Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2589013 A1 2006/06/08

(21) 2 589 013

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2005/11/28
(87) Date publication PCT/PCT Publication Date: 2006/06/08
(85) Entrée phase nationale/National Entry: 2007/05/31
(86) N° demande PCT/PCT Application No.: CN 2005/002034
(87) N° publication PCT/PCT Publication No.: 2006/058487
(30) Priorité/Priority: 2004/12/01 (CN200410084791.7)

(51) Cl.Int./Int.Cl. *A61K 36/67* (2006.01),
A61P 25/00 (2006.01)

(71) Demandeurs/Applicants:
WANG, EZHOU, CN;
JIANG, YI, CN

(72) Inventeurs/Inventors:
WANG, EZHOU, CN;
JIANG, YI, CN

(74) Agent: GOWLING LAFLEUR HENDERSON LLP

(54) Titre : EXTRAIT DE PIPER LAETISPICUM C.DC., PROCEDE D'ELABORATION ET APPLICATIONS
(54) Title: AN EXTRACTIVE OF PIPER LAETISIPICUM C.DC., ITS PROCESS AND ITS USES

(57) Abrégé/Abstract:
An extractive of Piper laetispicum C.DC., its process and its uses for preparing a medicine, health care product and food additive for treating and preventing diseases correlated with monoamine transmitter ,such as 5-HTA noradrenalin dopamine and so on. The extractive is processed through impregnating or percolating at common temperature or ≤70°C or using supercritical fluid extraction method, to obtain higher alkaloid content and higher biological activity, compared to using reflux method of prior art.

Abstract

The invention published the extract of *Piper laetispicum* C.DC., the methods of preparation and purification and the utility of the extract. Additionally, the extract prepared by these methods could be used as a (re)uptake inhibitor of monoamine neurotransmitters (5-HT and/or NA and/or DA.), and be used as a drug, health foods or food additives for prevention and/or treatment for psychiatric disorders which associated with monoamine neurotransmitters (5-HT,NA,DA.).

The preparation method for the extract of *Piper laetispicum* C.DC. , the extract and its use

Technical Field

The present invention relates to herb *Piper laetispicum* C.DC., more particularly, this invention relates to preparation method for the extract of *Piper laetispicum* C.DC., the obtained extracts by the said method, and the use thereof.

Background of the invention

In recent years, Psychiatric illness whose onset has been correlated to the metabolism disorders of monoamine neurotransmitter (5-HT, NA, DA) have become recognized as one of major public health problems. According to the data provided by WHO, the financial burden caused by psychiatric illness is only next to coronary heart disease in the developed countries, and it has been ranking in the first place in China, approximately 20% of the whole financial burden. For example, the studies in many communities and health care organizations showed that the morbidity of depressive disease and Anxious disease have higher rate of occurence, whose results showed that there are about 17.3%of the entire population who are thought to suffer from depressive disease and 24.5% have Anxious disease in certain period over the course of their lifetime. These results also found that there will be more people suffering from depressive disease or Anxious disease in the future. Some specilal groups, such as disabilities, patients with chronic diseases, drug abusers, people with alcohol dependence and celibates have a higher possibility to suffer from this disorders which is correlated to the metabolism disorders of monoamine neurotransmitter (5-HT, NA, DA). With the aging, the psychiatric illness morbidity of the aged people becomes worse and worse, among which dementia, Depressive disease and /or Anxious disease are most prominent.

It has been considered that agents with dual reuptake inhibition of 5-HT and /or NA and /or DA, have been come into being a particularly market in treating Depressive disease and Anxious disease with low side-effects and would bring huge economic benefit.

Piper laetispicum C.DC. is only recorded by “Flora of China” and “Herb of China”(published in 2000). According to “Herb of China”, this plant has been only used “as a wound-healing agent in antispasmodic, analgesic and anti-inflammatory by decoctummed or soaked in wine and with the warm properties and taste hot…” .

A series of research had recently showed that the extract from *Piper laetispicum* C.DC. have remarkable effect on anti-depressive activity and some effect on anxiolytic,

analgesic and sedative activities.

The inventors of the present invention have filed two Chinese applications related to *Piper laetispicum* C.DC., the first one (Appl. No. 00119452.6) has disclosed the use of *Piper laetispicum* C.DC. and its extract in preparation of pharmaceutical compositions. The second one (Appl. No. 03115911.7) has disclosed the preparation method of the active portion extacts from *Piper laetispicum* C.DC., the extract obtained by the said method were thought to possess antidepressive activity and might be a useful option for the prevention and treatment for psychiatric illness such as depressive disease or other disorders.

But further studies have found that some active components have been destroyed at a certain extent by the traditional procedure (water-heatup-refluence) which consists of heating, filtering, concentration and so on, which need numerous steps, long periods, wastes too much energy sources, and have more difficulty in practice. More importantly, the method of heat up-refluence results in reduction of its pharmacological activities and safe-evalution. Thus, it is necessary to research and develop a new method.

Aim of the invention

Firstly, the aim of the present invention is to overcome the shortcomings of the traditional methods(water-heat up-refluence) for preparation of extract from *Piper laetispicum* C.DC., thus provide an improved method to prepare extract from *Piper laetispicum* C.DC., which can avoid the destruction of active components by high temperature during extraction, as well as make the process more simple and easier to operate.

Secondly, the aim of the present invention is to provide the extract of *Piper laetispicum* C.DC. obtained by the said method.

