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Original Research Article

Studies on Anti-Depressant Activity of Four Flavonoids Isolated from *Apocynum venetum* Linn (Apocynaceae) Leaf in Mice

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Abstract

Purpose: *To investigate the anti-depressant activity of kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose isolated from Apocynum venetum Linn. (Apocynaceae) leaf and their mechanisms of action.*

Methods: *The four flavonoids were isolated from Apocynum venetum leaf by chromatography. Mice were divided into vehicle, fluoxetine, kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose groups (n = 10). Forced swimming (FST), tail suspension (TST) and locomotor activity (LAT) tests were used to evaluate the effects of the four flavonoids (0.35 mM/kg) on immobility time, monoamine neurotransmitters, viz, norepinephrine (NE), dopamine (DA) and 5-hydroxytryptamine (5-HT), as well as on the metabolite (5-HIAA) in mice brain and central nervous system (CNS) with the aid of video camera, HPLC-ECD and activity-monitoring system.*

Results: *The four flavonoids significantly ($p < 0.05$) reduced mice immobility time (72.58 - 90.24; 52.58 - 70.24 s), 5-HIAA levels (940.8 - 1244.7; 880.8 - 1164.1 ng/g) and 5-HIAA/5-HT ratio (1.77 - 4.76; 1.83 - 4.16), but increased NE, DA and 5-HT levels (238.7 - 405.7, 308.4 - 528.1, 261.4 - 531.9; 243.9 - 423.6, 296.7 - 534.9, 279.8 - 481.4 ng/g) in FST and TST, compared with control group (146.18, 126.18 s; 1363.4, 1240.9 ng/g; 7.43, 6.16; 138.4, 235.4, 183.4 and 143.7, 218.6, 201.4 ng/g). The effects of the four flavonoids on the above indices were significant ($p < 0.05$) and positively related to their polarity. They had no CNS-stimulating effects in LAT.*

Conclusion: *The anti-depressant activities of the four flavonoids are positively related to their polarity, and the mechanisms may be due to increased NE, DA and 5-HT and reduced 5-HT metabolism.*

Keywords: *Kaempferol, Quercetin, Forced swimming test, Tail suspension test, Locomotor activity test, Neurotransmitters*

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INTRODUCTION

Depression is a kind of mental disorders and its major symptoms include the reduced interest and pleasure, lowered mood and metabolic disorder of monoamine neurotransmitters in the central nervous system (CNS) [1]. World Health Organization (WHO) predicted that depression will be the second most common disease in 2020 [2].

Apocynum venetum Linn. (Apocynaceae family) is widely distributed in many provinces of China such as Xinjiang, Gansu, Shandong and Hebei

[3]. It's reported that *A. venetum* leaf has lots of pharmacological effects such as anti-oxidant [4,5], anti-hypertensive [6], anti-depressant [7] and anti-anxiety activities [8]. A series of studies [9-14] suggested that the active constituents of anti-depressant activity of *A. venetum* leaf are total flavonoids. The flavonoids of *A. venetum* leaf include tamarixetin, kaempferol, quercetin, hyperoside, trifolin, quercetin-3-O-(6''-O-malonyl)-β-D-glucose, kaempferol-3-O-β-D-glucose, quercetin-3-O-β-D-glucose, rutin, etc [15,16]. Although it is reported that quercetin and kaempferol have significant anti-depressant activities [17,18], differences in their anti-depressant activities as well as possible mechanisms still need further investigation. Thus, the main aim of the present study was to evaluate the differences in the anti-depressant activities of quercetin, quercetin-3-O-β-D-glucose, kaempferol and kaempferol-3-O-β-D-glucose isolated from *A. venetum* leaf.

EXPERIMENTAL

Plant material

A. venetum leaves were purchased from Chinese herbal medicine market of Xian in 2013, identified by Jun-Lian Lang. A voucher specimen (voucher no. BJHLGH20130164) was stored in pharmacy department of Beijing Huilongguan Hospital for future reference.

Chemicals and reagents

Analytical grade ethanol, methanol, chloroform, petroleum ether, ethyl acetate, n-butyl alcohol and silica gel were obtained from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China) and Sephadex LH-20 was purchased from H&E Co., Ltd (Beijing, China). HPLC grade methanol was purchased from Fisher (Fisher Scientific, Germany). Fluoxetine-HCl was obtained from Lilly (Lilly, USA, purity > 98 %). NE, DA, 5-HT, 5-HIAA and dihydroxy-benzoic acid (DHBA) were obtained from Sigma (Sigma, USA, purity > 98 %).

