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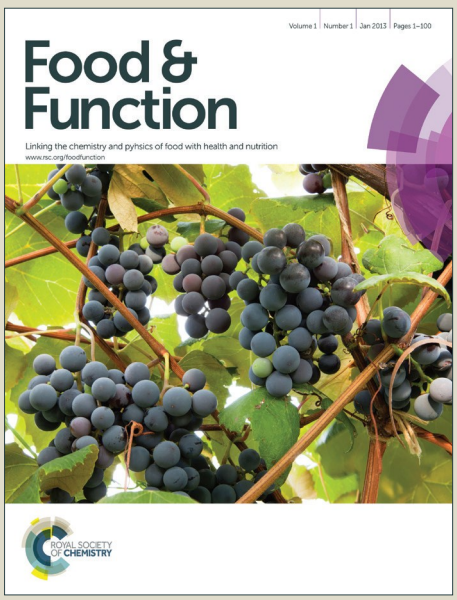

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Study on Quality Components and Sleep-promoting Effect of GABA Black Tea

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The aims of this study were to analyze the changes in quality components of gamma (γ)-aminobutyric acid (GABA) black tea during processing process, and to investigate the effect of three dosages of GABA black tea on sleep improvement. The results showed that GABA content was increased significantly up to 2.70 mg/g after vacuum anaerobic and aerobic treatment. In addition, the content of GABA after drying reached to 2.34 mg/g, which achieved to the standard of GABA tea. During the entire processing process of GABA black tea, the contents of tea polyphenols, caffeine and total catechins displayed a gradually descending trend, while the contents of free amino acids and GABA were firstly increased, and then reduced. The GABA black tea had significant effects on prolonging sleeping time with sodium pentobarbital (P<0.05) and significantly enhancing the sleeping rate induced by sodium pentobarbital at sub-threshold dose (P<0.05). But its effect on shortening the sleeping latency period induced by sodium barbital was not significant (P>0. 05). It had no effect on directly inducing sleep and the mouse body weight. The extract of GABA black tea improved the sleeping quality of mice to extend with optimal effect being found in the high dose-treated mice. **Keywords:** γ-aminobutyric acid; Black tea; Quality components; Sodium pentobarbital; Sleep-promoting effect

1 Introduction

Gamma (γ)-aminobutyric acid (GABA), $C_4H_9NO_2$, a non-proteinaceous amino acid, is one of the major inhibitory neurotransmitters in the sympathetic nervous system[1]. It is widely distributed in mammalian nervous system, bacteria[2] and higher plants including rice[3], soybean[4-5] and tea[6]. Several studies have demonstrated that

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GABA has a physiological role in many systems outside the central nervous system by acting as a natural relaxant to induce relaxation and diminish anxiety [7], such as regulation of cardiovascular and cerebrovascular functions[3,8], inhibition of metastasis of cancer cells [9], and modulation of the renal function[10]. With the discoveries of many of its beneficial physiological functions in GABA, GABA tea has become a hot research spot in tea field. In tea, the amount of GABA could be accumulated by the repeating and alternating anaerobic and aerobic treatments[11-13]. After anaerobic treatment, GABA content was significantly increased, and could be reached to the standard of GABA tea, whose content was higher than 1.50 mg/g[6].

Sleep is a complex neurological behavior essential to both life and health. Proper sleep is required for individual survival from adequate immune responses and metabolic maintenance to memory and cognitive. As life pace becomes faster and working pressure gets heavier, the problem of sleep disorders has shown an upward trend year by year, which has become one of the main diseases that threaten people's health and influence the quality of people's life. In the current society, insomnia is the most common phenomenon in sleep disorders. A study has shown that more than 27% of people worldwide suffer from insomnia[14], and 38% of Chinese may experience insomnia at certain point in their lives. At present, among the clinical chemosynthesis of sedative-hypnotic drugs for treatment of sleep disorders, benzodiazepines are the most common ones. Those drugs may cause different degrees of side effects such as dizziness, fatigue, sleepiness, inattention, anxiety, fear, irritability, hallucinations, even temporary amnesia and hangover while prolong sleep time. They have not really achieved the desired effect on improving sleep quality. Therefore, choosing a natural high-efficiency sedative-hypnotic product is an important way for the treatment of sleep disorders.