Finally, the aim of the invention is to provide the use of the extract from *Piper laetispicum* C.DC.

Summary of the invention

The procedures for preparation of the extract from *Piper laetispicum* C.DC. are as follows:

The roots, rhizomes, leaves and fruits or whole herb were pretreated, then macerated or diacolated(immerse or percolate) with organic solvent at room temperature(normal temperature) or ≤70□, then concentrated at ≤70□ and obtained the crude extract.

The crude extract also could be refined and the purification was obtained by concentration at ≤70□. further purificative manage means include but not limit to

macropore poly adsorbent column, polyamide column, silica gel column, ion exchange column, ethanoldistill-waterdeposit, aciddistill-alkalideposit and alkalimoist-organic soventextract and so on. Or supercritical fluid extraction (SFE) was applied directly after the herbs were pretreated.

Crude or purified extract is a cream, sticky and yellowish-brown or dark brown solid which is unsoluble in water.

The structures of the known alkaloid compounds include:

N-isobutyldeca-trans-2-trans-4-dienamide;

1-[(2E,4E)-2,4-Decadienoyl]pyrrolidine;

N-isobutyl-9(3',4'-methylenedioxyphenyl)-2E,8E-nonadienamide;

3',4'-methylenedioxycinnamicacid isobutylamide;

5'-Methoxy-3',4'-methylenedioxycinnamicacid isobutylamide;

1,2-Benzenedicarboxylic acid diisooctyl ester

1-[(2E)-5(3,4-Methylenedioxyphenyl)-2-pentenoyl]pyrrolidine;

5'-Methoxy-3',4'-methylenedioxycinnamicacid pyrrolidide.

ED_{50} of the refined extract in mice is 4.56mg/kg (forceswimmingtest) and 3.49mg/kg (tailsuspensiontest) respectively; ED_{50} in rats is 1.85mg/kg (forceswimmingtest). Acute toxicity of pharmacological experiments results showed that therapeutic index in mice is LD50/ED50=116-124 (forceswimmingtest).

Obvious advantages of the improved method is higher extraction rate, efficiency and better extract quality, compared with the traditional one. The experiments show that the antidepressive effect of the extract from *Piper laetispicum* C.DC. may be associated with the increase of monoaminergic neurotrasmitter and strengthening the monoaminergic function in the brain. The findings of the extract may have a potential utility in the treatment of human psychiatric disorders such as schizophrenia, brain organic mental disorders, obsession, mutism, mania, anxiety disorders, insomnia, depression, epilepsy, Parkinson syndrome, headache， neurodynia and senile dementia, which are associated with the disturbance of metabolism of the monoamine transmitter .This extract is promising as a new type of potent drug including antidepressant, antianxiety, sedative hypnotics and antisenile dementia by significantly inhibiting 5-HT, NA and DA uptake into synaptosomes. This extract can be used as one of activity ingredient in pharmaceutical agents, health foods and food additives.

Specific Examples:

We take the specific samples only to have a further explanation for the findings, without the intention of limiting the reach of the invention.

Example 1. Preparation of *Piper laetispicum* C.DC. extract

According to the table 1, the roots, rhizomes, vines, leaves, fruits or whole herb were prepared for the extract of *Piper laetispicum* C.DC.

Sample 1-1: The air-dried roots and rhizomes (1 kg) of *Piper laetispicum* C.DC. were cut into section and macerated in the 90% ethanol about 48h at room temperature and then added the same ethanol up to 20 L. The crude extract solution was percolated, and then added 15 L warm water and mixed. The diluted solution was chromatographed on D101 macropore poly adsorbent column (TianJin DaJun science and technology limited company), eluted with 40% and 90% ethanol consecutively. The 90% ethanol fraction was collected and refined by concentrating and drying at the temperature under 70□.

Sample 1-2: The air-dried roots and rhizomes (1 kg) of *Piper laetispicum* C.DC. were cut into section and macerated in the 80% ethanol about 48h at room temperature and then added the same ethanol up to 10 L. The crude extract solution was achieved by percolation at 50□, and concentrated to to 2L under reduced pressure at 60□. The diluted solution was chromatographed on D101 macropore poly adsorbent column (TianJin DaJun science and technology limited company), eluted with 50% and 80% ethanol consecutively. The 80% ethanol fraction was collected and refined by concentrating and drying at the temperature under 70□.

Sample 2: The air-dried and powdered roots and rhizomes (1 kg) of *Piper laetispicum* C.DC. were macerated in the 90% ethanol about 48h at room temperature and then added the same ethanol up to 16L. The crude extract solution was achieved by percolation and concentrated to to one-third volume under reduced pressure at 60□, and then added cool water with same volume and mixed. The mixture been perserved in a refrigerator for 3 days and then filtrated to remove the deposition. The extract was refined by concentrating and drying at the temperature under 70□.

Sample 3: The air-dried whole herb (1 kg) of *Piper laetispicum* C.DC. were cut into section and macerated in the 10L 0.5% acetic acid about 48h at room temperature. The crude extract solution was achieved by percolation and added 2% ammonia solution and mixed slightly. The mixture was extracted with $CHCl_3$ and chloroform and refined by concentrating and drying at the temperature under 70□.

