Original Article

Anthocyanin Extract From Purple Sweet Potato Improving Neurotransmitter and Locomotor in Chronic Stressed-mice

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ABSTRACT

Background: Prolonged stress plays an essential role in depression disorder through brain inflammation and neurotransmitter imbalances. The natural plant antioxidant is promising to resist the negative impact of stress.

Objectives: This study aimed to analyze the effect of total anthocyanin (ANC) extracts from purple sweet potatoes (PSP) on brain neurotransmitters, inflammation, and locomotor behavior in the chronic-stressed mice model.

Methods: Twenty male adult BALB/c mice were assigned to control, stress (STR), STR+ANC (10 mg/kg body weight [BW]), STR+ANC (20 mg/kg BW), and STR+ANC (40 mg/kg BW). Restraint stress was applied 2 h/d for 14 days. The enzyme-linked immunosorbent assay (ELISA) was performed to measure brain dopamine, gamma-aminobutyric acid (GABA), and corticosterone levels. The locomotor behavior was analyzed using an open field test before and after ANC treatment. In silico, molecular docking was carried out between ANC and monoamine oxidase-B (MAO-B) enzyme.

Results: Administration of ANC decreased brain corticosterone levels. The dopamine neurotransmitter decreased in the stress-induced group and increased following ANC treatment. Increased GABA levels were observed in the stressed and treated groups. Locomotor analysis showed reduced total distance movement and velocity after ANC treatment. Molecular prediction showed that ANC can inhibit the MAO-B enzymes.

Conclusion: The ANC extracted from PSP relieved brain inflammation and modified the neurotransmitters of dopamine and GABA, affecting the locomotor function of chronically stressed-induced mice. Furthermore, in vivo studies are necessary to evaluate the molecular mechanism of ANC from PSP in chronic stress exposure, particularly on MAO enzyme regulation.

Keywords: Anthocyanin (ANC), Brain, Dopamine, Gamma-aminobutyric acid (GABA), Stress

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Introduction

Stress is strongly associated with mental health deterioration, including depression (Juliana et al., 2022). Stress activates paraventricular nucleus (PVN) neurons in the hypothalamus to secrete a corticotrophin-releasing hormone (CRH), then activates the hypothalamic-pituitary-adrenal cortex axis; thereby, extra corticosterone is released into circulation. Similarly, stress triggers the sympathetic nervous system to produce noradrenalin and provokes the adrenal medulla to release adrenaline neurotransmitters (Chaves et al., 2021). Hormonal and neural mechanisms are assigned to maintain energy requirements against homeostatic disruption during stress (Hall, 2016).

Unfortunately, chronic stress blunts physiological responses to stress (Bloomfield et al., 2019) and dysregulates brain neurotransmitters of dopamine and gamma-aminobutyric acid (GABA) (Baik, 2020; Lowes et al., 2021). Dopamine is an important neurotransmitter responsible for the brain's motivation and locomotor function. Chronic stress attenuates dopamine levels in dopaminergic circuit areas and correlates with learning deficit symptoms (Baik, 2020; Jia et al., 2021). In addition, stress-induced disruption of GABA in the ventral tegmental area is involved in anhedonia symptom development in depression (Lowes et al., 2021).

Antidepressants play crucial roles in depression management (Solmi et al., 2021). Recently, the pharmacotherapy for depression has focused on serotonin as the target neurotransmitter. However, therapeutic limitations are challenging for further improvement because the recent treatment has a prolonged onset, residual symptoms, and partial remission (Vahid-Ansari et al., 2019). Neurotransmitter systems are proposed as the target of therapy to obtain better therapeutic outcomes (Liu et al., 2018). Alternative approaches are suggested from the plant's bioactive compounds. Several plants, such as *Panax ginseng*, *Rhodiola rosea*, *Schisandra chinensisc*, etc. are categorized as "adaptogen" that promotes recovery from stress conditions (Panossian et al., 2021). Previous work suggested the antidepressant activity of cyanidin from purple sweet potatoes (PSP) as an antidepressant by agonist binding to dopamine receptors. In addition, prior research demonstrated the behavioral benefit effects of anthocyanin (ANC) from PSP (Kurnianingsih et al., 2020; Kurnianingsih et al., 2021).

