Ph. D Dissertation

Antinociceptive, antidepressant, anxiolytic and toxicity studies on *Piper laetispicum* C. DC.

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Contents

Section 3

Abstract

Piper laetispicum C. DC. (Piperaceae), popularly known in folk as Xiao Chang-feng, Shan Hu-jiao, Ye Hu-jiao, is an endemic climbing, glabrous plant available in the southern part of China. As a folk medicine, this plant enjoys vast uses for invigorating circulation and reducing stasis, detumescence and analgesic. Besides, the aerial part of *P. laetispicum* (Hei Shagan in Dai dialect) is widely used in Dai Nationality, one of the 55 Ethnic Minorities of China, to treat epigastralgia, abdominal pain. After scanning more than 100 medicinal plants in our group, we focus on *P. laetispicum* because of its potential antidepressant activity. Based on this information and the folk use of *P. laetispicum*, the present study was designed to evaluate the antinociceptive, antidepressant and anxiolytic effects, as well as the mechanism of actions. The toxicity was studied preliminarily.

Part 1

The classical animal models were used in the research of pharmacological activities and mechanism of actions. The acetic acid-induced writhing test, formalin test, hot plate test, immersion test and xylene-induced ear edema test were used to verify the putative antinociceptive activity of *P. laetispicum*. The essential oils from different organs, the hydroethanol extract, the fractions and 2 individual compounds – laetispicine and d-sesamin were tested in these animal models. The opioid receptor

antagonist naloxone hydrochloride was used to test if the opioid receptors are involved in the antinociceptive activity of *P. laetispicum*.

Results:

1. The essential oils from stems, leaves, fruits and roots have antinociceptive effects. There exists a quantity-activity relationship between the quantity of sesqui-terpenes and the antinociceptive activity.
2. 95% hydroethanol extract promotes a dose-dependent antinociceptive effect, with a mechanism of action which probably involves the participation of both the peripharel and central system. But the peripheral antinociceptive effect is much better than the central antinociceptive activity.
3. Among the different fractions, petroleum ether fraction and chloroform fraction are observed significant antinociceptive in acetic acid writhing test. When it comes to the anti-inflammatory activity, all the petroleum ether fraction, chloroform fraction and ethyl acetate fraction have good activity.
4. For individual compounds, both laetispicine and d-sesamin have analgesic activity. Laetispicine has a dose-dependent central antinociceptive activity without the involvement of the opioid receptor, but d-sesamin acts mainly peripherally.

Conclusions:

1. The essential oils have good antinociceptive effects, the sesqui-terpenes compounds are the main compounds responsible for the activity. While the extract free from the essential oil, with amids alkaloids and lignant as the main compounds, also show a significant antinociceptive activity. There exists a

synergic analgesic effect between the sesqui-terpenes and amides and/or ligants.

2. Both the total extract and fractions show strong peripheral analgesic activity.
3. Leatispicine and d-sesamin are the two main compounds from the organic fractions of *P. laetispicum* extract. Laetispicine has a dose-dependent central antinociceptive activity without the involvement of the opioid receptor, but d-sesamin acts mainly peripherally. About the antidepressant effect, the study was undertaken to evaluate the influence of hydroethanol extract, the fractions, laetispicine and the derivatives of laetispicine on the duration of immobility in the forced swimming test (FST) and in the tail suspension test (TST). The mechanism underlying the antidepressant action of laetispicine was preliminarily studied.

Results:

1. HEPl at doses of 120, 240 and 480 mg/kg appears to produce a specific antidepressant-like behavioral effect after acute and chronic treatment.
2. The PEF, CHLF and EAF at the given does have a good antidepressant activity in a dose-dependent manner. The antidepressant activity of PEF and CHLF is stronger than EAF. The serotonergic mechanism may not be involved in the antidepressant-like effect of PEF and EAF. However, serotonergic system takes part in, at least partially, the mechanism of action of CHLF in anti-immobility time activity.
3. Laetispicine given orally is effective in producing significant dose-dependent antidepressant-like effects, when assessed in FST and in TST. Our data

demonstrate that the activation of the opioid system and serotonergic mechanism seem unlikely to be involved in the antidepressant-like effect of laetispicine, while the L-arginine-nitric oxide pathway might be partially involved in the antidepressant effect of laetispicine.

4. A series of laetispicine derivatives were synthesized and evaluated as potential antidepressants. After acute administration, ysy-5, ysy-7, ysy-10, ysy-11, ysy-12, ysy-16 show significant activity. After 7 days administration, ysy-5, ysy-7, ysy-10 and ysy-16 have better antidepressant activity without any drug tolerance.

Conclusions:

1. HEPl produces a specific antidepressant-like behavioral effect without drug tolerance after sub-chronic administration.
2. Serotonergic system takes part in the mechanism of action of CHLF in anti-immobility time activity, but not PEF and EAF.
3. The opioid system and serotonergic mechanism seem unlikely to be involved in the antidepressant-like effect of laetispicine, while the L-arginine-nitric oxide pathway might be partially involved in the antidepressant effect of laetispicine.
4. Among the derivatives, ysy-7, ysy-10 and ysy-16 have the potential for further development. And these data give us some information about the structure and activity relationship.
5. The mechanism of actions of extract, fractions, laetispicine and derivatives deserve further research.

A series of experiments are designed to verify the anxiolytic effect of ethyl acetate extract and laetispicine in the light/dark test, elevated plus maze test and hole-board test. In addition, the mechanism of action of the extract and laetispicine were preliminary studied using flumazenil, an antagonist of BZD receptor.

Results:

1. EAF and laetispicine have anciolytic activities.
2. EAF at 240 mg/kg has an obvious anxiolytic activity.
3. At the given doses, laetispicine at 10 mg/kg has a anxiolytic activity, while no significant activity was observed in laetispicine at the doses of 5 and 20 mg/kg in three animal models.
4. Flumazenil didn't block the anxiolytic effect of EAF and laetispicin.

