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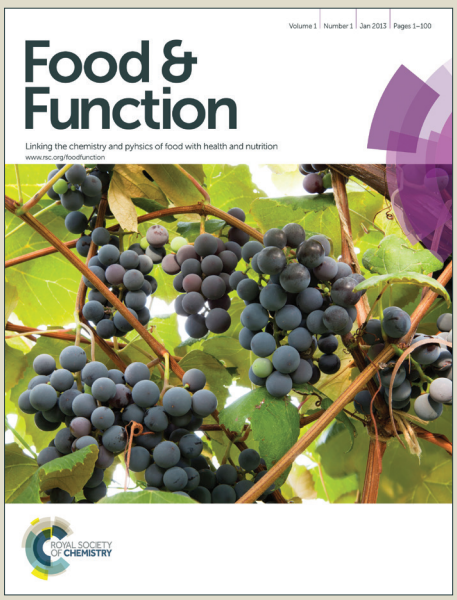

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The chemoprevention of blueberry anthocyanins extract on the acrylamide-induced oxidative stress in mitochondria: unequivocal evidence in mice liver

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Abbreviations Used: AA, acrylamide; BAE, blueberries anthocyanins extract; cyt c, cytochrome c; DCFH-DA, 2',7'-dichlorofluorescindiacetate; ETC, electron transport chain; $HO^{\cdot}$, hydroxyl radicals; MDA, malondialdehyde; MMP, mitochondrial membrane potential; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; -SH, thiol groups; SOD, superoxide dismutase.

Abstract:

Acrylamide (AA) is one of the most important contaminants occurring in heated food products. Accumulating evidences indicate that AA-induced toxicity is associated with oxidative stress and long-term exposure to AA induced mitochondria collapse and finally led to apoptosis. Whereas, anthocyanins are natural antioxidants and have strong ability to reduce oxidative damage *in vivo*. This study investigates the protection of blueberry anthocyanins extract (BAE) against the AA-induced mitochondrial oxidative stress in mice model. The activities of electron transport chain complexes, oxidative status, the structure and function of mitochondria were measured. Results showed that pretreatment with BAE markedly inhibited reactive oxygen species (ROS) formation, and prevented the successive events associated with the mitochondrial damage and dysfunction, including recovered activities of electron transport chain, ATPase and superoxide dismutase, ameliorated the depolarization of mitochondrial membrane potential and membrane lipid peroxidation, reduced release of cytochrome *c* and protected mitochondria against swelling. In a word, mitochondria are a key target at organelle level for the protective effect of BAE against AA toxicity. These results will be helpful to provide new clues for better understanding of AA toxicity intervention mechanism and exploring effective dietary constituents for intervention against AA toxicity.

Key word: Acrylamide; Anthocyanins; Mitochondria; Oxidative stress; Reactive oxygen species.

Introduction

Acrylamide (AA) is an important industrial chemical and widely used in many fields from industrial manufacturing to laboratory personnel work.[1, 2] Since 2002, AA has been found in various fried, deep-fried and oven-baked foods, [3] which make up one of the most important parts of the occidental diet. Several studies *in vivo* and *in vitro* have revealed that AA is neurotoxic and genotoxic,[4-7] and has the ability to cause toxic effects on development and reproduction.[8] It has been demonstrated that AA causes damage through forming adducts with DNA[1, 2] and has the clastogenic properties in rodents.[9, 10] Moreover, the increase of reactive oxygen species (ROS) which could lead to diverse toxicity was the consequence of AA-induced biotransformation.[11-14]

Moreover, the protection effects of several phytochemicals against the diverse AA toxicity due to their strong ability to scavenge ROS have been found in *vitro* and *vivo*, such as curcumin, hydroxytyrosol, resveratrol, tea polyphenols, and diallyl trisulfide.[13, 15-17] Anthocyanins are one class of abundant flavonoids, which are widely distributed in fruits and vegetables and have potential physiological activities.[18] In our previous study, anthocyanins significantly prevented the AA-induced ROS production in both cell lines[19] and mice models.[20] In light of these reports and our findings, we further postulate that anthocyanins may provide the intervention effect though scavenging excessive ROS in mitochondria.