PSP are a primary staple food with high ANC content. ANC from PSP was demonstrated as an antioxidant as opposed to oxidative stress on neuronal cells (Zhong et al., 2023) and had a neuroprotective effect on ischemia stroke model animals (Wati, et al., 2018). However, the impact of total ANC from PSP on neurotransmitter and locomotor behavior has remained unexplored. Therefore, further studies must be developed in animal stress models to provide intelligible biomechanism of ANC from PSP to improve the behavior through neurotransmitter regulation. Thus, this study aimed to evaluate the effect of ANC from PSP on brain corticosterone, dopamine, GABA level, and locomotor behaviors in chronic stress model mice. This study also provided computational prediction of ANC effect in inhibiting neurotransmitter degradation as monoamine oxidase (MAO) inhibitor by molecular docking interaction.

Materials and Methods

Animals

All animal procedures followed the Declaration of Helsinki for animal welfare. A total of 25 eight-week-old male BALB/c mice were housed in pairs at the Animal Experimental Laboratory, Institute of Bioscience, Universitas Brawijaya. The animals were maintained in a controlled temperature and humidity room with a 12:12 light-dark photoperiod. The standard laboratory food pellet and drink were provided ad libitum (Ghotbitabar et al., 2022; Nikjooy et al., 2022).

Experimental design

A total of 25 mice were divided into 5 groups: Control (CTRL), stress (STR), STR+ANC (10 mg/kg BW) (STR+ANC10), STR+ANC 20 (mg/kg BW) (STR+ANC20) and STR+ANC 40 (mg/kg BW) (STR+ANC40). Stress was exposed 2 h/d for 14 days. Total ANC extracts were administrated via intragastric tube at a frequency once a day under a duration of stress. Open field test behavioral assessment was conducted before and after treatment evaluation for distance traveled and movement velocity. All animal procedures were performed in a light period (Kurnianingsih et al., 2023; Resae et al., 2023).

Plant materials

A local cultivar of PSP (Antin-3 variety) was harvested from the Research Centre of Legume and Tuber Plant, East Java, Indonesia, at 4 months plantation age. Plant tuber root was freshly ground, followed by homog-

enization and maceration in acidic-methanol (99.9%; Sigma-Aldrich, Darmstadt, Germany) solvent pH 4.5 at room temperature for 24 hours. After filtration, the homogenates were evaporated at 50-60˚C using a rotary evaporator. The extracts were stored at 4˚C until further application (Dwiwibangga et al., 2022; Kurnianingsih et al., 2020).

Stress exposure

Restraint stress was carried out using a modified previous method by immobilizing each animal in a transparent, ventilated, and fitted diameter of an acrylic cylinder with free breathing. The restraint stress was applied as random schedules to avoid animal habituation (Reyhanditya et al., 2022).

Open field test

According to previous studies, behavioral assessment of open field test was carried out (Kurnianingsih et al., 2020). In brief, after room habituation, each animal was placed in the center of a 40×40×40 cm acrylic box. The movement of each animal was recorded for 6 minutes. The video was analyzed using Noldus EthoVision XT (Noldus Information Technology, Wageningen, Netherlands). to measure the total distance traveled, the velocity of movement, track-line and heatmaps visualization (Iturra-Mena et al., 2018; Khodadadeh et al., 2020).

Enzyme-linked immunosorbent assay (ELISA)

After 14 days of the experiment, mice were sacrificed as pre-euthanized, followed by brains removal. After weighing, the whole region of brain tissues was processed for ELISA analysis to measure dopamine, GABA, and corticosterone levels. The ELISA procedures were conducted as listed in the ELISAkit protocol. The catalog number was EM1603 (FineTest, Wuhan Fine Biotech Co., Ltd, China) for mouse-GABA analysis, EM1712 (FineTest, Wuhan Fine Biotech Co., Ltd, China) for mouse-dopamine analysis, and DEV9922 (Demeditec Diagnostics GmbH, Germany) for corticosterone analysis (Xie et al., 2013).

Molecular docking prediction

Ligand and protein preparation

Ligands of cyanidin (CID 128861), cyanidin-3-O-glucoside (CID 441667), and peonidin-3-O-glucoside (CID 443654) were retrieved from PubChem Database. Rasagiline (CID 3052776), as an MAO inhibitor drug, was used as a control ligand. The protein of human monoamine oxidase-B (MAO-B), PDB ID:2BK3, was downloaded from the Protein Data Bank database. Active sites of protein were predicted using Molegro Virtual Docker software, version 5.0 with molecular surface van der Waals no more than 5 (Bitencourt-Ferreira & de Azevedo, 2019).