Conclusions:

1. The anxiolytic activity of AEF is weak.
2. The dose response curve of laetispicine in the three animal models is bell-shaped, which has a relationship with its mechanism of action.
3. BZD receptor isn't involved in the mechanism of actions of EAF and laetispicine.

Open field test was arranged to avoid the effects on locomotor activity caused by central nervous system stimulants. The results showed that all the active doses in these three pharmacological researches did not significantly change locomotor activity.

Part 2

The acute and chronic oral toxicity effects of EAF extract of *P. laetispicum* stems was designed in mice to get some basic toxicity information.

Results:

1. The values of LD_{50} were 1530.0 and 538.0 mg/kg for oral and intraperitoneal administration of the EAF extract, respectively.
2. After 90 days treatment, there are no significant differences in body weight gain or behavior in mice between the drug-treated groups and the vehicle group, while there is a significant increase in liver weight in 500 mg/kg group.
3. After 90 days administration, the locomotor and memory abilities are not affected.

Conclusions:

1. Toxicity in EAF is weak.
2. Liver might be the target organ in the chronic administration.
3. Chronic administration doesn't affect the locomotor activity and memory ability.

In the present research work, 12 different animal models are used to verify the antinociceptive, antidepressant and anxiolytic effects of *Piper laetispicum* C.DC., an endemic medicinal plant in southern China, as well as the preliminary toxicity

research. The data obtained will be the basic scientific information for the further research and development of the fractions extracts, laetispicine and derivatives of laetispicine.

Key words: *Piper laetispicum*; antinociceptive; antidepressant; anxiolytic; toxicity; behavioral pharmacology

Résumé

Piper laetispicum C. DC (Piperaceae) est une plante connue dans l'usage traditionnel chinioise particulierement au niveau des provinces de Xiao-Chang Feng, Hu Shan-jiao, Ye Hu-jiao. *Piper laetispicum* C. DC (Piperaceae) est une espèce endémique montagneuse depouvue de poils, la plante est plus abondante dans la partie sud de la Chine. En médecine traditionnelle chonoise, cette plante est utilisées pour soigner de nombreuses maladies par exemple l'amélioration de la circulation et la réduction de la stase, la détumescence et contre la douleur. La partie aérienne de *P. laetispicum* (Hei Shagan en dialecte Dai) est largement utilisé chez le Dai, l'une des 55 ethnies minoritaires de la Chine, pour traiter les épigastralgies et les douleurs abdominales.

En effet, plus d'une centaine de plantes médicinales ont été étudiées dans notre laboratoire, notre attentions s'est portées sur le *P. laetispicum* pour ses potentielles propriétés antidépressives.

Sur la base des informations reçues auprès de tradi-praticiens, cette étude s'est portée sur l'évaluation des propriétés antidépresseurs, antinociceptives, les effets anxiolytiques ainsi que le mécanisme d'action et l'évaluation de la toxicité préliminaire.

Partie 1

Les modèles animaux classiques ont été utilisés dans la recherche d'activités pharmacologiques et du mécanisme d'action.

Les tests à l'acide acétique, au formol, à la plaque chauffante et au xylène ont été utilisés pour vérifier l'activité antinociceptive centrale et periphérique putative de *P. laetispicum.*

Les huiles essentielles provenant de différentes parties, l'extrait hydro éthanolique, les fractions ainsi que les deux composés individuels, laetispicine et D-sésamine, ont été testés sur ces modèles animaux.

Le chlorhydrate de naloxone, antagoniste des récepteurs opioïdes a été utilisé pour tester si ces récepteurs etaient impliqués dans l'activité antinociceptive de *P. laetispicum.*

Résultats:

Atravers cette étude il a été constaté que:

1. Les huiles essentielles obtenues à partir des tiges, des feuilles, des fruits et des racines ont des effets antinociceptifs. Il existe une corrélation entre l'intensité de l'activité antinociceptive et la quantité de sesquiterpènes.

2. 95% de l'extrait hydro éthanolique montre un effet dose-dépendant antinociceptif, avec un mécanisme d'action qui implique probablement la participation tant du système périphérique que centrale. Mais l'effet antinociceptif périphérique est bien

mieux que l'activité antinociceptive centrale.

3. Parmi les différentes fractions, la fraction d'éther de pétrole et la fraction du chloroforme ont montré une activité antinociceptive importante à travers le test de l'acide acétique. Pour l'activité anti-inflammatoire, l'ensemble de fractions de l'éther de pétrole, de chloroforme et d'acétate d'éthyle révèlent une bonne activité.

4. Pour les composés individuels; aussi bien la laetispicine que le D-sésamine révèlent une bonne activité analgésique. La laetispicine a une activité nociceptive central dose-dépendante sans l'implication des récepteurs opioïdes, mais le D-sésamine agit principalement en périphérie.

En conclusion:

Cette étude a montré que:

1. Les huiles essentielles et les extraits alcaloïdes ont une bonne activité antinociceptive; cette activité est principalement due aux sesquiterpènes pour les huiles essentielles et aux amides et aux lignans. Il pourrait exister un effet analgésique synergique entre les sesquiterpènes et les amides et / ou les ligants.

2. Il est observé qu'aussi bien les extraits totaux que les fractions, ceux –ci révèlent une forte activité analgésique périphérique.

3. La leatispicine et le d-sésamine sont les deux principaux composés contenues dans les fractions organiques de l'extrait de *P. laetispicum.*

La laetispicine induit une activité antinociceptive centrale dose-dépendante sans l'implication des récepteurs opioïdes, mais D-sésamine agit principalement en périphérie.