As a natural and safe drink, tea (*Camellia sinensis*) (*C. sinensis*) is one of the most popular beverage consumed around the world. Many studies have shown that tea has improved the sleeping function, which is attributed to its bioactive components such as polyphenols, catechins and water-soluble polysaccharides, etc[15]. However, previous studies mainly focused on the GABA green tea (non-fermentation tea), very few studies were conducted on GABA black tea, the fully fermentation tea. Thus, the purposes of the present work were to analyze the changes in quality components of GABA black tea during anaerobic treatment and to investigate whether or not the extract of GABA black tea has the sleep-promoting effect.

2 Materials and methods

2.1 Plant material

Fresh leaves (*C. sinensis*), consisted mainly of two leaves and a bud, were harvested from a tea cultivation base of South China Agriculture University in Guangdong, China in August 2013. The GABA black tea was manufactured as the follows. After being harvested, the fresh leaves were packed into a bag, vacuumed for 3 h, and removed out under aerobic conditions for 2 h[12,16]. The two steps were repeated twice and third respectively so that the amount of GABA could be accumulated by these repeating treatments of vacuum anaerobic and aerobic sequence fermentation. The leaves were followed by being rolled, fermented and dried at 110 ℃ until they were dried adequately to obtain GABA black tea.

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2.2 Chemicals and drugs

(-)-epigallocatechin gallate (EGCG), (-)-Epigallocatechin (EGC), (-)-gallocatechin (GC), (-)-catechin (C), (-)-epicatechin (EC), (-)-catechin gallate (CG), (-)-epicatechin gallate (ECG), gamma (γ)-aminobutyric acid (GABA) and caffeine were purchased from Sigma-Aldrich Chemical (St. Louise, MO, USA). HPLC grade methanol obtained from Tianjin Kemiou Chemical Reagent Co., Ltd (Tianjin, China) was used as the solvent. All the other chemicals and reagents used were of the highest grade available. Sodium pentobarbital was purchased from China National Pharmaceutical Group (Shanghai,China).Rotundine tablets were obtained from Sichuan Kofule Pharmaceutical Co., Ltd (Deyang, Sichuan, China).

2.3 Animals

Both male and female KM mice (25 ± 3 g) were purchased from the Laboratory Animal Center of Guangzhou University of Chinese Medicine (Guangzhou, Guangdong, China). All the animals were housed in cages at a constant temperature (23 ± 2 ^{o}C) and maintained on a 12 h light-12 h dark cycle with free access to food and water. Each animal was used only once. Mice were given free access to standard diet and water. The animals were maintained according to the rules and regulations of the Experimental Animal Ethics Committee of the Guangzhou University of Traditional Chinese Medicine.

2.4 Assays of biochemical compositions in GABA black tea

The samples would be detected, which were taken from the GABA black tea manufacturing process.About 3 g of tea powder was extracted with 500 ml of distilled water at 100^{o}C for 45 min with shaking. The extracted solutions were filtered through a filter paper. The residue was used to determine water extracts based on the oven drying method.The supernatants of the extracted solutions were collected for the analysis of other components. Contents of tea polyphenols were measured with ferrous tartrate method at 540 nm with the spectrophotometer as described previously[17]. The contents of total free amino acids were determined by the ninhydrin colorimetry at 570 nm through the spectrophotometer according to the method of He et al[17]. All the assay methods mentioned above were based on the National Standard of the People's Republic of China. The contents of catechins, caffeine and GABA were determined by high performance liquid chromatography (HPLC) according to the method developed by Dalluge et al[18] and Lu et al[19] with a slight modification, respectively. The column used was an Agilent Ecllpsc XDB-C18 column (4.6*150mm, 5um).

2.4.1 Behavioral testing

The test was performed in five groups of fifteen mice each. All of the behavioral tests and drug administrations were performed in the light cycle between 7:00 and 12:00 AM with experimental mice. Method of sleep-promoting effect were conducted in accordance with the Technical Standards for Testing & Assessment of Health Food formulated by the Chinese Ministry of Heath.

2.4.2 Observational test on primary sleep

The mice were administrated orally once daily with various doses of the extracted solution of GABA black tea at 0.83 mg/kg, 1.67 mg/kg and 3.33 g/kg or rotundine tablets (40.00 mg/kg) as a positive control for 15 consecutive days. Controlled mice were tested in parallel with the animals being given saline only. Body weight (BW) was measured daily during the fifteen day- experimental period. Primary sleep

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observation was recorded with an electronic thermometer in groups of mice 30 min before and after the administration of drugs. Each mouse was observed for the sleeping time and sleeping rate, with the criterion for sleep being loss of righting reflex.