Mitochondria are major producers of ROS and also the primary targets of oxidative damage. It has been demonstrated that xenobiotic-induced toxic effects are closely associated with the oxidative stress-induced mitochondrial dysfunction, which can trigger the negative events such as the disorder of energy metabolism and the activation of signaling pathways of cell apoptosis.[21-22] Recently, Chen *et al*. indicated AA could cause mitochondrial collapse and apoptosis in human astrocytoma cells.[23]

In this study, we hypothesized that protective effect of BAE against AA-induced oxidative stress is engendered directly by its strong ability to restore mitochondrial dysfunction. We investigated the BAE-caused changes in oxidative status and mitochondrial function in AA-exposed mice liver including the excessive ROS generation, function of electron transport chain (ETC), membrane permeability and potential, ATPase and superoxide dismutase (SOD) activities. Also, the protection mechanism of anthocyanins on AA-induced oxidative stress is proposed. Hopefully, it will be helpful to provide new evidences for understanding the prevention of BAE against AA toxicity *in vivo*.

2. Materials and Methods

2.1 Chemicals

The blueberry (*vaccinium uliginosum*) anthocyanins extract (BAE) was provided by the Daxing'anling Lingonberry Organic Foodstuffs Co., Ltd. (Daxinganling, China). Detailed information including characterization and quantification of anthocyanins by HPLC/MS method[24] was listed in our previous study.[20] The BAE has three types of anthocyanin including cyanidin-3-glucoside, peonidin-3-glucoside, cyanidin-3,5-diglucoside and the percentage of cyanidin-3-glucoside is up to 90. The total anthocyanin contents of BAE was 257.6 ± 2.3 mg/g.

All chemicals, reagents, and standards used in this study were observed from Sigma-Aldrich (St. Louis, MO, USA). MitoComplex I-V kit, mitochondrial swelling assay kit, Cyt *c* release assay kit and JC-1 staining kit were purchased from Genmed Scientifics Inc. (Wilmington, DE, USA). Mitochondrial ATPase kit, SOD activity kit, Malondialdehyde (MDA) content kit and Bradford protein assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

84 **2.2 Animals and treatments**

85 The animals were provided by the Beijing Vital River Laboratory Animal Technology Co.
86 Ltd (Beijing, China). Forty-eight male healthy Kunming (KM) mice weighing 25.0 ± 3.0 g (6 - 7
87 weeks old) were used. All mice were kept in the animal room of Supervision and Testing Center
88 for Genetically Modified Organisms Food Safety, Ministry of Agriculture (Beijing, China) with
89 the license number SYXK (Beijing) 2010-0036 and were provided with standard laboratory diet
90 (Beijing Branch Macau third-Feed Co. Ltd, Beijing, China) and tap water. Animal room was
91 maintained at a temperature of 23.0 ± 1.0 °C, relative humidity of 40 - 70%, artificially
92 illuminated with a 12-h light/dark cycle and air exchanges of 15 times/h. The animals were
93 allowed to acclimatize to the environment for 1 week before the experiment.

94 Mice were divided at random into six groups, including control (C) group, AA group, three
95 BAE intervention groups and BAE control group (BAE-C group), each of which consists of 8
96 animals. During the 14-day periods, all groups were allowed to freely access to food and water.
97 Control group was received by oral administration with 0.1 mL/10 g BW/day physiologic saline
98 for 14 days. AA group was obtained by intraperitoneal injection of 50 mg/kg BW/day AA for 7
99 days after 7-days oral administration of physiologic saline. For three intervention groups, BAE
100 was dissolved in water before use and the solution was orally administered to animals at 0.1
101 mL/10 g BW for 7 days at dosages of 50 mg/kg BW/day (BAE-L group), 150 mg/kg BW/day
102 (BAE-M group), and 250 mg/kg BW/day (BAE-H group) before exposure to AA, respectively.
103 Since the eighth day, the mice in all intervention groups were injected intraperitoneally with AA
104 at a level of 50 mg/kg BW/day along with different dosages of BAE by oral administration,
105 respectively. The mice in BAE-C group were given by oral administration of BAE once daily for
106 14 days at a dose of 250 mg/kg BW. All animals were sacrificed on the 15th day after fasting for

18 h. The care and treatment of the animals conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, and all the experiments were in accordance with animal ethics standards.