Sample 4: The air-dried roots and rhizomes (1 kg) of *Piper laetispicum* C.DC. were cut into section and macerated in the 55% ethanol about 24h at room temperature and then added the same ethanol up to 20 L. The crude extract solution was achieved by percolation at 60□, and concentrated. The solid crude extract was chromatographed on silica gel column. (silica gel H, ShanDong QingDao HaiYang chemical engineering company), eluted with cyclohexane-ethylacetate and petroleumether-acetone and collected petroleumether-acetone (10:1to2:1) fraction, The extract was refined by concentrating and drying at the temperature under 70□.

Sample 5: The air-dried and powdered roots and vines (1 kg) of *Piper laetispicum* C.DC. were macerated in the 5L aether about 24h at room temperature and collected the extract, which was repeated 3 times. The extract was combined and removed the aether under reduced pressure. The extract was dissolved with acetone and chromatographed on polyamide column (HunanXiangtan zhaoyang chemicalfactory), The extract was refined by concentrating and drying at the temperature under 70□.

Sample 6: The air-dried roots and rhizomes (1 kg) of *Piper laetispicum* C.DC. were cut into section and macerated in 5L 0.5% cold aceticacid about 24h and the heated slightly at the temperature under 70□. The mixture was chromatographed on ion exchange column (732 cation exchange resin, Hebi Xiangyang resin factory), then eluted with 2% ammonia solution. The extract was refined by concentrating and drying at the temperature under 70□.

Sample 7: The air-dried and powdered roots and vines (1 kg) of *Piper laetispicum* C.DC. were macerated in chloroform about 24h at room temperature, and then added chloroform to the volume of 10L. The mixed were removed the chloroform under reduced pressure and refined by concentrating and drying at the temperature under 70□.

Sample 8: The air-dried and powdered whole herb (1 kg) of *Piper laetispicum* C.DC. were macerated in ethylacetate about 24h at room temperature, and then added ethylacetate up to the volume of 10L. The ethylacetate was removed and the solid crude extract was dissolved with the ethanol. Then it was chromatographed on D860021 macropore poly adsorbent column (ShanDong LuKang medicine stock limited company) and eluted with 40% and 90% ethanol. 90% ethanol fraction was collected and refined by concentrating and drying at the temperature under 70□.

Sample 9: The air-dried roots (l kg) of *Piper laetispicum* C.DC. were cut into section and macerated in 2L 0.5% ammonia solution about 24h and then extracted with diethylether for 3 times.The extract was Combined and aether was removed under reduced pressure. The extract was refined by concentrating and drying at the temperature under 70□.

Sample 10 (control):
The air-dried and powdered roots and rhizomes (l kg) of *Piper laetispicum* C.DC. were water-heatup-refluxed with 60% ethanol 2 times. The mixture was filtrated and combined, and concentrated to 14% of total volume under reduced pressure.
A clear solution was obtained by adding 30% ethanol, which was chromatographed on macropore poly adsorbent column and eluted with 40%, 50% and 80% ethanol in order. The 80% ethanol fraction was collected and concentrated to acquire the the extract (According to Example 1 published in Chinese Patent 03115911.7).

Sample 11: the extract from whole herb was prepared by supercritical carbon dioxide fluid extraction (SFE) as follows.
The air-dried and powdered whole herb was placed into extract pot, the extract was prepared by supercritical carbon dioxide fluid extraction (SFE). The condition is as follows: $30Mp_a$, 40□, 2h and $20m^2/h$ (CO_2), 200g material each time. The extract was obtained when the pressure extract pot was reduced to normal.

Table 1 The preparation of extract from *Piper laetispicum* C.DC.

Sample	Material	Pretreatment	Organic solvent	Extraction	Refine method
1-1	root, rhizoma	cuting	ethanol	diacolation	macropore resin
1-2	root, rhizoma	cutting	ethanol	heat diacolation	macropore resin
2	vine, leaves	powder	ethanol	diacolation	ethanoldistill-waterdeposit
3	whole herb	Cutting	acid water	maceration	aciddistill-alkalideposit
4	root, rhizoma	Cutting	ethanol	heat diacolation	silica gel column
5	root,	Powder	diethylether	maceration	polyamide column

	vine				
6	root, rhizoma	Cutting	acid water	diacolation	ion exchange column
7	root, vine	Powder	chloroform	diacolation	-
8	whole herb	powder	ethylacetate	diacolation	macropore resin
9	Root	cutting	ammonia solution	maceration	organic solvent
10*	root, rhizoma	powder	ethanol	Waterheatup-refluxing	macropore resin
11	Whole herb	powder	supercritical fluid extraction(SFE)		

*as control extract by water-heatup-refluxing

Example 2. The main applications of the extract in pharmaceutical agents

The extract from *Piper laetispicum* C.DC. prepared in the **Example 1** can be used as the main ingredient in capsule, troche, granule, powder，dripping-pill，subtle-pill, injection, sterile injection powder, oral-preparation, sustained release or controlled release preparation and target drug and so on. extract (sample 1-2) was taken as example to show the application in pharmaceutical agents such as capsule, troche and granule.

2.1 Capsule

Formula: extract 10g; cornstarch 80g; stearic acid-magnesium 8g; antioxidant 2g.

Method: Mixed the extract, cornstarch, stearic acid-magnesium and antioxidant evenly, then sieved, compressed and filled it into empty 1000 capsules.one capsule which been filled material weights 100mg and includ 10mg of refined extract per piece.

2.2 Drug granules

Formula: extract 20g; amidulin 380g; british gum 200g.