2.4.3 Test on the duration of sodium pentobarbital-induced sleep

The mice were administrated with the extract of GABA black tea at 0.83 mg/kg, 1.67 mg/kg and 3.33 mg/kg while the mice in control group were given saline once daily for 15 consecutive days. The animals in the 5th group were given rotundine tablets (40.00 mg/kg) as a positive control. Thirty min later, sodium pentobarbital (47.50 mg/kg), which served as the reference point for the determination of pre-treatment, was administered by an intraperitoneal injection to each mouse to induce sleep. Each mouse was observed for the latency and duration of sleep. Sleep latency was recorded as the time of sodium pentobarbital injection to the time of sleep onset. Sleep duration, an index of hypnotic effect[20], was defined as the interval between loss and recovery of righting reflex.

2.4.4 Sub-threshold dose of sodium pentobarbital-induced hypnosis experiment

Mice were divided into five groups, i.e. one group received orally saline, three groups received the extract of GABA black tea at 0.83 mg/kg, 1.67 mg/kg and 3.33 mg/kg, respectively and one group received rotundine tablets (80.00 mg/kg). These mice were administrated with the drugs orally once daily for 15 consecutive days. Thirty min after the last administration, the mice were given an intraperitoneal injection of sodium pentobarbital (27.50mg/kg), with which the highest subthreshold dose was predetermined to make 80%-90% mice remain the righting reflex. The mice were observed for the number of sleeping mice and sleeping rate for 30 minutes. The judgment standard for sleep was the time between loss and recovery of righting reflex for more than 60 s.

2.5 Statistical Analysis

All data were subjected to analysis of variance using SPSS (version 19.0, SPSS, Inc.). The statistical analyses were performed using one-way analysis of variance (ANOVA). Data were expressed as mean ± standard deviation (SD) per group. Differences were considered significant at $p<0.05$.

3 Results

3.1 Biochemical compositions of GABA black tea

The changes in chemical component during GABA black tea processing were presented in Table1. Data showed that tea polyphenols, caffeine and total catechins were decreased gradually during the entire processing process of GABA black tea, while the tea polyphenols and total catechins were reduced more significantly due to oxidation and mutual transformation between catechin monomers. The contents of water extract and total free amino acids showed the same changing trend. Their contents were the highest after being dried, which were 46.57% and 2.74%, respectively. The total catechins contents after vacuum anaerobic and aerobic treatment was 8.32%, which was the hightest, but reduced in the subsequent processing obviously. Among all catechins monomers, GCG, ECG and CG increased in GABA black tea, and other components of catechins showed a downward trend. However, the effective chemical components were almost decreased and their loss ratios were under control. It showed that the resultant GABA black tea were rich in

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water extract, tea polyphenols, caffeine, amino acids and catechins, which are beneficial to quality.

We also found a similar correlation between the content of amino acids and GABA, and the tendency that increased firstly and then decreased. In general, the content of GABA in tea made by Jin Xuan variety is about 0.12 mg/g[20]. After vacuum anaerobic treatment, the content of GABA was significantly increased up to 2.70 mg/g. In addition, content of GABA after drying reached to 2.34 mg/g, which meets the required standard for GABA tea.

3.2 Primary sleep observation test

After treatments, mice pretreated with GABA black tea extracts and rotundine tablets were quiet and had less activity. No mice with loss of righting reflex were observed. Both GABA black tea extracts and rotundine tablets did not cause direct hypnotic effect in mice. According to the testing methods of slumber improvement, the follow-up experiments on GABA black tea extracts and rotundine tablets can be undertook.

The data for initial and final weights of animal in different groups were presented in Fig 1. There was no significant difference in BW between the groups at the beginning (0 day) of the experiment. At the end of the experiment (15 day), the changes in BW of mice in these groups were basically identical and the BW gains of mice in low dose of GABA black tea extract group was significantly higher than those of mice in all the other groups (P<0.05). However, no significant variations in BW were found between the tea extracts-treated groups and the NC group at the end (15 day) of the experiment.