2.3 Isolation of liver mitochondria

Mitochondria were isolated from the mice liver by differential centrifugation, as previously described elsewhere.[25] Briefly, tissue samples were homogenized in the buffer consisting of 0.01 M Tris-MOPS, 0.001 M EGTA/Tris and 0.2 M sucrose (pH 7.4), and then were centrifuged at 600 *g* for 10 min to discard nuclei and cell debris. Afterwards, the sediment was discarded and the supernatant was further centrifuged at 7000 *g* for 10 min to obtain the enriched mitochondria fraction. The mitochondrial pellet was washed twice and resuspended in a minimum volume of the buffer same as mentioned above. The whole procedure was carried out at 0-4 °C. The isolated mitochondria were used fresh for functional measurements. A portion of fresh isolated mitochondria was frozen at -80 °C for enzymatic activity measurements. Mitochondrial protein concentration was measured spectrophotometrically using Bradford protein assay kit.

2.4 Assays

Measurements of activities of individual complexes I-V of the ETC, ATPase, SOD, MDA contents, MMP, Cyt *c* distribution, mitochondrial swelling were performed by commercial kits according to the manufacturer's instructions.

ROS generation and NAO mitochondrial fluorescent assay were measured according to the method of Song *et al*.[19] and Maftan *et al*.[26], respectively. Briefly, DCFH-DA working solution was added directly to the medium and incubated with isolated mitochondria at 37 °C for 30 min. The reaction solution was kept on ice for immediate DCF detection with a fluorescence microplate reader (TECAN Infinite M200 PRO, Austria) at an excitation wave length of 485 nm

and an emission wave length of 530 nm. NAO dye binds to the cardiolipin of mitochondrial inner membrane and the levels of uptake of this dye reflect the integrity and functionality of inner membrane because cardiolipin is easily oxidized by ROS. After incubated in 1 mL NAO staining solution at 37 °C for 30 min, the suspension of isolated mitochondria were analyzed at an excitation wave length of 495 nm and an emission wave length of 519 nm with a fluorescence microplate reader (TECAN Infinite M200 PRO, Austria).

2.5 Statistical analysis.

Plotting and curves fitting were performed by Origin 8.0 (Origin Lab Co., MA, USA). The data are presented as Mean ± SE. Statistical analysis of data was performed using SPSS 17.0 statistical package, and the results with $p < 0.05$ were considered to be statistically different. The data were tested for homogeneity of variances by the test of Levene prior to calculate the significance of difference by One-way ANOVA test; for multiple comparisons, one-way ANOVA was followed by a Duncan test when variances were homogeneous or by the Tamhane test when variances were not homogeneous.

3. Results

3.1 Protective effect of BAE on AA-changed the activities of mitochondrial ETC and ATPase in mice liver

Impairments of electron transport chain in specific mitochondrial complexes were observed in the AA-treated mice (Table 1). The activities of mitochondrial complexes I, III, IV, V were significantly reduced by AA ($p<0.05$), while BAE pretreatment showed positive recovery effects on the activities of the complexes I, III, IV and V in a dose-dependent manner. However, complex II remained insignificantly altered in all groups. Similarly, compared with the control group, significant decline in liver mitochondrial ATPase activities was detected in AA-treated

mice as shown in Table 1. Moreover, the Na^+/K^+-ATPase activity and Mg^{2+}-ATPase activity in AA group were about 1/2 less than that in control group. BAE pretreatment significantly recovered these ATPase activities and high dose (250 mg/kg BW) of BAE basically resumed the ATPase activities to control level.

