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# Exploring the neurotoxicity and changes in life cycle parameters of *Drosophila melanogaster* exposed to arecoline

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## Abstract

**Background:** Arecoline (an alkaloid) has been reported as a potent cytotoxic as well as genotoxic agent but its neurotoxic effects are limited.

**Material and methods:** In the present study, we evaluated the neurotoxic effects and changes in life cycle parameters of *Drosophila melanogaster* on exposing the flies to 5, 10, 20, 40 and 80  $\mu\text{M}$  of arecoline for 5 days. After the exposure, the flies were assayed for climbing ability, activity pattern, oxidative stress markers, acetylcholinesterase (AChE), monoamine oxidase activity (MAO), caspase-3 and caspase-9 activities and apoptosis in the neuronal cells. For studying the effects on life cycle parameters, duration of pupation, emergence and life span were also studied.

**Results:** The exposure of flies to 5 and 10  $\mu\text{M}$  of arecoline did not show toxic effects, but the flies exposed to 20, 40 and 80  $\mu\text{M}$  showed a significant dose-dependent loss of climbing ability, activity pattern, MAO, glutathione (GSH) content and acetylcholinesterase activity and increase in the glutathione-S-transferase (GST), MAO activity, lipid peroxidation assay (LPO), protein carbonyl content (PCC), caspase-3 and caspase-9 activities and apoptosis in the neuronal cells. The exposure of flies to arecoline not only results in the delay of pupation but also delayed the emergence of flies. A significant reduction was also observed in the life span of flies exposed to 20, 40 and 80  $\mu\text{M}$  of arecoline. The eyes of flies emerged from the larvae exposed to various doses of arecoline were also analysed for the phenotypic abnormality. Clear dose-dependent deformities were observed in the eyes of the flies emerged from the exposure of larvae exposed to 20, 40 and 80  $\mu\text{M}$  of arecoline.

**Conclusion:** Arecoline not only is a neurotoxic agent but also affects the life cycle parameters of the fly.

**Keywords:** Arecoline, *Drosophila*, Oxidative stress, Pupation, Emergence, Life span, Apoptosis

## Background

Arecoline, a main areca alkaloid of the betel nut, has been reported to be cytotoxic as well as genotoxic in cultured cells (in vitro), mice and rats (in vivo) (Kumpawat, Deb, Ray, & Chatterjee, 2003; Chou et al., 2009; Kevekordess et al., 2001; Dasgupta et al., 2006; Zhou, Sun, Yang, & Zhang, 2014; Run-mei, Jun-jun, Jing-ya, Li-juan, & Yong, 2014). Arecoline is highly effective against tapeworms, cysticercus, *Fasciola hepatica*. It is also effective in treating cardiovascular diseases due to its vasorelaxation, anti-thrombosis and anti-antherogenic effects (Liu, Peng,

Hu, Xu, & Wu, 2016). The prolonged exposure of arecoline leads to the oral submucous fibrosis and oral cancers (Fareed, Afzal, & Siddique, 2011; Jyoti et al., 2014; Jyoti, Khan, Afzal, Naz, & Siddique, 2013; Jyoti, Siddique, Khan, Naz, & Ali, 2015). The pharmacology, toxicology and potential applications of arecoline have been extensively reviewed by Liu et al. (2016). It has also been reported to cross the blood brain barrier and is therefore used as a potential therapeutic action against the symptoms of schizophrenia and Alzheimer disease (Asthana et al., 1996; Herz, Fraling, Niedner, & Färber, 1967; Sullivan, Andres, Otto, Miles, & Kydd, 2007). But still its toxic effects on the central nervous system are warranted. In our study, it showed the toxic effects in the third instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*) *Bg*<sup>9</sup> (Shakya

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& Siddique, 2018). The study performed by Shih et al. (2010) on the cultured rat primary cortical neurons showed that it is potent in inducing apoptotic death by attenuating an antioxidant defence system and enhancing the oxidative stress. Arecoline at the concentration of about 0.1–97.39 µg/ml showed to induce reactive oxygen species (ROS) inflammation and oral carcinogenesis (Chang et al., 2004; Jeng et al., 2003; Shih et al., 2010). Hence, we decided to study the effect of arecoline on the adult *D. melanogaster* (Oregon R).

## Materials and methods

### *D. melanogaster* stock, culture and treatments

*D. melanogaster* (Oregon R) was obtained from *Drosophila* Genetic Resource Centre (DGRC), Kyoto Institute of Technology, Kyoto Stock Centre, Japan. The flies were cultured on the standard *Drosophila* food containing agar, corn meal, sugar and yeast at 25 °C (Siddique, Ara, Jyoti, Naz, & Afzal, 2011; Siddique, Jyoti, & Naz, 2014). Arecoline hydrobromide (Sigma, USA) was dissolved in distilled water and mixed in the diet to obtain the final concentrations of 5, 10, 20, 40 and 80 µM. Newly eclosed 1 to 3-day-old male flies were starved for 16 h and then placed on the diet having the desired concentration of arecoline. The flies were allowed to feed on it for 5 days (50 flies per vial; 5 replicates/treatment). Methyl methanesulphonate (10 µl/ml) was used as a positive control.

### Climbing assay

The climbing assay was performed as described by Pendleton, Parvez, Sayed, and Hillman (2002). Ten flies were placed in an empty glass vial (10.5 cm × 2.5 cm). A horizontal line was drawn 8 cm above the bottom of the vial. After the flies had acclimated for 10 min at room temperature, both controls and treated groups were assayed at random to a total of 10 trials for each. The flies were then gently tapped down to bottom of the vials. The numbers of flies above the mark of the vial were counted after 10 s of climbing and repeated 10 times to get the mean number.

### Activity pattern

The activity of newly eclosed starved male flies was analysed by using a *Drosophila* activity monitor (DAM) (TriTek, USA). The male flies were allowed to feed on the diet having the desired concentration of arecoline. The activity was recorded every 60 min for a total of 5 days and the data obtained was analysed by Actogram J Software. The results were presented as chi-square periodogram (Chiu, Low, Pike, Yildirim, & Edery, 2010; Rosato & Kyriacou, 2006).