Animals

Male mice (20 ± 2 g) were purchased from the SLRC Laboratory Animal Company (Shanghai, China). The animals were conditioned to standard laboratory conditions (12/12 h light /dark cycle at 25 ± 1 °C) and had free access to food and water. All experiments were strictly in accordance with the international guidelines for care and use of laboratory animals [19]. The experiments were carried out with the approval of the Animal Care and Use Committee of Beijing Huilongguan Hospital (protocol no: BJHLGH ACUC2013).

Extraction and isolation

The air-dried *A. venetum* leaves (10 kg) were finely cut and extracted with 75 % ethanol by percolation method at the speed of 20 mL/min. The 75 % ethanol solvent was combined and concentrated under vacuum to afford a crude extract (1.2 kg), which was then suspended in hot water and successfully partitioned with petroleum ether, ethyl acetate and n-butyl alcohol. The ethyl acetate fraction (180 g) was subjected to column chromatography (CC) over silica gel (74 - 149 μm), eluted with chloroform-methanol and chloroform-methanol-water to obtain nine fractions. Fraction 2 (32.2 g) was separated by CC over silica gel (chloroform-methanol) and purified on Sephadex LH-20 (chloroform-methanol) to provide compounds kaempferol (98 mg) and quercetin (173 mg). Fraction 5 (28.9 g) was separated by CC over silica gel (chloroform-methanol-water) and purified on Sephadex LH-20 (chloroform-methanol-water) to provide compounds kaempferol-3-O-β-D-glucose (67 mg) and quercetin-3-O-β-D-glucose (173 mg). The structures of the four compounds were identified by nuclear magnetic resonance (NMR) data, compared with the existing literatures [15,16], and their purities were more than 98 %, verified by area normalization method of high performance liquid chromatography with electrochemical detector (HPLC-ECD). Their chemical structures are shown in Figure 1.

Grouping and drug treatment

Mice were randomly divided into 6 groups (n = 10): negative control, positive control, kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose groups. Fluoxetine, kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose were separately dissolved in 0.5 % CMC-Na to obtain 2 mM fluoxetine, 35 mM kaempferol, 35 mM quercetin, 35 mM kaempferol-3-O-β-D-glucose and 35 mM quercetin-3-O-β-D-glucose. The vehicle solvent (0.5 % CMC-Na) and fluoxetine served as negative control and positive control groups, respectively. The doses of fluoxetine, kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose for mouse were 0.2, 0.35, 0.35, 0.35 and 0.35 mM/kg, respectively. The vehicle and drugs were intraperitoneally administered at volume of 0.1 mL/10 g 60 min prior to the test session.

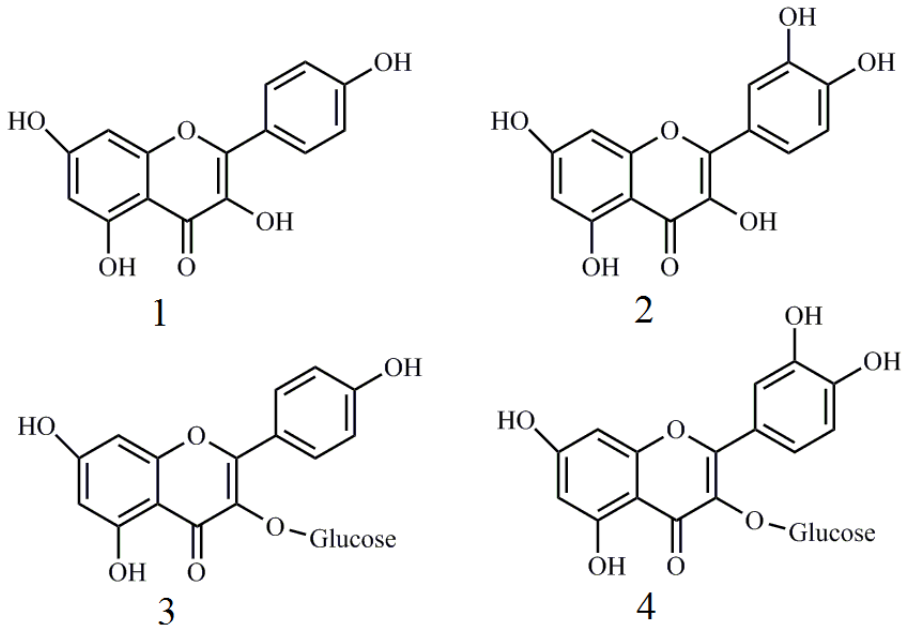

Figure 1: Chemical structure of kaempferol (1), quercetin (2), kaempferol-3-O-β-D-glucose (3) and quercetin-3-O-β-D-glucose (4); polarity: 1 < 2 < 3 < 4