A propos de l'effet antidépresseur, l'étude a été entreprise pour évaluer l'influence de l'extrait hydro éthanolique, des différentes fractions, de la laetispicine et des dérivés de laetispicine sur la durée d'immobilité dans le test de la nage forcée (FST) et dans le test de suspension par la queue (TST). Le mécanisme qui sous-entend l'action préliminaire antidépressive de la laetispicine a été aussi évalué.

Résultats:

1. Le HEPL, à des doses de 120, 240 et 480 mg / kg semble produire un effet comportemental antidépresseur spécifique après le traitement aigu et chronique.

2. Le DEP, CHLF et EAF ont montré une bonne activité antidépressive dose-dépendante. L'activité antidépressive du DEP et CHLF est plus fort que celle de l'EAF. Le mécanisme sérotonergique n'est pas impliqué dans l'activité antidépressive comme l'effet du DEP et de l'EAF. Cependant, le système sérotonergique est impliqué directement ou au moins partiellement au niveau du mécanisme d'action du CHLF dans le test de activité anti-immobilité.

3. La laetispicine donnée par voie orale est efficace pour produire une dose-dépendante, comme un effet antidépresseur, lorsqu'elle est évalué dans la TVF et la TST.

Nos résultats montrent que l'activation du système opioïde et du mécanisme sérotonergique semble peu protable dans l'effet antidépresseur de la laetispicine, tandis que la voie du L-arginine-oxyde nitrique pourrait être partiellement impliquée dans l'effet antidépresseur de la laetispicine.

4. Une série de dérivés de laetispicine ont été synthétisé et évalués comme antidépresseurs potentiels. Après l'administration aiguë, les dérivées suivant YSY-5, YSY-7, YSY-10, YSY-11, YSY-12, YSY-16 ont montré une activité significative et après 7 jours d'administration, les dérivées YSY-5, YSY-7, YSY et YSY-10-16 ont montrée un meilleur effet antidépresseur sans aucune tolérance aux médicaments.

Conclusions:

1. Après l'administration chronique, le HEPL produit un effet antidépresseur qui ressemble produire un changement trouble du comportement sans tolérance aux médicaments.

2. Le système sérotonergique prend part au mécanisme d'action de CHLF dans une activité le test anti-immobilité, mais pas sur le PEF et l'AEP.

3. Le système opioïde et le mécanisme sérotonergique semblent peu susceptibles d'être impliqués dans l'effet antidépresseur de laetispicine, tandis que la voie du L-arginine-oxyde nitrique pourrait y être partiellement.

4. Parmi les dérivés, seuls les YSY-7, YSY-10 et YSY-16 ont le potentiel de développement de l'effet antidépresseur. Ces données nous donnent quelques informations sur la structure et les relations de cette activité.

5. Le mécanisme d'action des extraits, des fractions, de la laetispicine et ses dérivés

méritent des recherches supplémentaires.

Une série d'expériences a été réalisé pour vérifier l'effet anxiolytique de laetispicine extraite par l'acétate d'éthyle notamment, le test de la chamber Claire/obscure, le test du labyrinthe et de la planche à trou. Aussi, les mécanismes d'actions préliminaires de l'extrait et de la laetispicine ont été étudiés à l'aide du flumazénil, un antagoniste du récepteur BZD.

Résultats:

1. Le EAF et le laetispicine ont des activités anxiolytiques,

2. Le EAF à 240 mg / kg a une activité anxiolytique évidente,

3. Aux doses données, la laetispicine à 10 mg / kg a une activité anxiolytique, même si aucune activité significative n'a été observée pour la laetispicine aux doses de 5 et 20 mg / kg en trois modèles d'animaux,

4. Le Flumazénil ne bloque pas l'effet anxiolytique du l'AEF et du laetispicin,

En conclusion dans cette etude nous constatons que:

1. L'activité anxiolytique d'AEF est faible,

2. La courbe dose-réponse de laetispicine dans les trois modèles animaux est en forme de cloche, ceci pourrait avoir une relation avec son mécanisme d'action,

3. Le récepteur BZD n'est pas impliquée dans le mécanisme d'action de l'AEF et du laetispicine. Un certain nombre d'essais ont été réalisé dans un milieu ambiant afin d'éviter les effets sur l'activité locomotrice provoquée par des stimulants du système nerveux central.

Les résultats ont montré que toutes ces doses actives dans ces trois recherches pharmacologiques n'ont guère changé significativement l'activité locomotrice.

Partie 2

Afin d'obtenir quelques informations de base sur la toxicité aiguë et chronique, de l'EAF extraits de *P laetispicum* de tiges, différentes doses ont été administrés aux souris par voie orale. Dans cette parties, les resultants trouvées montrent que:

1. Les valeurs de la DL_{50} sont 1530,0 et 538,0 mg / kg ont été obtenue après administration de l'extrait de l'AEP par voie orale et intra péritonéale, respectivement,

2. Après un traitement de 90 jours, il n'ya aucune différence significative au niveau du poids corporel chez la souris ou le comportement de la drogue entre les groupes traités et le groupe non traité, alors qu'il existe une augmentation significative du poids du foie chez les souris traitées à environ 500 mg / kg et par groupe,

3. Après administration de 90 jours, la locomotion et les capacités de la mémoire n'ont nullement été affectées,

Nous pouvons ainsi conclure que;

1. La toxicité du l'EAF est faible,

2. Par contre le Foie pourrait être l'organe cible de l'administration chronique,

3. Mais l'administration chronique n'affecte ni l'activité locomotrice ni la capacité de la mémoire,

Dans ces travaux, 12 différents modèles d'animaux sont utilisés pour vérifier l'activité antidépresseur, antinociceptive et les effets anxiolytiques de *Piper laetispicum* C.DC une plante endémique du sud de la Chine. Les résultats obtenus tant sur les effets anxiolytiques, antinociceptive, antidépresseur que sur la toxicité de différentes fractions, de la laestispicine et ses dérivées serviront de bases de données scientifiques pour leurs recherches et leurs développements.