Method: All the component were mixed and pressed, sieved and dryed,then divided it into 1000 pouches.

2.3 troche

Formula: extract 30g; starch 100g; british gum 30g; sucrose 20g; talcum powder 12g; stearic acid-magnesium 5g; antioxidant 3g.

Method: Mixed the extract, starch, british gum, sucrose before drying, and produced it into grain. Then drying and added the talcum powder , stearic acid-magnesium, antioxidant extrusion it into 1000.

Example 3 Inhibitory effect of the extract on reuptake of the 5-HT, NA and DA *in vivo*

Previous studies have showed that the mechanism by which norepinephrine in synaptic cleft is eliminated and inactivated is reuptaked by nerve terminal. the uptake process can be inhibited by Cocaine, some phenylethylamine compounds and antidepressants, which is one of the most essential mechanism of down-regulation of the activity of adrenergic receptor by anti-depressive Drug. Physiological functions of 5-HT-positive and dopaminergic nerve is similar to that of 5-HT. The effective anti-depressive drug in clinic therapy can inhibite the reuptake of the Norepinephrine and/or serotonin and/or dopamine. The primary purpose of this study was to determining the inhibitory effect of the compound on reuptake of the 5-HT, NA and DA by Rat brain synaptosomes and its value as anti-depressive drug.

Firstly, the preparation of brain synaptosomes was done accoding to the the method described previous by Whittaker as follows (Whittaker VP & Barker LA. The subcellular tractionation of brain tissue with special reference to the preparation of synaptosomes and their component organelles. In: Fried R. ed. In Methods in Neurochemistry, Vol. New York: Marcel Dakker, Inc, 1972). After rats were sacrificed by decapitation, the brains were rapidly removed, and placed into Precooled saline solution after Removing of leptomeningeal and vascular tissue. Cerebral cortex was collected and placed into cold sucrose solution. Brain synaptosomes were prepared by homogenating with Ultrasonic Disrupter and centrifugating.

The activity was evaluated as previous described in the literature of " Modern Medical Experimental Method" by Wang Q (1997) and "Modern Methodology in Pharmacological Experiment by" Zhang J T (1998).

The preparation of brain synaptosomes were resuspended in Tris-Krebs buffer. Then 10μl of the extract of *Piper laetispicum* C.DC. (sample 1-1) was added into the solution, which was incubated at 37□ for 5min after mixing. The mixture was placed at 4□, in which 10μl of 3H-5-HT, 3H-NA or 3H-DA was added rrespectively (final concentration 300nM), and then incubated at 37□ for 5min. The uptake reaction was terminated by addition of procooled Tris-Krebs buffer followed by immediate filtration through glass fiber filters using a cell collection system. The filters was cleaned with the same volume of wash solution. After dried, the filter membranes was placed into scintillation flask, into which the liquid scintillation liquid containing toluene was add, the result was elucidated by using beta-liquid scintillation counter.

Table 3 Inhibitory effect of the extract on reuptake of the 5-HT, NA and DA

Sample 1-1 (final concentration)	positive control	Inhibitory effect on reuptake of monoamine (counts per minute, CPM)		
		5-HT	NA	DA
	0□	1114	1519	1265
	37□	**1362**	**1671**	**1606**
	Fluoxetine (0.1mM)	**1101**		
	Desipramine (0.1mM)		**1499**	
	Pargyline (0.1mM)			**1197**
0.064 μg/ml		**1341**	**1674**	**1587**
0.32 μg/ml		**1329**	**1580**	**1326**
1.6 μg/ml		**1261**	**1490**	**1201**
8 μg/ml		**1191**	**1159**	**1003**
40 μg/ml		**927**	**1144**	**956**
200 μg/ml		**692**	**975**	**735**
1000 μg/ml		**465**	**680**	**487**

As shown in Table 3, compared with the uptake of 5-HT, NA and DA at 37□, the extract of *Piper laetispicum* C.DC. had significant inhibitory effect on the on reuptake of the three monoamines at the dose of higher than 0.064 μg/ml (with lower CPM than the normal), inhibited the reuptake of 5-HT by brain synaptosomes completely at the dose of 40μg/ml, whose effect is comparable with Fluoxetine. when the concentration reach at the range of 200~1000μg/ml, the value of CPM is lower than that of CPM at 0□, which showed that non-specific diffusion of 5-HT by brain synaptosomes was inhibited completely (IC_{50}=4.2μg/ml, final concentration).

The extract had more potent inhibitory effect on NA, which can inhibit the uptake of NA almost completely at the dose of 1.6 μg/ml whose effect is comparable with 0.1mM Pargyline. Similarly, non-specific diffusion of 5-HT by brain synaptosomes was inhibited when the concentration is more that about 200 μg/ml (IC_{50}=0.34μg/ml, final concentration). The inhibitory effect of extract on DA is between that on 5-HT and NA (IC_{50}=1.1μg/ml, final concentration).

The results showed that the extract from *Piper laetispicum* C.DC. has the potential to

develop as drug, health food or food additive for treating and/or preventing the disease relating to the metabolism of 5-HT, NA and DA (psychotic disease).

Example 4 5-HTP-induced head twitches test in mice

5-HTP is the precursor of 5-HT. Pargyline, the monoamine oxidase (MAO) inhibitor, can inhibit the biotransformation of 5-HT from 5-HTP. Head twitches response in mice were observed when treated with 5-HTP in combination with antidepressant drugs.

