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ORIGINAL PAPER

Ilex paraguariensis Attenuates Changes in Mortality, Behavioral and Biochemical Parameters Associated to Methyl Malonate or Malonate Exposure in *Drosophila melanogaster*

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Abstract

Methylmalonic acidemia is a genetic disease characterized by accumulation of organic acids, such as methylmalonic (MMA) and malonic (MA) acids. Considering that the accumulation of MMA and MA causes several damages due to oxidative stress, antioxidants are thought to play a pivotal role in preventing deleterious effects associated with exposure to such compounds. *Ilex paraguariensis* (IP) was used here to test the hypothesis that supplementation with the aqueous extract of this plant could exert protective effect against MMA or MA induced mortality, behavioral and/or biochemical changes in *Drosophila melanogaster* (DM). Initially, a curve time- and dose–response to MMA (1–10 mM), MA (1–10 mM) and IP (63–500 µM) was performed. Thereafter, flies were concomitantly exposed to MA (5 mM), MMA (5 mM) and/or IP (250 µg/mL) during 15 days for survival assay, and for 48 hs to MA (1 or 5 mM), MMA (1 or 5 mM) and/or IP (250 µg/mL) for subsequent investigations. Both MMA and MA exposure resulted in higher incidence of mortality, a worse performance in the negative geotaxis assay and increased locomotion in open-field test as compared with control group. Furthermore, a marked increase in non-protein thiol (NPSH) and in thiobarbituric acid reactive substances (TBARS) levels, decrease in superoxide dismutase (SOD), catalase and acetylcholinesterase (AChE) activities, and decrease in MTT and resazurin reduction were noted in MMA or MA treated groups. IP treatment offered significant protection against all alterations associated to MMA or MA exposure. This study confirm the hypothesis that supplementation with IP offers protection against changes associated to MMA or MA exposure in DM, due, at least in part, to its antioxidant effect.

Keywords Methylmalonic acid · Malonic acid · *Ilex paraguariensis* · Drosophila · Oxidative stress

Introduction

Methylmalonic acidemia, a group of autosomal recessive genetic disorders, is generally caused by mutations in the MUT gene (609058). In the process, a series of enzymes and bimolecules are affected and leads to the formation of methylmalonic acid (MMA) [1, 2]. Evidences suggests that methylmalonic acidemia is associated with mitochondrial dysfunction, energy disturbance, hinders in ATP production and diminishes various enzymatic activities like NADH:cytochrome c oxireductase and succinate:cytochrome c oxireductase [2–5].

Similarly, it is important to note that MMA is not the only major toxic metabolite in methylmalonic acidemia. In fact, patients with methylmalonic acidemia also presented elevated levels of malonic acid (MA) [2, 6]. Involvement of MA has

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been reported in mitochondrial dysfunction, superoxide radical generation, secondary excitotoxicity mediated by Ca^{2+} influx and reactive species-induced apoptosis [7, 8].

In the stated context, antioxidants are thought to play a pivotal role in preventing MMA and MA oxidative activity. Accordingly, there is a growing interest in the use of natural antioxidants, including polyphenols found in medicinal and dietary plants that might prevent damage associated with such conditions [9–14].

In view of the above, *Ilex paraguariensis* (IP), a plant from the aquifoliaceae family, possess antioxidant, anti-inflammatory, antimutagenic, antiobesity, antidiabetic, diuretic, antifungal, stimulant and antidepressant-like activities [15–17]. Biochemical characterization of IP, revealed the presence of a diverse range of chemical compounds like polyphenols, methylxanthines, caffeoyl derivatives, saponins and minerals, which may be responsible for its biological efficacy [11, 17]. Mechanistically, IP acts via scavenging of reactive species (RS) and modulating the expression of genes and antioxidant enzymes [17]. However, the modulation of oxidative stress in MMA or MA-induced toxicity by aqueous extract of IP remains to be explored.

Considering the necessity to counteract oxidative stress damage associated to methylmalonic acidemia (a condition in which MMA or MA accumulation occurs), the development of simple animal models is profoundly needed. Accordingly, invertebrate organisms such as *Drosophila melanogaster* (DM) emerged as useful animal model for the study of molecular mechanisms involved in human neuronal dysfunction [18, 19]. It can be attributed to the simplicity of its nervous system, rapid life cycle, easy genetic manipulation and sequenced genome.

The present study was designed to not only explore the involvement of oxidative stress in MMA as well in MA-induced toxicity but also to test the hypothesis that supplementation with the aqueous extract of IP could exert protective effect against changes associated to both agents in *Drosophila melanogaster*. In this context, we designed for the first time a short-term dietary regimen model, where DM was exposed to different concentrations of MMA, MA and/or IP. Thereafter, we evaluated several behavioral and biochemical determinations such as mortality and locomotor deficit, superoxide dismutase (SOD), catalase, acetylcholinesterase (AChE) activities, MTT and resazurin reduction capacity, non protein thiol (NPSH) contents and TBARS levels in head and entire body homogenates. Using HPLC, we also characterized the IP extract, which may give us significant insights about its major phytochemical constituents.

Materials and Methods

Chemicals

Malonic Acid (MA), Methylmalonic acid (MMA), Thiobarbituric acid (TBA), acetylthiocholine iodide, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), quercetin, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 2,4-dinitrophenyl hydrazine (DNPH), gallic acid, catechin, chlorogenic acid, caffeic acid, caffeine, epigallocatechin, rutin, quercetin and kaempferol were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and acetic acid were purchased from Merck (Darmstadt, Germany). All the other chemicals were commercial products of the highest purity grade available.

Plant Material and Extract Preparation

Ilex paraguariensis marketed and distributed by Ponche Verde Industrial do Mate Ltda. – Arvorezinha/RS/Brazil was obtained from local commercial source. Accordingly, an independent batch (#07) was randomly purchased and used in this study. The aqueous extract was prepared as infusion immediately before use as described previously [11]. Accordingly, 1 g of the IP sample was placed in 10 mL (in a proportion of 10 g/100 mL) of distilled hot water (96 °C) for 10 min and cooled at room temperature (20–25 °C). Thereafter, extracts were diluted in distilled water for subsequent use.