### Preparation of homogenate for biochemical assays

At the end of the treatment period, the heads of male flies (50 heads/treatment; 5 replicates/group) were collected, weighted and homogenised in 0.1 M phosphate buffer (pH 7.4) and centrifuged at 4 °C at 6000 rpm for 10 min. Subsequently, the supernatant was separated from the pellet into an Eppendorf tube kept in ice, and used for the determination of glutathione (GSH), glutathione-S-transferase (GST), lipid peroxidation assay (LPO), protein carbonyl content (PCC), monoamine oxidase (MAO), caspase-9, caspase-3 and acetylcholinesterase (AChE) activities.

### Estimation of glutathione (GSH) content

The glutathione (GSH) content was estimated colorimetrically using Ellman's reagent (DTNB) according to the procedure described by Jollow, Mitchell, Zampaglione, and Gillette (1974). The supernatant was precipitated with 4% sulphosalicylic acid (4×) in the ratio of 1:1. The samples were kept at 4 °C for 1 h and then subjected to centrifugation at 5000 rpm for 10 min at 4 °C. The assay mixture consisted of 550 µl of 0.1 M phosphate buffer, 100 µl of supernatant and 100 µl of DTNB. The OD was read at 412 nm and the results were expressed as micromoles of GSH per gram tissue.

### Determination of glutathione-S-transferase (GST) activity

The glutathione-S-transferase activity was determined by the method of Habig et al. (1974). The reaction mixture consists of 500 µl of 0.1 M phosphate buffer, 150 µl of 10 mM 1-chloro-2,4-dinitrobenzene (CDNB), 200 µl of 10 mM reduced glutathione and 50 µl of supernatant. The OD was taken at 340 nm and the enzyme activity was expressed as micromoles of CDNB conjugates per minute per milligram per protein.

### Lipid peroxidation assay (LPO)

Lipid peroxidation was measured according to the method described by Ohkawa, Ohishi, and Yagi (1978). The reaction mixture consisted of 5 µl of 10 mM butyl-hydroxy toluene (BHT), 200 µl of 0.67% thiobarbituric acid, 600 µl of 1% O-phosphoric acid, 105 µl of distilled water and 90 µl of supernatant. The resultant mixture was incubated at 90 °C for 45 min and the OD was measured at 535 nm. The results were expressed as micromoles of TBARS formed per hour per gram tissue.

### Estimation of protein carbonyl content (PCC)

The protein carbonyl content was estimated according to the protocol described by Hawkins, Morgan, and Davies (2009). The brain homogenate was diluted to a protein concentration of approximately 1 mg/ml. About 250 µl of each diluted homogenate was taken in Eppendorf centrifuge tubes separately. To it 250 µl of 10 mM 2,4-dinitrophenyl hydrazine (dissolved in 2.5 M HCl) was added,

vortexed and kept in dark for 20 min. About 125  $\mu$ l of 50% (*w/v*) trichloroacetic acid (TCA) was added, mixed thoroughly and incubated at  $-20^{\circ}\text{C}$  for 15 min. The tubes were then centrifuged at  $4^{\circ}\text{C}$  for 10 min at 9000 rpm. The supernatant was discarded and the pellet obtained was washed twice by ice cold ethanol: ethyl acetate (1:1). Finally, the pellets were re-dissolved in 1 ml of 6 M guanidine hydrochloride and the absorbance was read at 370 nm.

#### Determination of acetylcholinesterase (AChE) activity

Acetylcholinesterase activity was estimated according to the method of Ellman, Courtney, Andres Jr, and Featherstone (1961). The reaction mixture consisted of 650  $\mu$ l of 0.1 M phosphate buffer (pH 7.4), 100  $\mu$ l of 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and 100  $\mu$ l of supernatant. The content was mixed thoroughly and the absorbance was measured at 412 nm. After having a stable absorbance value, 10  $\mu$ l of 0.075 M acetylthiocholine was added and the change in absorbance per minute was recorded, to calculate the enzyme activity.

#### Estimation of monoamine oxidase (MAO)

The method described by McEwan (1965) was used to estimate the monoamine oxidase activity. The assay mixture consisted of 400  $\mu$ l of 0.1 M phosphate buffer (pH 7.4.), 1300  $\mu$ l of distilled water, 100  $\mu$ l of benzylamine hydrochloride and 200  $\mu$ l of brain homogenate. The assay mixture was incubated for 30 min at room temperature and then 1 ml of 10% perchloric acid was added and centrifuged at 1500g for 10 min. The OD was taken at 280 nm.

#### Spectrophotometric assay for caspase-9 (Dronc) and caspase-3 (Drice) activities

The assay was performed according to the manufacturer protocol with some modification (Bio-Vision, CA, USA). The assay was based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) obtained after specific action of caspase-3 and caspase-9 on tetrapeptide substrates, DEVD-pNA and IETD-pNA, respectively. The assay mixture consisted of 50  $\mu$ l of fly head homogenate and 50  $\mu$ l of chilled cell lysis buffer incubated on ice for 10 min. After incubation, 50  $\mu$ l of 2 $\times$  reaction buffer (containing 10 mM DTT) with 200  $\mu$ M substrate (DEVD-pNA for Drice, and IETD-pNA for Dronc) was added and incubated at  $37^{\circ}\text{C}$  for 1.5 h. The reaction was quantified at 405 nm.

#### Apoptotic index (AI)

After isolating the brains (20/treatment; 5 replicates per group) in PBS from treated and control group, the brain cell suspension was prepared by exposing the isolated brain to collagenase (0.5 mg/ml) for 2 min. The cells were centrifuged to remove cellular debris and the suspended cells were incubated in a cocktail of acridine

orange (1  $\mu$ g/ml) and ethidium bromide (1  $\mu$ g/ml) for 3 min. The cells were washed with PBS and observed under a fluorescent microscope. For the quantification of extent of apoptosis, a total of 100 cells were scored. The live cells appeared green, apoptotic cells appeared orange and necrotic cells red (Singla & Dhawan, 2013).

#### Effect of pupation and emergence of flies

To study the effect of arecoline on the pupation and emergence of flies, the arecoline was mixed in diet at a final concentration of 5, 10, 20, 40 and 80  $\mu$ M. To these doses, 50 first instar larvae were introduced. Triplicate sets of each treatment group (including the untreated and positive control) were employed in the study. Each day the numbers of larvae pupate followed by number of flies emergence were recorded separately and the data was expressed as mean of three replicates (Podder and Roy, 2014).