Docking simulation

Molegro Virtual Docker software, version 5.0 was used to interact protein and ligand with a specific grid (X=54.30; Y=147.7; Y=22.01; radius=16). The docking parameter of Molegro Virtual Docker was function moldock score (grid) with these variables: Grid resolution of 0.30; algorithm MolDock SE; number of runs of 10, max iteration of 1500; maximum population size of 50; pose generation energy threshold of 100, tries between 10 and 30; simplex evolution maximum steps of 300; neighbor distance factor of 1.00; multiple pose number of 5; energy threshold of 0.00; and cluster similar poses RMSD threshold of 1 (Bitencourt-Ferreira & de Azevedo, 2019)

Molecular docking analyses

The docking complex from Molegro Virtual Docking version 5 was superimposed with PyMol, software, version 2.2. Complex interaction visualization was displayed in 3D and 2D views using the Discovery Studio software, version 21.1.1 program (Bitencourt-Ferreira & de Azevedo, 2019).

Statistical analysis

The results were expressed as Mean±SEM. The differences between treatments were assessed using two-way analyses of variance (ANOVA) followed by least significant difference (LSD) multiple comparisons using GraphPad Prism software, version 9.0.0. The significance level was $P<0.05$ (Kurnianingsih et al., 2023).

Results

Chronic stress exposure for 14 days significantly reduced brain weight (0.40±0.004 g) compared to control (0.43±0.003 g). The ANC from PSP increased brain weight by 0.42±0.002 g and 0.42±0.001 g with doses of 20 and 40 mg/kg body weight (BW), respectively (Figure 1A). Stress dramatically increased brain corticosterone levels (141.80±2.44 ng/mL) higher than the control (82.63±4.51 ng/mL). Treatment of ANC reduced this corticosterone level dose-dependently at 9.45%, 13.08%, and 27.23% for 10, 20, and 40 mg/kg BW, respectively (Figure 1B). Brain neurotransmitters of GABA increased among stress and ANC-treated groups

(P=0.004) (Figure 1C). Meanwhile, brain dopamine levels in stress-exposed mice (10.54±0.19 ng/mL) were lower than control (12.95±0.17 ng/mL). However, ANC administration increased brain dopamine higher than control for a dose of 10 mg/kg BW (14.59±0.45 ng/mL) and 20 mg/kg BW (13.42±0.24 ng/mL) (Figure 1D).

Locomotor analysis revealed decreased distance traveled and movement velocity after stress exposure. Stressed mice have 495.37 cm shorter distance movement than the control. The ANC reduced the delta of traveled distance by about 306.61 cm, 248.82 cm, and 231.06 cm in a dose-dependent manner at the post-test (Figure 2A). Stress significantly declined the velocity of movement. Although ANC did not increase movement velocity, the post-test evaluation showed that the velocity among ANC-treated groups was higher than the post-stressed-only group (Figure 2B).

The movement of animals is visualized as a heatmap and track line. Lack of movement was shown after stress exposure. Reduced movement in the central zone is demonstrated among stress and ANC-treated animals. At the pre-test, the evaluation exhibits more heatmap spots visualized as green-yellow-red. The heatmap color represents longer time spent at the point of track lines. Post-test evaluations qualitatively show a decline of a green-yellow-red color spot in heatmap observation (Figure 2C).

