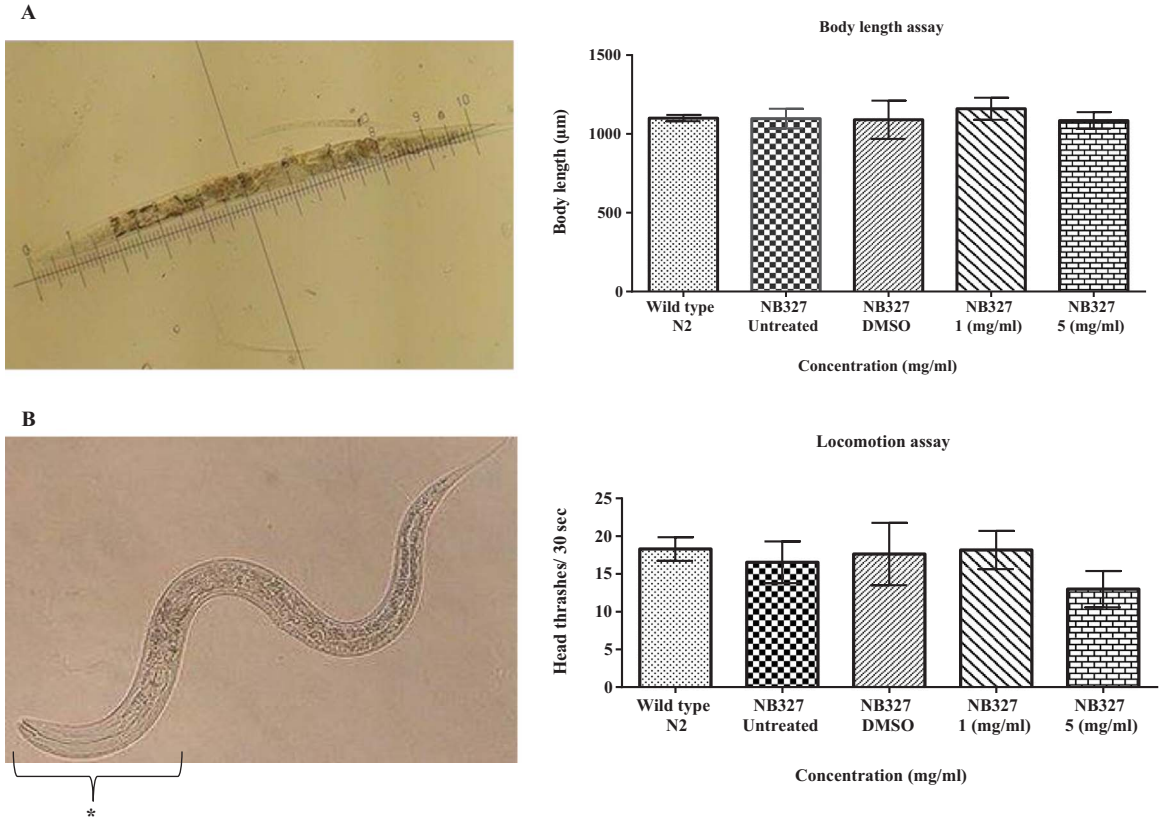

Fig. 2. Body length and locomotion of NB327 mutant and N2 wild strain. A. Body length of NB327 mutant strain in reticle microscope, comparison of the NB327 mutant and N2 wild strain body length without extract, exposed EEAML (1 mg/ml and 5 mg/ml) concentrations and control with DMSO 0.2%. Significant effects on length were not observed as being produced by EEAML. **B.** Undulation photography taken as a reference of the NB327 mutant strain locomotion (*Undulation head) and locomotion comparison between NB327 mutant and N2 wild strain. A significant decrease in locomotion was observed when using EEAML 5 mg/ml concentration. The tests were performed in triplicate and analysis was performed using Graph Pad Prims program version 6 (One-way ANOVA). Significant differences were determined as $P < 0,05$. Photographs taken by the Olympus microscope light CX31 40X and Leica DM IL inverted microscope 40X.

suggested that it could be due to cell division inhibition during embryogenesis, and the possible alteration of nematode vital structures [47].

The possible effect of EEAML on nematode locomotion coincides with research carried out since 2002, where the neurotoxic effect *in vivo* or *in vitro* of *A. muricata* extract has been reported. It has been found that this effect could be produced by the Acetogenins, specially the Annonacin [47,48]. Additionally, it has been reported the Acetogenins, as an environmental neurotoxin, could be responsible for atypical sporadic Parkinson's and dementia in tropical zones (French West Indies) [49].

Finally, EEAML showed negative effects on the NB327 inherent strain phenotype, *C. elegans*, resulting in two additional adverse effects: neurotoxicity (locomotion), and the possible alteration of egg position and hatching.

Conflicts of interest

No conflicts of interest are reported for any authors.

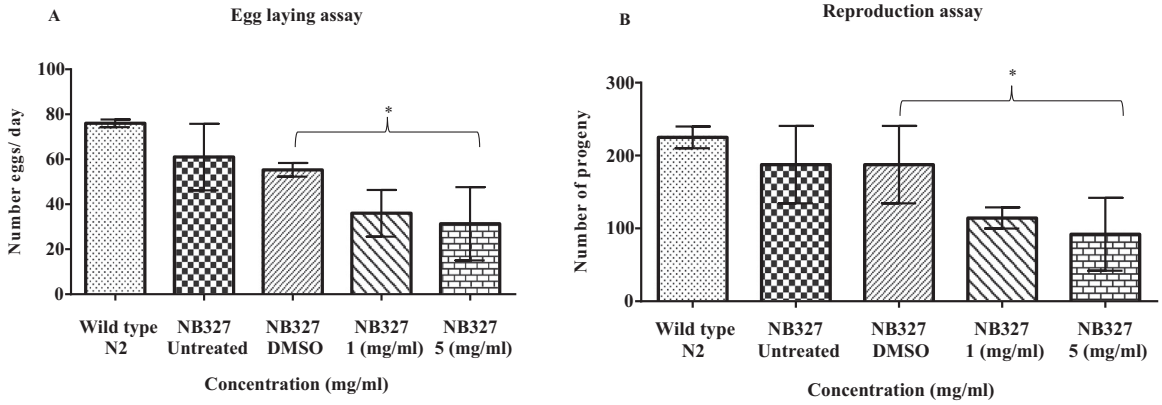

Fig. 3. Reproductive behaviour of the NB327 mutant and N2 wild strain. A. Reproductive behaviour of the NB327 mutant and N2 wild strain expressed in the egg laying; tests performed without extract, using EEAML (1 mg/ml and 5 mg/ml) concentrations, and control with DMSO 0.2%. A significant decrease in the number of eggs is observed using EEAML (1 mg/ml and 5 mg/ml) concentrations. **B.** Reproduction assay. A reduction in progeny numbers is observed in the strain NB327, exposed to EEAML (1 mg/ml and 5 mg/ml). All assays were performed in triplicate and analysis was performed with the Graphpad Prism program version 6 (One-way ANOVA), showing statistical significance $P = 0.0682$ ($P > 0.05$).

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Appendix A. Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.bbrep.2017.04.016

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