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Full Length Research Paper

In vitro and *in vivo* protective action of ethanolic extract of *Triphala* on LDL against glycation-oxidation

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The modified low-density lipoprotein (LDL; glycated and oxidized) is more atherogenic and are poorly recognized by LDL receptor. In the present study, an attempt has been made to evaluate the effects of *Triphala* on LDL modification in experimentally treated rats and in human LDL. The effect of ethanolic extract of *Triphala* on LDL oxidation susceptibility in high fat diet fed and STZ induced rats was evaluated .The inhibitory effect of *Triphala* on LDL oxidation and glycation was investigated using copper and glucose induced human LDL, respectively *in vitro*. The co-presence of *Triphala* extract significantly increased lag phase and minimized the conjugated dienes formation in dose dependent manner. Copper mediated LDL oxidation was characterized by elevated indices of thiobarbituric acid reactive substance (TBARS), whereas co-presence of *Triphala* extract significantly minimized the production of TBARS. The results of this investigation shows that *Triphala* probably with their antioxidant properties inhibited LDL glycation and oxidation and provide scientific reasons regarding the possible medical benefits of using *Triphala* to prevent diabetic and cardiovascular complications.

Key words: *Triphala*, low-density lipoprotein (LDL) oxidation, LDL glycation, high fat diet, streptozotocin (STZ).

INTRODUCTION

Atherosclerosis, a cardiovascular disorder posing main cause for the death in developed as well as developing countries (Stachura and Pierzynowski, 2009). Atherosclerosis is characterized by low-density lipoprotein (LDL) deposition in the arterial wall which finally ends in the formation of lesions. Rupture of lesions clinically leads to heart attack and stroke. Atherosclerosis is a complication associated with diabetes. In patients with diabetes, the risk of atherosclerosis is three to five folds greater than in non diabetics. LDL is a major risk factor in this regard.

The LDL consists of 25% apo B-100 protein and 75% of lipid consisting of cholesterol esters and some triglycerides. LDL is the main cholesterol carrying lipoprotein in plasma (Kalyanaraman, 1995). Linoleate is the polyunsaturated fatty acids (PUFAs) present in LDL, which are associated with cholesteryl ester, are very susceptible to oxidative damage. LDL modifications (oxidation and glycation) are strongly related to diabetic complications, atherosclerosis, and other cardiovascular diseases (Lyons, 1993; Hunt, 1991; Li et al., 1996; Picard, 1995). Glycated LDL is more susceptible to oxidation

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than unglycated LDL, and oxidized LDL is more prone to modification by glycation (Picard, 1995; Albertini et al., 2002). Oxidation of LDL is crucial in plaque formation and onset of atherosclerosis (Steinberg, 1997).

Triphala ['three' (tri) 'fruits' (phala)] is a traditional Ayurvedic herbal formulation consisting of the dried fruits of three medicinal plants, *Terminalia chebula, Terminalia bellirica*, and *Phyllanthus emblica*, also known as the 'three myrobalans'. This formulation is a frequently used Ayurvedic medicine to treat many diseases such as anemia, jaundice, constipation, asthma, fever and chronic ulcers. It is an important medicine of the 'rasayana' group and is believed to promote health, immunity and longevity. It corrects constipation, cleanses and tonifies the gastrointestinal tract and also detoxifies the whole body, and improves digestion and assimilation (Nadkarni, 1976). It exhibits anti-viral, anti-bacterial, anti-fungal and anti-allergic properties (Mehta et al., 1999; Singh, 2003). *Triphala* and its constituents act as cardio-tonic, control blood pressure, improve blood circulation and reduce cholesterol levels (Thakur et al., 1988; Tariq et al., 1977). *Triphala* shows immunomodulatory properties and helps in improving the body's defense system (Srikumar et al., 2005). In recent years there are also several reports in the literature which suggest that *Triphala* possesses antimutagentic, and radio protecting activity (Kaur et al., 2005, 2002; Vani et al., 1997; Jagetia et al., 2002, 2004a, b; Arora et al., 2003; Kumar et al., 1996; Naik et al., 2005). *Triphala* has been reported to be a rich source of Vitamin C, ellagic acid, gallic acid, chebulinic acid, bellericanin, β-sitosterol and flavanoids (Jagetia et al., 2002).

Since LDL oxidation and glycation are closely interrelated and both are important factors for the development of atherosclerosis, proper agents with antioxidative and antiglycative properties may provide medical benefits. This formulation is rich in antioxidant and there is no evidence to support *Triphala* has an inhibitory role on LDL oxidation and glycation. Thus, this work was undertaken to investigate the effect of *Triphala* on the modification of LDL both in isolated LDL from human and experimental rat.

MATERIALS AND METHODS

Animals and diet

Male Sprague-Dawley rats (230 ± 20 g) were used in this study. The animals were housed in large spacious cages, bedded with husk and were given food and water *ad libitum*. The animal room was well ventilated with a 12-h light/dark cycle throughout the experimental period. Animal experimentation was conducted according to the current institutional regulations (IAEC no.: IAEC/39/05/CLBMCP/2013).

Chemicals

Disodium ethylene diamine tetraacetate (Na_2 EDTA), sodium chloride (NaCl), and disodium hydrogen phosphate (Na_2HPO4) were purchased from Sigma chemical. *Terminalia chebula*, *Terminalia bellerica*, and *Phyllanthus emblica*, were purchased from the local market.

Extraction

T. chebula, *T. bellerica* and *P. emblica* were bought from the local market and identified by botanist in CSMDRIA, Chennai. The fruits were dried and pulps were coarsely powdered. One kilogram powder was soaked in ethanol for 7 days with intermittent shaking and the solvent was filtered with Whatmann filter paper. The filtrate was evaporated under vacuum drier and the brown mass residue obtained was stored at -4°C for further use(s).

Experimental design

In vitro experiments

To study the effect of *Triphala* on human LDL oxidation and glycation, *in vitro* experiments were conducted on LDL isolated from human plasma.

Blood sampling

Fasting blood samples were collected from normal healthy volunteers. To obtain fresh plasma, blood samples were centrifuged (3000 rpm for 10 min at 4°C) as soon as the samples were collected to avoid auto oxidation.

LDL isolation

Plasma LDL was isolated by precipitation method using heparin-citrate buffer (Ahotupa et al., 1998). The precipitation buffer consisted of 0.064 M trisodium citrate adjusted to pH 5.05 with 5 N HC1, and contained 50,000 IU/L heparin. Plasma sample and precipitation reagents were allowed to equilibrate to room temperature before precipitation of LDL. To 100 µl of sample, 1 ml of the heparin-citrate buffer was added and mixed using Vortex mixer, and was incubated in room temperature for 10 min; then, was centrifuged at 1000 g for 10 min to remove insoluble lipoproteins. The pellet was resuspended in 1 ml of 0.1 M Na-phosphate buffer, pH 7.4, containing 0.9% of NaCl.

LDL oxidation and glycation

The oxidation of LDL was done by incubating 100 µg LDL with $CuSO_4$ 10 mmol/L for 4 h at 37°C (Ghaffari and Ghiasvand, 2006). The ox-LDL was then extensively dialyzed overnight in phosphate buffered saline containing ethylenediaminetetraacetic acid 0.05 mmol/L. The glycation of LDL was induced by incubating with glucose (25 mM) for 7 days at 37°C (Li et al., 1996) with various concentration of *Triphala* extract followed by dialysis and $CuSO_4$ induced oxidation for 4 h. The degree of oxidation and glycation were measured as the amount of thiobarbituric acid-reactive substances produced.

LDL oxidation kinetics

The total protein was measured after the LDL isolation by Lowry's method. 0.1 ml of native LDL and glycated LDL (100 µg of protein) was diluted to 0.9 ml with PBS and was incubated with or without

0.1 ml of *Triphala* extract (1, 5 and 10 µg/ml) at 37°C for 30 min. At the end of incubation period, oxidation was initiated by adding 0.01 ml of freshly prepared 10 mM $CuSO_4$. The LDL oxidation kinetics was measured by continuously monitoring the change in absorbance for 4 h (at 37°C) every 20 min at 234 nm. The lag phase was calculated by drawing a tangent to the slope of propagation phase and extrapolation into the intercept of initial-absorbance axis. The lag phase represents the antioxidant-protected phase during oxidation by *Triphala* extract.

Assay for conjugated dienes and products of lipid peroxidation

At the end of 4 h incubation, the formation of conjugated dienes was calculated as conjugated dienes equivalent content (nmol/mg-protein) at 240 min. The conjugated dienes concentration was calculated by using the extinction coefficient for diene conjugates at 234 nm (29500 L/mol.cm).