Chromatographic Analyses

High performance liquid chromatography (HPLC–DAD) was performed following the method described previously [20], with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

In short, IP aqueous extract at a concentration of 10 mg/mL were injected by means of a model SIL-20A Shimadzu Auto sampler. Separations were carried out using Phenomenex C_{18} column (4.6 mm × 250 mm × 5 µm particle size). The sample and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. The mobile phase was water with 0.5% acetic acid (v/v) (solvent A) and HPLC grade methanol (solvent B) at a flow rate of 0.6 mL/min and injection volume 40 µL. The composition gradient was: 5% solvent B reaching 15% at 10 min; 30% solvent B at 25 min, 65% solvent B at 40 min and 98% solvent B at 45 min, followed by 50 min at

isocratic elution until 55 min. At 60 min the gradient reached the initial conditions again. In order to verify if plant antioxidants and the organic acids (MA or MMA) are making complexes in the media, HPLC fingerprinting of the aqueous extract of IP in the presence of MMA (5 mM) or MA (5 mM) was also carried out (Data not shown).

Stock solutions of standards references were prepared in the methanol at a concentration range of 0.025–0.400 mg/mL. Quantifications were carried out by integration of the peaks using the external standard method, at 254 nm for gallic acid; 280 nm for catechin, epigallocatechin and caffeine; 327 nm for chlorogenic acid and caffeic acid; and 366 for quercetin, kaempferol and rutin. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 700 nm). All chromatography operations were carried out at ambient temperature and in triplicate.

Drosophila Culture Condition

DM wild-type (Harwich strain) were obtained from the National Center species, Bowling Green, Ohio, USA. The flies were maintained and reared on corn meal medium (1% w/v brewer's yeast, 1% w/v sucrose, 1% w/v powdered milk, 1% w/v agar, and 0.08% v/w methylparaben) at constant temperature and humidity (22 ± 1 °C; 60% relative humidity, respectively) under 12 h dark/light cycle conditions. Flies were maintained in this medium during developmental period until treatment (1–3 days old). Thereafter flies were transferred to "treatment medium" containing agar, sucrose 1% and the respective treatment (see details on 2.5. MMA or MA exposure and treatment with IP section). All the experiments were carried out with the same fly strain.

MMA or MA Exposure and Treatment with IP

Fifty flies (1–3 days old; both genders) were exposed to each different MA or MMA concentrations (1–10 mM) and evaluated each 24 h for the longevity, until day 15. The medium were replaced each 48 h. This protocol was repeated at least ten times, and was carried out at room temperature and in quadruplicate. Of particular importance, 48 h of exposure at 5 mM of both organic acids were chosen for the biochemical tests since in these conditions, mortality was significantly enhanced by both MMA and MA treatments.

Fifty flies (1–3 days old) were also exposed to each different extract IP concentrations (63–500 µg/mL) and evaluated each 24 h for the longevity, until day 15. The medium were replaced each 48 h. This protocol was repeated at least four times, and was carried out at room temperature and in quadruplicate. Of note, IP (250 µg/mL) was chosen to subsequent experiments once that no mortality and no significant behavioral changes were found at this concentration, and also because it was the lowest concentration capable of preventing the behavioral changes associated to both MMA (5 mM) or MA (5 mM) exposure during 48hs (Data not Shown).

Fifty flies (1–3 days old; both genders) were also exposed to MA (5 mM) or MMA (5 mM) and/or IP (250 µg/mL) and evaluated each 24 h for the longevity, until day 15 (the medium were also replaced each 48 h). For subsequent set of experiments, DM (both genders) of 1–3 days old were divided as follow: (1) control (no treatment) (2) IP 250 µg/mL (3) MA (1 or 5 mM) (4) MA (1 or 5 mM) + IP (250 µg/mL) (5) MMA (1 or 5 mM) and (6) MMA (1 or 5 mM) + IP (250 µg/mL). Flies were exposed a diet containing MA, MMA and/or IP for 48 h for mortality, behavioral and biochemical assays. This treatment protocol was independently repeated, at least 4 times and in triplicate, to each assay.

In Vivo Assays

Negative Geotaxis

Locomotor ability of flies was performed with a negative geotaxis assay as described previously [21]. In short, flies (15 per group from each independent experiment) were sorted under a brief ice anesthesia and placed in a vertical glass column (length: 10 cm, diameter: 1.5 cm/ 10 flies each). After the recovery from cold exposure (approximately 15 min) the flies were gently tapped to the bottom of the column. The flies that reached the top of the column and the flies that remained at the bottom were counted separately during 6 s. The scores represent the mean of the numbers of flies at the top (ntop) as percentage of the total number of flies (ntot). The values represent the mean of five independent experiments. The results are expressed as percentage of flies that escaped beyond a minimum distance of 6 cm in 6 s during four independent experiments. Data are expressed as a % of flies that reach the top in 6 s.

Open-Field Test

Open-field test was performed according to the method described previously [22]. Accordingly, three flies from each group were kept in an arena divided by squares (1 cm × 1 cm) measuring 9 cm of diameter, which can be covered by petri dish. The fly's activity were recorded with a video camera and the number of squares crossed by each single fly, during a given time-window (30 s), was analyzed. The values represent the mean of five independent experiments.

Ex Vivo Assays

Homogenate Preparation

At the end of the treatment period (48 h), flies were anesthetized in ice. Heads were separated from the body using a sharp blade/cutter. Afterward, heads and bodies were homogenized in 0.9% NaCl solution, 1:5 (flies/volume; i.e. 50 heads or 50 bodies to 250 µL). The homogenates were centrifuged at 2500×*g* for 10 min at 4 °C, and the supernatant was used for biochemical assays. All biochemical determinations were performed in duplicates in 3–5 independent experiments.