Mots-clés: *Piper laetispicum*; antinociceptive, antidepresseur; anxiolytique; toxicité; comportement pharmacologique

Preface

The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main sources of drugs. Till now, according to the World Health Organization (WHO), about 65%～80% of the world's population in developing countries, due to the poverty and lack of access to modern medicine, depend essentially on plants for their primary health care. The Industrial Revolution and the development of organic chemistry resulted in a preference for synthetic products for pharmacological treatment. However, even if we only consider the impact of the discovery of penicillin, on the development of anti-infection therapy, the importance of natural products is clearly enormous. About 25% of the drugs prescribed worldwide, 121 such active compounds being in current use, come from plants. Of the 252 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors. It is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin. The vast majority of these drugs cannot yet be synthesized economically and are still obtained from wild or cultivated plants. In addition, compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicines and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies.

China has a vast territory, with the third richest species of 24500 spermatophyte, belonging to 253 families and 3184 genus. China is also known as a herbal kingdom with the most diversity medicinal plants of 11118 species, from 385 families and 2312 genus. Among them, more than 90% is spermatophyte. Obviously, spermatophyte is the main resources of medicinal plants in China. It is well known that the development and utilization of chinese herbal medicine has a long history, from legend of ShenNong tasting hundreds of herbs to the Eastern Han Dynasty famous pharmacology work ShenNongBenCaoJing, from the Ming Dynasty's BenCaoGangMu to modern ZhongHuaBenCao, vast literature of herbal medicines not only reflects the use of medicinal plant resources of people experiences, but also provides good references for the researchers. Compared with the synthesized medicine, herbal medicines have an advantage in the treatment of common diseases, chronic diseases, senile diseases, various incurable diseases, and persistent disease.

Pain transmission is a mechanism that involves a very complex interaction of peripheral and central structures from the skin surface to the central cerebral cortex. In accordance with the International Association for the Study of Pain, pain has been defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.

Pain is also one of the major reasons patients seek medical care. According to the survey, more than half of the patients go to hospital because of some kind of pain, and more than one third of the population suffers from persistent or recurrent pain in the world.

There are several pain types, namely 'nociceptive', 'neuro-genic', 'neuropathic' and 'psychogenic', which are associated with a stimulation of nociceptors, damage to neuronal tissue, dysfunction of a nerve, or psychological factors, respectively. When it comes to analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs) play a prominent role in the treatment of many pain states and opioids are still our most powerful analgesics up to now, while present analgesics have several serious adverse effects, for instance, NSAIDs can cause gastric injury and ulceration, renal damage and bronchospasm, and opioids are associated with nausea and vomiting.

Our understanding of the pain processes has progressed dramatically in recent years, in great part due to clarification of the mechanisms underlying the afferent fibre physiology and synaptic processing in the dorsal horn of the spinal cord. Many of these molecules and pathways are attractive therapeutic targets for the treatment of pain. Notwithstanding these advances, the mechanisms of pain continue to be incompletely understood. Therefore, a need arises for the development of newer analgesics from natural sources with more powerful activity and with fewer side effects as substitutes for chemical therapeutics.

Depression is a common mental disorder that presents with depressed mood, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, low energy, and poor concentration. These problems can become chronic or recurrent and lead to substantial impairments in an individual's ability to take care of his or her everyday responsibilities. At its worst, depression can lead to suicide, a tragic fatality associated with the loss of about 850 000 thousand lives every year. Depression is the

leading cause of disability as measured by YLDs and the 4th leading contributor to the global burden of disease (DALYs) in 2000. By the year 2020, depression is projected to reach 2nd place of the ranking of DALYs calcuated for all ages, both sexes. Today, depression is already the 2nd cause of DALYs in the age category 15～44 years for both sexes combined.

Since the 1960s, depression has been diagnosed as "major depression" based on symptomatic criteria set forth in the Diagnostic and Statistical Manual (DSMIV, 2000). Milder cases are classified as "dysthymia," although there is no clear distinction between the two. Epidemiologic studies show that roughly 40%～50% of the risk for depression is genetic. This makes depression a highly heritable disorder, at least as heritable as several common complex medical conditions (type II diabetes, hypertension, asthma, certain cancers), which are often thought of as genetic. In addition, vulnerability to depression is only partly genetic, with nongenetic factors also being important. Nongenetic factors as diverse as stress and emotional trauma, viral infections (e.g., Borna virus), and even stochastic (or random) processes during brain development have been implicated in the etiology of depression.

In contrast to our limited understanding of depression, there are many effective treatments. 50 years ago, two classes of agents were discovered—entirely by serendipity—to be effective antidepressants: the tricyclic antidepressants and the monoamine oxidase inhibitors. The discovery that depression can be treated with these medications provided one of the first clues into the types of chemical changes in the brain that regulate depressive symptoms.

Indeed, much depression research over the last half-century was based on the notion that understanding how these treatments work would reveal new insight into the causes of depression. However, with the numerous second generation medications, today's treatment remain sub-optimal, and only about 50% of all patients demonstrate complete remission, although many more (up to 80%) show partial responses. Therefore, research for new antidepressants with greater effectiveness without any (or with lower) adverse effects is still desirable.

Anxiety is defined as a psychological and physiological condition that includes various components (behavioral, cognitive, emotional and genetic/somatic), which merge and result in feelings such as nervousness, panic and discomfort. Anxiety is one of the most common mental disorders affecting mankind. Its prevalence is increasing in recent years due to the rather tense lifestyle imposed on man by the competitive and inhumane atmosphere pervading everyday life. It is estimated that at any given time, around 13% of the population will suffer from an anxiety disorder of one kind or another.

There are six principal categories of anxiety according to the DSMIV: specific and social phobias, panic disorder, generalized anxiety disorder, obsessive-compulsive disorder, post-traumatic stress disorder and acute stress disorder. These disorders fill people's lives with overwhelming anxiety and fear. Unlike the relatively mild, brief anxiety caused by a stressful event such as a business presentation or a first date, anxiety disorders are chronic, relentless, and can grow progressively worse if not treated.