After 4 h incubation, samples were incubated with 0.5 ml of 20% trichloroacetic acid and 1 ml of 0.67% aqueous solution of thiobarbituric acid (Scoccia et al., 2001). The reaction mixtures were heated at 100°C for 20 min and then centrifuged at 2000 g for 5 min. The absorbance of the supernatant fractions was estimated at 532 nm. The concentration of TBARS was calculated using the extinction coefficient of 165,000 mol/cm and expressed as nmol of malondialdehyde equivalents per mg LDL protein

***In vivo* study**

The study was divided into two phases: the atherosclerotic progression and regression studies. The models used were the male Sprague-Dawley rats. The animals were randomly divided into 8 groups of six animals each. The total duration of the experiment is 8 weeks.

Group 1: Control (0.5% dimethyl sulfoxide [DMSO]); Group 2: Control + extract (200 mg/kg body weight in 0.5% DMSO); Group 3: high fat diet (HFD) + STZ; Group 4: HFD + extract (100 mg/kg body weight in 0.5% DMSO) + STZ; Group 5: HFD + extract (200 mg/kg body weight in 0.5% DMSO) + STZ; Group 6: HFD + STZ + extract (100mg/kg body weight in 0.5% DMSO); Group 7: HFD+ STZ + extract (200 mg/kg body weight in 0.5% DMSO); Group 8: HFD + STZ + 2 mg/kg body weight atorvastatin in 0.5% DMSO.

Progression study

For the progression study, 12 standard deviation (SD) rats were randomly divided into two groups(4 and 5), fed with high fat diet and received *Triphala* 100 and 200mg/kg of body weight orally along with HFD. After 4 weeks, the animals were subjected to an overnight fast and injected with STZ (35 mg/kg body weight in 0.1 M citrate buffer, pH 4.5). Animals had free access to food and water after the STZ injection. The animals continued on their HFD diet for the duration of the study.

Regression study

Twelve SD rats were randomly divided into two groups (6 and 7), fed with HFD. After 4 weeks, the animals were subjected to an overnight fast and injected with STZ (35 mg/kg body weight in 0.1 M citrate buffer, pH 4.5) and received *Triphala* 100 and 200 mg/kg of body weight orally. The animals continued on their HFD diet for the duration of the study.

At the end of the first and second phases of the experimental period, the rats were fasted overnight, anesthetized with phenobarbital (25 mg/kg intravenously) and sacrificed. The tissues were removed quickly, washed with cold saline, and stored at 20°C until analyzed. Various biochemical analysis and histopathologic analysis of the aorta were performed.

LDL isolation and effect of *Triphala* on LDL oxidation

Plasma LDL was isolated by precipitation method using heparin-citrate buffer (Ahotupa et al., 1998) as described earlier. The total protein was measured after the LDL isolation by Lowry's method. 0.1 ml of LDL (100 µg of protein) was diluted to 0.9 ml with phosphate buffer saline (PBS) and was incubated with or without 0.1 ml of *Triphala* extract (1, 5 and 10 µg/ml) at 37°C for 30 min. At the end of incubation period, oxidation was initiated by adding 0.01 ml of freshly prepared 10 mM $CuSO_4$.

Assay for conjugated dienes and products of lipid peroxidation

At the end of 4 h incubation, the formation of conjugated dienes was calculated as conjugated dienes equivalent content (nmol/mg-protein) at 240 min. The conjugated dienes concentration was calculated by using the extinction coefficient for diene conjugates at 234 nm (29500 L/mol.cm).

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Statistical analysis

Each data value was presented the mean ± (SD) of three experiments performed in duplicate. Results were expressed as mean ± SD. Statistical evaluation of the data was done by one way ANOVA followed by Duncan's multiple range test (DMRT) test. Differences were considered significant at $P<0.05$.

RESULTS

Oxidation kinetics of both glycated and oxidized LDL was measured by change in absorbance at 234 nm for every 20 min as shown in Figures 1A and 2A. It clearly shows copper increased LDL oxidation. The conjugated dienes formation, marker of the LDL oxidation, was decreased by the incubation with *Triphala* extract. Lag phase was also calculated as shown in Figures 1B and 2B. Four hours after copper addition, the extent of LDL oxidation was estimated from TBARS and conjugated dienes measurements as shown in Figures 1C, 1D, 2C and 2D. TBARS production was decreased in the presence of 1 µg, 5 g and 10 µg of *Triphala* concentrations. As shown in Figures 1C, 2C and 3, this reduction was dependent on *Triphala* concentrations.

The TBARS and conjugated dienes formation in experimental animals was also studied and measurements were given in graph 3A and B. *In vitro* LDL oxidation studies revealed that HFD+STZ treated rats showed

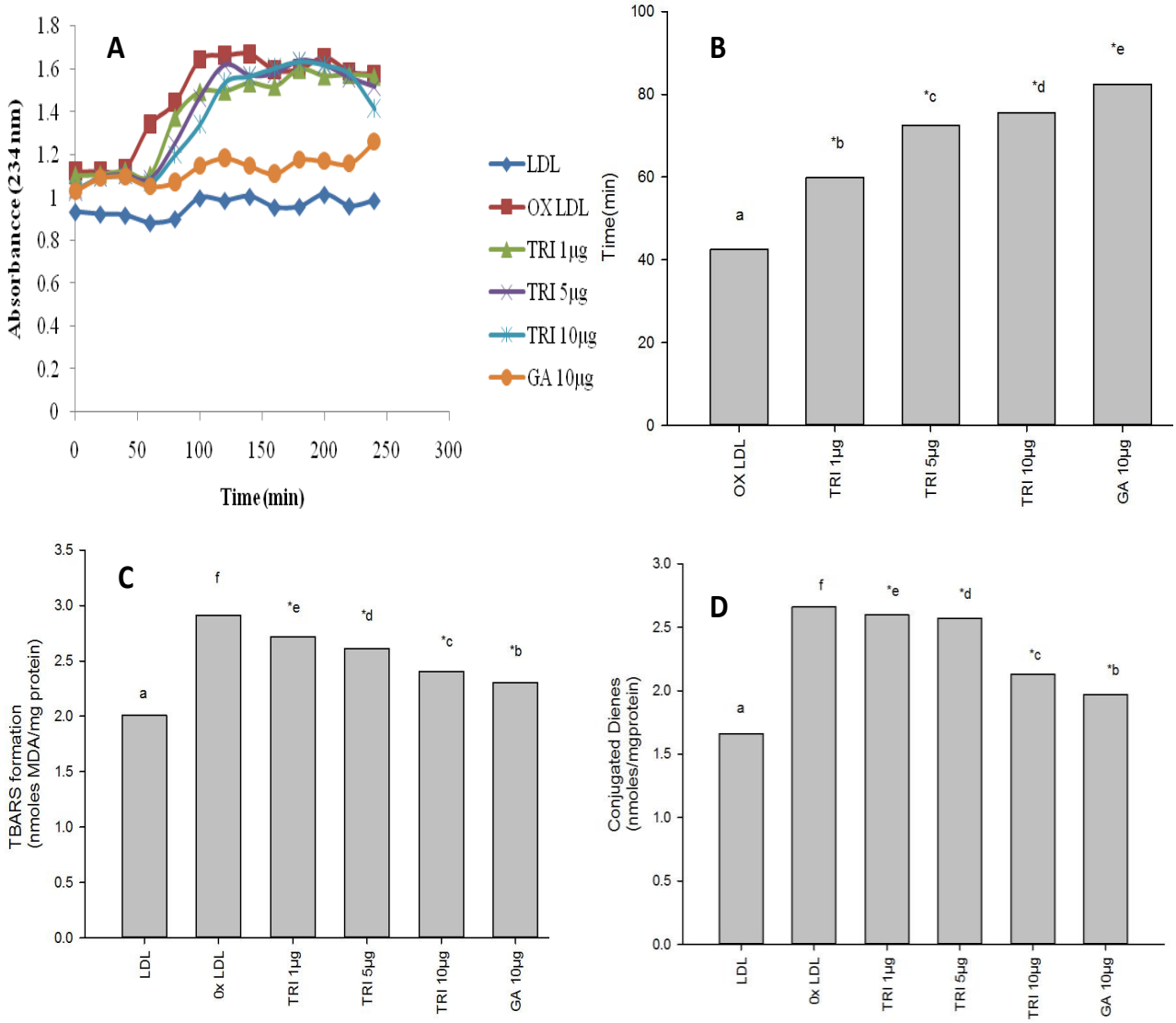

Figure 1. (A) The effect of *Triphala* extract on the LDL oxidation kinetics. Each point represents the mean of three experiments. (B) The effect of *Triphala* extract on the lag time (The lag phase represented the length of the antioxidant - protected phase during LDL oxidation by extract). Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Ox-LDL. (C) The effect of *Triphala* extract on the formation of TBARS. Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Ox-LDL. (D) The effect of *Triphala* extract on the formation of conjugated dienes. Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Ox-LDL.

significantly lowered lag phase time, increased CD concentration and significantly elevated levels of TBARS, whereas no significant change was observed in lag phase of *Triphala* alone treated controls rats as compared to untreated control rats (group 1). However, supplementing *Triphala* simultaneously along with HFD (groups 4 and 5) significantly increased the lag phase, lowered the CD formation and TBARS level as compared to the rats supplemented with *Triphala* after four weeks treatment with HFD and induction of diabetes with STZ (groups 6 and 7).