3.2 Protective effect of BAE on mitochondria subjected to oxidative stress

A maintaining balance between the ROS formation and the removal is important to the mitochondrial function. AA caused the significant accumulation of ROS in mitochondria (Fig. 1A). This is in agreement with the previous studies,[13, 16, 27]which linked the AA-induced diverse toxicity with oxidative stress. The generation of ROS was markedly inhibited by different dosages of BAE (50-250 mg/kg BW). Especially, the ROS levels in the BAE groups with the dose of 150 and 250 mg/kg BW were recovered to the control group. SOD is an important member of oxidative defense system and plays an important role in ROS removal.[28] Results showed the AA-induced decrease in SOD activity was reversed in BAE intervention groups, indicating BAE may show to provide a parallel protection by enhancing the activity of SOD (Fig. 1B). Simultaneously, AA significantly caused membrane lipid oxidation and decreased the cardiolipin level in mitochondria membrane, suggesting oxidative damage in *vivo* occurred when mice were exposed to AA. The levels of mitochondrial cardiolipin (Fig.1A) and membrane lipid oxidation (Fig.1C) were markedly recovered in all the BAE groups and the mitochondrial cardiolipin concentration in the BAE-H group was raised and similar to those observed in control group.

3.3 Protective effect of BAE on AA-changed membrane function in mice liver

The MMP was assayed by JC-1 staining and the low ratio of red to green was used to indicate the MMP collapse. Fig. 2A showed that AA group had the lowest ratio among all the

groups, suggesting that AA induced MMP disruption. The BAE pretreatment presented a dose-dependent protection effect on the mitochondrial membrane. Especially, the MMP in BAE-H group restored to the control level. The release of cyt *c* from damaged mitochondria is an important marker to evaluate the integrity of mitochondria.[29] Remarkably, AA treatment resulted in the increased release of cyt *c*, while BAE pretreatment greatly inhibited this subcellular shift (Fig. 2B). After the release of cyt *c*, mitochondrial swelling occurs. Fig. 2C showed that the A_{520} values in all groups declined under the induction with reaction buffer (Ca^{2+}), indicating mitochondrial swelling in different extent was caused by abnormal osmotic pressure. The BAE pretreatment markedly reduced the amplitude of the swelling and BAE-H restored the rise to the control level ($p < 0.05$) (Fig. 2D).

4. Discussion

This is the first study shows BAE protects mitochondrial structure and function against the AA-induced oxidative stress by scavenging excessive ROS in mice liver. Furthermore, we believe that the novelty of the data presents here reflects that mitochondria are target organelles for AA toxicity intervention.

Mitochondria play a prominent role in the production of the energy currency of the cell and regulating cellular metabolism.[29] It is well known that the mitochondrial matrix enclosed by the inner membrane is the site for a series of important reactions including ETC and oxidative phosphorylation.[7] Therefore, the integrated structure and compartment of mitochondria are essential for it to maintain the normal physiological state of cells. Several researchers found the AA-induced formation of excessive ROS in *vitro* studies and proposed that AA toxicity could be related to the oxidative stress.[13, 16, 30] In the present study, we provided the unequivocal evidence that BAE could protect mitochondria against AA-induced oxidative stress in mice liver.

In mitochondria, the ETC complexes I, III and IV guide the electrons through a series of redox reactions utilizing the free energy released to create a proton motive force that drives ATP synthesis by complex V. Moreover, ATPase catalyzes the decomposition of ATP into ADP and Pi, which is the main source of energy in organism.[29] Our results showed that pretreatment with BAE markedly recovered the activities of mitochondrial ETC complexes and Na^+-K^+-ATPase in AA-treated mice and the protection effect was the dose-dependent. Thus, the AA-induced leak of electrons from complex I and III was inhibited, and then the AA-induced ROS generation and energy metabolism were regulated to normal range. In line with our results, Muralidhara *et al*.[31] also suggested the beneficial effects of spice active principles in the AA-induced down-regulation of ATPase activity in rats and further demonstrated the energy disorder was the main reason to cause neurotoxicity.