#### *Drosophila* life span determination

Newly eclosed flies were placed in the culture tubes (10 flies per tube) containing desired concentration of the arecoline. The flies were transferred to a new diet at every third day containing desired concentration of the arecoline till the last one died (Long, Gao, Sun, Liu, & Zhao-Wilson, 2009).

#### Phenotypic analysis of eye

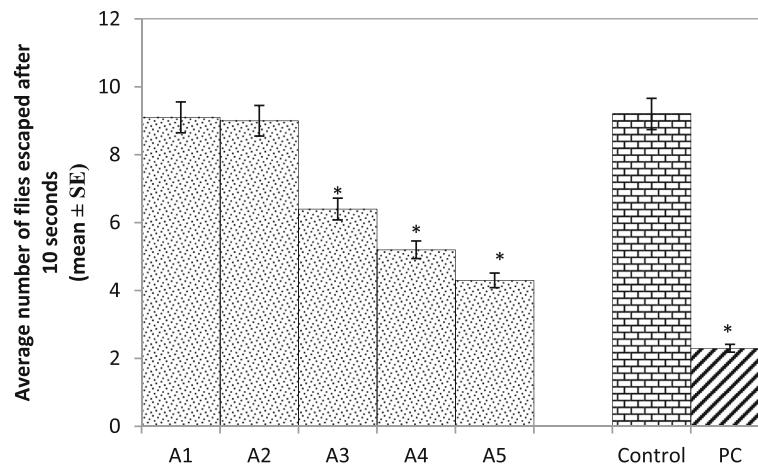
The newly emerged flies, from the larvae which were allowed to feed on the 5, 10, 20, 40 and 80  $\mu$ M of arecoline, were analysed for the phenotypic anomaly in eyes. The heads (20 heads/treatment; 5 replicates/group) were collected in PBS, and the eye caps were separated and stained by 1% toluidine blue for 2 min and observed for any deformity in the ommatidia under a light microscope.

#### Statistical analysis

All data were expressed as mean  $\pm$  standard error, and Dunnet ANOVA test was used for the analysis by using software SPSS 16. Statistical significance was considered at the 5% level.

#### Results

The results obtained for the climbing ability showed a dose-dependent significant loss of 1.43, 1.76 and 2.13 folds in the flies exposed to 20, 40 and 80  $\mu$ M of arecoline, respectively, compared to control (Fig. 1;  $p < 0.05$ ). The flies exposed to 5 and 10  $\mu$ M of arecoline did not show any loss in the climbing ability compared to control (Fig. 1;  $p < 0.05$ ). The positive control was associated with the fourfold decrease in the climbing ability compared to control (Fig. 1;  $p < 0.05$ ). The results obtained for activity pattern (Additional file 1: Figures S1-S7) showed a dose-dependent loss in the activity of flies exposed to 20, 40 and 80  $\mu$ M of arecoline compared to



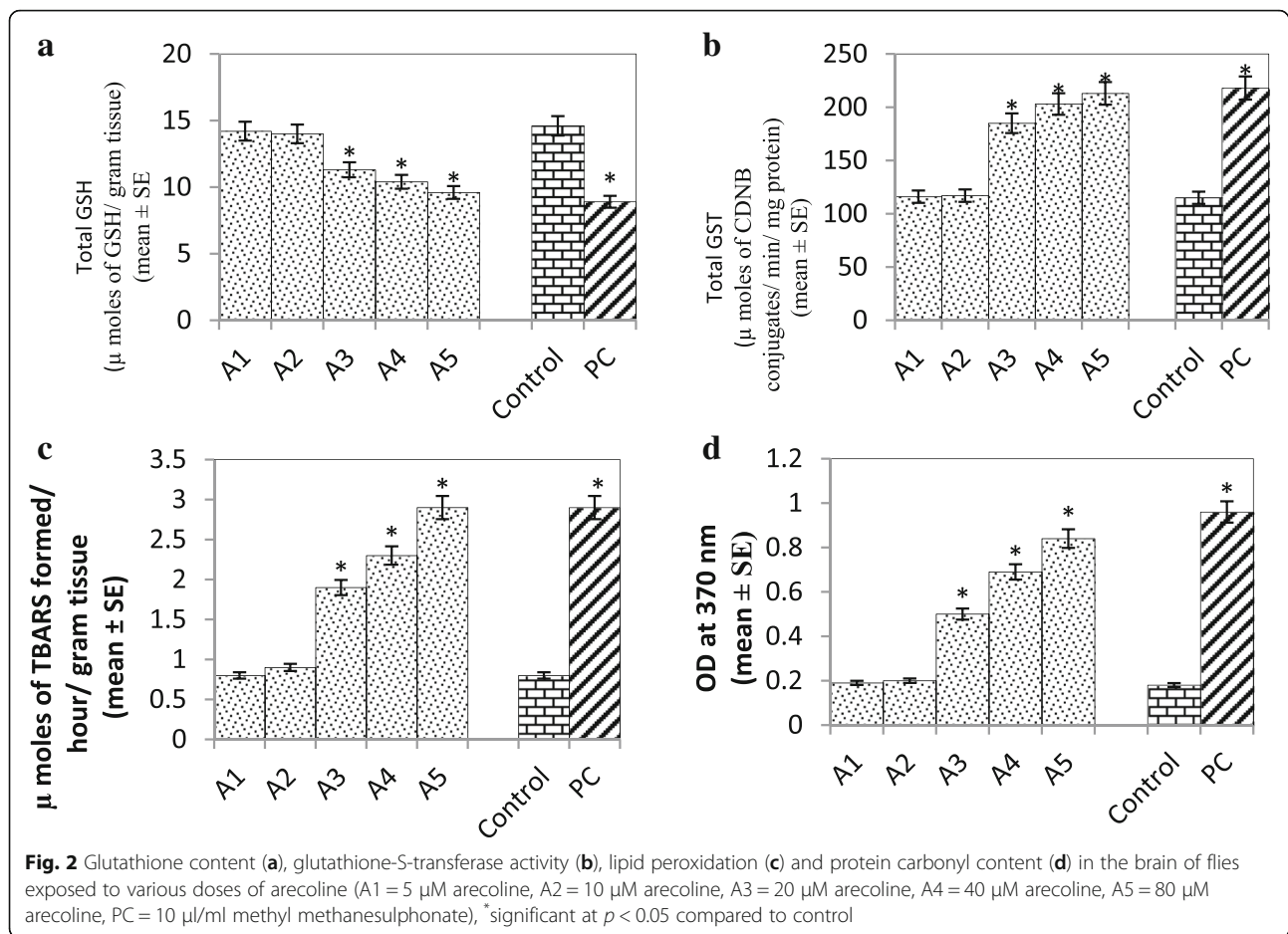
**Fig. 1** Climbing assay performed on flies exposed to various doses of arecoline (A1 = 5  $\mu$ M arecoline, A2 = 10  $\mu$ M arecoline, A3 = 20  $\mu$ M arecoline, A4 = 40  $\mu$ M arecoline, A5 = 80  $\mu$ M arecoline, PC = 10  $\mu$ l/ml methyl methanesulphonate), \*significant at  $p < 0.05$  compared to control