DISCUSSION

Natural antioxidants and lipid lowering interventions such as food supplements, spices, and herbs find extensive application in prevention of atherosclerosis, because of their ability to prevent *in vitro* LDL oxidation (Chang et al., 2006; Chu et al., 2009) and plaque formation (Ho et al., 2010).

LDL oxidation is mediated by free radical and the process is initiated by the removal of hydrogen atom from a methylene (CH_2) group of PUFA moiety of LDL. The

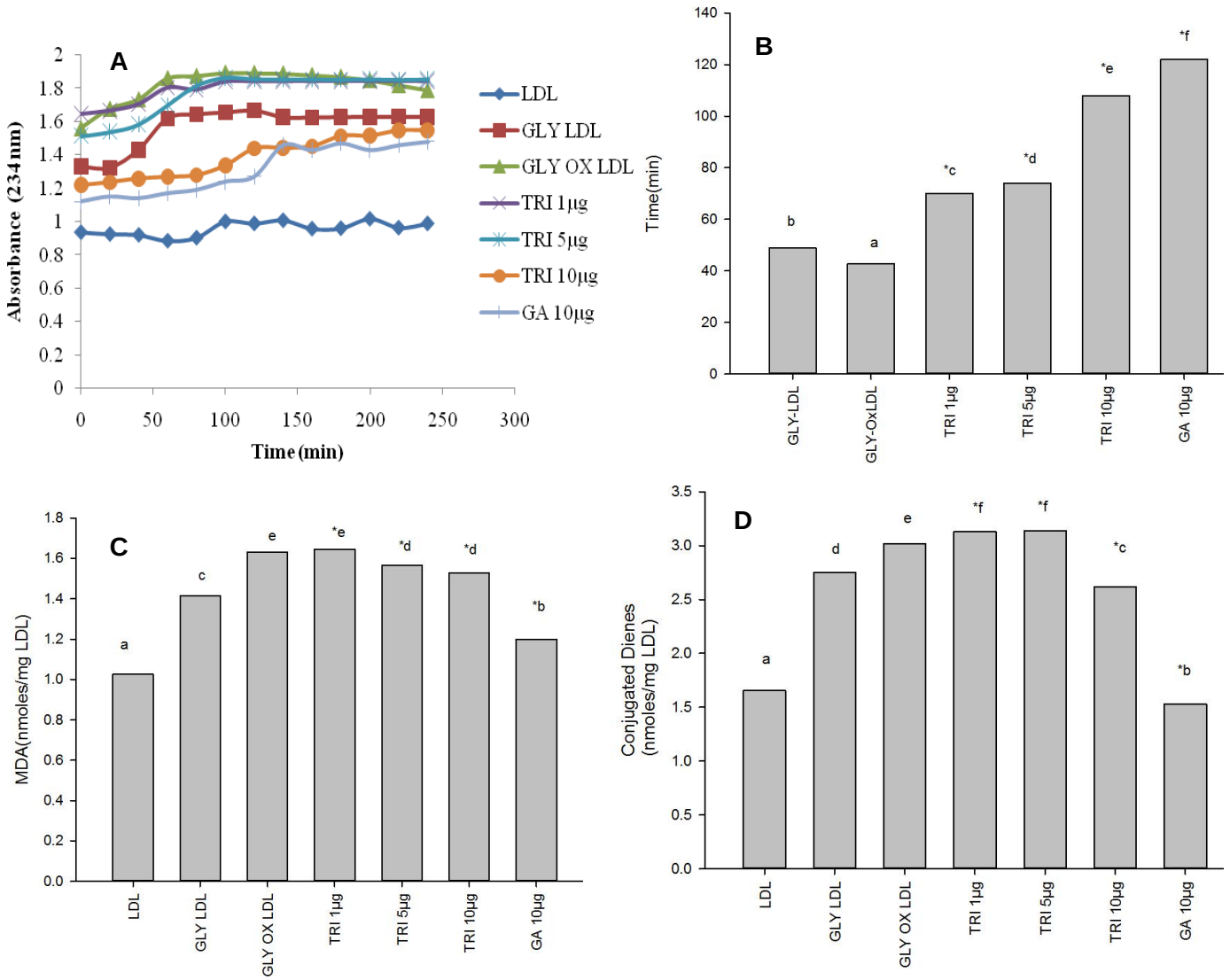

Figure 2. (A) The effect of *Triphala* extract on oxidation kinetics of glycated- oxidized LDL. Each point represents the mean of three experiments. (B) The effect of *Triphala* extract on the lag time on Gly-Ox-LDL (The lag phase represented the length of the antioxidant-protected phase during LDL oxidation by extract). Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Ox-LDL. (C) The effect of *Triphala* extract on the formation of TBARS in glycated-oxidized LDL. Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Gly-Ox-LDL. (D) The effect of *Triphala* extract on the formation of conjugated dienes in glycated-oxidized LDL. Each point represents the mean of three experiments. (*) is the symbol for significant difference compared to Gly-Ox-LDL.

resultant unstable carbon atom undergoes rearrangement to form stable conjugated diene. Further, conjugated diene will react with oxygen to form peroxyl radical that further abstracts hydrogen atom form adjacent PUFA and cholesterol to form lipid hydrogen peroxide and oxysterols, respectively. The lipid hydrogen peroxide is cleaved to form short aldehyde chain, that is, malondialdehyde (MDA) and 4-hydroxynonenal. Later, these aldehydes will form covalent adduct to lysine residue in Apo-B in LDL. This derivatization introduces a negative charge to the LDL molecule and it is recognized by the scavenger receptor of macrophages, resulting in increased LDL uptake by macrophages and their transformation into foam cells (Young and McEneny, 2001). This is a hallmark event to onset of atherosclerosis. Foam cell formation is thought to be mediated by modified LDL. The formation of foam cells *in vitro* can be induced by OxLDL and acetylated LDL (AcLDL). OxLDL can induce proliferation of peripheral macrophages *in vitro.* OxLDL induces PPAR-γ expression.