Mice were randomly assigned to 4 different groups, and each group was administered with the extract A (sample 1-1)of *Piper laetispicum* C.DC prepared as Example 1 or saline solution as control by p.o. for seven consecutive days as specified in Table 4. Sixty min after p.o. administration of the test substances, the 4 groups were administered by intradermal injection of pargyline hydrochloride. The number of head twitches was counted ninety min after tail vein injection of 5-HTP.

Table 4 Enhancing effect of the extract A on head twitches response induced by 5-HTP (n=10, Ridit test)

Group	Dose (mg/kg/d)	Number of head twitches response				
		0	□	□	□	□
Control	-	6	**4**	**0**	0	0
A	20	0	3	1	2	4
A	10	0	3	3	2	2
A	5	2	5	3	0	0

Ratio of control with high dose: 2.05, $P<0.05$

Ratio of control with media dose: 2.05, $P<0.05$

Ratio of control with low dose: 1.06

15 min after administration of 5-HTP, The 4 groups of mice showed head twitches response in varying degree, but the response in the groups administrated with extract A are more significant than that in the control group. Among them, the response in the group with the dose of 20 mg/ml is the most significant, with the most animals showing positive respond. After 2 hours, the group with the dose of 20mg/ml showed the head twitches response, while the control group had returned to normal. The results showed that the he extract A (sample 1-1) of *Piper laetispicum* C.DC can Enhance the head twitches h response induced by 5-HTP, and demonstrated the effect on the uptake of 5-HT in vivo.

Example 5 Yohimbine induced lethality test in mice

As the Antagonist of α_2 receptor, yohimbine can block the binding of NA with α2 receptor by binding the receptor. Antidepressant drugs, with the mechanism of inhibition of the reuptake and deactivation of NA, induced lethality by enhanced the NA when administrated in combination with yohimbine. The purpose of the present study was to investigated the effect of extract A (sample 1-1) on the reuptake of NA by examination in vivo.

Mice were randomly assigned to 5 different groups, and each group was administered with the extract A (sample 1-1) or saline solution as control by p.o. for seven consecutive days as specified in Table 5. Sixty min after p.o. administration of the test substances, the 5 groups were administered by intradermal injection of yohimbine and then the lethality of each group was observed and recorded at 1, 2, 4, 5, 24 hr, rrespectively.

Table 5 Enhancing effect of the extract A on lethal toxicity induced by Yohimbine (n=10)

Group	Dose(mg/kg/d)	Lethality in mice				
		1h	2h	4h	5h	24h
Control	-	0	0	0	0	0
A	20	2	0	0	0	0
A	10	1	0	0	0	0
A	5	0	0	0	0	0
A	2.5	0	0	0	0	0

20 min after administration of yohimbine, the 5 groups of mice showed the increasing of activity of central nerve system in varying degree, with the symptoms of Irritability, muscle tremors and occasional oronasal bleeding. The respond in the group with the dose of 20 mg/ml is the most significant.

As shown in the Table 5, death occurred in the groups with the dose of 10 mg/ml and 20 mg/ml 60 min after administration of yohimbine. The results suggested that the extract A can enhance the lethality induced by yohimbine, and demonstrated the inhibitory effect of the extract A on reuptake of the NA.

Example 6 Inhibitory effect of the extract A on MAO in mice

Monoamine oxidase (MAO) has the bioactivity of adjusting the metabolism of the endogenous and exogenous catecholamine neurotransmitters such as noradrenaline,

adrenaline and dopamine. MAO-A and MAO-B. According to the specificity of substrate and inhibitor, MAO exist two isomers. Dopamine and tyramine are the substrates of both MAO-A and MAO-B; 5-HT and noradrenaline are the substrates of MAO-A; β-phenylethylamine and benzylamine are the substrates of MAO-B. Clorgyline is the selective inhibitor of MAO-A, while deprenyl and pargyline is the selective inhibitor of MAO- B. The inhibitor of MAO has the anti-depressive activity. This study was carried out to determine whether the extract A can inhibit the activity of MAO and investigate the mechanism of anti-depressive.

Mice were randomly assigned to 5 different groups, and each group was administered with the extract A (sample 1-1), saline solution as negative control, or pargyline as positive control by p.o. for forteen consecutive days as specified in Table 6. Sixty min after p.o. administration of the test substance, mice were sacrificed by cervical dislocation and the brain was removed. According to the method described in the literature by Zhen L (Journal of China Pharmaceutical University, 2002, 33(2):138-141), the value of optical density (OD) was determined by Ultraviolet-visible spectrophotometer.

Table 6 Inhibitory effect of the extract A with different concentration on MAO in mice. (n=10, $\bar{x} \pm s$)

Group	Dose(mg/kg)	MAO-A (OD)	MAO-B (OD)
Control	-	0.025±0.007	0.04±0.007
Pargyline	25	0.012±0.005**	0.025±0.008**
A	20	0.024±0.008	0.041±0.008
A	10	0.026±0.009	0.039±0.008
A	5	0.026±0.007	0.041±0.006

**P<0.01 compared with control group

The result showed that extract A did not inhibit the activities of either MAO-A or B. No relationships were found between the anti-depressive effect of the extract A and the activities of MAO-A and B.