control flies (Additional file 1: Figures S3-S5). The flies exposed to 5 to 10  $\mu$ M of arecoline did not show any change in the activity pattern as is clearly evident from peaks near the midline compared to control (Additional file 1: Figures S1-S2). The result obtained for GSH content showed that the flies exposed to 5 and 10  $\mu$ M of arecoline did not show any significant decrease in the GSH content compared to control flies. The flies exposed to 20, 40 and 80  $\mu$ M of arecoline showed a significant dose-dependent decrease of 1.29, 1.40 and 1.52 folds compared to control flies (Fig. 2a;  $p < 0.05$ ). The flies exposed to 5 and 10  $\mu$ M of arecoline did not show any significant increase in the activity of GST compared to control flies (Fig. 2b;  $p < 0.05$ ). The flies exposed to 20, 40 and 80  $\mu$ M of arecoline showed a significant dose-dependent increase of 1.60, 1.76 and 1.85 folds in the GST activity compared to control flies (Fig. 2b;  $p < 0.05$ ). The flies exposed to 5 and 10  $\mu$ M of arecoline did not show any significant increase in LPO compared to control flies (Fig. 2c;  $p < 0.05$ ). The flies exposed to 20, 40 and 80  $\mu$ M of arecoline showed a significant dose-dependent increase of 2.37, 2.87 and 3.62 folds in LPO as compared to control flies (Fig. 2c;  $p < 0.05$ ). The flies exposed to 5 and 10  $\mu$ M of arecoline did not show any significant increase in protein carbonyl content (PCC) as compared to control flies (Fig. 2d;  $p < 0.05$ ). The flies exposed to 20, 40 and 80  $\mu$ M of arecoline showed a significant increase of 2.77, 3.83 and 4.17 folds, respectively, compared to control flies (Fig. 2d;  $p < 0.05$ ). The flies exposed to 5 and 10  $\mu$ M of arecoline did not show any significant decrease in the activity of acetylcholinesterase (AChE) in the brains of flies as compared to control (Fig. 3a;  $p < 0.05$ ). The flies exposed to 20, 40 and 80  $\mu$ M of arecoline showed a dose-dependent significant decrease of 1.68, 1.94 and 2.38 folds respectively as compared to control flies (Fig. 3a;  $p < 0.05$ ). The

results obtained for MAO activity showed no increase compared to control flies in the brain of flies exposed to 5 and 10  $\mu$ M of arecoline (Fig. 3b;  $p < 0.05$ ). The flies exposed to 20, 40 and 80  $\mu$ M of arecoline showed a significant dose-dependent increase of 1.61, 2.16 and 2.51 folds respectively in the activity of MAO compared to control flies (Fig. 3b;  $p < 0.05$ ). Figure 4 (A1-A5) shows apoptotic cells in the brain of flies exposed to various doses of arecoline. A dose-dependent increase in the apoptotic cells was observed in the brain of flies exposed to 20, 40 and 80  $\mu$ M of arecoline (Fig. 4, A3-A5) compared to control (Fig. 4c). The exposure of flies to 20, 40 and 80  $\mu$ M of arecoline showed a dose-dependent increase of 2.57, 3.28 and 3.71 folds in the activity of caspase-3 respectively as compared to control (Fig. 5a;  $p < 0.05$ ). The flies exposed to 5 and 10  $\mu$ M of arecoline did not show any increase in the activity of caspase-3 (Fig. 5a;  $p < 0.05$ ). Similarly for caspase-9, the flies exposed to 20, 40 and 80  $\mu$ M of arecoline showed a dose-dependent significant increase of 1.81, 2.31 and 2.87 folds, respectively, compared to control (Fig. 5b;  $p < 0.05$ ). The exposed flies to 5 and 10  $\mu$ M of arecoline did not show any increase in the activity of caspase-9 (Fig. 5b;  $p < 0.05$ ). The exposure of 5 and 10  $\mu$ M of arecoline did not show any significant increase in apoptotic index (Fig. 5c). The exposure of 20, 40 and 80  $\mu$ M of arecoline to the flies showed a dose-dependent significant increase of 3.31, 5.42 and 8.52 folds in the apoptotic index compared to control (Fig. 5c;  $p < 0.05$ ).