3.3 Sodium pentobarbital-induced sleep time test

After being given sodium pentobarbital (47.50 mg/kg), mice showed loss of writhing reflex within 5 min after drug administration in Fig 2. The sleep latency of mice in the tea extracts-treated groups (GBTH, GBTM and GBTL) were all lower than that of mice in the NC group but was close to that of the PC group. However, there was no statistically significant difference in sleep latency among NC, PC and all the tea extracts-treated groups.

Compared with that of control group, rotundine tablets (40.00 mg/kg), a central depressant drug as a positive control for sleep, prolonged the duration of sodium pentobarbital-induced sleep. The tea extracts at 0.83-3.33 g/kg prolonged dose-dependently the duration of pentobarbital-induced sleep in mice as compared to the NC group in Fig 3. The duration of sleep of mice administrated with the extract of GABA black tea at 3.33 g/kg was prolonged significantly (P<0.05) by 55.44%, which was better than that in mice treated with rotundine tablets. Also, the extracts of GABA black tea at 1.67 and 0.83g/kg also prolonged the sleep duration but the effects were no significant, Their rations were 23.40% and 9.20%, respectively.

3.4 Sub-threshold dose of sodium pentobarbital-induced hypnosis

Intraperitoneal administration of mice with sodium pentobarbital (27.50 mg/kg) induced sleep in mice after oral treatment with the extracts of GABA black tea and rotundine tablets in Table 2. The sleeping rates of mice received the extract of GABA black tea at doses of 0.83 g/kg, 1.67 g/kg and 3.33 g/kg were 0.00%, 6.67% and 20.00%, respectively. It is noteworthy noting that the doses of GABA black tea may be commensurate with the effect of promoting sleep. Sleeping rate was

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significantly increased in higher dose of GABA black tea extracts-treated groups as compared with that in the NC group and was near to that of the PC group.

4 Discussions

This study has demonstrated that GABA black tea rich in water extract, tea polyphenols, caffeine, free amino acids, GABA and catechins, with the relative contents of 46.57%, 15.47%, 3.86%, 2.74%, 2.34% and 1.47%, respectively, could be manufactured. These active components are beneficial to the tea quality.

After the whole processing, the biochemical compositions in resultant GABA black tea including water extract content and amino acids content were increased whereas the contents of tea polyphenols, caffeine, GABA and catechins would be decreased under control. Fermentation and drying process bring about marked chemical changes including protein degradation to produce free amino acids and condensations of catechins in tea polyphenols to form theaflavins and thearubigins[22-23]. In addition, other macromoleculars are hydrolyzed to soluble substances so that the water extract contents are increased, reflecting the abundance in taste of black tea soup. The content of GABA was increased firstly and then decreased. This is probably related to formation mechanism of GABA [12]. The glutamic acid (Glu) content in fresh tea leaves increased after harvested and the glutamate decarboxylase (GAD) activity was activated under anaerobic stress conditions. Both of these changes created a condition for glutamic acid decarboxylation reaction, which transforms Glu into GABA. The increased cell rupturing rate in rolling made GAD have more chance to contact with Glu as a precursor substance of GABA. On the one hand, the rate of GABA synthesis was decreased because of a shortage of Glu supply. On the other hand, leaf water content was decreased excessively, leading to the inactivation of GAD in the follow-up process. Thus it can be seen that GABA content was significantly increased after alternative sequences of anaerobic and aerobic treatment, and reached to the standard of GABA tea[6].

Sleep-wakefulness cycle is considered to be the result of an interaction between excitatory neurons (generate awakening and maintain neurons) and inhibitory neurons (generate sleep and maintain neurons). Many kinds of neurotransmitters such as GABA, Glu et al are involved in sleep in the sleep center system. Among these neurotransmitters, GABA and Glu are the most important ones in the central nervous system and they play both the inhibitory and excitatory roles, respectively. Several studies have shown that GABA, which is hardly transported across the blood brain barrier, a selective barrier mainly formed by the brain capillary endothelial cells, could play an important role in preventing paracellular transport of small and large water-soluble compounds from blood to the brain[24]. But Glu can be converted to GABA under the action of GAD[25]. Moreover, the GABA neurons play an important role in the alternation from wakefulness to sleep. Thus, it can be seen that as an inhibitory neurotransmitter, GABA helps to maintain overall balance in the nervous system by dampening neurons ability to respond to excitatory messages from other cells.