Example 7 Forced Swimming Test in mice

Porsolt propose that mouse model of behavioral despair can be effective evaluation model to evaluate the effect of anti-depressive drug. Researchers who study depression in lab animals use a behavioral test called the "forced swim test." It works like this: Normal rats are put in a tub of water. Typically, they swim hard for 10 minutes, then give

up and float until researchers take them out, which express the behavioral despair of animal. anti-depressive drug can inhibit the behavioral despair of model animal.

Mice were randomly assigned to 13 different groups, and each group was administered with the extract A (sample 1-1, 2.5, 5, 10 mg/kg), extract B (sample 10, 10, 30, 60 mg/kg), extract C (sample 7, 30 mg/kg), extract D (sample 3, 30 mg/kg), extract E (sample 5, 30 mg/kg), extract F (sample 9, 30 mg/kg), extract G(sample 11, 30 mg/kg), saline solution as negative control, or Fluoxetine (25 mg/kg) as positive control by p.o. for forteen consecutive days, rrespectively, as specified in Table 7. Sixty min after p.o. administration of the test substance, the mice were place individually in glass cylinders (10×20 cm, depth of water: 8~10CM, water temperature: 22~24□). The total immobility time was recorded for 4 minutes 2 min after test.

Table 7. The result of Forced Swimming Test in mice (n=10, $\bar{x}$ ±s)

Group	Dose(mg/kg/d)	Total immobility time (s)
Control	-	121.4±17.7
Fluoxetine	30	73.7±17.2**
A	10	84.0±15.3**
A	5	93.5±13.3**
A	2.5	107.1±24.8
B	10	112.7±13.0
B	30	107.7±12.5
B	60	96.6±16.7*
C	30	79.3±16.5**
D	30	68.4±12.6**
E	30	81.8±8.3**
F	30	76.7±13.3**
G	30	73.9±18.2**

**P<0.01, *P<0.05, compared with control group; ED_{50}(A)=4.56mg/kg

In the mice forced-swimming test, the immobility time was significantly reduced in the groups with administered with the extract A (2.5 mg/kg, 5 mg/kg and 10mg/kg) and Fluoxetine, compared with control group (P<0.01). The extract A at dose of 2.5 mg/kg showed significant anti-depressive effect, with a good dose-responses relationship. The extracts C-G have comparable effect with the extract A at the dose of 30mg/kg, and are more effective that the extract B at the same dose.

The result suggested that the extract A and C-G have significant anti-depressive effect. In addition, the activity of the extract refined by diacolation is more significant than that of the extract B prepared by reflux extraction.

Example 8 Forced Swimming Test in rats

Mice were randomly assigned to 6 different groups, and each group was administered with the extract A (sample 1-1, 2.5, 5, 10 mg/kg), extract B (sample 10, 20 mg/kg), saline solution as negative control, or Venlafaxine (30 mg/kg) as positive control by p.o. for forteen consecutive days, rrespectively, as specified in Table 8. 4 days after starved, the mice were administered with the test substance and place individually in glass cylinders (40×18 cm, depth of water: 15CM, water temperature: 25□). The total immobility time was recorded for 5 min.

Table 8 The result of Forced Swimming Test in rats (n=10, $\bar{x} \pm s$)

Group	Dose(mg/kg/d)	Total immobility (s)
Control	-	243.8±20.1
Venlafaxine	30	211.2±24.7*
A	10	187.1±38.2**
A	5	197.4±47.9*
A	2.5	222.7±20.2*
B	20	238.6±22.4

**P<0.01, *P<0.05, compared with control group; ED_{50}=1.85 mg/kg

In the present study, Venlafaxine and A (2.5, 5,10 mg/kg) significantly reduced immobility time in the FST, compared with control group (P<0.05).The intensity of immobility reduction was stronger at a dosage of 2.5 mg/kg(A). Our results also indicate that the activity of A in the FST was more effective at a dosage (2.5 mg/kg) than that of B (20 mg/kg) and antidepressant activities showed good dose-effect relationship.

Obviously, the extract A has higher active ingredients than extract B, because of purification achieved by diacolation is a better extract quality than by water-heatup-refluxing.

Example 9 The Tail Suspension Test in mice

The method of the Tail Suspension Test (TST) in mice was first reported by Stern for assessing antidepressant activity in 1985. The immobility displayed by rodents when subjected to unavoidable stressful situations is thought to reflect a behavioral despair, which is similar the depressive disorders in human. The immobility time can be reduced by the effective clinical antidepressant significantly.

Mice were randomly assigned to 10 different groups, and each group was administered with the extract A (sample 1-1, 2.5, 5, 10, 20 mg/kg), extract B (sample 10,

20 mg/kg), extract C (sample 7, 20 mg/kg), extract D (sample 3, 20 mg/kg), extract E (sample 5, 20 mg/kg), saline solution as negative control, or Venlafaxine (50 mg/kg) as positive control by p.o. for forteen consecutive days, rrespectively, as specified in Table 9. Sixty min after p.o. administration of the test substance, the mice were performed Tail Suspension Test, and the immobility time was recorded for 6 min.