Forced swimming test (FST)

FST was carried out on mice according to the existing method with some modifications [18,20,21]. Mice were individually placed into glass cylinders (height 25 cm, diameter 10 cm), which were filled with water (temperature: 25 ± 1 °C; depth: 18 cm). Twenty-four hours after the pretest, mice were forced to swim in the glass cylinders for 6 min. After 2 min swimming, the duration of immobility was recorded during the remaining 4 min using a video camera. Mice were judged to be immobile when no additional activity was observed other than that those movements necessary to keep its head above the water. Additionally, the observer recording the immobility of mice was blinded to the drug treatments and after each test, the glass cylinders was refilled with fresh water.

Tail suspension test (TST)

TST was carried out on mice according to the existing method with some modifications [22]. Briefly, each mouse was individually suspended by the tail (20 mm from the end) for 6 min in a box (250 × 250 × 300 mm^3) with the head 50 mm from the bottom. Testing was carried out in a darkened room with minimal background noise. The duration of immobility was recorded during the final 4 min of the test using a video camera. Mice were judged to be immobile only when they were passively hanged and completely motionless. Additionally, the observer recording the immobility of mice was blinded to the drug treatments.

Locomotor activity test (LAT)

To access the effects of drugs on locomotor activity, mice were individually placed into cylinders (height 25 cm, diameter 15 cm) at the same time 60 min after drug administration and then the total locomotor activity of each mouse was automatically measured and recorded in 5 min separately by an activity-monitoring system (Institute of Materia Medica, Chinese Academy of Medical Sciences) [1].

Determination of monoamine neurotransmitter and metabolite

The neurotransmitter concentrations in mice brain were simultaneously determined according to the existing method with some modifications [23]. The vehicle group (negative control group) without any stress was added as vehicle-stress group in this part. After the FST and TST were completed, mice were sacrificed by decapitation in the animal laboratory, and their brain tissues were immediately obtained on ice and stored at -80 °C until biochemistry assay. The brain tissue of each mouse was homogenized in ice-cold 0.02 M perchloric acid including 1.0 μM DHBA (internal standard). Then the homogenates were centrifuged at 14000 rpm for 15 min at 4 °C, and the supernatant was separated and filtered through a 0.22 μm membrane filter prior to HPLC analysis. The HPLC analysis was carried out on Agilent 1200 (Ageilent, USA), equipped with ECD (Model 5300A CoulArray Detector) and connected to an Agilent ChemStation. The chromatography was performed on a Diamonil

C18 column (4.6 mm × 25 mm, 5 μm). The mobile phase consisted of 11% methanol and 0.1 M NaH2PO4 aqueous solution including 0.5 mM EDTA•Na_2 and 0.85 mM OSA. The pH of mobile phase was adjusted to 3.4 using phosphate acid. Then mobile phase were filtered through 0.22 μm membrane prior to HPLC analysis. The flow rate, injection volume, column temperature and working potential were 0.8 mL/min, 20 μL, 25 °C and +80 mV, respectively. The quantifications of NE, DA, 5-HT and 5-HIAA in sample were analyzed by standard curve method. Additionally, the levels of NE, DA, 5-HT and 5-HIAA were represented as ng/g per brain wet weight.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM, n = 10). One-way ANOVA (LSD test) was used to analyze differences among different groups on SPSS (version 20.0). Differences were recognized as statistically significant at $p < 0.05$.