In the present study, an attention has been paid to the

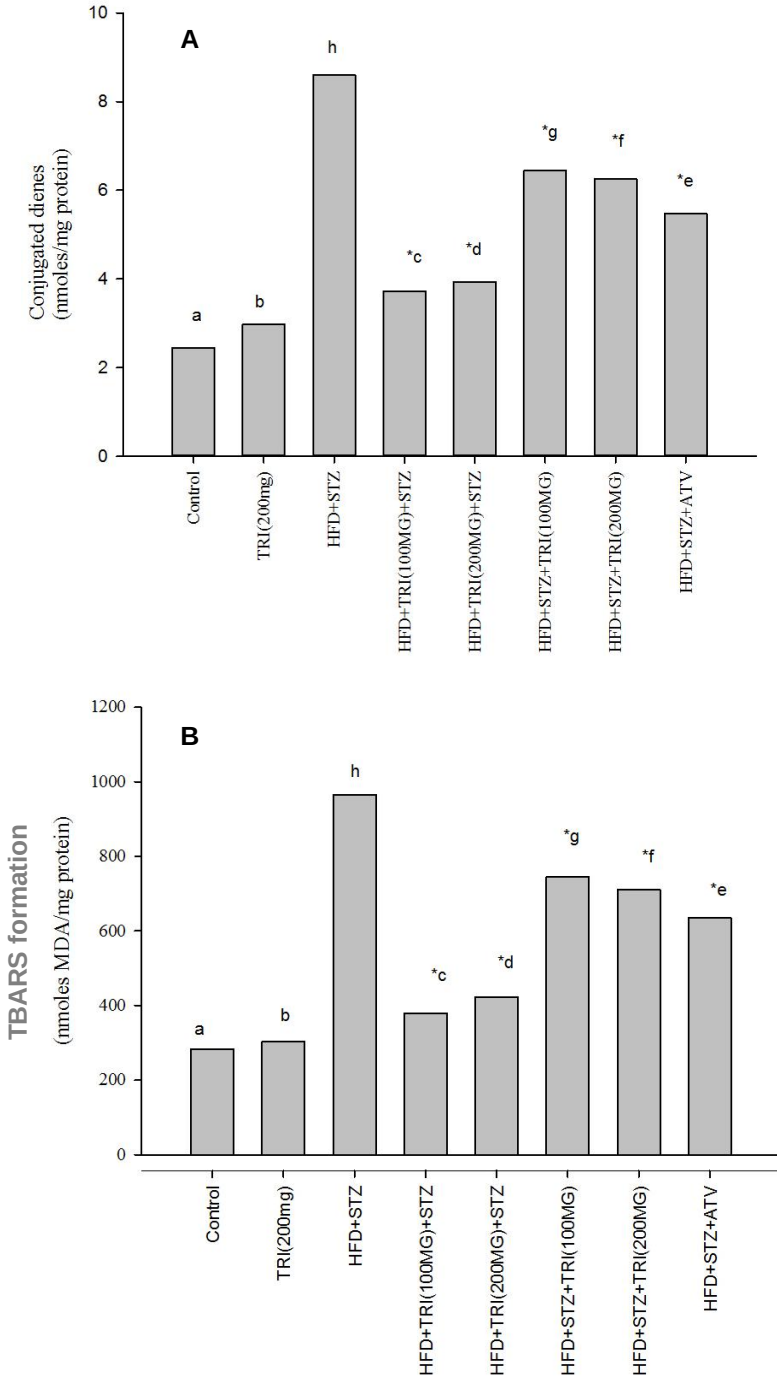

Figure 3. (A) The effect of *Triphala* extract on the formation of conjugated dienes in experimentally treated rats with HFD and STZ (n=6). (*) is the symbol for significant difference compared to HFD+STZ treated rats. (B). The effect of *Triphala* extract on the formation of TBARS in experimentally treated rats with HFD and STZ (n=6). (*) is the symbol for significant difference compared to HFD+STZ treated rats.

antioxidant effect of *Triphala* towards the oxidation and glycation of LDL, which is thought to be a key process in diabetes and atherosclerosis. Our result show *Triphala* extract showed prolonged lag phase in copper mediated native LDL and glycated LDL oxidation kinetics. It also reduced the conjugated dienes formation, and also, significant reduction in the formation of various LDL oxidation intermediary products (MDA). This indicates the

role of *Triphala* extract as a chain breaking antioxidant.

Also, this study demonstrated that *Triphala* at a dose of 100 and 200 mg/kg body weight per day modulated the conjugated dienes (CDs) and TBARS levels in rats fed with HFD and treated with STZ in progression phase more significantly than in regression phase of the study. These results can be attributed to the established antioxidant and free radical scavenging property of *Triphala* extract (Deepa et al., 2013). Previous study from our laboratory has established that *Triphala* extract is rich in polyphenols (Deepa et al., 2013). The present study shows that *Triphala* is a potent antioxidant in protecting plasma LDL against oxidation and glycation.

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Full Length Research Paper

Antidepressant-like effects of flavonoids extracted from *Apocynum venetum* leaves in mice: the involvement of monoaminergic system in mice

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The present study investigated a possible antidepressant-like activity of flavonoids extracted from *Apocynum venetum* leaves using two predictive tests for antidepressant effect on rodents: the forced swimming test (FST) and the tail suspension test (TST). Additionally, the monoaminergic mechanisms involved in the antidepressant-like effect of AV-extract in the mouse forced swimming test (FST) were evaluated. The extract (25, 50 and 100 mg/kg, i.g.) produced antidepressant-like effects in the FST and TST, without accompanying changes in ambulation distances (open-field test). The antidepressant-like effects of AV-extract (50 mg/kg, i.g.) was prevented by the pretreatment of mice with ketanserin (5 mg/kg, s.c., a serotonin 5-HT_{2A} receptor antagonist), cyproheptadine (3 mg/kg, i.g., a serotonin 5-HT_2 receptor antagonist), prazosin (1 mg/kg, i.g., an α_1-adrenoceptor antagonist), yohimbine (1 mg/kg, i.g., an α_2-adrenoceptor antagonist) or propranolol (2 mg/kg, i.g., aβ-adrenoceptor antagonist), SCH23390 (0.05 mg/kg, i.g., a dopamine D_1 receptor antagonist) and sulpiride (50 mg/kg, i.g., a dopamine D_2 receptor antagonist). By contrast, pretreatment of mice with WAY 100635 (0.1 mg/kg, s.c., a serotonin 5-HT_{1A} receptor antagonist) did not counteract the antidepressant-like effect of AV-extract in the TST. It can be concluded that the AV-extract produces an antidepressant-like effects in the FST and in the TST through interaction with the serotonergic (5-HT_{2A} and 5-HT_2 receptors), noradrenergic (α_1-adrenoceptor, α_2-adrenoceptor, a β-adrenoceptor) and dopaminergic (D_1 and D_2 receptors) systems. Taken together, our results suggest that AV-extract deserves further investigation as a putative alternative therapeutic tool that could help the conventional pharmacotherapy of depression.

Key words: Antidepressant, AV-extract, serotonergic, noradrenergic, dopaminergic, tail suspension test, forced swimming test.

INTRODUCTION

Depression is a major psychiatric disorder affecting nearly 17% of the world population and imposes a substantial health burden on society (Nemeroff, 2007; Yu et al., 2002). According to the monoamine theory, which is a widely accepted explanation for depression, the major neurochemical process in depression is the

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impairment of monoaminergic functions and the decrease of serotonin, noradrenaline and dopamine levels (Delgado, 2000). Antidepressants increase the availability of these monoamines at the synapse, which may induce longer-term adaptive changes by modulating the monoaminergic functions and promoting neurogenesis (Elhwuegi, 2004; Dailly et al., 2004). Serotonergic, noradrenergic and dopaminergic systems represent the major targets of current therapeutic treatments and drug development. Yet, there are a few major issues with conventional antidepression drugs. (1) Low remission rate: Although they are mostly effective, only about 50% of individuals with depression showed full remission (Berton and Nestler, 2006). (2) Low side effects: There are antidepressants that are not suitable for many patients because of the side effects. (3) Slow action: For many of these drugs, it takes several weeks to achieve their clinical efficacy. Hence, there is a growing interest in complementary and alternative medicine (CAM) among depression patients, with the general belief that "Natural is better" (Pilkington et al., 2006; Sarris and Kavanagh, 2009). Examples of plants with confirmed anti-depression effects include *Akebiae quinata* (Zhou et al., 2010), *Albizzia julibrissin* (Kim et al., 2007) and *Bupleurum falcatum* (Kwon et al., 2010).

Discovering new sources of natural products for depression treatment will continue to be an important field of research. *Apocynum venetum* L. (Apocynaceae) has shown a great promise for its antidepressant-like effects. A flavonoid extract from its leaves was found to markedly shorten the immobility time in a forced swimming test (FST), indicating antidepressant activities (Butterweck et al., 2001). The main flavonoids in *A. venetum* leaves are hyperoside and isoquercitrin (Butterweck et al., 2001). There is evidence that the *A. venetum* extract affects monoamine levels (Butterweck et al., 2003), we are interested in investigating the underlying mechanisms. Recently, we demonstrated that flavonoids from *A. venetum* leaves could generate a neuroprotective effect on corticosterone-induced neurotoxicity in PC12 cells (Zheng et al., 2011).

The purpose of the present study was to gain further insight into a possible involvement of noradrenergic, dopaminergic and serotonergic systems using laboratory mice.

MATERIALS AND METHODS

Plant material and preparation of the flavonoids extracted from Apocynum venetum leaves

Dry Leaves of *A. venetum* leaves was purchased from Tong Ren Tang Co., Beijing. A sample of 10 g of leaves was extracted three times in a refluxed condenser for 1 h each with 200 ml 70% ethanol. The combined extract was evaporated until dryness, dissolved in 20 ml hot water, adjusted to pH 3.0 with sulfuric acid, and then filtered. The filtrate was chromatographed on a macroporous resin D101 column (10 × 80 cm, Naikai Chemical Co.China) and eluted sequentially with 100 ml water and 70% 100 ml ethanol. The aqueous ethanol fraction was evaporated to dryness to obtain *A. venetum* leaves extract. The extract contained 3.5% hyperoside and 3.7% isoquercitrin, respectively. High performance liquid chromatography (HPLC) analytical conditions were as follows: Column: SHISEIDO CAPCELL PAKC18 (UG) 4.6 mm i.d. 3150 mm, detector at 330 nm, mobile phase: 0.1% TFA in water/0.1% TFA in acetonitrile 85: 15, flow rate: 1.0 ml/min. Equipment: Waters 2695 with a Waters 2998 DAD detector. Hyperoside appeared at 33.8 min and isoquercitrin at 39.7 min.