Drugs with sedative-hypnotic properties have been known to prolong the duration of sleep induced by barbiturates[26-27]. Furthermore, the extract of GABA Maoyecha green tea possesses the sleep-promoting effect, and the neuropharmacological properties are possibly and mainly mediated via the GABAergic

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neurotransmission[15]. In this study, we observed that the extract of GABA black tea significantly prolonged sleeping time with sodium pentobarbital and significantly enhancing the sleeping rate induced by sodium pentobarbital at sub-threshold dose. However, it had no significant effect on shortening sleeping latency period induced by sodium barbital (P>0. 05). According to the method of The Chinese Ministry of Heath for improving sleep function testing, these results indicated that GABA black tea has both a sleep-promoting effect[28] and central nervous system depressant activity[29]. The result indicated that the extracts of GABA black tea possess a certain sedative effects, which may be regulated by neurotransmitter systems, such as $GABA_A$, and dopamine D-2 receptors[30-33]. The precise molecular mechanisms remain to be further investigated.

5 Conclusions

The results obtained from this study have indicated that after repeating vacuum anaerobic treatments, GABA content in black tea is increased significantly, reaching the standard of GABA tea. The changes in the contents of tea polyphenols, caffeine and total catechins displayed a gradually decreasing trend, while the contents of free amino acids and GABA were increased first, and then reduced. The GABA black tea was rich in water extract, tea polyphenols, caffeine, amino acids, GABA and catechins, whose relative contents were 46.57%, 15.47%, 3.86%, 2.74%, 2.34% and 1.47%, respectively. These active components are beneficial to quality.

Additionally, although they did not shorten significantly the sleeping latency period induced by sodium pentobarbital (47.50 mg/kg), the extracts of GABA black tea still increased significantly the sleeping rate in mice induced by sub-threshold dose of sodium pentobarbital (27.50 mg/kg) as well as prolonged significantly sleeping time induced by sodium pentobarbital (47.50 mg/kg). More importantly, the extract of GABA black tea conferred the sleep-promoting effect.

Table 1 The changes in biochemical compositions of GABA black tea

	Vacuum anaerobic	Rolling	Fermentation	Drying
Tea polyphenols (%)	30.79 ±0.24 a	28.19 ±0.17 b	20.36 ±0.37 c	15.47 ±0.40 d
Caffeine (%)	4.11 ±0.02 a	4.00 ±0.06 b	3.96 ±0.05 b	3.86 ±0.01 c
Water extract (%)	38.75 ±0.17 b	39.32 ±0.41 b	34.34 ±0.19 c	46.57 ±0.82 a
Amino acids (%)	1.69 ±0.06 c	1.75 ±0.02 b	1.60 ±0.01 d	2.74 ±0.02 a
GABA(mg/g	2.70 ±0.03 b	2.76 ±0.03 a	2.44 ±0.06 c	2.34 ±0.05 d
Total catechins(%)	8.32 ±0.09 a	6.51 ±0.07 b	1.92 ±0.03 c	1.47 ±0.03 d
EGCG(%)	3.51 ±0.05 a	2.61 ±0.13 b	0.68 ±0.01 c	0.13 ±0.01 d
GCG(%)	0.57 ±0.01 a	0.30 ±0.02 b	0.04 ±0.01 c	0.36 ±0.00 b
EGC(%)	1.03 ±0.11 a	1.04 ±0.04 a	0.60 ±0.01 b	0.05 ±0.00 c
ECG(%)	0.77 ±0.04 a	0.59 ±0.06 b	0.23 ±0.00 c	0.42 ±0.01 b
EC(%)	0.94 ±0.03 a	0.86 ±0.05 a	0.35 ±0.00 b	0.18 ±0.00 c
GC(%)	0.63 ±0.06 a	0.38 ±0.01 b	trace.	0.02 ±0.00 c
C(%)	0.54 ±0.01 a	0.38 ±0.00 b	trace.	0.03 ±0.01 c
CG(%)	0.32 ±0.11 a	0.35 ±0.08 a	0.20 ±0.03 b	0.27 ±0.04 a

Each value is expressed as mean ± standard deviation (n=3). Values with different letters in the same row are significantly different (P<0.05) from each other. Those that share the same letters are not significantly different (P>0.05).