Anxiolytic substances, mostly belonging to the benzodiazepine group, occupy a prominent post in the ranking of the most utilized drugs by man to minimize stress, tension and anxiety. However, the anxiolytic drugs have an unfavorable risk/benefit ratio, as they produce anterograde amnesia, dependence, abstinence syndrome, paradoxical reaction in humans and decay of psychomotor functions. These symptoms can lead to an increased possibility of car accidents and of fractures. As at present the etiologic factors responsible for anxiety and tension are not expected to decrease; there is a need for new anxiolytic drugs with less potential to induce adverse reactions.

It seems that pain, depression and anxiety are three different diseases, while a growing body of literature has focused on the co-morbidity between pain and depression, between depression and anxiety or among the three. Although the mechanisms of co-morbidity is not fully understood, the new drug research based on the mechanisms of co-morbidity maybe a shortcut for more effective and fewer side effects agents. This is also the main reason for us to study the activities of *P. laetispicum* on three diseases.

The purpose of this study is to find more effective and newer natural drugs or precursors as analgesic, antidepressant or anxiolytic agents from Chinese ethno-medicinal plants. After a scanning program of more than 100 plants for the potential antidepressant material, *Piper laetispicum* became the focus of attention.

In addition, because of a dramatic rise in use of ecological medicine approaches to health all over the world, especially for chronic illness or for the disease prevention,

more and more medicinal and food plants have been reported to have diversity activities in recent years, while there is limited evidence about the assessment of the quality, safety and efficacy of the food and medicinal plants materials. Tremendous amount work is still needed to evaluate the toxicity of the medicinal plant with the good pharmacological activities.

We hope that the results in this research work can provide some basis for the future research and development of *P. laetispicum* and relative franctions, individual compounds or the derivatives as new drugs or natural precursors for pain and psychiatric diseases.

1.0. Chapter 1. The introduction of the phytochemistry, pharmacological effects of Piper genus

The genus *Piper,* belonging to Piperaceae, is the biggest family in the Piperaceae and has over 2000 species widely distributed in the tropical and subtropical regions of the world. They are erect or scandent herbs, shrubs or infrequently trees. There exist more than 60 species in China, mainly distributed in Guangdong, Hainan, Yunnan and Taiwan province.

The *Piper* species have high commercial, economical and medicinal importance. Economically the Piperaceae is important for the pepper in the worldwide spice markets. The ripened fruit of *P. nigrum* is the source of white pepper, while the unripe fruit of the same species is the source of black pepper. A narcotic beverage is produced in Oceania from the roots of *P. methysticum.* Several species of *Piper* are grown domestically as house plants for their foliage.

Piper species, widely distributed in the tropical and subtropical regions of the world are used medicinally in various manners. Plants belonging to the genus *Piper* are reputed in the Indian Ayurvedic system of medicine for their medicinal properties and in folklore medicine of Latin America and West Indies.

1. The phytochemistry of *Piper* genus

The chemistry of *Piper* species has been widely investigated and the phytochemical investigations from all parts of the World have led to the isolation of a number of physiologically active compounds, viz. alkaloids/amides, lignans, neolignans,

propenylphenols, terpenes, steroids, kawapyrones, piperolides, chalcones, dihydrochalcones, flavones and flavanones. Amide alkaloids, lignans and neolignans are characteristic metabolites of the *Piper* genus.

1.1. Alkaloids/amides

Amide alkaloids are characteristic constituents of *Piper* genus, which can be classified as isobutyl, pirrolydine, pirydonil and piperydines (Table 1).

1.2. Lignans and neolignans

Lignans and neolignans constitute a large fraction of the bioactive compounds.

1.2. 1.Lignans

In recent years, *Piper* species have received considerable attention because of their reputation for producing lignans with PAF antagonist activity. The lignans isolated from different *Piper* species have been included in Table 2.

1. 2. 2. Neolignans

The neolignans isolated from different *Piper* species have been included in Table 3.

1.3. The pharmacological activities of *Piper* genus

1.3.1. Antiplatelet activities

(+)-Kavain, a 4-methoxy-α-pyrone prepared from *Piper methysticum* Forst. Exerts

Table 1: Alkaloids/ amides in *Piper* species

Compounds	Plant(s)
Aduncamide	1
Alatamide	2
Aristolactam A II	3,4,5,6
Auranamide	7
Aurantiamide	7
Aurantiamide acetate	7,8
Aurantiamide benzoate	7
(+)-N-Benzoylphenylalanine	7
Brachyamide A	8
Brachyamide B	8,9
Brachystamide A	8
Brachystamide B	8
Brachystine	8
Cepharadione A	3,10,11,12,13,4,5,6,14,15,16,17,18
Cepharadione B	19,11,3,12,4,5,6,17,18
Cepharanone B	11,3,4,20,6,18
3-Chloro-l-hydroxy-2-piperidone	21
N-Cinnamoylpyrrole	11
N-Cinnamoylpyrrolidine	11,15,22,18

N-p_Coumaroyltyramine	11
Cyclobutane-2-(1,3-benzodioxol-5-methoxy-6-y1)-4-(1,3-benzodioxol-6-yl)-1,3- dicarboxapyrrolidide	23
Cyclobutane-2,4-bis (1,3-benzodioxol-5-methoxy-6-yl)-1,3-dicarboxapyrrolidide	23
Cyclopiperstachine	24
Cyclostachine A	24
Cyclostachine B	24
2,4Decadienoylpiperidine	0
Dehydropipemonaline	6
Dihydropipericide	9
$\triangle^{\alpha,\beta}$-Dihydropiperine	25,26
4,5Dihydropiperlonguminine	27,25,9,28
8,9-Dihydropiplartine	29,30
$\triangle^{\alpha,\beta}$-Dihydrowisanidine	25
$\triangle^{\alpha,\beta}$-Dihydrowisanine	25
3,4-Dihydroxy-6-(N-ethylamino)benzamide	9
N-(3,4-Dimethoxybenzoyl)isobutyl-amine	27
N-(3'4'-Dimethoxycinnamoyl)-isobutylamine	27
N-(3'4'-Dimethoxycinnamoyl)- $\triangle^{3}$ piperidin-2-one	19
iV-(3'5'-Dimethoxycinnamoyl)-pyrrolidine	27
3-(3'4'-Dimethoxyphenyl)propionamide	31