Animals

Male ICR mice weighing 18 to 22 g were purchased from the Experimental Animal Center, Changchun Institute of Biological Products, Jilin, China. Animals were under a normal 12 h/12 h light/dark schedule (with the lights on at 07:00 a.m). Ambient temperature and relative humidity were maintained at 22 ± 1°C and at 55 ± 5%, respectively. All mice had free access to tap water and food pellets, and were given a standard chow and water *ad libitum* for the duration of the study. All procedures were performed in accordance with the published guidelines of the China Council on Animal Care (Regulations for the Administration of Affairs Concerning Experimental Animals, approved by the State Council on October 31, 1988 and promulgated by Decree No. 2 of the State Science and Technology Commission on November 14, 1988). All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

Chemicals

The following drugs were used: hyperoside, isoquercitrin, WAY-100635 (a serotonin5-HT1A receptor antagonist), ketanserin (a serotonin 5-HT_{2A} receptor antagonist), cyproheptadine (a serotonin 5-HT2 receptor antagonist), prazosin (an α_1-adrenoceptor antagonist), propranolol (a β-adrenoceptor antagonist), yohimbine (an α_2-adrenoceptor antagonist), sulpiride (a dopamine D2 receptor antagonist), SCH23390 (a dopamine D1 receptor antagonist) were purchased from Sigma-Aldrich (St.Louis, MO, USA). Fluoxetine hydrochloride was purchased from Shanghai Zhongxi Pharmaceutical Co., Ltd. (Shanghai, China). All other chemicals were of high-purity analytical grade obtained from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China).

Drug treatments

Different groups of mice, 10 animals per group, were used for drug treatment and for each test. All drugs were administered by intragastric (i.g.) in a constant volume of 10 ml/kg body weight, except fluoxetine that were administered by oral (p.o.) gavage, and WAY 100635 and SCH23390 which were administered by subcutaneous route (s.c.). Appropriate vehicle treated groups were also assessed simultaneously. The animals were used only once in the test.

Experiment 1: To text the antidepressant-like effect of AV-extract

Animals were divided into five experimental groups: one 0.9% saline control group, one fluoxetine group (5 mg/kg) and three AV-extract treatment groups (25, 50, and 100 mg/kg). The administration volume was 20 ml/kg-body weight. To investigate its possible antidepressant-like effect, AV-extract was administrated by oral route 60 min before the forced swimming test (FST), tail suspension test (TST) or OFT.

***Experiment 2:* To assess the involvement of the serotonergic system in the antidepressant-like effect of AV-extract**

To assess the involvement of the serotonergic system in the antidepressant-like effect of AV-extract in the TST, mice were pre-treated with WAY 100635 (0.1 mg/kg, s.c., a serotonin5-HT_{1A} receptor antagonist), cyproheptadine (3 mg/kg, i.g., a serotonin 5-HT_2 receptor antagonist), ketanserin (5 mg/kg, i.g., a serotonin 5-HT_{2A} receptor antagonist) or vehicle. After 60 min, they received AV-extract (50 mg/kg, i.g.) or vehicle, and were tested in the TST 60 min later.

***Experiment 3: T*o investigate the possible involvement of the noradrenergic system in the antidepressant-like effect of AV-extract**

To investigate the possible involvement of the noradrenergic system in the antidepressant-like effect of AV-extract in the TST, animals were pretreated with prazosin (1 mg/kg, i.g., an α_1-adrenoceptor antagonist), yohimbine (1 mg/kg, i.g., an α_2-adrenoceptor antagonist), propranolol (2 mg/kg, i.g., a β-adrenoceptor antagonist) or vehicle. After 60 min they received AV-extract (50 mg/kg, i.g.) or vehicle which were tested in the TST 60 min later.

***Experiment 4:* To test the possible involvement of the dopaminergic system in the antidepressant like effect of AV-extract**

To test the possible involvement of the dopaminergic system in the antidepressant like effect of AV-extract in the TST, animals were pretreated with SCH23390 (0.05 mg/kg, s.c., a dopamine D_1 receptor antagonist), sulpiride (50 mg/kg, i.g., a dopamine D_2 receptor antagonist) or vehicle. After 60 min they received AV-extract (50 mg/kg, i.g.) or vehicle and were tested in the TST 60 min later. The doses of the drugs used above were determined according to literature data, which were reported not to increase the locomotor activity (Gay et al., 2010).

Forced swimming test

Mice were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 25 ± 1°C; the total amount of time each animal remained immobile during a 6-min session was recorded (in seconds) as immobility time, as described previously (Machado et al., 2009). Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. A decrease in the duration of immobility is indicative of an antidepressant-like effect (Porsolt et al., 1977).

Tail suspension test

The tail suspension test (TST) was conducted as previously described (Steru et al., 1985), with some modifications. Briefly, mice were individually suspended by the tail with a clamp (10 mm from the tail tip in a box (250 × 250 × 300 mm) with the head 50 mm from the bottom. Testing was carried out in a darkened room with minimal background noise. Each mouse was suspended for a total of 6 min, and the duration of immobility was recorded during the final 4 min interval of the test. Mice were considered immobile only when they hung passively and completely motionless. The test sessions were recorded by a video camera and scored by an observer blind to treatment.

Open-field test

To assess the effects of the extract from AV-extract on locomotor activity, the mice were individually housed in a rectangular container made of dark polyethylene (40 × 40 × 25 cm) in a dimly lit room equipped with a video camera above the center of the floor, as described previously (Kim et al., 2007) with slight modification, and locomotor activity was measured. The locomotor activity was monitored by a computerized video-tracking system using the S-MART program (Pan Lab, Barcelona, Spain). The animals were allowed to adapt for 1 h in the container, and the distance they traveled was recorded during the last 10 min of a total 20 min test. The locomotor activity was measured in centimeters. The floor surface of each chamber was thoroughly cleaned with 70% ethanol between tests.

Statistical analyses

All data were expressed as mean ± standard error of mean (SEM). To compare experimental and control groups, we used one- or two-way ANOVA, followed by post-hoc Dunnett's test using the SPSS software (SPSS Inc., Chicago, USA). A value of $P < 0.05$ was considered statistically significant for analysis. The figures were obtained by the Statistical Analysis System (Graph Pad Prism 4, Graph Pad Software, Inc., San Diego, CA).

RESULTS

Effects of AV-extract on the immobility time in the FST and TST and on the locomotor activity in the OFT

AV-extract or the conventional antidepressant fluoxetine given by oral route significantly decreased the immobility time in the FST [$F(4, 45) = 16.25$, $P < 0.001$] and TST [$F(4, 45) = 22.37$, $P < 0.001$]. Administration of AV-extract (20, 50,100 mg/kg) and fluoxetine (5 mg/kg) resulted in a significant decrease in the immobility time. As shown by the OFT, AV-extract (25, 50 and 100 mg/kg) did not lead to significant changes in ambulation distance (2212.6 ± 48.6, 2253 ± 89.9, and 2275.2 ± 98.9 mm, respectively) relative to the vehicle group (2297.3 ± 100 mm). This indicates a lack of apparent association of immobility in the tests with changes in locomotor activity.

Involvement of the serotonergic system

The result presented in Figure 2A showed that the pretreatment of animals with ketanserin (5 mg/kg, i.g., a 5-HT2A receptor antagonist) was able to prevent the anti-immobility effect of AV-extract (50 mg/kg, i.g.) in the TST. A two-way ANOVA revealed significant differences of AV-extract treatment [$F(1, 36) = 14.46$, $P < 0.01$] and ketanserin pretreatment × AV-extract treatment interaction [$F(1, 36) = 13.68$, $P < 0.01$], but not of ketanserin pretreatment [$F(1, 36) = 2.47$, $P > 0.05$]. Figure 2B showed that the pretreatment of mice with cyproheptadine