Moreover, evidences suggested that MMP is the key point in mitochondrial function. When the depolarization of MMP induced by oxidative stress, the mitochondrial dysfunction could not be reversed.[32-34] Also, excessive mitochondrial ROS generation initiates mPTP opening which causes the release of cyt *c* from mitochondria into the cytosol and it is an important marker to evaluate the integrity of mitochondria.[28,29] Our results showed that BAE significantly inhibited the AA-induced depolarization of MMP, reduced the mPTP opening and the release of cyt *c,* and protected mitochondria against AA-induced swelling, which could be explained by the theory that antioxidants could inhibit mPTP opening through protecting -SH in mitochondria membrane against oxidation.[35] Besides, excessive ROS damaged membrane of cell and organelle, which often manifested as lipid and protein peroxidation.[36]BAE pretreatment significantly decreased the MDA level and increased the cardiolipin level in AA-treated mice, suggesting that BAE protected the mitochondrial membrane lipid against oxidative damage (Fig.1A and Fig. 1C).

Clearly, BAE pretreatment prevented AA-induced oxidative stress via decreasing ROS generation and recovering the mitochondrial function.

Taken together, the intervention mechanism of BAE on AA-induced mitochondrial damage in mice liver was schematically illustrated in Fig. 3. The complexes I and III are identified as the major ROS generators in the mitochondrial ETC for AA exposure. When superoxide radical ($O_2^{\cdot}$-) are produced, SOD catalyzes the dismutation of $O_2^{\cdot}$- to H_2O_2, which is converted to highly reactive hydroxyl radicals ($HO^{\cdot}$) via the ferrous ions (Fe^{2+})-dependent Fenton reaction. Then, ROS oxidize thiol groups (-SH) of the mPTP complex, lead to mPTP opening and promote membrane permeability through lipid oxidation. Eventually, AA-induced oxidative damage in mitochondria occurs, and the above-mentioned events result in autophagy or apoptosis in mice liver cells. Whereas, BAE protected the structure and function of mitochondria against AA-induced oxidative damage through its antioxidant actions including directly scavenging excessive ROS, limiting net ROS availability, improving the activity of antioxidant enzymes like SOD and protecting -SH groups in mPTP. The similar protection of anthocyanins against the oxidative damage in the restraint stressed mice and endothelial cells have been demonstrated.[37, 38] They found that anthocyanins played a positive role in relieving the disorder of mitochondrial function and balancing the energy metabolism, and interfered in the regulation of apoptotic intracellular signaling mechanisms mediated by mitochondria. Moreover, Rugină et al.[39] explained why anthocyanins have significant antioxidant activity in cells. They suggested that the ortho-3', 4'-dihydroxy groups on the B ring in anthocyanins seems to be crucial to show antioxidant capacity during the interaction between cells and anthocyanins. The meta-5,7-dihydroxy arrangements in the A ring and the 3-hydroxyl group in C ring increase the antioxidant potential of anthocyanins. This would provide a possibility for predicting the

chemoprevention of other bioactive components on the AA-induced oxidative stress from chemical structure.

In conclusion, our findings clearly demonstrated that BAE provide significant protection against AA-induced mitochondrial damage through preventing the oxidative stress and balancing the disorder of metabolism. We believe that the novelty of the data presents here reflects that mitochondria are target organelles for AA toxicity intervention. Moreover, this study provided an effective way for the intervention of AA toxicity through offering anthocyanins enriched foods in our daily life. However, the further research is needed to explore a possible intercellular signal apoptosis pathway of AA-induced liver toxicity in molecular level, which would be beneficial to find the highly efficient targeted intervention methods.

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The authors declare they have no actual or potential competing financial interests.