RESULTS

Effects of four flavonoids on duration of immobility

As shown in Figures 2 and 3, after treatment with fluoxetine at dose of 0.2 mM/kg, the duration of immobility (58.9 and 38.59 sec) in FST and TST was significantly ($p < 0.05$) reduced, compared with the vehicle group (146.18 and 126.18 sec), and the results indicated that the FST and TST models were successfully established. After treatment with kaempferol, quercetin, kaempferol-3-O-β-D-glucose or quercetin-3-O-β-D-glucose at dose of 0.35 mM/kg, the duration of immobility (72.58 - 90.24 and 52.58 - 70.24 sec) was significantly ($p < 0.05$) reduced in FST and TST, compared with the vehicle group (146.18 and 126.18 sec). Moreover, the inhibitory effects of the four flavonoids on the duration of immobility in FST and TST were significantly ($p < 0.05$) different (kaempferol < quercetin < kaempferol-3-O-β-D-glucose < quercetin-3-O-β-D-glucose).

Effects of four flavonoids on locomotor activity

As shown in Figure 4, the locomotor activity counts did not show any significant differences among the vehicle, fluoxetin, kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose groups. The results suggested that the effects of drug treatments on the reductions of immobility time in FST and TST were not induced by possible CNS-stimulating effects.

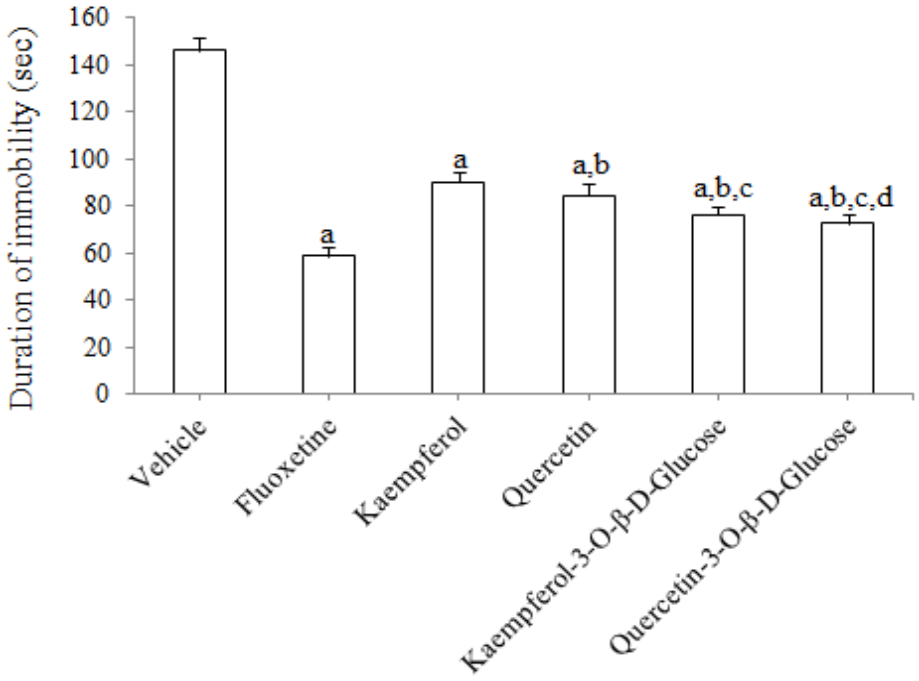

Figure 2: Effects of kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose on the duration of immobility in FST; [a]$p < 0.05$ vs. the vehicle group, [b]$p < 0.05$ vs. the kaempferol group, [c]$p < 0.05$ vs. the quercetin group, [d]$p < 0.05$ vs. the kaempferol-3-O-β-D-glucose group