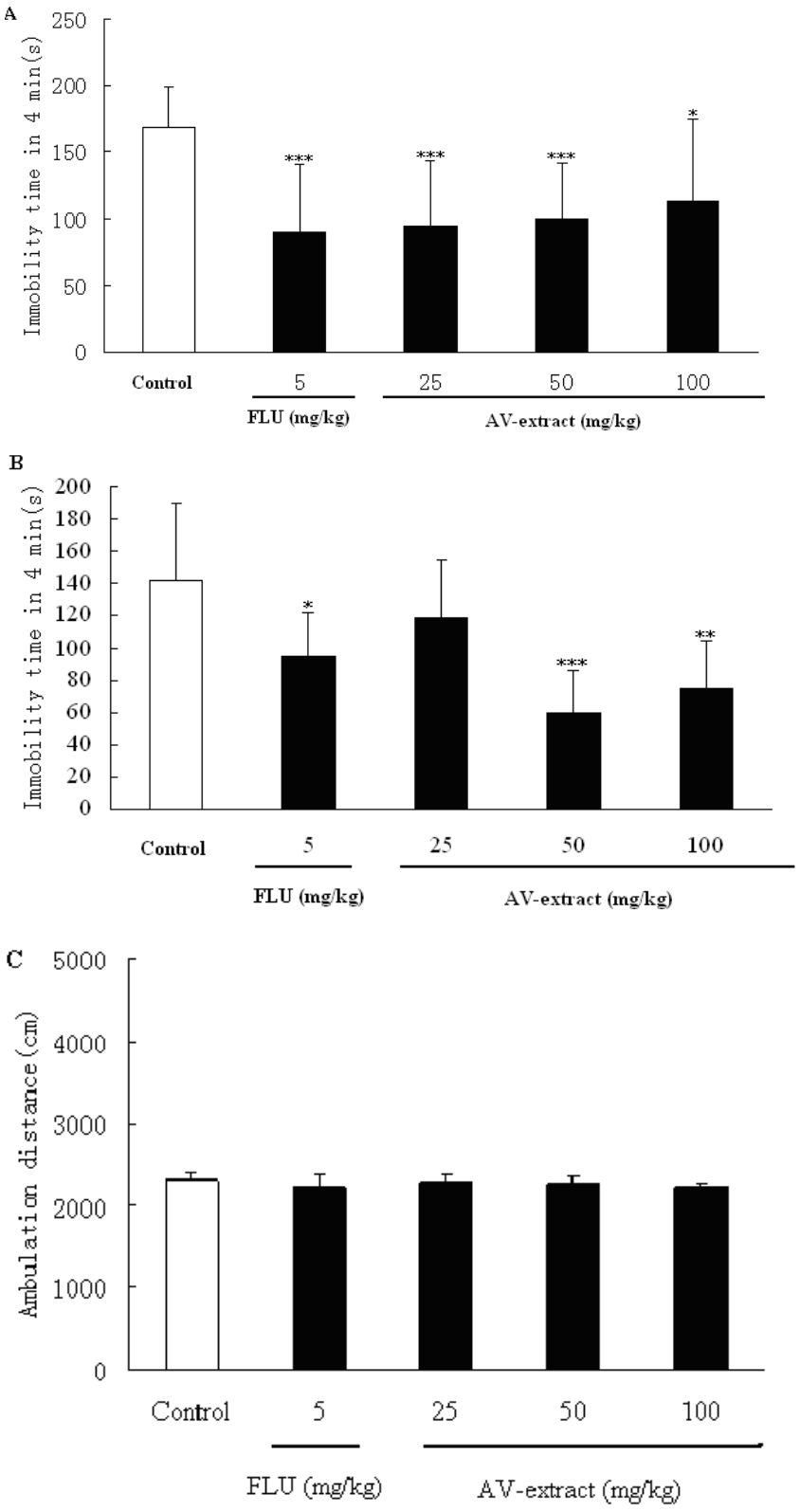

Figure 1. Effects of AV-extract and fluoxetine on immobility time in the mouse FST (A), TST (B), and OFT (C).

The immobility time was measured in mice receiving 0.9% saline (vehicle), 25, 50 and 100 mg/kg AV-extract, 5 mg/kg fluoxetine for 10 days. The number of mice in each group was 10. Data were expressed as mean ± S.E.M. **P* < 0.05 and ***P* < 0.01 and ****P* < 0.001 vs FST or TST or OFT with vehicle group.

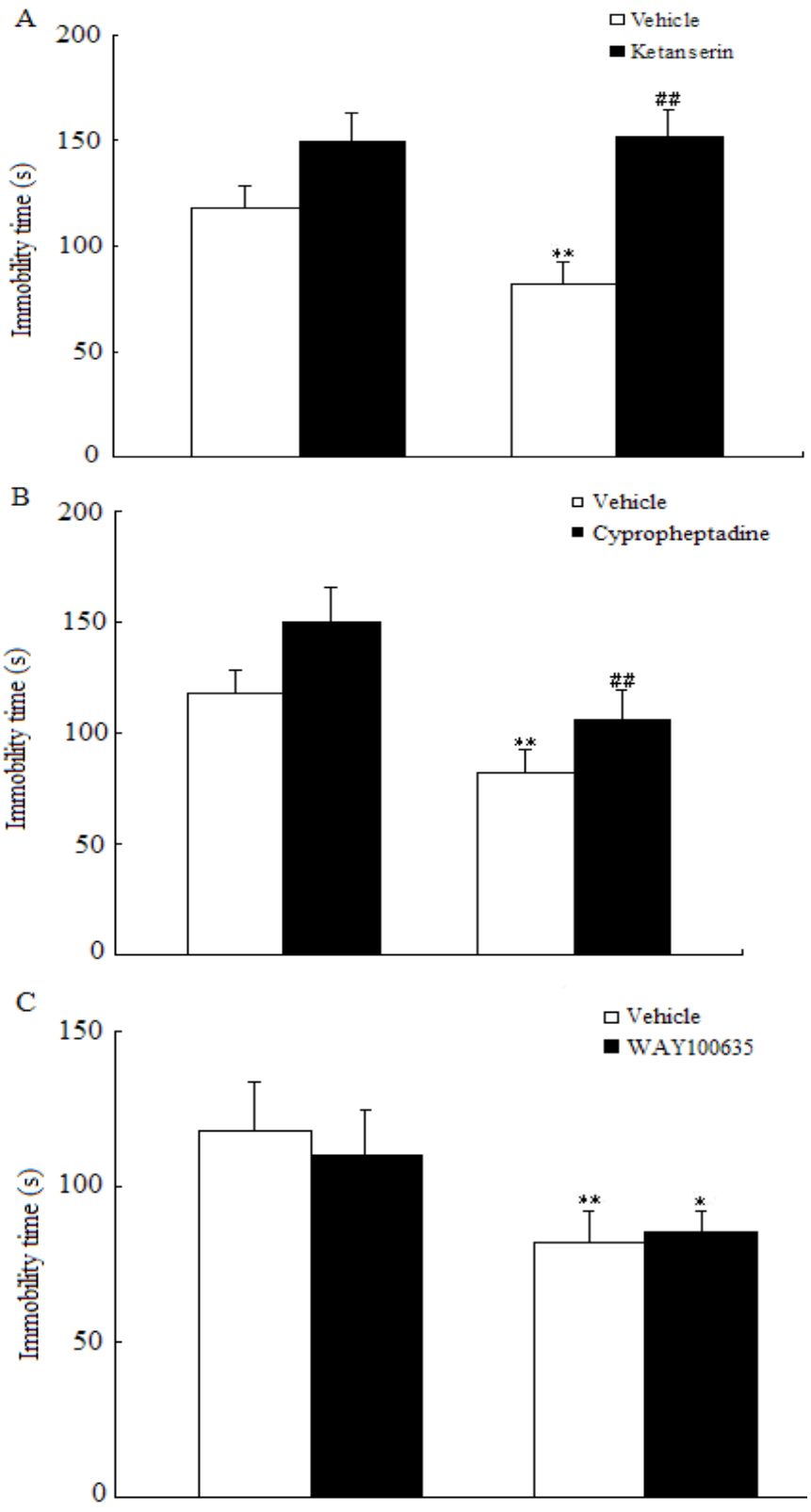

Figure 2. Effects of pretreatment with ketanserin (5mg/kg, i.p., A), cyproheptadine (3 mg/kg, i.p., B) and WAY 100635 (0.1 mg/kg, s.c., C) on the AV-extract (50 mg/kg, i.g.)-induced reduction in immobility time in the mouse TST.

The number of animals in each group was 10. Data were expressed as mean ± S.E.M. ***P*<0.01 vs. TST with vehicle group, $^{\#\#}P$<0.01 vs. AV-extract group pretreated with vehicle.