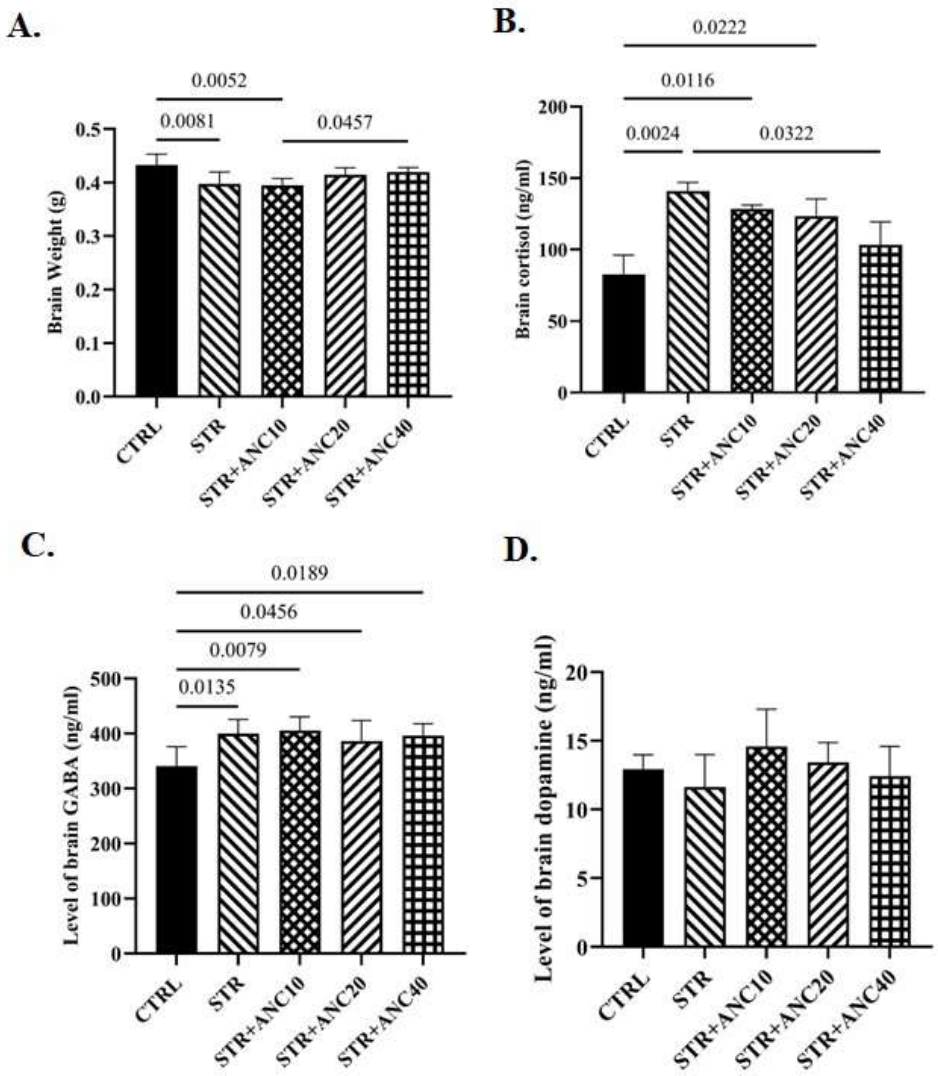

Figure 1. A) The enhancement of brain weight following ANC treatment; B) Brain corticosterone level increased after stress application, the administration of ANC reduced the cortisol level; C) Upregulation of brain dopamine level after ANC treatment among stressed mice; D) The incline of brain GABA level both in stressed and ANC treated mice

Note: Data are presented as Mean±SEM. Significant P between groups was denoted above each bar of groups.