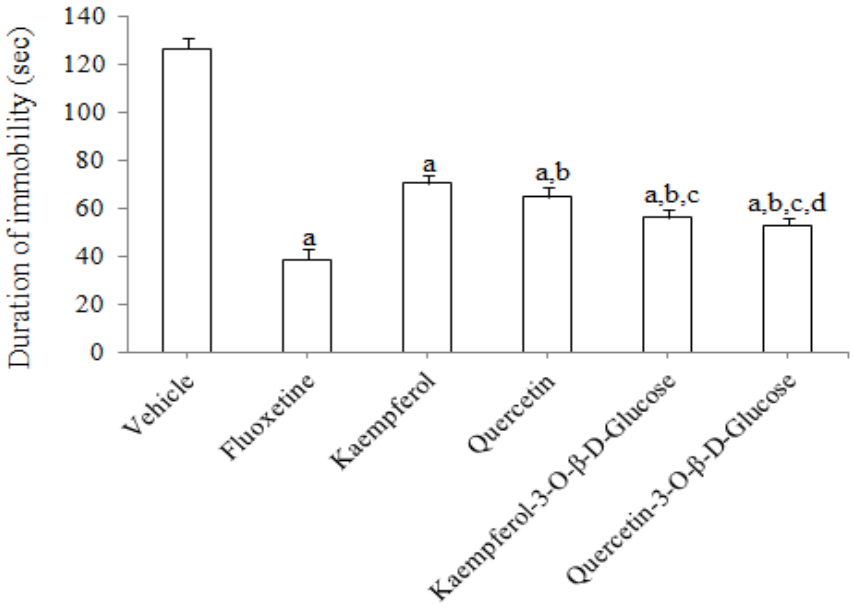

Figure 3: Effects of kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose on the duration of immobility in TST; [a]$p < 0.05$ vs. the vehicle group, [b]$p < 0.05$ vs. the kaempferol group, [c]$p < 0.05$ vs. the quercetin group, [d]$p < 0.05$ vs. the kaempferol-3-O-β-D-glucose group

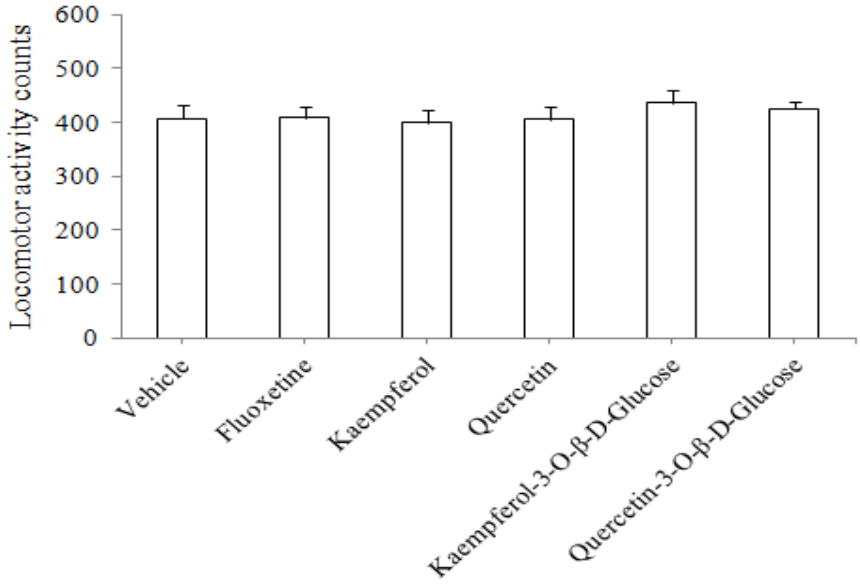

Figure 4: Effects of kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose on locomotor activity of mice

Effect of four flavonoids on monoamine neurotransmitter and metabolite

The quantifications of monoamine neurotransmitter and metabolite concentrations in sample were evaluated according to the standard curves of NE (y = 0.045800x+0.003712, r=0.9999), DA (y=0.054806x+0.006300, r=0.9998), 5-HT (y=0.048102x+0.001102, r=0.9994) and 5-HIAA (y=0.050893x+0.004106, r=0.9999). The chromatogram of standard substances and sample are shown in Figure 5.

The effects of drug treatments on the levels of NE, DA, 5-HT and 5-HIAA in FST and TST are presented in Tables 1 and 2. The effects of drug treatments on the ratio of 5-HIAA/5-HT in FST and TST are shown in Figures 6 and 7. Compared with the vehicle-stress group, the levels of NE, DA, 5-HT of vehicle group were significantly ($p < 0.05$) reduced in FST and TST, and the level of 5-HIAA and the ratio of 5-HIAA/5-HT of vehicle group were significantly ($p < 0.05$) increased in FST and TST. After treatment with fluoxetin at dose of 0.2 mM/kg, the

levels of NE, DA and 5-HT were significantly (p < 0.05) increased in FST and TST, compared with the vehicle group, and the level of 5-HIAA and the ratios of 5-HIAA/5-HT were significantly (p < 0.05) reduced in FST and TST, compared with the vehicle group. The results of the vehicle-stress, vehicle and fluoxetin groups indicated that the FST and TST models were successfully established. After treatment with kaempferol, quercetin, kaempferol-3-O-β-D-Glucose or quercetin-3-O-β-D-glucose at dose of 0.35 mM/kg, the levels of NE, DA and 5-HT were significantly (p < 0.05) increased in FST and TST, compared with the vehicle group, and the level of 5-HIAA and the ratios of 5-HIAA/5-HT were significantly (p < 0.05) reduced in FST and TST, compared with the vehicle group. Moreover, the reversed effects of the four flavonoids on the levels of NE, DA, 5-HT and 5-HIAA and the ratio of 5-HIAA/5-HT were significantly (p < 0.05) different in FST and TST (kaempferol < quercetin < kaempferol-3-O-β-D-glucose < quercetin-3-O-β-D-glucose).