Determination of TBARS

The lipid peroxidation end products were quantified as thiobarbituric acid reactive substances (TBARS) according to established previously [23] with some modifications. In brief, an aliquot of homogenate (100 µL) was incubated per 1 h at 37 °C in a water bath. Thereafter samples were incubated at 100 °C for 120 min in 200 µL of a medium containing equal volumes of trichloroacetic acid (10%, w/v) and thiobarbituric acid (0.6%, w/v) in 0.1 M HCl for color development. After boiling step, 20 µL 8.1% SDS was added. The reaction product was determined at 532 nm and the results were expressed as % of control after correction by the protein content.

Determination of Non-protein Thiol (NPSH)

The NPSH level was determined in the control and treated flies according to the method previously described [24]. For NPSH assay, 70 µL homogenates were precipitated with 70 µL TCA 10% (1:1 v:v) followed by centrifugation at 3000×*g* for 10 min at 4 °C. The reaction system was made up of 940 µL of 1 M dibasic potassium phosphate buffer, 50 µL of sample, and 10 µL of 5 mM DTNB. At the end of 10 min incubation at room temperature (25 °C), the absorbance was measured at 412 nm. The results were expressed as % of control after correction by the protein content.

Activity of Superoxide Dismutase (SOD)

The assay consists in the inhibition of superoxide driven oxidation of quercetin by SOD [25]. Briefly, the reaction medium contained 200 µL of buffer (160 µL 0.02 M phosphate buffer/0.08 mM EDTA (pH 7.4) mixed with 200 µL TEMED), 10 µL of 0.05 µM quercetin and 40 µL of sample (1:5 dilution). The reaction was analyzed in time 0 and 20 min at 406 nm. The results expressed as % of control after correction by the protein content.

Activity of Catalase

The catalase activity was measured spectrophotometrically according to the method of [26], by monitoring the disappearance of H_2O_2. Briefly, the reaction medium contained 960 µL of 0.05 M phosphate buffer (pH 7.0), 20 µL of 1 M H_2O_2, and 20 µL of sample (1:5 dilution). The reaction was analyzed for 2 min (15 s intervals) at 240 nm. Results were expressed as percentage of control after correction by the protein content.

Activity of Acetylcholinesterase (AChE)

AChE activity was determined according previously determined [26]. Briefly, the assay medium consisted of 70 µL of distilled water, 100 µL of system (0.5 M potassium phosphate buffer (pH 7.4) with 10 mM DTNB), 10 µL of sample and 20 µL of 8 mM acetylthiocholine as substrate. The degradation of acetylthiocholine iodide was analyzed for 2 min (30 s intervals) at 412 nm. The results were expressed as % of control after correction by the protein content.

MTT and Resazurin Reduction Assays

Dehydrogenases activity was measured by two different methods. Firstly, its activity was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay described previously [22]. Briefly, an aliquot of sample (50 µL) was incubated during 30 min (37 °C) with 20 µL MTT and 5 mM succinate. Thereafter, reaction was stopped by addition of 200 µL DMSO and incubated per 30 min (37 °C) to dissolve formazan salts. Then, the samples were centrifuged to 2000 rpm for 5 min, and the absorbance was monitored at 630 nm and 545 nm. To calculate the MTT reduction was used: $|545|\text{nm} - |630|\text{nm} = \text{Result} \times 100$. The results were expressed as % of control after correction by the protein content.

The second method used was the resazurin reduction assay which was performed as previously described [27]. In short, 20 flies were homogenized in 1 mL 20 mM Tris buffer (pH 7.0) and centrifuged at 2000 rpm for 10 min at 4 °C. After that, the supernatant was incubated in ELISA plates with 20 mM buffer Tris (pH 7.0) and resazurin for two hours. Fluorescence was recorded using EnsPireR multimode plate reader (Perkin Elmer, USA) at $_{ex}$579 nm–$_{em}$584 nm.

Protein Determination

The protein content was determined as described previously using bovine serum albumin (BSA) as standard [28].

Statistical Analysis

Behavior parameters were analyzed using Kruskal–Wallis (kw) followed by Dunn's multiple comparisons test when appropriate, once Kolmogorov–Smirnov's test indicated the absence of homogeneity of variance. Survival data were evaluated using Kaplan–Meier analysis and Gehan–Breslow–Wilcoxon test. Other data were analyzed by one-way ANOVA followed by Tukey's multiple range tests when appropriate. Differences between groups were considered significant when $P < 0.05$. Data of non-parametric analysis are represented as box and whisker plots (min to max); and data of parametric analysis as means and S.E.M. Graphics were created using Graph Pad Prisma 6.0.

Results

Phytochemical Composition

HPLC fingerprinting of the aqueous extract of IP revealed the presence of a series of major and minor components. We have confirmed gallic acid ($t_R = 9.83$ min, peak 1), catechin ($t_R = 15.07$ min, peak 2), chlorogenic acid ($t_R = 24.01$ min, peak 3), caffeic acid ($t_R = 26.54$ min, peak 4), caffeine ($t_R = 28.17$ min, peak 5), epigallocatechin ($t_R = 34.20$ min, peak 6), rutin ($t_R = 38.91$ min, peak 7), quercetin ($t_R = 49.76$ min, peak 8) and kaempferol ($t_R = 60.11$ min, peak 9) as shown in Fig. 1 and Table 1.