(3 mg/kg, i.g., a serotonin 5-HT2 receptor antagonist) was able to block the antidepressant-like effect of AV-extract (50 mg/kg, i.g.) in the TST. A two-way ANOVA revealed significant differences of AV-extract treatment [$F(1, 36) = 14.46$ $P < 0.01$], cyproheptadine pretreatment [$F(1, 36) = 15.63$, $P < 0.01$] and cyproheptadine pre-treatment × AV-extract treatment interaction [$F(1, 36) = 29.42$, $P < 0.01$]. Figure 2C showed that the pretreatment of mice with WAY 100635 (0.1 mg/kg, s.c., a serotonin 5-HT_{1A} receptor antagonist) did not block the antidepressant-like effect of AV-extract in the TST. A two-way ANOVA revealed significant differences of AV-extract treatment [$F(1, 36) = 10.2$, $P < 0.01$], but not of WAY 100635 pretreatment [$F(1, 36) = 3.31$, $P > 0.05$], and WAY 100635 pretreatment × AV-extract treatment interaction [$F(1, 36) = 0.48$, $P > 0.05$]. Post hoc analyses indicated that the pre-treatment of mice with ketanserin and cyproheptadine but not WAY 100635 prevented the decrease in immobility time in the TST produced by the administration of the AV-extract.

Involvement of the noradrenergic system

The result presented in Figure 3A showed that the pretreatment of animals with prazosin (1 mg/kg, i.p., an α1-adrenoceptor antagonist) was able to inhibit the anti-immobility effect of AV-extract (50 mg/kg, i.g.) in the TST. A two-way ANOVA revealed significant differences of AV-extract treatment [$F(1, 36) = 14.42$, $P < 0.01$], prazosin pretreatment [$F(1, 36) = 4.25$, $P < 0.05$] and prazosin pretreatment × AV-extract treatment interaction [$F(1, 36) = 6.47$, $P < 0.01$]. Figure 3B showed that the pretreatment of mice with yohimbine (1 mg/kg, i.p., an α2-adrenoceptor antagonist) was able to prevent the antidepressant-like effect of AV-extract (50 mg/kg, i.g.) in the TST. A two-way ANOVA revealed significant differences of AV-extract treatment [$F(1, 36) = 7.21$, $P < 0.01$], and Yohimbine pretreatment × AV-extract treatment interaction [$F(1, 36) = 19.24$, $P < 0.01$], but not of yohimbine pretreatment [$F(1, 36) = 0.55$, $P > 0.05$]. Figure 3C showed that the pretreatment of mice with propranolol (2 mg/kg, i.p., aβ-adrenoceptor antagonist) was able to prevent the antidepressant-like effect of AV-extract (50 mg/kg, i.g.) in the TST. A two-way ANOVA revealed significant differences of AV-extract treatment [$F(1, 36) = 7.28$, $P < 0.01$] and propranolol pretreatment × AV-extract treatment interaction [$F(1, 36) = 27.18$, $P < 0.01$], but not of propranolol pretreatment [$F(1, 36) = 0.73$, $P > 0.05$]. Post hoc analyses indicated that the anti-immobility effect of the AV-extract was completely prevented by pre-treatment of animals with prazosin, yohimbine or propranolol.

Involvement of the dopaminergic system

The result presented in Figure 4A showed that the pretreatment of animals with SCH23390 (0.05 mg/kg, s.c., a dopamine D1 receptor antagonist) was able to prevent the anti-immobility effect of AV-extract (50 mg/kg, i.g.) in the TST. A two-way ANOVA revealed significant differences of AV-extract treatment [$F(1, 36) = 5.71$, $P < 0.05$] and SCH23390 pretreatment × AV-extract treatment interaction [$F(1, 36) = 22.85$, $P < 0.01$], but not of SCH23390 pretreatment [$F(1, 36) = 0.26$, $P > 0.05$]. Figure 4B showed that the pretreatment of mice with sulpiride (50 mg/kg, i.p., a dopamine D2 receptor antagonist) was able to block the antidepressant-like effect of nobiletin (50 mg/kg, i.g.) in the TST. A two-way ANOVA revealed significant differences of AV-extract treatment [$F(1, 36) = 4.42$, $P < 0.05$], sulpiride pretreatment [$F(1, 36) = 5.19$, $P < 0.05$] and sulpiride pretreatment × AV-extract treatment interaction [$F(1, 36) = 8.35$, $P < 0.01$]. Post hoc analyses indicated that the pre-treatment of mice with SCH23390 and sulpiride prevented the decrease in immobility time in the TST produced by the administration of the AV-extract.

DISCUSSION

Behavioral study is an important approach in evaluating anti-depressant drugs, and forced swimming test (FST) and tail suspension test (TST) are popular tools. The characteristic behavior scored in these tests, termed "immobility", is an indicator of the degrees of severity of behavioral despair; the lower are the scores, the less severe is the despair. Antidepressant drugs are able to reduce the immobility time in rodents (Porsolt et al., 1977). The antidepressant-like effect of *A. venetum* was first demonstrated by Butterweck et al. (2001) using a forced swimming test (FST) in rats, and the present study has provided more detailed behavioral data using mice. Our results showed that administration at 100, 50 and 25 mg/kg (i.g.) for 10 days significantly reduced immobility time in FST ($P < 0.001$, $p < 0.05$ and $P < 0.01$, respectively; Figure 1A). Administration at 100, 50 mg/kg (i.g.) also significantly reduced immobility time in TST ($P < 0.01$ and $P < 0.001$, respectively; Figure 1B). In line with these observations, treatments with AV-extract at 100, 50 and 25 mg/kg did not cause significant changes in amulation distance (2212.6 ± 48.6, 2253 ± 89.9 and 2275.2 ± 98.9 mm, respectively) compared to the vehicle group (2297.3 ± 100 mm; Figure 1C). These effects were comparable to those found with fluoexetine treatment. Taken together, AV-extract possesses a clear antidepressant-like effect in all animal models used.

It is generally accepted that improving brain mono-aminergic functions is effective in treating depression, and the serotonergic, noradrenergic or dopaminergic systems have become the targets for development of antidepressants (Lambert et al., 2000; Esposito, 2006). The serotonergic system has long been implicated in the pathogenesis of anxiety and depression (Heninger et al., 1996). Some of the most compelling evidence involves the