Table 1: Effects of kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose on the levels of NE, DA, 5-HT and 5-HIAA in FST

Group	NE (ng/g)	DA (ng/g)	5-HT (ng/g)	5-HIAA (ng/g)
Vehicle	138.4±9.8	235.4±43.1	183.4±12.4	1363.4±47.2
Fluoxetine	463.2±24.9[a]	640.1±52.8[a]	479.3± 4.7[a]	835.5±45.6[a]
Kaempferol	238.7±35.6[a]	308.4±34.2[a]	261.4±20.4[a]	1244.7±41.2[a]
Quercetin	312.4±47.1[a,b]	391.0±23.5[a,b]	387.6±18.5[a,b]	1128.9±54.6[a,b]
Kaempferol-3-O-β-D-glucose	358.1±39.5[a,b,c]	469.4±25.7[a,b,c]	467.2±30.1[a,b,c]	1054.4±54.7[a,b,c]
Quercetin-3-O-β-D-glucose	405.7±41.2[a,b,c,d]	528.1±23.8[a,b,c,d]	531.9±25.7[a,b,c,d]	940.8±50.3[a,b,c,d]
Vehicle-stress	512.4±30.2[a]	654.8±34.7[a]	645.7±52.4[a]	808.9±32.1[a]

[a]p < 0.05 vs. the vehicle group, [b]p < 0.05 vs. the kaempferol group, [c]p < 0.05 vs. the quercetin group, [d]p < 0.05 vs. the kaempferol-3-O-β-D-glucose group

Table 2: Effects of kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose on the levels of NE, DA, 5-HT and 5-HIAA in TST

Group	NE (ng/g)	DA (ng/g)	5-HT (ng/g)	5-HIAA (ng/g)
Vehicle	143.7±11.2	218.6±32.5	201.4±23.7	1240.9±56.3
Fluoxetine	441.8±19.4[a]	596.1±46.7[a]	498.5± 34.1[a]	838.5±38.9[a]
Kaempferol	243.9±26.1[a]	296.7±26.1[a]	279.8±23.2[a]	1164.1±58.2[a]
Quercetin	298.4±37.5[a,b]	388.6±30.3[a,b]	321.3±29.5[a,b]	1040.9±45.7[a,b]
Kaempferol-3-O-β-D-glucose	368.7±20.2[a,b,c]	473.1±31.2[a,b,c]	391.6±38.6[a,b,c]	962.4±40.1[a,b,c]
Quercetin-3-O-β-D-glucose	423.6±28.8[a,b,c,d]	534.9±35.3[a,b,c,d]	481.4±45.5[a,b,c,d]	880.8±39.3[a,b,c,d]
Vehicle-stress	512.4±30.2[a]	637.5±54.6[a]	645.7±52.4[a]	897.9±52.1[a]

[a]p < 0.05 vs. the vehicle group, [b]p < 0.05 vs. the kaempferol group, [c]p < 0.05 vs. the quercetin group, [d]p < 0.05 vs. the kaempferol-3-O-β-D-glucose group