The results obtained for the pupation of larvae showed no significant change in the rate of pupation in the larvae exposed to 5 and 10  $\mu$ M of arecoline compared to control (Fig. 6a;  $p < 0.05$ ). The larvae exposed to 20, 40 and 80  $\mu$ M of arecoline showed a significant dose-dependent delay in the rate of pupation compared to control (Fig. 6a;  $p < 0.05$ ). The results obtained for the



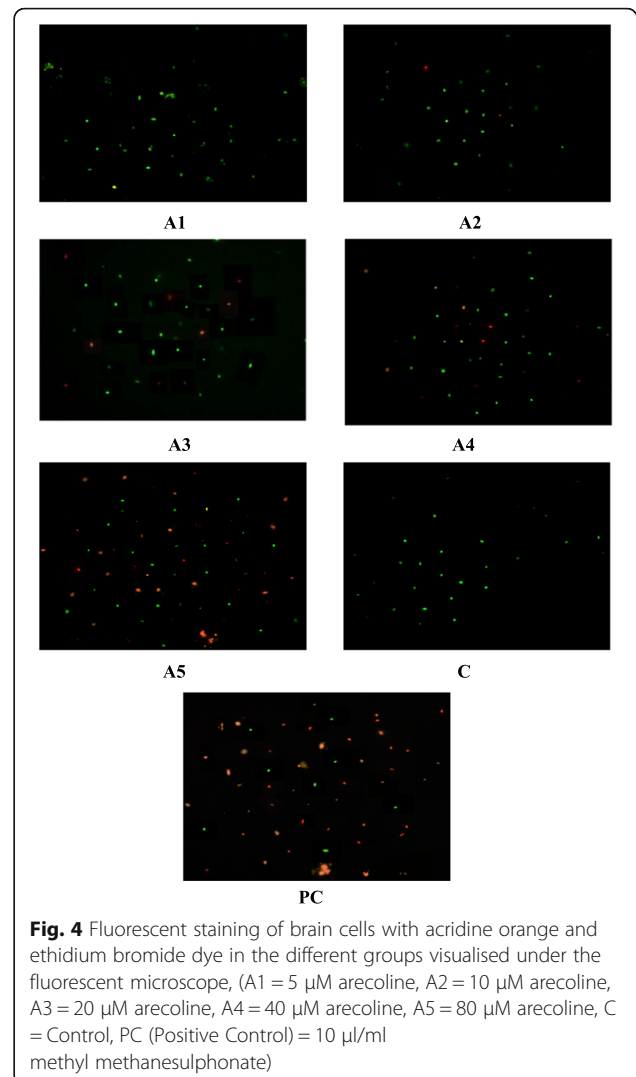
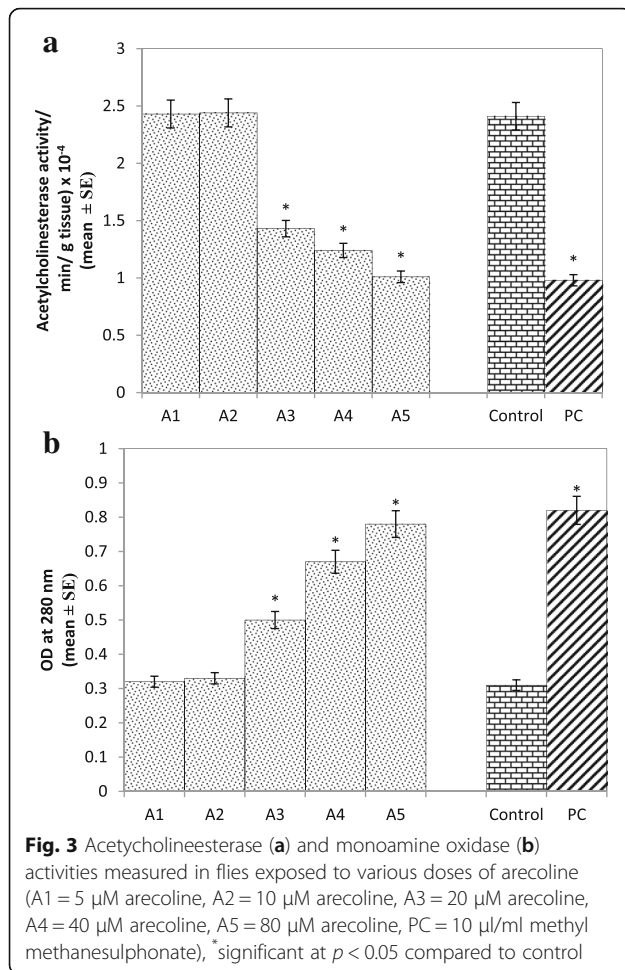


emergence of flies showed no significant change at the doses of 5 and 10  $\mu$ M of arecoline compared to control (Fig. 6b;  $p < 0.05$ ). The pupa formed from the larvae exposed to 20, 40 and 80  $\mu$ M of arecoline showed a dose-dependent significant delay in the emergence of flies compared to control (Fig. 6b;  $p < 0.05$ ). The result obtained for the survival assay is shown in Fig. 6c. No significant change in the life span of the flies exposed to 5 and 10  $\mu$ M of arecoline was observed compared to control flies (Fig. 6c;  $p < 0.05$ ). The flies exposed to 20, 40 and 80  $\mu$ M of arecoline showed a dose-dependent decrease in the survival rate compared to control flies (Fig. 6c;  $p < 0.05$ ). The flies emerged from the larvae exposed to 5 and 10  $\mu$ M of arecoline did not show any deformity in eyes. Each ommatidium was uniform and exhibits a normal hexagonal arrangement. There were regular polarised inter ommatidial bristles (Fig. 7, A1, A2). However the flies emerged from the larvae exposed to 20, 40 and 80  $\mu$ M of arecoline showed dose dependent deformities in the eyes. Ommatidia were round, inter-ommatidial bristles were disoriented and of variable length (Fig. 7, A3-A5). At highest dose the inter-ommatidial bristles were absent and the ommatidia

clearly exhibited the round texture (Fig. 7, A5). The hexagonal arrangement was lost in a dose dependent manner as compared to control (Fig. 7, c).