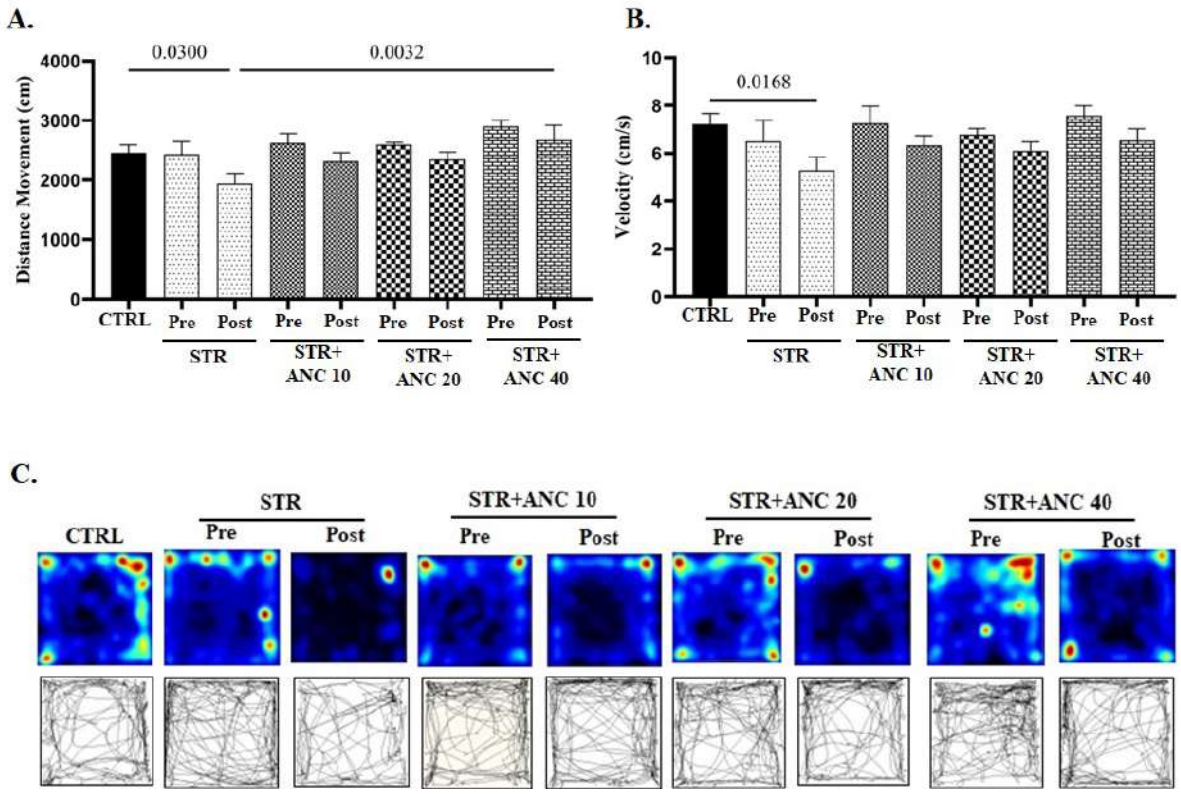

Figure 2. Locomotor assessment of ANC treated mice

Note: A) Stress reduced the distance movement of mice. The decline of distance movement among ANC-treated mice is lower than stressed-only mice; B) The velocity of stressed mice was lower than control. Total anthocyanin extract treatment in stressed mice tends to reduce movement velocity; C) The differences in heatmap pattern and track of movement animals among groups were visualized with EthoVision XT software; Data presented as Mean±SEM. Significant P between groups denoted above each bar of groups.

To predict ANC roles in dopamine neurotransmitter regulation, molecular docking was performed to interact with MAO-B with selected ANC. Molecular docking demonstrated cyanidin, cyanidin-3-O-glucoside, and peonidin-3-O-glucoside have similar interaction sites with the control ligand (Rasagiline) (Figure 3). Amino residues of ALA263 and PRO265 from MAO showed interaction with cyanidin. Meanwhile, peonidin-3-O-glucoside binds at amino residues of ALA439 and ILE14. All ANC have interaction on residue ARG42. The interaction of MAO and cyanidin resulted in the most positive energy binding, that is -370 kJ/mol. Furthermore, the energy binding of MAO with cyanidin-3-O-glucoside and peonidin-3-O-glucoside was -492 kJ/mol and -498.4 kJ/mol, respectively. Compared to ANCs, rasagiline as a control ligand has an energy binding of 264.8 kJ/mol, indicating a more positive value than ANCs (Table 1). The left panels show a 3D interaction structure between ligand and MAO protein. Central panels show ligand interaction, which is visualized as a type of interaction in the right panel.

Discussion

The systemic effect of stress is mediated by ultradian corticosterone release from the adrenal cortex. This stress hormone quietly crosses the blood-brain barrier to bind to glucocorticoid receptors. The broad site of glucocorticoid receptor activation by corticosterone then affects neuronal activities, including neurotransmitter signaling (Joëls, 2018). High corticosterone concentration previously used as severe stress mimicked basolateral amygdala cell culture. Neuronal firing enhancement is well documented because of higher corticosterone stimulation. The combination of high-dose corticosterone and β-adrenoceptor agonist isoproterenol long-lasting enhanced postsynaptic current (Karst & Joëls, 2016). Therefore, in this study, the escalation of brain corticosterone in stress-exposed groups strongly suggests a significant response toward restraint stress procedure.

Total ANC extract from PSP demonstrated a favorable effect on reducing brain corticosterone levels. Previ-