(2E,4E)-N-Dodecadienoyl pyrrolidine	9
(2E,4E)-N-Eicosadienoyl piperidine	32
(2E,14E)-N-Eicosadienoyl pip&dine	32
2,l I-Eicosadienoylpyrrolidine	27
3,6-Eicosadienoylpyrrolidine	27
Eicosanoylpyrrolidine	27
2-Eicosenoylpyrrolidine	27
3,4-Epoxy-8,9-dihydropiplartin	33
N-cis-Feruloyltyramine	11
N-trans-Feruloyltyramine	11,9
Filfiline	34
(E)- and (Z)-Formouragines	11
(E)- and (Z)-N-Formylnornuciferines	11
N-Formylpiperidine	9
Futoamide	35,21
Guineensine	3,8,25,21,6,9,34,32,36
3,6-Hexadecadienoylpyrrolidine	27
Hexadecanoylpyrrolidine	27
2-Hexadecenoylpyrrolidine	27
N-(4-Hydroxyphenyl) ethylene cinnamamide	37

(2E,4E)-N-5-[(4-Hydroxyphenyl)-pentadienoyl]pipedine	9
2-Hydroxy-l-methoxy-4H-dibenzo-(de,g)quinoline-4,5-(6H)-dione	3,4,6
4-Hydroxy-3-methoxy-N-methylpiperolactam	38
(2E,4E)-N-Isobutyldecadienamide	3,0,25,21,6,38,9,26,16,23,43,8,18
(2E,4E)-N-Isobutyldodecadienamide	25,23
(2E,4E)-N-Isobutyleicosadienamide	25,6,39,9
(2E,4E,82)-N-Isobutyl-eicosatrienamide	6,9,34
(2E,4E,14E)-N-Isobutyleicosatrienamide	32
(2E,4E)-N-Isobutylhexadecadienamide	25
(2E,4E)-N-Isobutyl-7-(3,4-methylenedioxyphenyl)heptadienamide	40,41
(2E,4E)-N-Isobutyloctadecadienamide	11,25,6
(2E,4E,l2&V-Isobutyloctadecatrienamide	32
(2E,4E)-N-Isobutyloctadienamide	42,9,26
Longamide	6
I-(o-Methoxycinnamoyl)pyrrolidine	15
6-Methoxypiperoylisobutylamine	27
3'4'-Methylenedioxycinnamoylisopentylamine	27
3'4'-Methylenedioxycinnamoyl-n-pentylamine	27
N-3'4'-Methylenedioxycinnamoylpiperidine	26
3'4'-Methylenedioxycinnamoylpyrrolidine	27

17-(3,4-Methylenedioxyphenyl)-16-heptadecenoylpyrrolidine	27
(2E,8E-N-9-(3,4-Methylenedioxyphenyl)nonadienoylpiperidine	6,9,32
15-(3,4-Methylenedioxyphenyl)-14-pentadecenoylpyrrolidine	27
(2E,4E,8E)-N-9-(3,4-Methylenedioxyphenyl)nonatrienoylpyrrolidine	9
(2E,4E,lOE)-N-l l-(3,4-Methylenedioxyphenylhmdecatrienoylpiperidine	9
(2E)-4-[(2-Methylpropyl)amino]-4-oxo-butenoic	21
Norcepharadione B	3,4,5,6
I-(2E,4E)-Octadecadienoylpiperidine	32
3,6-Octadecadienoylpyrrolidine	27
Octadecanoylpyrrolidine	27
I-(2E,4E, 12E)-Octadecatrienoylpiperidine	32
2-Octadecenoylpyrrolidine	27
9-Octadecenoylpyrrolidine	27
Peepuloidin	23
N-(3-Phenylpropanoyl)pyrrole	43
Piperadione	3,5,6
Piperamide	9
Piperamine	0,9
Pipercallosidine	44
Pipercallosine	44,45

Pipereicosahdine	**32**
Piperettine	**7,9**

Table 2: Lignans in *Piper* species

Compounds	Plant(s)
Andamanicin	46
(+)-Asarinine	8,6,46
Aschantin	47,25
(2R,3R)-[2-(1,3-Benzodioxol-5-yl)thyI-3-(3,4,5-trimethoxyphenylmethyl]-butan-1,4-diol	48
(+)-Calopiptin	22,18
(-)-Clusin	47,48
(-)-Cubebin	47,48,49,50,9,38,24
(-)-Cubebinin	47,48
(-)-Cubebininolide	47,48
(-)-Cubebinone	47
(+)-Diaeudesmin	6,23,8
(+)-Diayangambin	19
(-)-Dihydroclusin	47
(-)-Dihydroeubebin	47,48,23,24
(-)-Dihydrotrichostin	24
(-)-3,4-Dimethoxy-3,4-desmethylenedioxycubebin	9
(-)-3'4'-Dimethoxy-3',4'-desmethylenedioxycubebin	9
rel-(7S,8S,7'S,8'S)-3',4'-Dimethoxy-3,4-methylen~ioxy-7.0.7',8.8'-lignan	18
(2S,3R,4S,5S)-Diveratryldimethyltetrahydrofuran	51
(+)-epi-Excelsin	19