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Figure and Table Legends

Fig.1 The oxidative damage of liver mitochondria in mice with different treatments. A. ROS accumulation and cardiolipin concentration. B. SOD activity. C. MDA level. Bars depicted are means ± standard error of mean for 8 independent experiments (provided by 8 different mitochondrial preparations from 8 different animals). Different uppercase and lowercase letters denote significant difference of complexes activities from each group, $p < 0.05$. Relative Fluorescence Units, RFU; Reactive oxygen species, ROS; Malondialdehyde, MDA; Superoxide dismutase, SOD.

Fig. 2 Mitochondrial membrane potential (A), Integrity of mitochondrial membrane (B), Typical traces for the mitochondrial swelling (C), and The decrease rates of absorbance within two minutes (D) of liver mitochondria in mice with different treatments. Bars depicted are means ± standard error of mean for 8 independent experiments (provided by 8 different mitochondrial preparations from 8 different animals). Different uppercase and lowercase letters denote significant difference of complexes activities from each group, $p < 0.05$.

Fig.3 The mitochondrial mechanism of AA-induced oxidative stress: Schematic diagram of the relevant mitochondrial components involved in mitochondrial respiration and antioxidant defenses. Bold letters are items which have been partially damaged after AA exposure. I -V represent the complexes in ETC; $O_2^{\cdot}$-, superoxide anion free radical; $HO^{\bullet}$, hydroxyl free radical; SOD, superoxide dismutase; ROS, reactive oxygen species; mPTP, mitochondrial permeability transition pore; -SH, thiol groups; NAD^+, nicotinamide adenine nucleotide; Cyt *c*, cytochrome *c*; NADH, reduced NAD^+; H^+, proton; e^-, electron; O_2, oxygen molecule; Pi, inorganic phosphorus;

348 Fe^{2+}, ferrous ions.

349 Table 1 The activity of complexes I -V (units of nanomole per minute per milligram of protein)

350 and ATPase activities (units of micromole Pi per hour per milligram of protein) of liver

351 mitochondria in mice with different treatments.