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Table 2 Influence of the oral treatment of GABA black tea extract on sleeping rate in mice

Group	Mouse (N)	Sleeping mice (N)	Sleeping rate (%)
NC	15	0	0.00
PC [80.00 mg/kg rotundine]	15	5	33.33*
GBTH [3.33 g/kg]	15	3	20.00*
GBTM [1.67 g/kg]	15	1	6.67
GBTL [0.83 g/kg]	15	0	0.00

*P<0.05, **P<0.01, significantly different as compared to the normal group. NC, normal control; PC, positive control; GBTH, high dose group of GABA black tea extract; GBTM, middle dose group of GABA black tea extract; GBTL, low dose group of GABA black tea extract

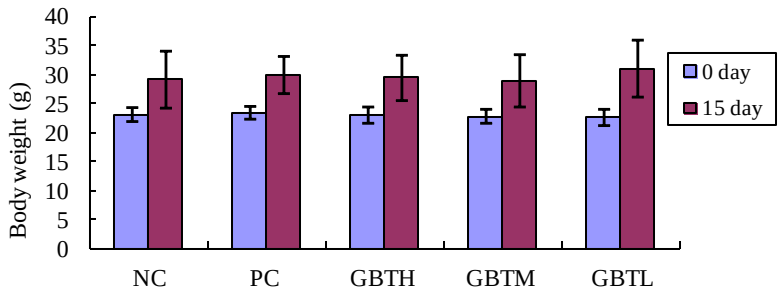

Fig.1 Influence of the oral treatment of GABA black tea extract on body weight in mice

*P<0.05, **P<0.01, significantly different as compared to that of the normal group. NC, normal control; PC, positive control; GBTH, high dose group of GABA black tea extract; GBTM, middle dose group of GABA black tea extract; GBTL, low dose group of GABA black tea extract.

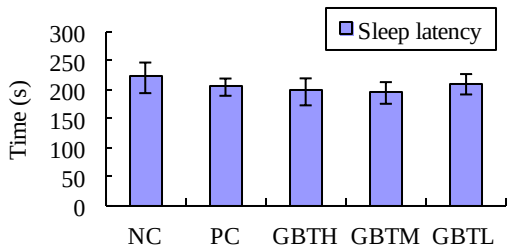

Fig.2 Influence of the oral treatment of GABA black tea extracts on latency of sleep in mice

*P<0.05, **P<0.01, significantly different as compared to that of the normal group. NC, normal control; PC, positive control; GBTH, high dose group of GABA black tea extract; GBTM, middle dose group of GABA black tea extract; GBTL, low dose group of GABA black tea extract.

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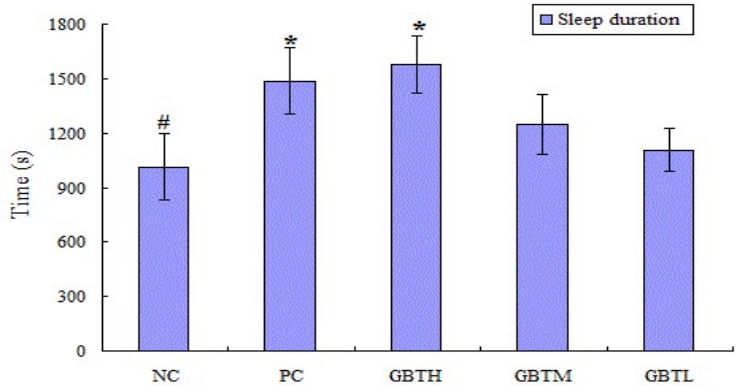

Fig.3 Influence of the oral treatment of GABA black tea extracts on duration of sodium pentobarbital induced sleep in mice

*P<0.05, **P<0.01, significantly different as compared to that of the normal group. #P<0.05, ##P<0.01, significant compared to the positive group. NC, normal control; PC, positive control; GBTH, high dose group of GABA black tea extract; GBTM, middle dose group of GABA black tea extract; GBTL, low dose group of GABA black tea extract.

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† Electronic Supplementary Information (ESI) available:
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‡ Footnotes should appear here.
Abbreviations: GABA, gamma(γ)-aminobutyric acid; HPLC, high pressure liquid chromatography; BW, body weight; NC, normal control; PC, positive control; GBTH, high dose group of GABA black tea extract, GBTM, middle dose group of GABA black tea extract, GBTL, low dose group of GABA black tea extract; Glu, glutamic acid; GAD, glutamate decarboxylase

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