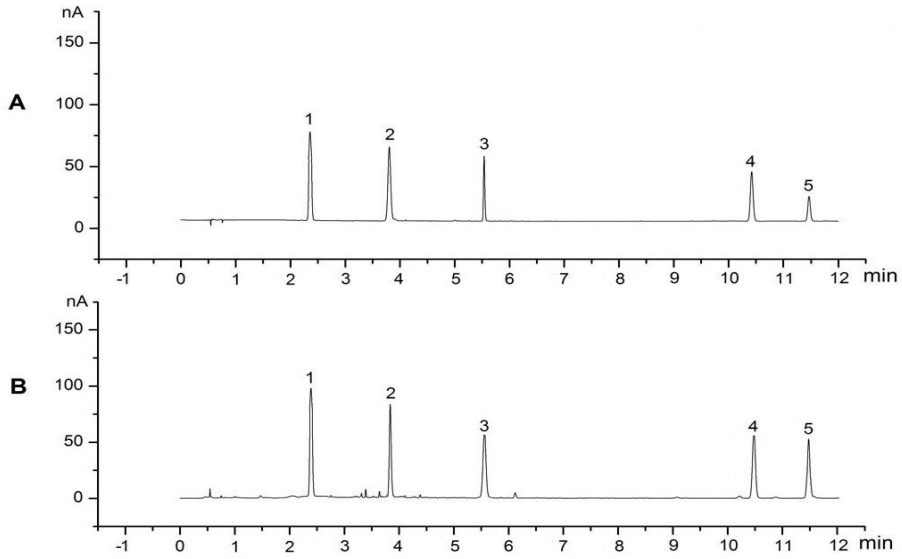

Figure 5: Chromatogram of standard substances (A) and sample (B); peak 1: NE, 2: DHBA, 3: DA, 4: 5-HT, 5: 5-HIAA

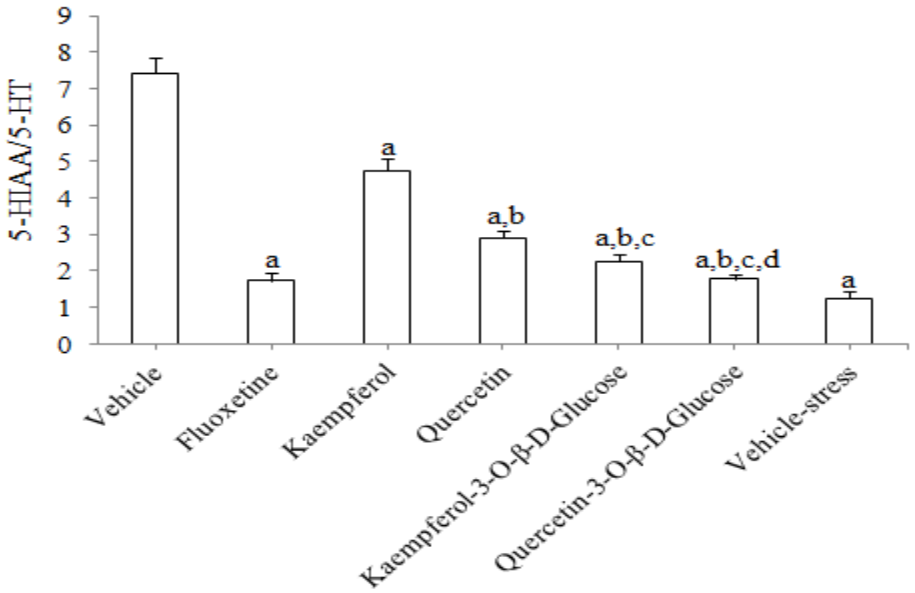

Figure 6: Effects of kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose on the ratio of 5-HIAA/5-HT in FST; [a]$p < 0.05$ vs. the vehicle group, [b]$p < 0.05$ vs. the kaempferol group, [c]$p < 0.05$ vs. the quercetin group, [d]$p < 0.05$ vs. the kaempferol-3-O-β-D-glucose group