352

Graphical Abstract

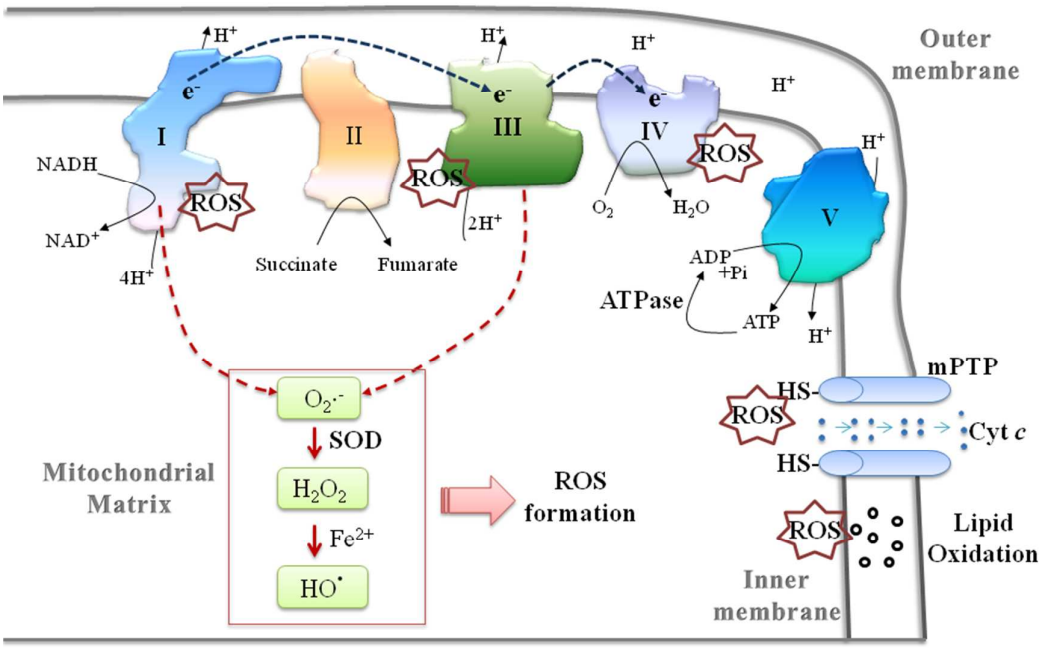


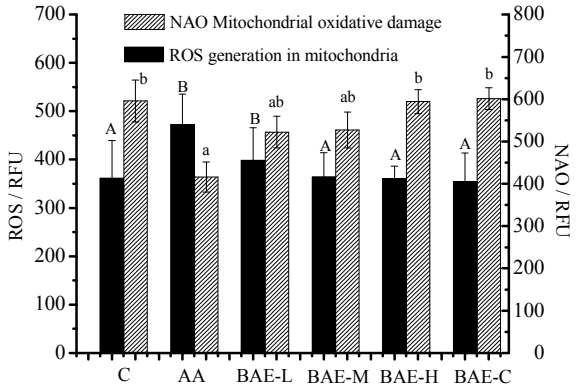
Table 1

Groups	Complex I (NADH coenzyme Q reductase)	Complex II (succinate dehydrogenase)	Complex III (cytochrome bc1 complex)	Complex IV (cytochrome c oxidase)	Complex V (F_0-F_1 ATP synthase)	Total-ATPase	Na^+-K^+-ATPase	Mg^{2+}-ATPase
C	216.11 ± 38.3^{c}	1899.34 ± 119.38	53.82 ± 4.77^{c}	34.23 ± 2.47^{b}	54.86 ± 7.72^{c}	33.44 ± 4.53^{c}	15.77 ± 1.20^{c}	16.11 ± 1.59^{b}
AA	68.15 ± 15.78^{a}	1791.81 ± 102.01	33.55 ± 4.62^{a}	23.20 ± 2.46^{a}	33.39 ± 3.18^{a}	17.08 ± 2.10^{a}	7.85 ± 0.88^{a}	8.28 ± 0.99^{a}
BAE-L	123.19 ± 22.82^{b}	1797.12 ± 367.28	40.45 ± 9.89^{ab}	26.39 ± 4.64^{a}	38.54 ± 6.12^{a}	25.79 ± 4.16^{b}	12.63 ± 1.56^{b}	13.95 ± 1.27^{b}
BAE-M	149.91 ± 20.28^{b}	1716.22 ± 273.71	50.15 ± 12.28^{bc}	27.04 ± 4.25^{a}	49.71 ± 6.88^{b}	24.64 ± 3.54^{b}	12.01 ± 1.71^{b}	14.65 ± 1.39^{b}
BAE-H	202.03 ± 20.91^{c}	1874.91 ± 189.54	53.95 ± 4.46^{c}	33.19 ± 3.43^{b}	59.63 ± 9.05^{c}	32.14 ± 3.98^{c}	16.15 ± 1.93^{c}	16.53 ± 1.82^{b}
BAE-C	209.48 ± 39.35^{c}	1958.11 ± 262.80	55.42 ± 6.14^{c}	35.80 ± 2.17^{b}	62.08 ± 10.88^{c}	33.12 ± 3.14^{c}	16.41 ± 2.23^{c}	16.46 ± 1.49^{b}

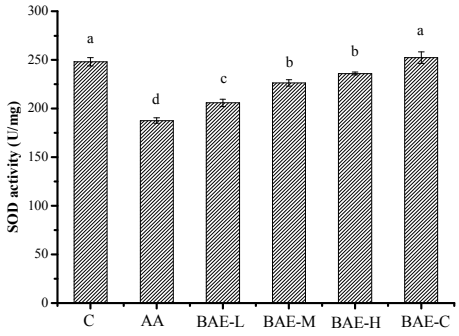
The results represent the mean ± S.E. of values obtained from 8 animals in each group.

Different lowercase letters denote significant difference of complexes activities from each group, $p < 0.05$.