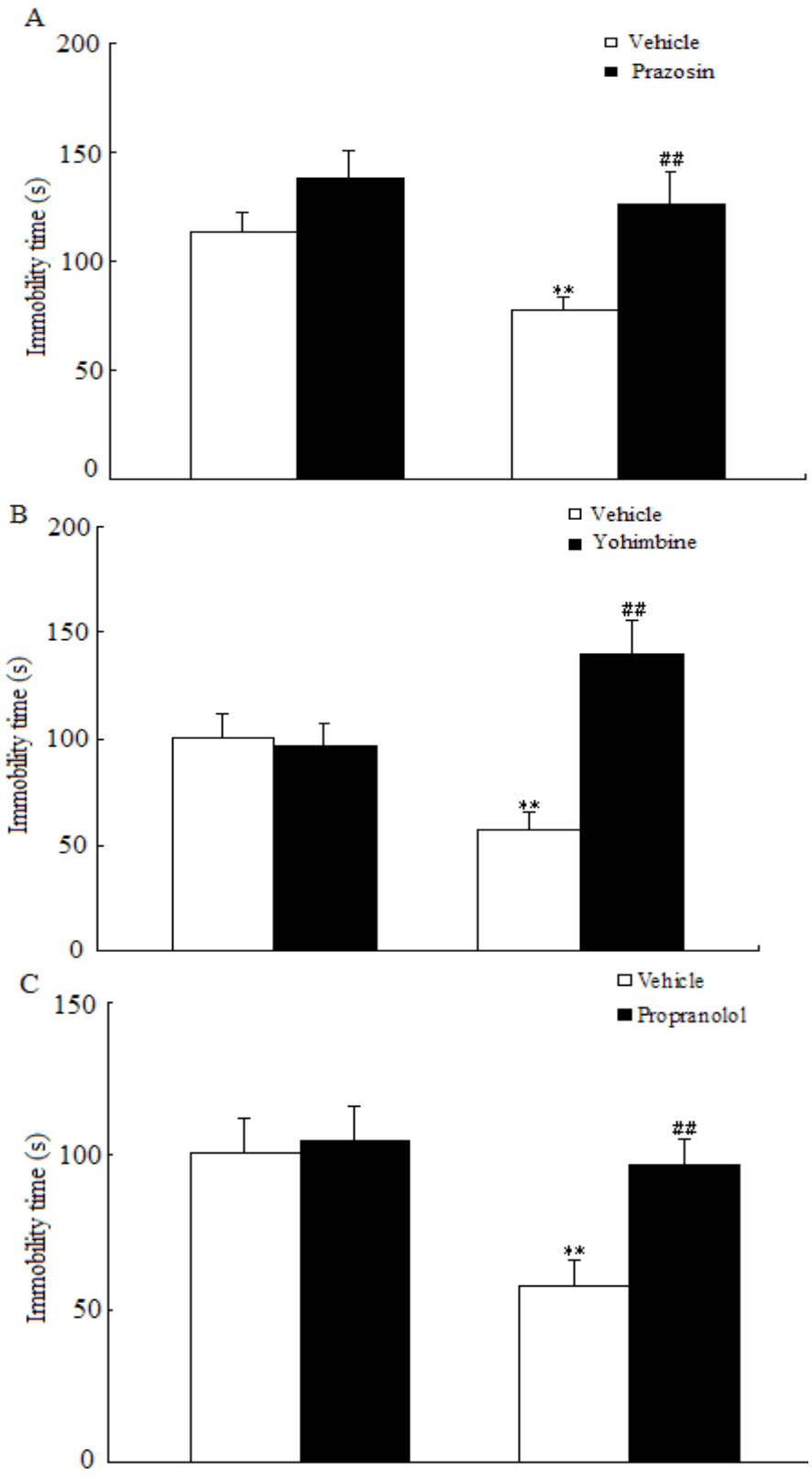

Figure 3. Effects of pretreatment with prazosin (1 mg/kg, i.p., A), yohimbine (1mg/kg, i.p., B) and propranolol (2 mg/kg, i.p., C) on the AV-extract (50 mg/kg, i.g.)-induced reduction in immobility time in the mouse TST. The number of animals in each group was 10. Data were expressed as mean ± S.E.M. $^{**}P<0.01$ vs. TST with vehicle group, $^{\#\#}P<0.01$ vs. AV-extract group pretreated with vehicle.

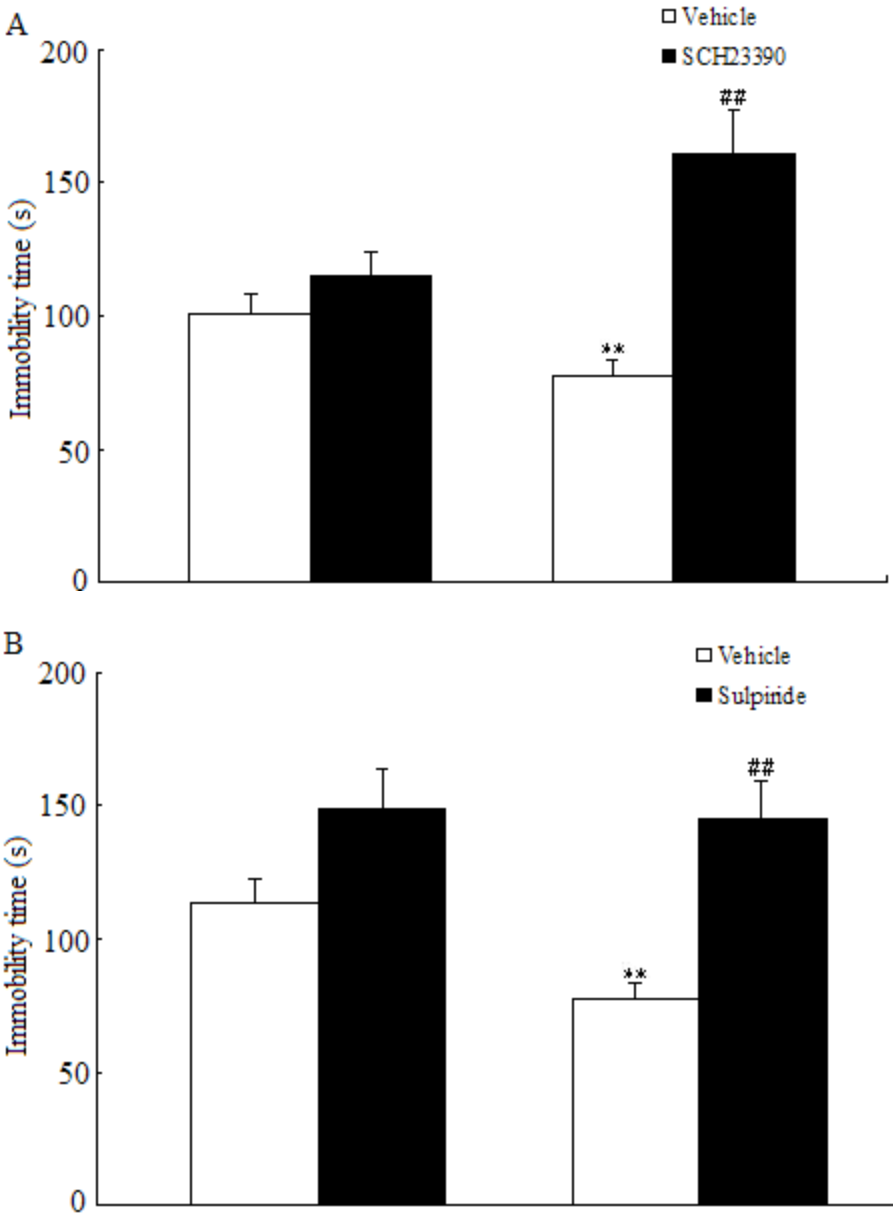

Figure 4. Effects of pretreatment with SCH23390 (0.05 mg/kg, s.c., A) and sulpiride (50mg/kg, i.g., B) on the AV-extract (50 mg/kg, i.g.)-induced reduction in immobility time in the mouse TST.
The number of animals in each group was 10. Data were expressed as mean ± S.E.M. ***P*<0.01 vs. TST with vehicle group, $^{\#\#}P$<0.01 vs. AV-extract group pretreated with vehicle.

alleviation of depression caused by serotonin selective reuptake inhibitors (SSRIs), which increase the availability of serotonin at the synapse (Malagie et al., 2002). The serotonergic system is a neuromodulatory system interacting with other neurotransmissions and participating in the elaboration of an adapted response of the central nervous system to external stimuli (Grimaldi et al., 1999). Depressions are often associated with a down regulation of 5-HT_{1A} receptors in the hippocampus and in the temporal lobe (Gross et al., 2000). 5-HT_2 receptors, especially 5-HT_{2A} and 5-HT_{2C} subtypes, have been shown to be involved in neurochemical changes mediated by antidepressants (Cryan and Lucki, 2000; Elhwuegi, 2004). Preclinical reports showed that the preferential 5-HT_{2A} receptor agonist DOI enhances the antidepressant-like effect of some compounds (Zomkowski et al., 2004) in the mouse FST. Accordingly, antidepressant desipramine treatment decreased $5HT_2$ receptors densities in the rat brain (Goodnough and Bake, 1992). In the present study, the pretreatment with pretreatment with

5-HT_2 receptor antagonist cyproheptadine and 5-HT_{2A} receptor antagonist ketanserin prevent the anti-immobility effect induced by AV-extract in the TST, whereas WAY 100635 was ineffective in reversing the immobility time in the TST; these results are indicative of participation of 5-HT_2 and 5-HT_{2A} receptors, but not 5-HT_{1A} receptor, in the antidepressant-like effect of AV-extract in the mouse TST (Figure 2).

Noradrenergic system is another target for antidepressants (Maj et al., 2000). Increased levels of α_1-adrenoceptor were found in the prefrontal cortex of depressed individuals (García-Sevilla et al., 1999). A down regulation of a_2-adrenergic receptors was documented in depression (Brunello et al., 2003). There is an up-regulation of β-adrenoceptor in depressed patients and a down-regulation after chronic antidepressant treatment in mice (Leonard et al., 1997). In our study, pretreatment of mice with prazosin and yohimbine or propranolol was able to reverse the antidepressant-like effect of the AV-extract, suggesting participation of α_1-, α_2- and αβ-adrenoceptors in the antidepressant-like effect of the extract in mouse (Figure 3).

The relationship between dopaminergic system and depression was confirmed by the fact that antidepressants act on the dopaminergic system (Klimek et al., 2002). Common symptoms of depression such as anhedonia, dysphoria and avolition are likely caused by a functional deficit of dopaminergic transmission (Heinz et al., 1994). Many antidepressant drugs such as SSRIs, imipramine (a tricyclic antidepressant) and bupropion (an atypical antidepressant that inhibits the reuptake of dopamine) act activating dopamine D_1 and D_2 receptors (Renard et al., 2001; Yamada et al., 2004). In our experiments, the pretreatment with SCH23390 or sulpiride inhibited the anti-immobility effect of AV-extract. Thus, our results suggested a participation of both dopamine D_1 and D_2 receptors in the antidepressant-like effects of AV-extract.

In summary, our results provided pharmacological and biochemical evidence for the previously reported antidepressant-like effects of AV-extract in the FST (Butterweck et al, 2001). We have further shown that these effects are apparently related to noradrenergic (α_1-adrenoceptor, α_2-adrenoceptor, aβ-adrenoceptor), dopaminergic (D_1 and D_2 receptors) and serotonergic (5-HT_{2A} and 5-HT_2 receptors).

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