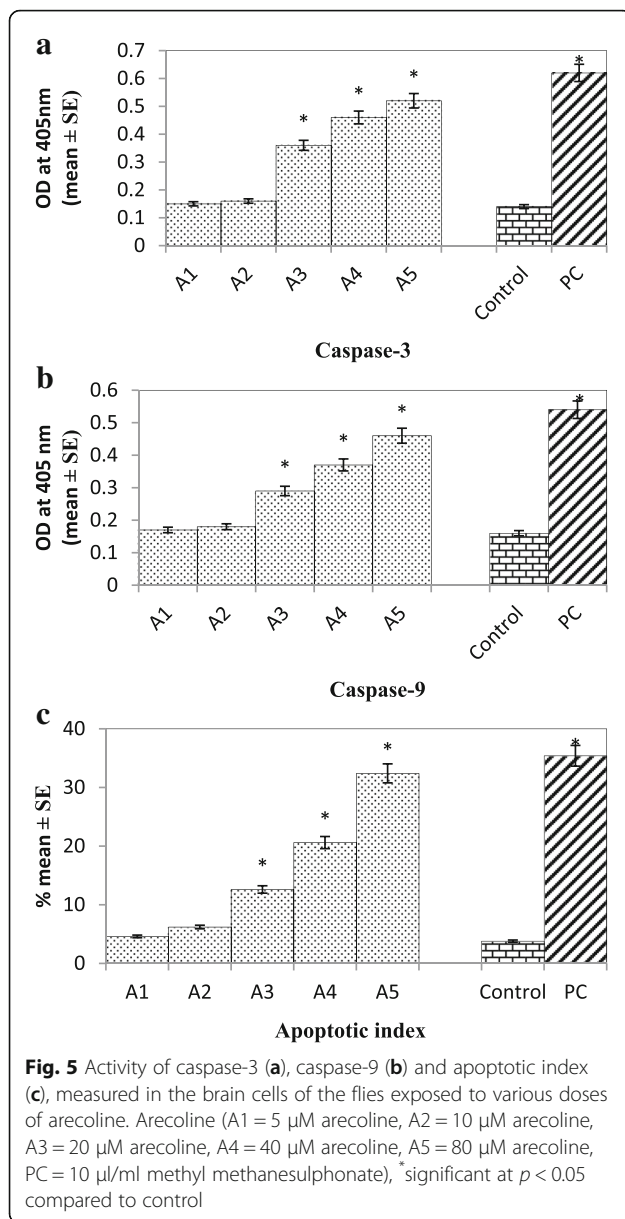
## Discussion

The results of the present study reveal that arecoline is toxic at 20, 40 and 80  $\mu$ M. In our present study, the exposure of flies to 20, 40 and 80  $\mu$ M showed a dose-dependent significant decrease in the climbing ability of flies. This may be due to the disruption of the coordination between the nervous system and muscular activity. Such impairment has also been observed in the flies exposed to pesticides, mainly organophosphates (Rajak, Dutta, Khatun, Mandi, & Roy, 2017) and 4-vinylcyclohexane (Abolaji et al., 2014). Similarly, the flies exposed to 20, 40 and 80  $\mu$ M of arecoline showed a dose-dependent reduction in the overall activity as is evident from the activity pattern. However, arecoline has also been reported to improve cognitive performance in Alzheimer's disease (Raffaele et al., 1996). Hence, it becomes important to explore its effects on the cognitive ability and the oxidative stress markers. Arecoline showed neurotoxic effects through increase in reactive



oxygen species (ROS). It also decrease antioxidants levels (Shih et al., 2010). The results obtained in our present study supports the results of Shih et al. (2010) as the flies exposed to higher doses of arecoline showed a decline in the climbing ability as well as in the overall activity of the flies. GSH is a most abundant thiol present in the eukaryotic cells. Arecoline react with free thiols such as GSH and *N*-acetyl-cysteine (NAC) to produce a common cysteine.  $\beta$ -alkylation adduct losing the methyl ester group present in neural aqueous solution, hence leading to the depletion of the GSH content (Boylard & Nery, 1969). Thus, depletion in the levels of GSH leads to cytotoxicity (Jeng et al., 1999). In our study, also the low levels of GSH were observed in the flies exposed to 20, 40 and 80  $\mu$ M of arecoline. GST takes part in adding -SH group to the phase I metabolised products for its proper excretion. The elevated levels of GST at higher doses of arecoline are a clear indication of the detoxification process. Reactive oxidants are generated continuously in biological systems via both endogenous processes and a result of exposure to external factors such as drugs, chemicals and pollutants (Hawkins et al.,

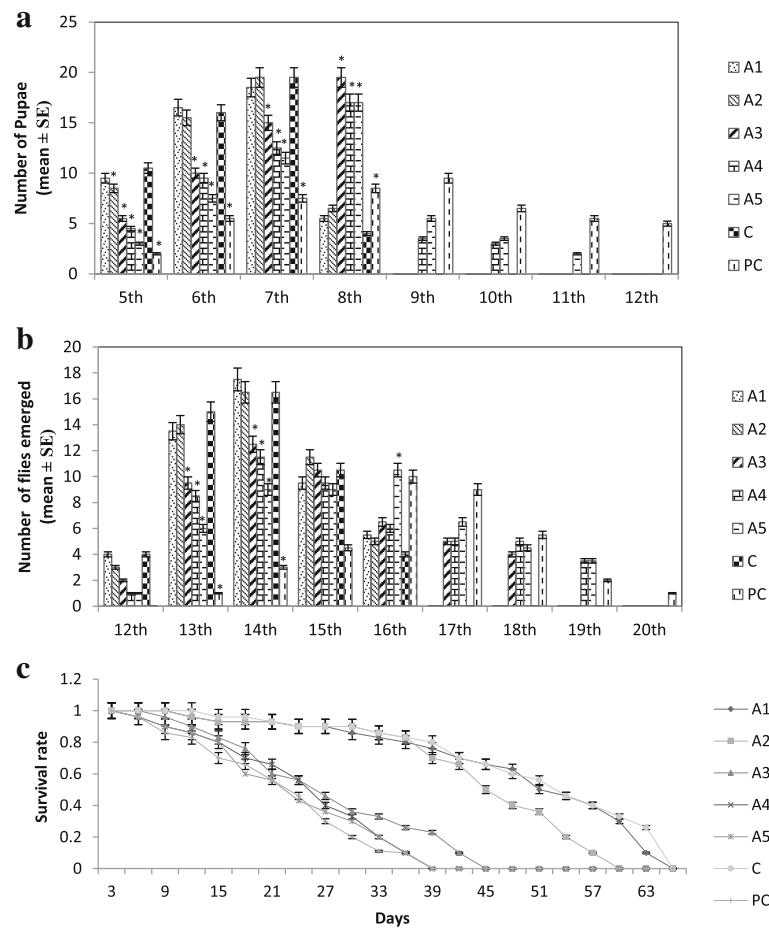
2009). Damage has been reported to occur on all components of biological systems due to high reactivity of many oxidants. Proteins and lipids are likely to be major targets due to their abundance in the living system. The metabolic activation of arecoline has been reported to generate reactive oxygen species (ROS) (Giri et al., 2006). This ROS may lead to the oxidation of protein and lipid membrane resulting in the carboxylation of protein and malondialdehyde (MDA) (a key product of LPO), respectively. Thus, increase in the PC content as well as MDA in the flies exposed to higher doses of arecoline supports the generation of ROS at higher doses of arecoline leading to oxidative stress. The activity of acetylcholinesterase (AChE) showed a decrease at the exposure of higher doses of arecoline. AChE hydrolyses the neurotransmitter acetylcholine to terminate the synaptic transmission. Any decrease in the AChE is a good marker for neural pathology. There are also reports of an increase in AChE activity upon the exposure of