(2S,3R,4R)-[2-Ethoxy-3-(3,4,5-trimethoxyphenyl)methyl-4-(1,3-benzodioxol-5yl)methyl]tetrahydrofurano1(194)	48
a-O-Ethylcubebin	47,48
β-O-Ethylcubebin	47
Fargesin	40,8,6
Galgravin	35,21,52,53
(+)-Galbelgin	3,35,54,18
(+)-Grandisin	55
Hemiariensin	47
Heterotropan	47
(-)-Hinokinin	47,48,38,24
(-)-Jsoyatein	47
Kusunokinin	47
(-)-Machilin G	22,18
Magnosalin	47
(2R,3R)[2-(7-Methoxy-l,3-benzodioxol-5-yl)methyl-3-(3,4,5-trimethoxyphenyl)methyl]butan-1,4-diol	48
(-)-5"-Methoxyhinokinin	47,24
(2R,3R)-2-(3",4"-Methylenedioxybenzyl)-3-(3',4'-dimethoxybenzyl)butyrolactone	47
Pluviatilol	8,6
Sesamin	40,8,48,47,23,8,32
(+)-Sylvatesmin	8
(+)-Sylvone	8
(-)-Thujaplicatin trimethylether	47
(-)-Trichostin	24
(+)-Veraguensin	57,35,52
(+)-Yangambin	23
(-))-Yatein	48,47

(-)-Zuonin	22

Table 3: Neolignans in *Piper* species

Compound	Plant(s)
(7R,8R,3'R)-7-Acetoxy-3'4'-dimethoxy-3,4-methylenedioxy-6'-oxo-$\triangle^{1',4',8'}$- 8.3'lignan	18
(7R,8R,3'R)-7-Acetoxy-3,3',4,4'-dimethoxy-3,4-methylenedioxy-6'-oxo-$\triangle^{1',4',8'}$- 8.3'lignan	18
2'-Acetoxy-4'-oxo-3,4,5'-trimethoxy-$\triangle^{5',8'}$8.3',7.4'-lignan	58
4-[2-(l,3-Benzodioxol-5-yl)-2-hydroxy-l-methylethyl]-2,5-dimethoxy-2-(2-propenyl)-3,5cyclohexadiene-l-one	11
rel-(7S,8S)-A8?3,4,3?4?Bis-methylene-dioxy-7.O.2?8.3?lignan (225)	59
(+)-Burchellin	52,18
Clarkinol	48
Conocarpan	60
Cuneifolin	57
Denudatin B	48,21,53
$\triangle^{8'}$-l'2'Dihydro-3,4,3'4'bis-methylenedioxy-2'-oxo-8.l'lignan	59
iso-$\triangle^{8'}$-l'2'Dihydro-3,4,3'4'bis-methylenedioxy-2'-oxo-8.l'lignan	59
1'2'-Dimethoxy-3,4-methylenedioxy-4'-oxo- $A^{2'5'8'}$-8.5' lignan	18
4'5'Dimethoxy-3, 4-methylenedioxy-2'-oxo-$A^{3'5'8'}$-8. 1-lignan	11,18
Iso-4'5'Dimethoxy-3, 4-methylenedioxy-2'-oxo-$A^{3'5'8'}$-8. 1-lignan	18
Eupomatene	45
Eupomatenoid-5	60
Eupomatenoid-6	60
Eupomatenoid-7	38
Fargesone A	18
Fargesone B	18

Futoenone	35
Futoquinol/Hancinone D	11,35,22,21,18
Hancinol	21
Hancinone	21,18
Hancinone B	21
Hancinone C	21,55,53
Hookerinone A	61
Isodihydrofutoquinol A	35, 61,22,18
Isodihydrofutoquinol B	4,35,5,61,22,18
Isofutoquinol A	35
lsofutoquinol B	11,35
Kadsurenin B	58
Kadsurenin C	58
Kadsurenin I	58
Kadsurenin J	58
Kadsurenin K	58
Kadsurenin L	58
(+)-Kadsurenone	35,21,53
Kadsurin A	11,3,4,47,35,5,22,18
Kadsurin B	11,5,35,22,18
Lancifohn C	11,22,18
(+)-Lancifolin D	11,55,22,18

0 P. chaba 1 P. aduncum 2 P. guayranum 3 P. attenuatum 4 P. boehimerifolium 5 P. hamiltoni 6 P. longum 7 P. aurantiacum 8 P. brachystachyum 9 P. nigrum 10 P. brachystachyum 11 P. argyrophylum 12 P. auritum 13 P. betle 14 P. manuausense 15 P. methysticum 16 P. pedicellosum 17 P. sanctum 18 P. wightii 19 P. aborescens 20 P. chiadoense 21 P. hancei 22 P. schmidlii 23 P. peepuloides 24 P. trichostachyon 25 P. guineense 26 P. novae hollandiae 27 P. amalago 28 P. tuberculatum 29 P. bartlingianum 30 P. rugosum 31 P. arboricola 32 P. retrofractum 33 P. verrucosum 34 P. officinarum 35 P. futokadrura 36 P. sylvaticum 37 P. steerni 38 P. ribesoides 39 P. macropodum 40 P. austrosinense 41 P. falconeri 42 P. banksii 43 P. sarmentosum 44 P. callosum 45 P. interruptum 46 P. sumatranum 47 P. cubeba 48 P. clusii 49 P. cuneifolium 50 P. Iacunosum 51 P. clarkii 52 P. puberculum 53 P. wallichii 54 P. thomsoni 55 P. polysyphorum 56 P. longum 57 P. cuneifolium 58 P. kadsura 59 P. capense 60 P. decurrens 61 P. hookeri

Its assume antithrombotic action on human platelets which was deduced from its ability to suppress arachidonic acid (AA)-induced aggregation, exocytosis of ATP, and inhibition of cyclooxygenase (COX) and thromboxane synthase (TXS) activity (Johannes et al., 1997).