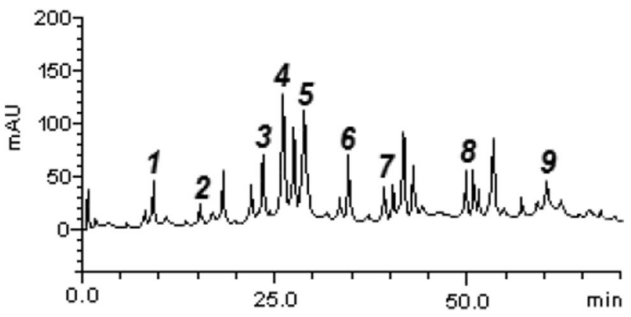

Fig. 1 Representative high performance liquid chromatography profile of IP extract. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), caffeine (peak 5), epigallocatechin (peak 6), rutin (peak 7), quercetin (peak 8) and kaempferol (peak 9)

Table 1 Components of *Ilex paraguariensis*

Compounds	*I. paraguariensis* mg/g
Gallic acid	1.43 ± 0.02
Catechin	0.51 ± 0.05
Chlorogenic acid	2.26 ± 0.01
Caffeic acid	5.57 ± 0.01
Caffeine	5.08 ± 0.02
Epigalloca.techin	2.39 ± 0.03
Rutin	1.17 ± 0.01
Quercetin	1.42 ± 0.04
Kaempferol	1.09 ± 0.01

Results are expressed as mean ± standard deviations (SD) of three determinations

Effect of Treatments on Behavioral Profile in DM

Effect of IP Extract on MMA or MA Caused Mortality

Gehan–Breslow–Wilcoxon test from Kaplan–Meier survival curve revealed a significant difference among groups from MMA (Fig. 2a), MA (Fig. 2b), or IP (Fig. 2c) and controls. A significant effect of IP against MMA (Fig. 2d) or MA (Fig. 2e) toxicity was also found.

The survival rate in the groups exposed to MMA for 15 consecutive days was as follow: Control (34%), MMA 1 mM (5.5%), MMA 5 mM (3.62%) and MMA 10 mM (2.5%). Accordingly, MMA at all concentrations significantly increase fly mortality as compared to control. In turn, the median survival was as follow (in days): Control (11.5 days), MMA 1 mM (8 days), MMA 5 mM (6.5 days) and MMA 10 mM (7 days) (Fig. 2a).

The survival rate in the groups exposed to MA for 15 consecutive days was as follow: Control (33.5%), MA 1 mM (0%), MA 5 mM (0%) and MA 10 mM (0%). Accordingly, MA at all concentrations significantly increase fly mortality as compared to control. In turn, the median survival was as follow (in days): Control (11.4 days), MA 1 mM (6 days), MA 5 mM (6 days) and MA 10 mM (6 days) (Fig. 2b).

The survival rate in the groups exposed to IP for 15 consecutive days was as follow: Control (34.7%), IP 63 µg/mL (48.75%), IP 125 µg/mL (52.4%), IP 250 µg/mL (54.3%) and IP 500 µg/mL (55.5%). Accordingly, IP at 250 and 500 µg/mL significantly decrease spontaneous fly mortality. In turn, the median survival was as follow (in days): Control (11.6 days), IP 63 µg/mL (15 days), IP 125 µg/mL (15 days), IP 250 µg/mL (> 15 days) and IP 500 µg/mL (> 15 days) (Fig. 2c).

IP (250 µg/mL) protected against MMA (5 mM) induced fly mortality (Fig. 2d). The survival rate in the groups exposed to MMA and/or IP for 15 consecutive days was as