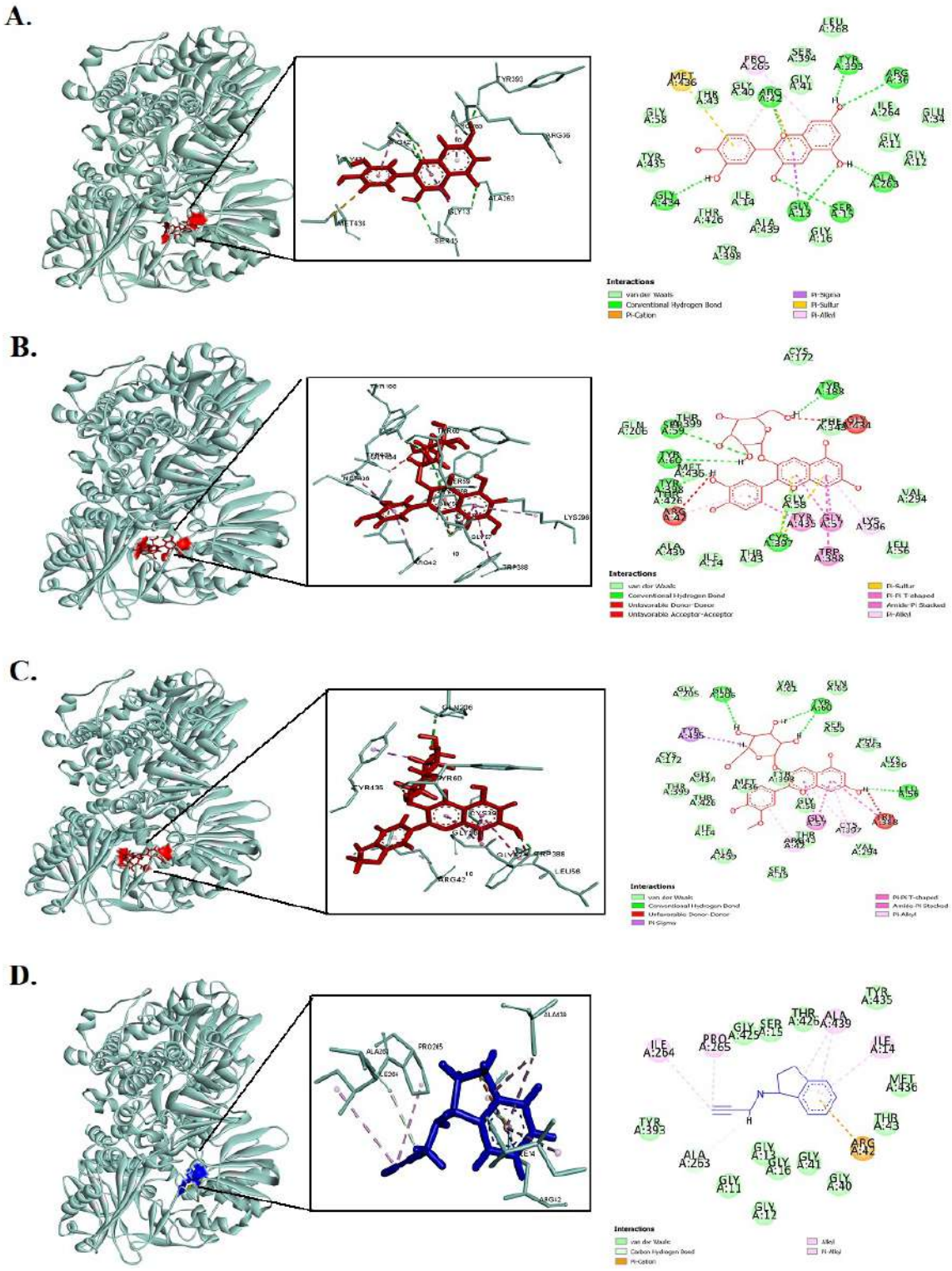

Figure 3. Molecular docking interaction prediction of anthocyanins and rasagiline as control ligand to MAO

A) cyanidin-MAO complex, B) cyanidin-3-O-glucoside-MAO complex, C) peonidin-3-O-glucoside-MAO complex, D) rasagiline-MAO complex