arecoline (Jeng et al., 1999), but a decrease in AchE activity has also been reported in the brain ganglia of the *Drosophila* larvae that were temperature shocked and exposed to certain chemicals at higher concentrations (Mukhopadhyay, Saxena, & Chowdhuri, 2003). According to cholinergic hypothesis, reduction of acetylcholine is responsible for the development of Alzheimer's diseases (AD) (Kumar & Singh, 2015). Inhibition of acetylcholinesterase could be a better strategy in improving the cognitive defects among AD patients (Holden & Kelly, 2002). In our study, although the exposure of arecoline results in a significant dose-dependent decrease in the activity of acetylcholinesterase, the activity of flies as measured by *Drosophila* activity monitor (DAM) as well as the climbing ability showed a marked

decline. Monoamine oxidase is an iron containing enzyme that catalyses bioactive monoamines (Richardson, 1993). It has also been reported to maintain the neuron firing rate and is involved in the metabolism of dopamine. The metabolism of dopamine could also lead to the generation of ROS that could further enhance the neuronal damage. The reduction in the climbing ability as well as the reduced activity of flies exposed to higher doses of arecoline could be due to the enhanced metabolism of dopamine by MAO because the dopamine is a main component involved in neuromuscular coordination (Mazzoni, Shabbott, & Cortés, 2012). Although the mammalian system of cytochromes may be entirely different from the insect system, the basic function of the cytochromes may have similarity in the mode of action. Midgut tissues of insects have high microsomal oxidase activity (Giri et al., 2006). Caspases, a group of cysteine proteases, constitute the effector arm of the cell death machinery. The genome sequence of *D. melanogaster* predicts a total of seven caspases in flies (Kumar & Doumanis, 2000). A comparison of the amino acid sequences of fly and mammalian caspases reveals the evolutionary relationship between caspases. In a mammalian system, the release of cytochrome c into the cytoplasm leads to the decrease in the Bcl-2 activity. The decrease in the Bcl-2 activity enhanced proapoptotic Bax levels which finally initiates apoptosis by activating caspase-3 activity (Singla & Dhawan, 2013). However, there are conflicting reports of cytochrome C release from the mitochondria of *Drosophila* cells during apoptosis (Kumar & Doumanis, 2000). Due to the exposure to various toxicants, the increase in the activity of caspase-9 (Dronc) and caspase-3 (Drice) has been reported earlier (Singh & Chowdhuri, 2017; Danish et al., 2015; Pandey, Chandra, Chauhan, Narayan, & Chowdhuri, 2013). In our study, the exposure of arecoline results in an increase in the activity of caspase-3 and caspase-9. Further, acridine orange and ethidium bromide staining also revealed an increase in the number of apoptotic neuronal cells (orange coloured due to ethidium bromide stain) as compared to green neuronal cells (acridine orange stained). A dose-dependent significant increase in the apoptotic index also supports that the exposure of arecoline increases the number of apoptotic cells. The increase in the number of apoptotic cells is likely due to nuclear fragmentation, cell shrinkage and nuclear condensation in neuronal cells (Singla & Dhawan, 2013). A dose-dependent increase in the apoptotic cells was observed in the brain cells of flies exposed to 20, 40 and 80  $\mu$ M of arecoline.

In the present study, arecoline hydrobromide was used for the first time to study whether its exposure was associated with toxic effects using *D. melanogaster* as a model. It has been reported that about 75% of human disease-causing genes have functional homology in *D.*

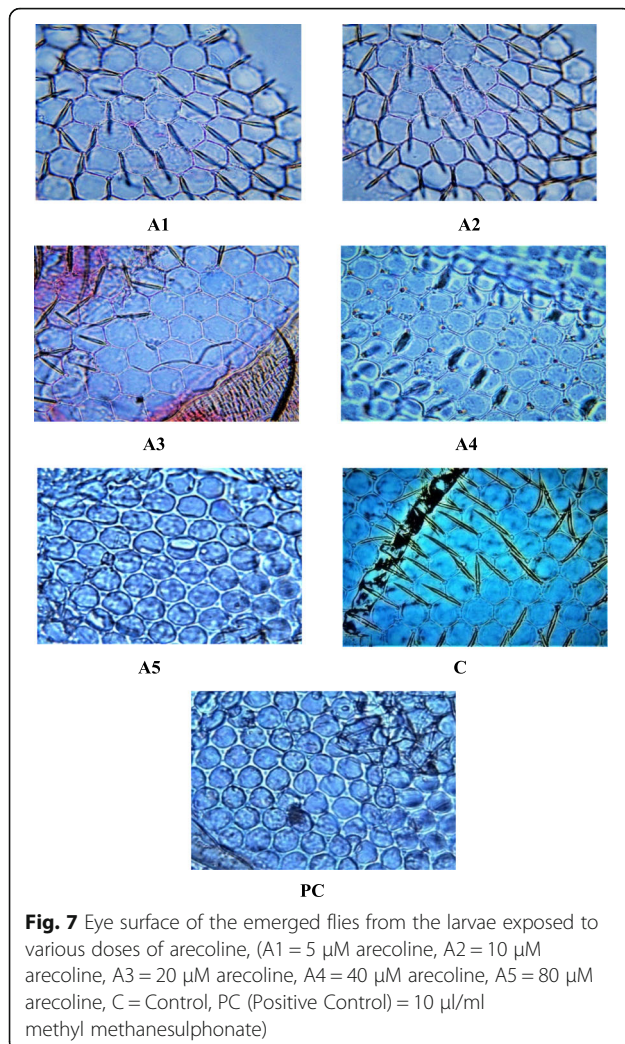


**Fig. 6** Effect on the pupation (a), emergence (b) and life span (c) of the flies after the exposure to various doses of arecoline (A1 = 5  $\mu$ M arecoline, A2 = 10  $\mu$ M arecoline, A3 = 20  $\mu$ M arecoline, A4 = 40  $\mu$ M arecoline, A5 = 80  $\mu$ M arecoline, PC = 10  $\mu$ l/ml methyl methanesulphonate), \*significant at  $p < 0.05$  compared to control