Fig. 1 A



B



C

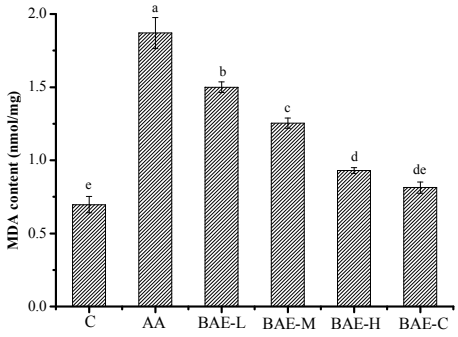


Fig. 2

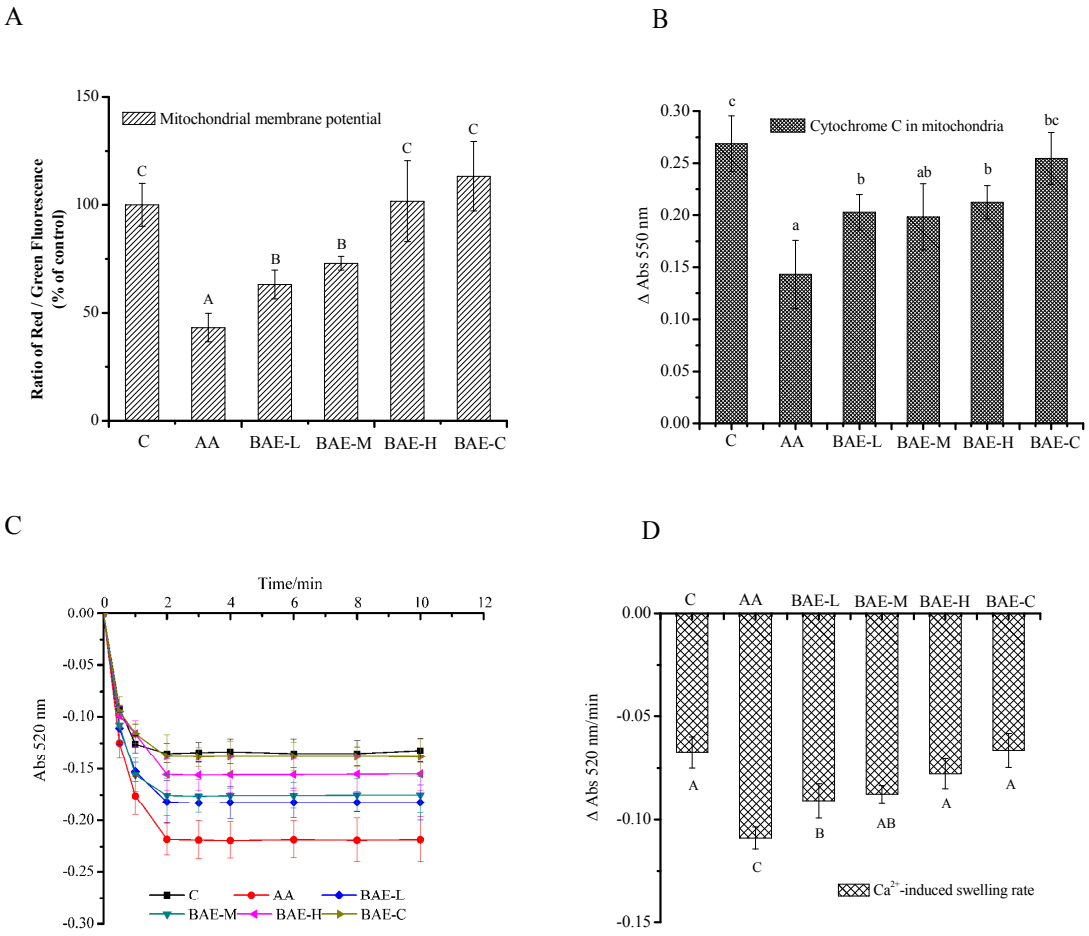


Fig 3