Table 1. Interaction between anthocyanins and rasagiline on human MAO-B

Ligand	Binding Energy (kJ/mol)	Interaction	Distance (A)	Category	Types
Cyanidin	-370	A:GLY13:N-:10:O3	2.98682	Hydrogen bond	Conventional hydrogen bond
		A:SER15:N-:10:O2	3.07293	Hydrogen bond	Conventional hydrogen bond
		A:ARG36:NH1-:10:O4	3.13127	Hydrogen bond	Conventional hydrogen bond
		A:ARG42:N-:10:O1	3.03441	Hydrogen bond	Conventional hydrogen bond
		A:ARG42:NE-:10:O1	3.15469	Hydrogen bond	Conventional hydrogen bond
		:10:H8-A:ALA263:O	1.61827	Hydrogen bond	Conventional hydrogen bond
		:10:H9-A:TYR393:O	1.73229	Hydrogen bond	Conventional hydrogen bond
		:10:H10-A:GLY434:O	2.3035	Hydrogen bond	Conventional hydrogen bond
		A:ARG42:NH2-:10	4.29851	Electrostatic	Pi-cation
		A:GLY13:CA-:10	3.71408	Hydrophobic	Pi-sigma
		A:MET436:SD-:10	5.98818	Other	Pi-sulfur
		:10-A:ARG42	4.87147	Hydrophobic	Pi-alkyl
		:10-A:PRO265	5.37395	Hydrophobic	Pi-alkyl
		:10-A:ARG42	4.29901	Hydrophobic	Pi-alkyl
Cyanidin-3-O-glucoside	-492	A:SER59:N-:10:O5	3.3335	Hydrogen bond	Conventional hydrogen bond
		A:CYS397:SG-:10:O7	3.4316	Hydrogen bond	Conventional hydrogen bond
		:10:H10-A:TYR60:O	2.60322	Hydrogen bond	Conventional hydrogen bond
		:10:H11-A:TYR188:OH	1.84323	Hydrogen bond	Conventional hydrogen bond
		:10:H20-A:TYR398:O	2.15947	Hydrogen bond	Conventional hydrogen bond
		A:CYS397:SG-:10	4.68116	Other	Pi-sulfur
		A:CYS397:SG-:10	4.53842	Other	Pi-sulfur
		A:TRP388-:10	5.30088	Hydrophobic	Pi-Pi T-shaped
		A:GLY57:C,O;GLY58:N-:10	3.74136	Hydrophobic	Amide-Pi stacked
		A:GLY57:C,O;GLY58:N-:10	3.70119	Hydrophobic	Amide-Pi stacked
		A:TYR435:C,O;MET436:N-:10	4.82201	Hydrophobic	Amide-Pi stacked
		:10-A:LYS296	5.02574	Hydrophobic	Pi-alkyl
		:10-A:ARG42	4.11246	Hydrophobic	Pi-alkyl
		A:ARG42:NH1-:10:H20	2.38389	Unfavorable	Unfavorable donor-donor
		A:GLY434:O-:10:O6	2.73868	Unfavorable	Unfavorable acceptor-a ccep-tor

Ligand	Binding Energy (kJ/mol)	Interaction	Distance (A)	Category	Types
Peonidin-3-O-glucoside	-498,4	:10:H8-A:TYR60:O	2.25123	Hydrogen bond	Conventional hydrogen bond
		:10:H9-A:GLN206:OE1	2.58876	Hydrogen bond	Conventional hydrogen bond
		:10:H10-A:TYR60:O	2.26974	Hydrogen bond	Conventional hydrogen bond
		:10:H19-A:LEU56:O	2.3069	Hydrogen bond	Conventional hydrogen bond
		:10:H21-:10:O11	2.38268	Hydrogen bond	Carbon hydrogen bond
		:10:H4-A:TYR435	2.5646	Hydrophobic	Pi-sigma
		A:TRP388-:10	5.55153	Hydrophobic	Pi-Pi T-shaped
		A:GLY57:C,O;GLY58:N-:10	4.15828	Hydrophobic	Amide-Pi stacked
		A:GLY57:C,O;GLY58:N-:10	3.54895	Hydrophobic	Amide-Pi stacked
		A:ALA439-:10:C22	3.65013	Hydrophobic	Alkyl
		:10:C22-A:ILE14	3.3547	Hydrophobic	Alkyl
		:10:C22-A:MET436	4.51516	Hydrophobic	Alkyl
		:10-A:CYS397	4.94376	Hydrophobic	Pi-alkyl
		:10-A:CYS397	4.43016	Hydrophobic	Pi-alkyl
		:10-A:ARG42	4.0933	Hydrophobic	Pi-alkyl
		A:TRP388:NE1-:10:H19	2.55109	Unfavorable	Unfavorable donor-donor
Rasagiline	-264.8	:10:H10-A:ALA263:O	3.03662	Hydrogen bond	Carbon hydrogen bond
		A:ARG42:NH2-:10	4.71293	Electrostatic	Pi-cation
		A:ALA439-:10	4.09578	Hydrophobic	Alkyl
		:10:C12-A:ILE264	5.17272	Hydrophobic	Alkyl
		:10:C12-A:PRO265	5.37402	Hydrophobic	Alkyl
		:10-A:ILE14	4.99427	Hydrophobic	Pi-alkyl
		:10-A:ARG42	4.93827	Hydrophobic	Pi-alkyl
		:10-A:ALA439	4.46751	Hydrophobic	Pi-alkyl

ous animal studies reported similar results: ANC-supplemented food limited the corticosterone level in rest and activities (Casagrande et al., 2020). A study on lipopolysaccharide induced-inflammation in BV-2 murine microglial cells showed ANC fractions reduced inflammation by declining cyclooxygenase-2 (COX-2), tumor necrosis factor-α, and nuclear factor κB (Poulose et al., 2012).