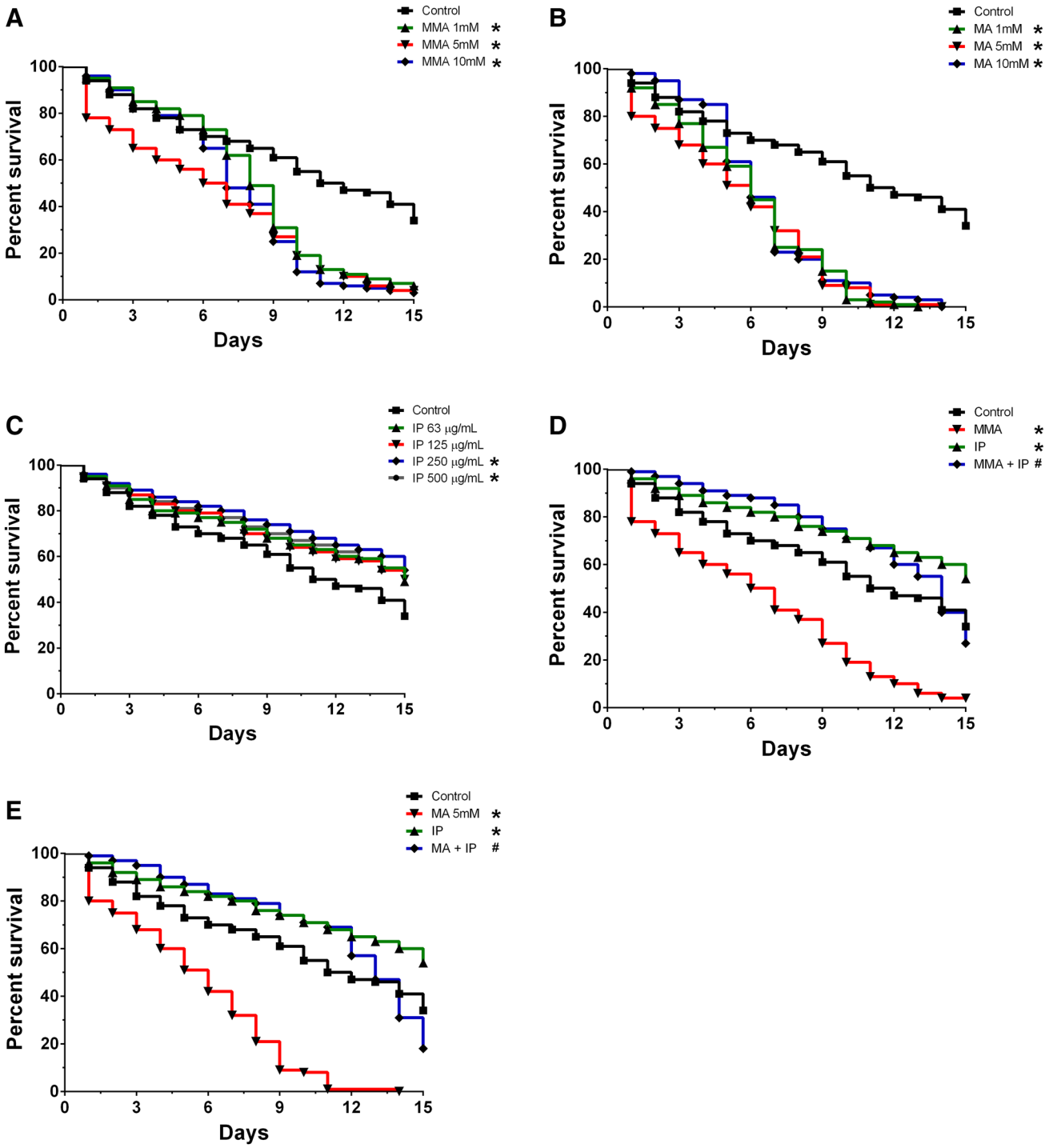

Fig. 2 **a** Effect of MMA (1–10 mM) on the survival rate of treated flies. **b** Effect of MA (1–10 mM) on the survival rate of treated flies. **c** Effect of IP (63–500 µg/mL) on the survival rate of treated flies. **d** Effect of IP (250 µg/mL) and/or MMA (5 mM) on the survival rate of treated flies. **e** Effect of IP (250 µg/mL) and/or MA (5 mM) on the survival rate of treated flies. Data were collected every 24 h for each group during 15 days and analyzed using a Kaplan–Meier survival plot. The total number of flies (200 per group) represents the sum of four independent experiments. The numbers of dead flies are represented as % of control. Statistical differences among groups were calculated by log-rank (Gehan–Breslow–Wilcoxon) test (*) represent significant difference of the respective curve as compared with the curve of control group. (#) represents significant difference between curves of MA vs. MA + IP or significant difference between curves of MMA vs. MMA + IP. Values are expressed as percentage

follow: Control (33.9%), MMA (2.65%), IP (50.06%) and MMA + IP (27%). In turn, the median survival was as follow (in days): Control (11.5 days), MMA (6.5 days), IP (> 15 days) and MMA + IP (14 days).

IP (250 µg/mL) also protected against MA (5 mM) induced fly mortality (Fig. 2e). The survival rate in the groups exposed to MA and/or IP for 15 consecutive days was as follow: Control (33.9%), MA (0%), IP (50.06%) and MA + IP (17.67%). In turn, the median survival was as follow (in days): Control (11.7 days), MA (6 days), IP (> 15 days) and MA + IP (13 days).

Effect of IP Extract on MMA or MA Induced Alterations in Negative Geotaxis and Open-Field Tests

The climbing behavior of flies in MMA and MA treated groups was significantly lower as compared to control group both at 1 mM (Fig. 3a; a condition in which no lethal toxicity was found) and at 5 mM (Fig. 3c). The effect of either MMA or MA was completely abolished by IP treatment. Additionally, no significant difference was observed in IP and control group.

In open-field test, Dunn's multiple comparisons test revealed that the flies treated with MMA and MA presented a significant increase in the number of crossings when compared to the control group both at 1 mM (Fig. 3b; a condition in which no lethal toxicity was found) and at 5 mM (Fig. 3d). Likewise on negative geotaxis test, the effect of MMA and MA was completely abolished by IP treatment. Statistically no significant difference in IP and control group was noted.