Table 9 The result of tail suspension test in mice (n=10, $\bar{x} \pm s$)

Group	Dose(mg/kg/d)	Total immobility (s)
Control	-	156.4±78.6
Venlafaxine	50	57.5±43.0**
A	20	80.1±41.1*
A	10	86.1±43.6*
A	5	87.5±59.6*
A	2.5	106.7±40.6
B	20	148.6±33.9
C	20	93.3±12.4*
D	20	89.4±16.3*
E	20	92.4±12.5*

**P<0.01, *P<0.05, compared with control group; ED_{50}=3.49 mg/kg

In the Tail Suspension Test, the immobility time was significantly reduced in the groups with administered with the extract A (5 mg/kg, 10 mg/kg and 20 mg/kg) and Venlafaxine, compared with control group (P<0.01). The extract A at dose of 5 mg/kg showed significant anti-depressive effect, with a good dose-responses relationship. The extract A at different dose show almost identical anti-depressive effect, which are slightly weaker than the effect of Venlafaxine but without statistically significant difference.

The extracts extract A at the dose of 2.5 mg/kg showed more significant effect than extract B at the dose of 20 mg/kg, which demonstrated that the activity of the extract refined by diacolation is more significant than that prepared by water-heatup-reflux extraction used in patent 03115911.7.

Example 10 The Four-Plate Test in Mice.

Mice were randomly assigned to 9 different groups, and each group was administered with the extract A (sample 1-1, 10, 20 mg/kg), extract B (sample 10, 20, 40 mg/kg), extract C (sample 7, 20 mg/kg), extract F (sample 3, 20 mg/kg), extract G (sample 5, 20 mg/kg), saline solution as negative control, or Diazepam (1 mg/kg) as positive control by p.o., respectively, as specified in Table 10. Thirty min after p.o.

administration of the test substance, the mice were placed on the plate. After a 15s latency period, the mouse was subjected to an electric shock (0.35mA, 0.5s) every time when it went from one plate to another, which result in significant avoidance response. If the mouse kept running for 3 min, the mouse was not subjected to electric shock any more. The number of electric shock of every mouse was recorded for 10 min.

Table 10. The result of Four-Plate Test in mice (n=8, $\bar{x} \pm s$)

Group	Dose (mg/kg/d)	Number of electric shock
Control	-	4.80±1.63
Diazepam	1	12.78±2.05**
A	20	8.64±2.14*
A	10	7.43±1.32*
B	20	5.08±1.05
B	40	7.32±1.01*
C	20	7.65±2.09*
F	20	8.98±3.21*
G	20	9.67±1.72*

**P<0.01, *P<0.05, compared with control group;

The results suggested that electric shocks can decrease the action of mice significant. Diazepam, extract A (10, 20 mg/kg), C (20 mg/kg), F (20 mg/kg) and G (20 mg/kg) can increase the number of electric shocks, which indicated that the extract of *Piper laetispicum* C.DC has potent anxiolytic effect, with more significant effect than that prepared by water-heatup-reflux extraction (extract B).

.

Example 11 Measurement of sedative effect of extract by the open field test

Mice were randomly assigned to 6 different groups, and each group was administered with the extract A (sample 1-1, 20, 40 mg/kg), extract B (sample 40 mg/kg), extract C (sample 7, 40 mg/kg), saline solution as negative control, or kavalactones (100 mg/kg) as positive control by p.o., respectively, as specified in Table 11. Thirty min after p.o. administration of the test substance, the mice were placed individually into a 35 cm high cylindrical box at height, whose bottom was divided into square with length of 5 cm. 3 min after adaptation, crossings of the square were counted for 5 min.

Table 11 The result of open field test (n=8, $\bar{x} \pm s$)

Group	Doses(mg/kg)	Total crossings of the square
Control	-	167.38±8.70

Kavalactones (kavepyrons)	100	136.43±6.86*
A	40	133.87±9.41*
A	20	154.74±10.23
C	40	139.78±9.61
B	40	151.33±5.72

**P<0.01, *P<0.05, compared with control group

The results indicated that the extract of *Piper laetispicum* C.DC. and kavalactones (kavepyrons) can reduce the crossings of the square, which demonstrated that the extract has rather potent anxiolytic effect, with more significant effect than that prepared by water-heatup-reflux extraction (extract B).

Example 12 Measurement of the analgesic effect by the abdomen writhing test

A chemical irritant with certain concentration is injected into the abdomen of mice, which stimulate the visceral peritoneum layer, caused inflammatory pain at deep larger area for a longer period of time, nduced abdominal contraction, which is called as writhing response. 15 min after the injection, the reaction occur frequently, therefore, the number of writhing 15 min after the injection is the indicators of the pain.

Mice were randomly assigned to 6 different groups, and each group was administered with the extract A (sample 1-1, 40, 80 mg/kg), extract B (sample 80 mg/kg), extract C (sample 7, 80 mg/kg), saline solution as negative control, or aspirin (50 mg/kg) as positive control by p.o., respectively, as specified in Table 12. The abdomen writhing is induced by intraperitoneal injection of 0.2 mL 0.02% aqueous solution of benzoquinone to each mouse 30 min after p.o. administration of test substances. Immediately after injection of benzoquinone, each mouse was placed into an individual observation box and the number of abdominal contortions was counted within 10 min.

Table 12 Effects on benzoquinone -induced writhing in mice (n=10, $\bar{x} \pm s$)

Group	Dose(mg/kg)	Number of writhing
Control	-	29.25±2.62
Aspirin	50	9.89±1.09**
A	80	10.34±1.44**
A	40	14.25±2.47*
C	80	16.25±0.90*
B	80	20.97±1.23

**P<0.01, *P<0.05, compared with control group

The results indicated that the extract of *Piper laetispicum* C.DC. and aspirin can reduce the number of writhing induced by aqueous solution of benzoquinone, which demonstrated that the extract has potent Analgesic activity, with more significant effect than that prepared by water-heatup-reflux extraction (extract B).