*melanogaster*, and therefore, it is being used as a model for toxicological studies (Rand, 2010). Further, we also investigated the effect of arecoline hydrobromide on pupation and emergence of flies. A significant dose-dependent increase in the duration of pupation was observed at 20, 40 and 80  $\mu$ M of arecoline and lower doses fail to express any dose-dependent increase in the duration of pupation. This delay in the pupation of larvae at higher doses may be due to the activation of some drug metabolising enzymes (Pai, 1983; Podder & Roy, 2015). Similarly, the emergence of the flies was also significantly delayed in a dose-dependent manner compared to the control flies. Similar results have been obtained by the treatments of cryolite and fluorinated insecticide (Podder & Roy, 2015. Gupta, Siddique, Saxena, & Chowdhuri, 2005). The delay in the emergence of flies is possible due to the activation of enzymes involved in the detoxification of the drug during the pupation. Besides these effects, arecoline has also been shown to induce various kinds of genetic damages, such as chromosomal

aberrations, sister chromatid exchange, DNA breaks and DNA protein cross-links on different target cells (Jeng, Chang, & Hahn, 2001). Arecoline also exhibits cytotoxicity and inhibits the growth of oral fibroblasts and keratinocytes (Chang et al., 1998; Tseng et al., 2012). The eyes of emerged flies were also analysed for the phenotypic abnormalities. Clear dose-dependent deformities were observed in the flies emerged from the exposure of larvae to 20, 40 and 80  $\mu$ M of arecoline. The flies showed the loss of hexagonal texture in a dose-dependent manner, bristles were also lost and the ommatidia were of round shape. The studies on insecticides and pesticides have revealed well-defined effects on the life cycle, hatching ability and the emergence of *D. melanogaster* (Gupta et al., 2005; Podder & Roy, 2015). The exposure of arecoline not only increased the duration of pupation but also delayed the emergence of flies. It also decreased the life span of flies exposed to 20, 40 and 80  $\mu$ M of arecoline in a dose-dependent manner.





**Fig. 7** Eye surface of the emerged flies from the larvae exposed to various doses of arecoline, (A1 = 5  $\mu$ M arecoline, A2 = 10  $\mu$ M arecoline, A3 = 20  $\mu$ M arecoline, A4 = 40  $\mu$ M arecoline, A5 = 80  $\mu$ M arecoline, C = Control, PC (Positive Control) = 10  $\mu$ l/ml methyl methanesulphonate)

This reduction is due to the neuronal damage which may be due to the exposure to arecoline. Betel chewers typically achieve salivary concentrations of arecoline ranging from 40 to 400  $\mu$ M and 90% of betel chewers have a residual level of at least 400 nM prior to chewing (Cox, Vickers, Ghu, & Zoellner, 2010; Papke, Horenstein, & Stokes, 2015). It has been suggested that arecoline would not be a good candidate for further development in the direction of therapeutic potential due to its strong muscarinic activity and potential involvement in the carcinogenesis (Papke et al., 2015). Arecoline at concentration above 50  $\mu$ M has been shown to cause neuronal injury by causing an increase in oxidative stress and suppression of the anti-oxidant system of the nervous system. A higher concentration may also lead to the cell death (Shih et al., 2010).

## Conclusion

It is concluded from the results obtained in our study that arecoline not only is a neurotoxic agent but also affects the life cycle parameters of the fly.

## Additional file

**Additional file 1:** Figure S1. a and b showing the average activity pattern and chi square periodogram, respectively for the flies exposed to 5  $\mu$ M of arecoline. Figure S2. a and b showing the average activity pattern and chi square periodogram, respectively for the flies exposed to 10  $\mu$ M of arecoline. Figure S3. a and b showing the average activity pattern and chi square periodogram, respectively for the flies exposed to 20  $\mu$ M of arecoline. Figure S4. a and b showing the average activity pattern and chi square periodogram, respectively for the flies exposed to 40  $\mu$ M of arecoline. Figure S5. a and b showing the average activity pattern and chi square periodogram, respectively for the flies exposed to 80  $\mu$ M of arecoline. Figure S6. a and b showing the average activity pattern and chi square periodogram, respectively for the control flies. Figure S7. a and b show the average activity pattern and chi square periodogram, respectively for the flies exposed to 10  $\mu$ l/ml of methyl methanesulphonate. (DOCX 627 kb)

## Abbreviations

AChE: Acetylcholinesterase; AI: Apoptotic index; BHT: Butyl-hydroxy toluene; CDNB: 1-Chloro-2,4, dinitrobenzene; DAM: *Drosophila* activity monitor; DNA: Deoxyribonucleic acid; DTNB: 5,5'-Dithiobis-2-nitrobenzoic acid; GSH: Glutathione; GST: Glutathione-S-transferase; HCl: Hydrochloric acid; LPO: Lipid peroxidation assay; MAO: Monoamine oxidase; MDA: Malondialdehyde; NAC: N-Acetyl-cysteine; PBS: Phosphate buffer solution; PCC: Protein carbonyl content; ROS: Reactive oxygen species; TCA: Trichloroacetic acid

## Acknowledgements

We are thankful to the Chairman of the Department of Zoology for providing the Laboratory facilities and UGC for providing non-net fellowship to Ms. Barkha Shakya, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, Uttar Pradesh, India. We are also thankful to Dr. Smita Jyoti, Rahul and Falaq Naz for their support and technical help.

## Funding

None

## Availability of data and materials

Not applicable

## Authors' contributions

YHS wrote the paper and performed experiments. BS performed the experiments and analysed data. Both authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not required

## Consent for publication

Not applicable

## Competing interests

The authors declare that they have no competing interests.

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Received: 31 May 2018 Accepted: 21 October 2018

Published online: 14 November 2018

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