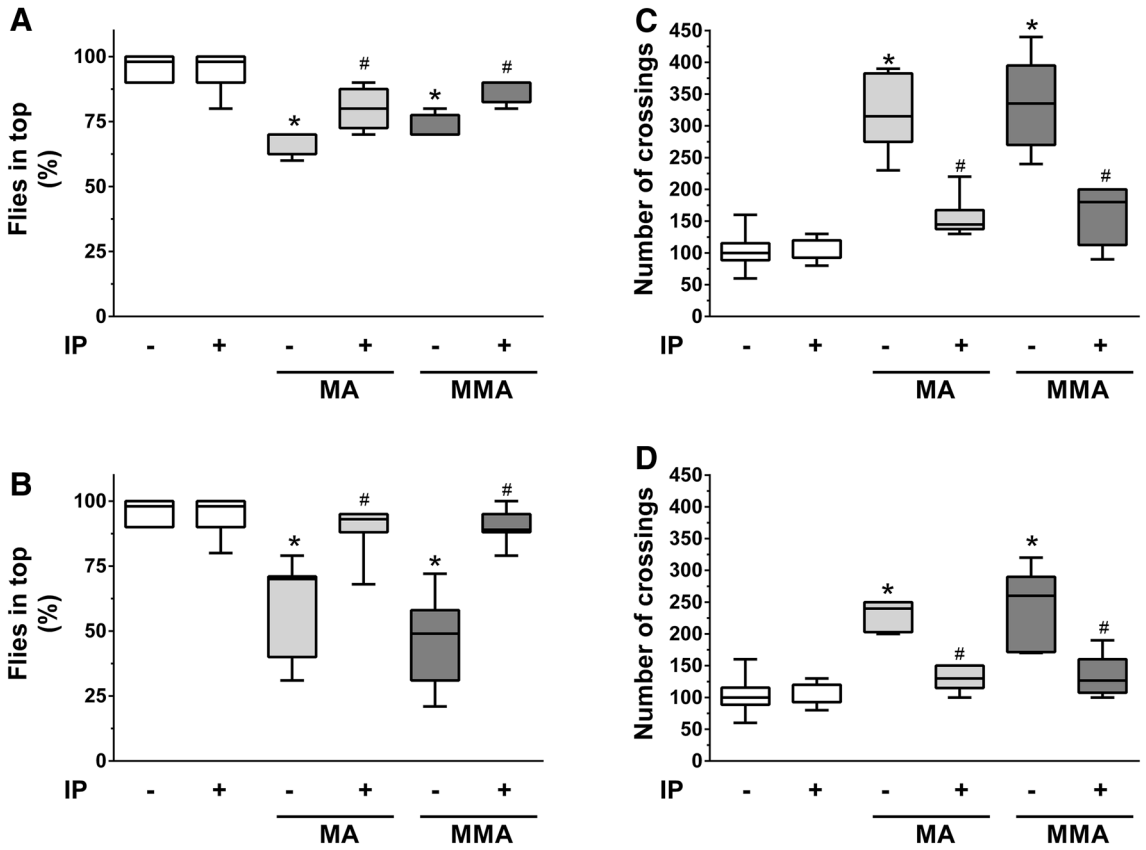

Fig. 3 **a** Effect of IP (250 µM/mL), MMA (1 mM) and MA (1 mM); and **b** Effect of IP (250 µM/mL), MMA (5 mM) and MA (5 mM) on geotaxis response (climbing). **c** Effect of IP (250 µM/mL), MMA (1 mM) and MA (1 mM); and **d** Effect of IP (250 µM/mL), MMA (5 mM) and MA (5 mM) on locomotor activity evaluated by open-field test. Data were collected after 48 h of exposure. The total number of flies (75 per group in negative geotaxis and 15 per group in open-field) represents the sum of five independent experiments. Values are expressed as median and range (interquartile interval). (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP (Kruskal–Wallis test followed Dunn' multiple comparisons test, $P < 0.05$)

Effect of Treatments on Ex Vivo Biochemical Analyses in DM

Effect of IP Extract on TBARS and NPSH Levels in MA or MMA Treated Flies

Tukey's multiple comparisons test revealed that treatment with MMA and MA caused a significant increase in TBARS levels and NPSH content as compared with control group. In fact, the increase was observed both in the head (Figs. 4a, 5a) and body regions (Figs. 4b, 5b), respectively. Of particular importance, the effect of MMA and MA on TBARS levels was normalized by IP treatment both in head and in body (Fig. 4a, b) regions, respectively. Similarly, the NPSH levels were also normalized in head and body (Fig. 5a, b) regions, respectively. Finally, there was no significant difference in

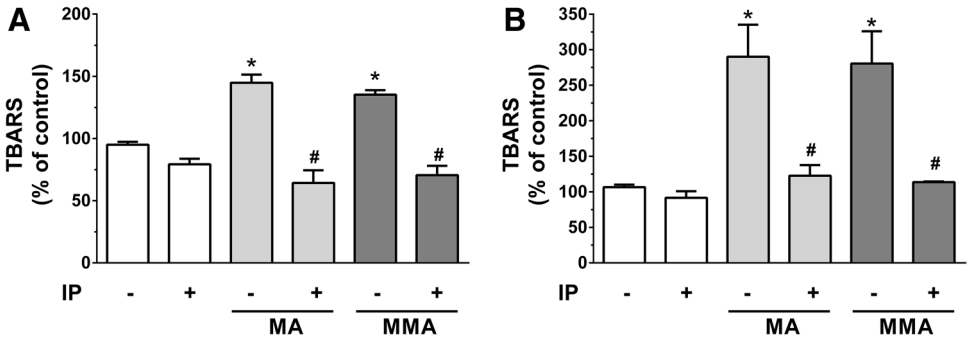

Fig. 4 Effect of IP on TBARS in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 4)

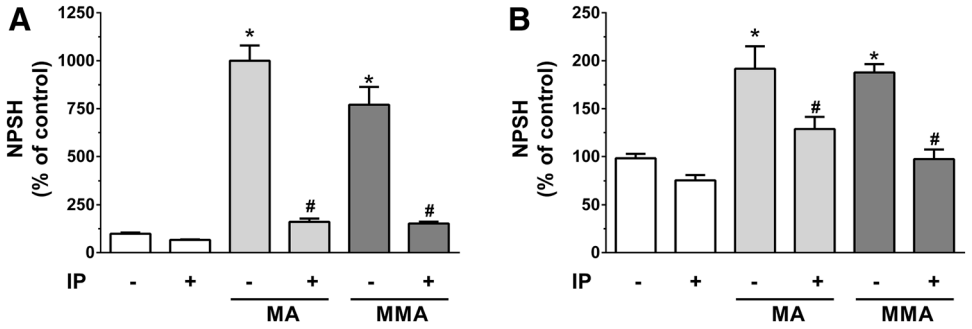

Fig. 5 Effect of IP on NPSH content in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 5)

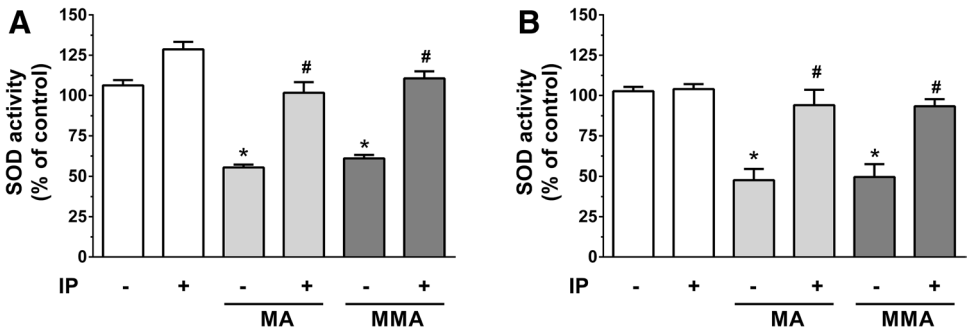

Fig. 6 Effect of IP on SOD activity in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 4)

TBARS (Fig. 4) and NPSH (Fig. 5) levels, in IP (alone) and control group.

Effect of IP Extract on SOD, Catalase and AChE Activities in MA or MMA Treated Flies

Treatment with MMA and MA caused a significant decrease in SOD, catalase and AChE activities, both in head (Figs. 6a, 7a, 8a) and body (Figs. 6b, 7b, 8b) regions, respectively. Treatment with IP normalized SOD (Fig. 6a, b), catalase (Fig. 7a, b), and AChE activities (Fig. 8a, b) in both MMA and MA treated groups. Statistically, no significant alteration was noted in SOD (Fig. 6), catalase (Fig. 7) or AChE (Fig. 8) activities in IP (alone) and control group.