The extract of *Piper laetispicum* C.DC., which was prepared by water-heatup-refluxing method published in the previous Chinese patents (NO: 00119452.6 and 03115911.7), was of less biological activities and lower safety because of the high temperature. In this patent, the preparation of the extract was performed by diacolating and concentrating under the low temperature, by which alkaloid, lignans and other compounds with bioactivity were preserved and the. biological activities of the extract were more significant than before.

All results of the present studies showed that the extract of *Piper laetispicum* C.DC. prepared by the method published in this invention, could inhibit reuptake of monoaminergic neurotransmitters such as 5-HT, NA and DA significantly, so the extract could be developed as drug, health foods or food additives for prevention and /or treatment for metabolism-disorders(pneuma) which is associated with the disturbance of monoamine neurotransmitter..The mental disorders which is associated with the disorder of monoamine neurotransmitter include schizophrenia, mania, mind-impediment, mutism ,organic mentalsyndrome, coerce, depression, anxiety, insomnia, epilepsy, Parkinson syndrome, headache, neurodynia and senile dementia.

Claims

1. Preparation method for the extract of *Piper laetispicum* C.DC., wherein the method includes the following steps: the herb of *Piper laetispicum* C.DC. is pretreated, then add in organic solvents to maceration or diacolate the herb, after that the crude extract is obtained.

2. The method according to claim 1,wherein the herb of *Piper laetispicum* C.DC. includes the roots, rhizoma, vines, leaves, fruits and whole herbs.

3. The method according to claim 1, wherein the organic solvents used to maceration or diacolate the herb include but not limit to ethanol, methanol, chloroform， ethylacetate

**P<0.01, *P<0.05, compared with control group

The results indicated that the extract of *Piper laetispicum* C.DC. and aspirin can reduce the number of writhing induced by aqueous solution of benzoquinone, which demonstrated that the extract has potent Analgesic activity, with more significant effect than that prepared by water-heatup-reflux extraction (extract B).

The extract of *Piper laetispicum* C.DC., which was prepared by water-heatup-refluxing method published in the previous Chinese patents (NO: 00119452.6 and 03115911.7), was of less biological activities and lower safety because of the high temperature. In this patent, the preparation of the extract was performed by diacolating and concentrating under the low temperature, by which alkaloid, lignans and other compounds with bioactivity were preserved and the. biological activities of the extract were more significant than before.

All results of the present studies showed that the extract of *Piper laetispicum* C.DC. prepared by the method published in this invention, could inhibit reuptake of monoaminergic neurotransmitters such as 5-HT, NA and DA significantly, so the extract could be developed as drug, health foods or food additives for prevention and /or treatment for metabolism-disorders(pneuma) which is associated with the disturbance of monoamine neurotransmitter..The mental disorders which is associated with the disorder of monoamine neurotransmitter include schizophrenia, mania, mind-impediment, mutism ,organic mentalsyndrome, coerce, depression, anxiety, insomnia, epilepsy, Parkinson syndrome, headache, neurodynia and senile dementia.

Claims

1. Preparation method for the extract of *Piper laetispicum* C.DC., wherein the method includes the following steps: the herb of *Piper laetispicum* C.DC. is pretreated, then add in organic solvents to maceration or diacolate the herb, after that the crude extract is obtained.

2. The method according to claim 1,wherein the herb of *Piper laetispicum* C.DC. includes the roots, rhizoma, vines, leaves, fruits and whole herbs.

3. The method according to claim 1, wherein the organic solvents used to maceration or diacolate the herb include but not limit to ethanol, methanol, chloroform， ethylacetate

and diethyl aether.

4. The method according to anyone of claims 1-3, further includes the step of purify the crude extract obtained by maceration or diacolation.

5. The method according to claim 4, the methods of purification include but not limit to macropore poly adsorbent column, polyamide column, silica gel column, ion exchange column, ethanoldistill-waterdeposit, aciddistill-alkalideposit and alkalimoist-organic soventextract.

6. Preparation method for the extract of *Piper laetispicum* C.DC., wherein the method includes the following steps: the herb of *Piper laetispicum* C.DC. is pretreated, then treated with supercritical fluid extraction (SFE) to botain the extract.

7. The extract of *Piper laetispicum* C.DC. obtained by the methods according to anyone of claims 1-6.

8. The use of the extract of *Piper laetispicum* C.DC. according to claim 7, wherein the extract of *Piper laetispicum* C.DC. is used to prepare drugs, health foods or food additives for prevention and/or treatment for metabolism-disorders(pneuma) which is associated with the disturbance of monoamine neurotransmitter.

9. The use according to claim 8, wherein the monoamine neurotransmitter include 5-HT, NA and DA.

10. The use according to claim 8,wherein the mental disorders which is associated with the disorder of monoamine neurotransmitter include but not limit to schizophrenia, mania,mind-impediment, mutism ,organic mentalsyndrome, coerce, depression,anxiety, insomnia,epilepsy, Parkinson syndrome, headache， neurodynia and senile dementia.

11. The use according to claim 8,wherein the dosages of the drugs include but not limit to capsule, troche, granule, powder， dripping-pill， subtle-pill, injection, sterile injection powder, oral-preparation, sustained release or controlled release preparation and target drug.