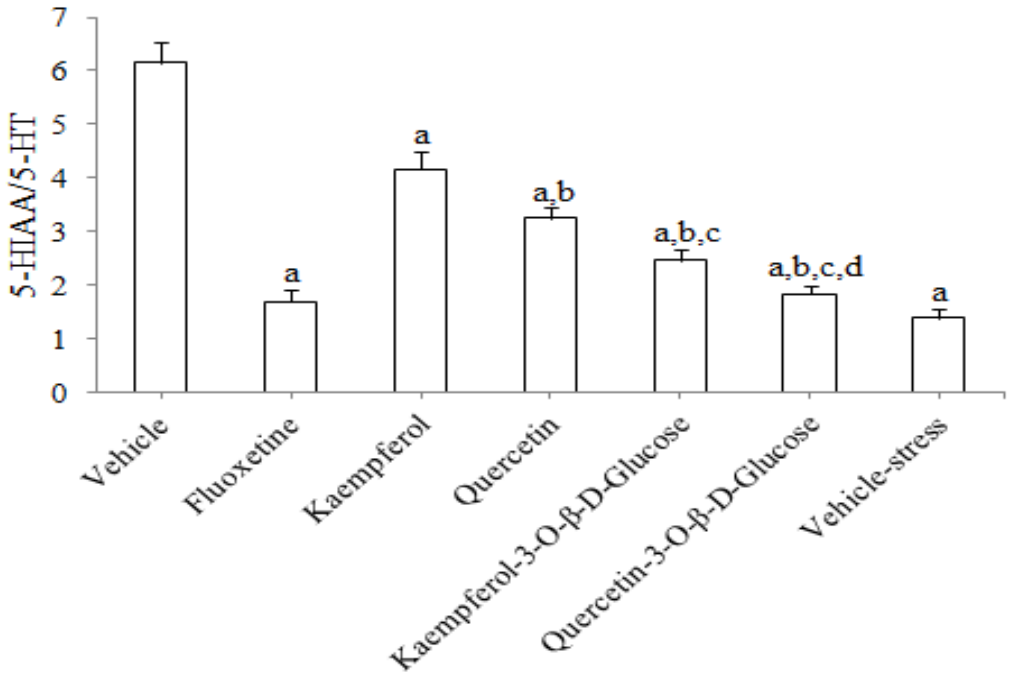

Figure 7: Effects of kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose on the ratio of 5-HIAA/5-HT in TST; [a]$p < 0.05$ vs. the vehicle group, [b]$p < 0.05$ vs. the kaempferol group, [c]$p < 0.05$ vs. the quercetin group, [d]$p < 0.05$ vs. the kaempferol-3-O-β-D-glucose groupffects of kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose on locomotor activity of mice

DISCUSSION

In the present study, the differences of anti-depressant activities of quercetin, quercetin-3-O-β-D-glucose, kaempferol, kaempferol-3-O-β-D-glucose from *A. venetum* leaf and the possible mechanisms were investigated by FST, TST and LAT.

The FST and TST are two accepted stress models used to evaluate the anti-depressant activities of drugs [1,24]. If a drug can induce the locomotor activity, a false positive activity in FST and TST will be observed and thus LAT should be carried out to rule out the false positive activity [25]. As shown in Figures 2 - 4, the results of FST, TST and LAT indicated that kaempferol, quercetin, kaempferol-3-O-β-D-

glucose and quercetin-3-O-β-D-glucose had significant anti-depressant activity by reducing the immobility time of mice, and the inhibitory effects of the four flavonoids on the immobility time were positively related to their polarity.

The monoamine theory is a widely admitted explanation for depression, which can lead to the impairment of monoaminergic functions and reduce the levels of monoamine neurotransmitters (NE, DA and 5-HT) [26]. The symptom of depression can be alleviated by up-regulating the levels of monoamine neurotransmitters (NE, DA and 5-HT) in the CNS [27]. The tricyclic antidepressants, monoamine oxidase inhibitors and selective 5-HT reuptake inhibitors are now widely used in the clinic, and fluoxetine, a kind of selective 5-HT reuptake inhibitor, are more popular because of its lower toxicity [28,29]. So, the fluoxetine was selected as the positive control in the study. The ratio of neurotransmitter/its metabolite can be considered as an index of neurotransmitter metabolism, and the reduction of the ration indicates the reduction of neurotransmitter metabolism [29,30]. As shown in Tables 1 and 2 and Figures 6 and 7, kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose significantly increased the levels of NE, DA and 5-HT and reduced the levels of 5-HIAA and the ratio of 5-HIAA/5-HT in the mice brain of FST and TST. Namely, their possible mechanisms of anti-depressant activity were related to the increase of NE, DA and 5-HT as well as reduction of 5-HT metabolism in mice brain. Moreover, the reversed effects of the four flavonoids on the levels of NE, DA, 5-HT and 5-HT metabolism were positively related to their polarity.

CONCLUSION

Kaempferol, quercetin, kaempferol-3-O-β-D-glucose and quercetin-3-O-β-D-glucose from *A. venetum* leaf have significant anti-depressant activity that is positively related to their polarity. The mechanism may be related to the increase of NE, DA and 5-HT as well as reduction of 5-HT metabolism. The study provides the evidence to support that *A. venetum* leaf has anti-depressant activity and the reference for studying the structure-function relationship of flavonoids on anti-depressant activity.

ACKNOWLEDGEMENT

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