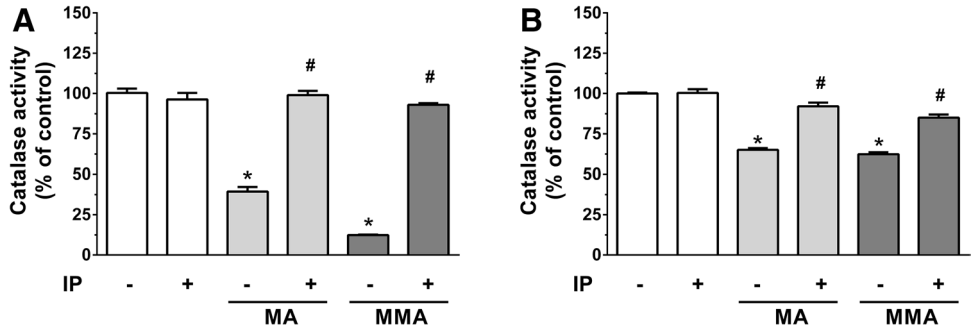

Fig. 7 Effect of IP on catalase activity in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 4)

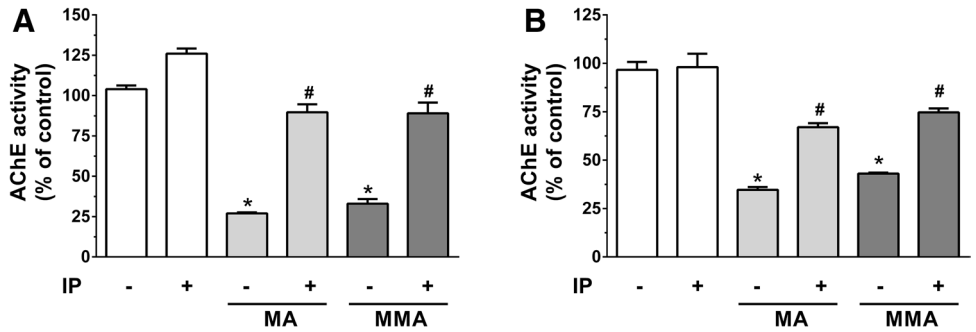

Fig. 8 Effect of IP on AChE activity in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 5)

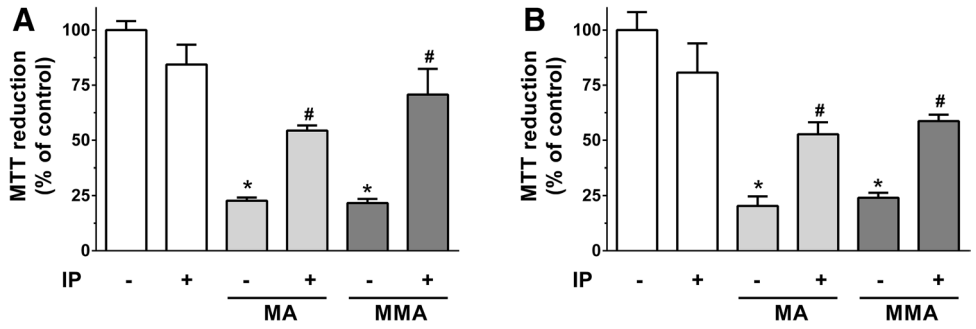

Fig. 9 Effect of IP on MTT reduction in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 5)

Effect of IP Extract on MTT and Resazurin Reduction Assays in MA or MMA Treated Flies

The Fig. 9 represents the MTT reduction assay. Exposure to MMA and MA caused a significant decrease in MTT reduction, both in head (Fig. 9a) and body (Fig. 9b) respectively, which was partially normalized by IP treatment. No significant change in MTT reduction assay was noted in IP (alone) and control group (Fig. 9). The Fig. 10 represents the resazurin reduction assay. Exposure to MMA caused a significant decrease in resazurin reduction in head (Fig. 10a), whereas MA exposure resulted in a significant decrease in resazurin reduction in body (Fig. 10b). Treatment with IP normalized resazurin reduction in both MMA (Fig. 10a) and MA (Fig. 10b) treated groups. No significant change in resazurin reduction assay was noted in IP (alone) and control group (Fig. 10).

Discussion

Contributing to the scientific literature, this is the first report to not only describe the adverse effects of MMA and MA but also the protective efficacy of the aqueous extract of IP in DM model. One of the highlights in present study was that treatment with MMA and MA caused a time dependent increase in the cumulative number of dead flies. The MMA or MA induced mortality could be attributed to the cytotoxic effect of both drugs which has been previously reported. Involvement of oxidative stress, mitochondrial dysfunction, neuroinflammation and apoptotic processes are some of the reported mechanisms of their (MMA and MA) induced toxicities in mammals [29, 30].

Locomotor activity is a complex behavior that may be influenced by different neural systems in flies [31]. Of particular importance, it may be altered by toxicants [11, 12, 22, 26]. Here, in order to accurately evaluate behavioral changes, we choose either a condition in which the mortality was not significantly enhanced by MMA or MA treatment (i.e. at MMA or MA 1 mM; 48 h) and a condition in which the mortality was significantly enhanced by MMA or MA treatment (i.e. at MMA or MA 5 mM; 48 h). Accordingly, we found that either MMA or MA—at both concentrations—significantly impaired the performance of flies in the negative geotaxis assay (Fig. 3a, b). Furthermore, MMA and MA (either at 1 and 5 mM) significantly increased the number of crossings in the open-field test (Fig. 3c, d). Although, we cannot directly elaborate the mechanism (s) by which MMA and MA lead to changes in locomotor performance, this is the first report where we described such alterations in an invertebrate model (DM). Notably, nicotine-exposed flies presented reduced climbing activity that was associated to locomotor hyperactivity [32–35]. Of note, in the negative geotaxis, the nicotine effect was found to be mediated by dopaminergic system and cyclic adenosine monophosphate (cAMP)/cAMP-response element binding (CREB) pathway [32, 34]. In turn, dopaminergic neurons and a protein in the decapping complex (decapping protein 2) were found to play crucial roles in mediating nicotine-induced locomotor hyperactivity [33, 35]. So, considering that different pathways seems to be involved in the control of locomotor activity in flies, we suggest that MMA or MA could act by interfering with some of that, which deserves further investigation.