In the present study, corticosterone levels were reduced by increased dopamine neurotransmitters among the ANC-treated group. Corticosterone, as a glucocorticoid hormone, increases blood glucose levels to enhance metabolism during stress, accompanied by releasing reactive oxygen species as free radicals. Therefore, chronic glucocorticoid exposure during a stress-induced oxidative state affects neurogenesis (Spiers et al., 2015). An additional study showed an elevation of brain dopamine in psychological stress-induced oxidative stress mice. Pre-treatment with ANC increases dopamine levels in several brain areas, including the cerebellum. Considering the motor coordination function of the cerebellum, the increase in dopamine might accelerate motor skills and movement (Rahman et al., 2008). However,

the present study analyzed whole brain tissue, so further evaluation of the specific functions of the dopamine-related areas is recommended.

Therefore, the locomotor behavior of mice was explored following ANC treatment. The present study showed decreased locomotor skills among stress- and ANC-treated mice. The GABA level was examined to analyze the potential inhibition effect of ANC. Accordingly, the GABA level of stress and ANC-treated mice were simultaneously higher than the control. Our previous study showed the possible binding of cyanidin as a major ANC component from PSP as an agonist towards the $GABA_B$ receptor (Kurnianingsih et al., 2020). ANCs are absorbed into intestinal epithelial cells and then modulate the synthesis of GABA neurotransmitters via the intestinal microbiota pathway. ANCs promote the growth of neurotransmitter-producing bacteria such as *Lactobacillus* and *Bifidobacterium* species. The bacteria transform short-chain fatty acids into GABA, serotonin, and glutamate neurotransmitters. Phenol katabolic products from ANCs are a source of short-chain fatty acid production. This pathway indicates the involvement of ANCs in brain behavior functions (Zhong et al., 2023).

Despite the potential effect of ANC on dopamine and GABA neurotransmitters, molecular docking prediction showed the potential of ANCs from PSP to inhibit the MAO. MAO enzymes degrade biogenic amine neurotransmitters in neuronal cells. Inhibition of the MAO enzyme increases the concentration of monoamine neurotransmitters in the synaptic cleft including dopamine (Patetas & Gartner, 2006). Our previous study revealed that cyanidin, cyanidin-3-O-glucoside, and peonidin-3-O-glucoside as major ANCs from PSP from our local cultivar (Kurnianingsih et al., 2021). This study also demonstrated the prediction of ANCs binding with MAO protein in a similar site to Rasagiline as an inhibitor. ANCs are then assumed to be MAO inhibitors that have the potential to inhibit monoamine degradation neurotransmitters.

Certain animal behaviors are influenced by the modulation of brain-specific receptors, anti-inflammatory agents, and specific protein treatment. Spatial memory observation shows that inhibiting the orexin 1 receptor in the nucleus accumbens impairs spatial memory consolidation in rats. Other treatment agents, such as anti-inflammatory drugs, inhibit seizure behavior in rat models of acute epilepsy by intracerebroventricular injection. Per nostril application of prolactoliberin decreases time spent in open arms at elevated plus maze test, thus indicating increased anxiety behavior in rats (Fazlelahi et al., 2023; Ghazi Ghanim et al., 2023; Zokaei et al., 2023). The modulation of plant extract on neurotransmitter system and organ weight indicated the importance of future research in animal models (Chukwu et al., 2022; Nikjooy et al., 2022). Therefore, further evaluations are necessary to reveal the molecular mechanism of ANC from PSP to inhibit the adverse effect of stress.

Conclusion

This study concludes that ANC from PSP reduces brain inflammation, thus enhancing the dopamine and GABA neurotransmitters, followed by a decrease in the movements of chronic restraint-stress-induced mice. In addition, molecular prediction shows that ANCs from PSP can interact with the MAO enzyme as an inhibitor.

Ethical Considerations

Compliance with ethical guidelines

All procedures were approved by the Ethics Scientific Committee of the Brawijaya University (Code: 029-KEP-UB-2022).

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Authors' contributions

Conceptualization and study design: Nia Kurnianingsih and Retty Ratnawati; Supervision: Nia Kurnianingsih, Novita Titis Harbiyanti, Agwin Fahmi Fahanani and Retty Ratnawati; Data collection: Ariella Ramadhini Hakim, Daffa Salsabila, and Agwin Fahmi Fahanani; Writing the original draft and data analysis: Nia Kurnianingsih; Final approval: All authors.

Conflict of interest

All authors declared no conflict of interest.

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