Dietary supplementation with aqueous extract of IP significantly reduced the MMA or MA mediated toxicity. IP not only decreased the mortality rate but also ameliorated the behavioral alterations associated with both drugs. Our results are strongly supported by an earlier report, where IP significantly extended lifespan and increased ability to resist to environmental stresses in flies [36], and also protected against paraquat induced mortality in *Caenorhabditis elegans* [37]. Importantly, caffeic acid, galic acid and epigallocatechin (some of the major content of IP) increased the lifespan and restored the impaired movement activity induced by paraquat in DM [38]. IP extract was also found to improve the locomotor deficits induced by the treatment with reserpine and MPTP in rodents, which was, at least in part, attributed to caffeine present in the extract [39]. Previous report also suggested that some polyphenols in IP extract are able to enhance GABAergic activity [40]. Therefore, the involvement of GABAergic pathway in IP effect cannot be denied.

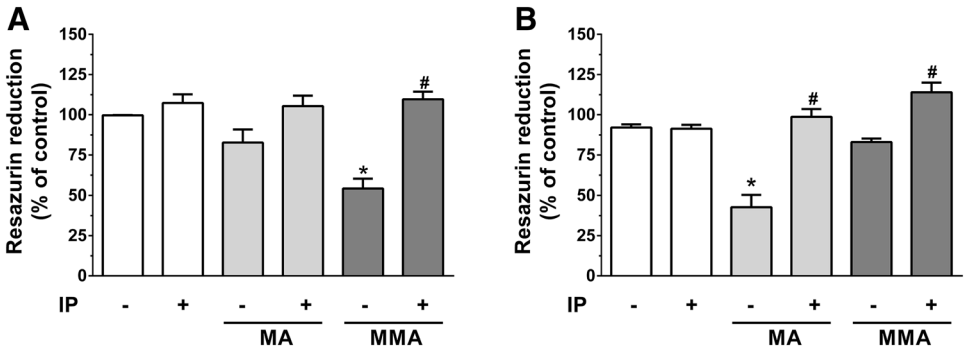

Fig. 10 Effect of IP on resazurin reduction in **a** head and **b** body of flies exposed to MMA or MA. (*) represent significant difference as compared with control group; (#) represents significant difference between MA and MA + IP or MMA and MMA + IP ($P < 0.05$). (One-way ANOVA followed by Tukey's multiple range tests). Values are expressed as mean ± S.E.M. (n = 5)

In an attempt to better understand the putative biochemical mechanism(s) of MMA and MA induced toxicity, we performed a series of assays to address the involvement of mitochondria and oxidative stress in both head and body regions of the treated flies. It is known that exposure to both MMA and MA can significantly influence mitochondrial dysfunction, increase TBARS, reactive species, total protein carbonylation and decrease in sulfhydryl content in mammals [2, 4, 5, 41, 42]. However, to the best of our knowledge, there is no data available about the effects of MMA or MA on flies. Thus, we observed that exposure to MMA and MA caused significant increase in TBARS levels, decreased SOD and catalase activities and also decreased the ability to reduce MTT and resazurin, either in head and body of treated flies. In strong contrast to above, treatment with MMA and MA resulted in increased NPSH levels in head and body regions, which may represent a primary compensatory response to oxidative insults.

Moreover, acute exposure to MMA and MA caused a significant decrease in AChE activity. Although, we cannot describe the exact mechanism by which MMA or MA decreased the enzymatic activity or their interference in cholinergic system, there are some reports which stated that MA can cause destruction of the basal forebrain cholinergic neurons [43]. In rats with renal failure treated to gentamicin, MMA was also found to increase susceptibility of activation of brain AChE, suggesting the involvement of cholinergic system on MMA toxicity [44].

Particularly important, dietary supplementation with IP extract significantly normalized all parameters (TBARS levels, SOD and catalase activity, NPSH levels and MTT or resazurin involved assay), thus suggesting the protective effect of IP against MMA or MA-induced mitochondrial impairment and/or oxidative stress in the treated flies. Notably, both caffeic acid and caffeine (two of major metabolites present in IP extract) were previously found to present antioxidant activity as well as to protect against mitochondrial dysfunction [45].

In order to explore some of the major constituents of IP, which may have a potential role against the MMA and MA induced damages, we performed HPLC analysis. As shown in (HPLC Fig. 1; Table 1), the IP extract contains gallic acid, catechin, chlorogenic acid, caffeic acid, caffeine, epigallocatechin, rutin, quercetin and kaempferol with concentrations ranging from 5.57 to 0.51 mg/g (for different constituents). In the present model we observed that dietary supplementation with IP significantly restored behavioral and biochemical parameters. Of particular importance, HPLC spectra of plant extract, as well as the UV–vis spectra, revealed that neither MA nor MMA were able to interact with the plant constituents, supporting the notion that plant antioxidants and the organic acids (MA or MMA) are not making complexes in the media (Data not Shown). So, we can assume that phenolic composition of IP extract may offer significant protection against MMA and MA induced damages.

In summary, IP protected against MMA and MA-induced toxicity in DM via reduction in mortality, inhibition of oxidative stress indices and maintenance of mitochondrial function. Moreover, dietary IP supplementation was associated with neuroprotection characterized by improvement in the AChE activity and locomotor function in flies. These observations highlights that IP extract may be a promising candidate against acute MMA or MA induce toxicity.

Conclusion

In fact, the use of IP can be of pharmacological importance because of the ease of availability and also the presence of different compounds that may have synergistic effects when combined. Additionally, our data reinforce the importance of DM model to toxicological studies and, based on presented data, to explore new therapeutic strategies to counteract MMA or MA toxicity under conditions where they may be present and/or causing deleterious effects, such as methylmalonic acidemia. However, additional studies are needed to understand the exact metabolic and neurological pathways involved in the protective role of IP.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

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