Piperbetol, methylpiperbetol, piperol A, and piperol B, isolated from *Piper betle*, selectively inhibited the washed rabbit platelet aggregation induced by platelet activating factor (PAF) in a concentration-dependent manner. The inhibitory potency of ginkgolide B was about 2.8, 1.2, 22.8, and 1.4 times higher than those of piperbetol, methylpiperbetol, piperol A, and piperol B. However, the aggregation of washed rabbit platelets induced by threshold ADP and arachidonic acid were unaffected by piperbetol, methylpiperbetol, piperol A, and piperol B. Furthermore, piperbetol,

methylpiperbetol, piperol A, and piperol B had no effects on the cAMP contents in rest washed rabbit platelets (Hua et al., 1997).

1.3.2. Anti-inflammatory activities

The experiments in vitro showed plants of *Piper* genus have inhibition effects on main enzymes of inflammatory processes. *P. kadsura* inhibited COX-1 with the same IC_{50} as aspirin (IC= 251μg/ml). The inhibition for PLA2, 12-LO and COX-2 were 147μg/ml, 85μg/ml and 631μg/ml, respectively (Rachel et al., 2003). While the crude extract *Piper sormentosum* was found to possess cyclooxygenase-1 (COX-1) and 5-lipoxygenase (5-LO) inhibitory activity (COX-1 IC_{50} = 19 μg/ml; 5-LO IC_{50} = 10 μg/ml) (Stohr et al., 1999). Inhibition effects of piperlactam S isolated from *Piper kadsura* on phytohemagglutinin (PHA) stimulated cell proliferation. The inhibitory action of piperlactam S was not through direct cytotoxicity. Cell cycle analysis indicated that piperlactam S arrested the cell cycle progression of activated T cells from the G transition to the S phase. Thus, the suppressant effects of piperlactam S on proliferation of T cells activated by PHA seemed to be mediated, at least in part, through inhibition of early transcripts of T cells, especially those of important cytokines, IL-2, IL-4, and blocking c-Fos protein synthesis (Kuo et al., 2002). The *n*-hexane extracts of 19 *Piper* species, predominantly from China, were screened for their 5-lipoxygenase (5-LOX) and cyclooxygenase-1 (COX-1) inhibitory potential. Many of them showed considerable inhibitory activity against at least one of these two key enzymes of the arachidonic acid metabolism, especially against COX-1

(Jochen et al., 2001). Hashimoto et al isolated 5, 6-Dehydrokawain (desmethoxyyangonin) and yangonin kawa *(Piper methysticum)*, which can significantly inhibited TNF-a release with IC_{50} values of 17 μM and 40 μM (Hashimoto et al., 2003).

1.3.3. Antinociceptive and sedative activities

Piperovatine, a sialogogic, piscicidal, and buccal local anesthesia producing isobutyl amide from the amazonian piscicidal and toothache-relieving plant, *Piper piscatorum* Trelease et Yuncker, was evaluated for its ability to induce changes in neuronal intracellular calcium concentration (McFerren et al., 2002). Ethanol extract of *P. nigrum* showed a significant anticonvulsant, sedative and antinociceptive effects at the dose of 2 g/kg (i.p.) (Hu et al., 1996). The extract of *P. methysticum* also has the similar effects, but the mechanism of actions might be affecting the dopamine level in rat brain (Baum et al., 1998).

1.3.4. Anti-infective activities

For many years, plants from *Piper* genus have been focused on anti-infective role. A variety of chemical constituents has found have anti-bacterial, antifungal and anti-viral activities. Generally, alkaloids and flavonoids are responsible for these activities. *N*-[7- (3', 4'-methylenedioxyphenyl)-2 (*Z*), 4 (*Z*)-heptadienoyl] pyrrolidine from leaves of *Piper hispidum* (Alberto et al., 1998), 5 amides from *Piper arboretum*, and 8 compounds from *Piper tuberculatum* (Renata et al., 2002), and flavonoids and

bioflavonoids coming from *Piper crassinervium* (Danelutte et al., 2003) showed antifungal activity against *Cladosporium sphaerospermum*. Pepper is known to be antibacterial. Two new phenolic compounds reported to be present in green pepper but absent in black, were tested for their antibacterial activity against the food borne pathogens, Salmonella typhimurium, Staphyloccus aureus, Bacillus cereus and *Escherichia coli*. The compounds 3,4-dihydroxyphenyl ethanol glucoside (A) and 3,4-dihydroxy-6-(N-ethylamino) benzamide (B) were found to inhibit the growth of all of the four bacteria tested (Pradhan et al., 1999).The ethanol extract of leaves of *Piper porphyrophyllum* N.E. Br. showed a broad spectrum of antibacterial activity (Wiart et al., 2004). 5 compounds with the antibacterial activity have been isolated from *Piper aduncum* (Orjala et al., 1993). Bioactivity-guided fractionation of a methanol extract from the leaves of *Piper lanceaefolium* resulted in the isolation of four new benzoic acid derivatives, together with taboganic acid, pinocembrin, and pinocembrin chalcone. Lanceaefolic acid methyl ester and pinocembrin chalcone displayed activity against *Candida albicans* with a minimal inhibitory concentration value of 100µg/ mL in both cases (Lopez et al., 2002). The ethyl acetate extract presented a good activity against *S. aureus* and *B. subtilis* with MIC and MBC at 15.62 µg/ ml (Pessini et al., 2003). *Piper aduncum* was found active on Poliovirus (Lohezic et al., 2005).

1.3.5. Antioxidant activities

In the structure analysis of the compounds of the genus *Piper*, five phenolic amides from *Piper nigrum*, seven compounds from *P. retrofractum*, and two compounds from *P. baccatum* were found to possess significant anitoxidant activities that are more effective than the naturally occurring antioxidant, a-tocopherol.

One amide, feruperine, has antioxidant activity as high as the synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Nakatani et al., 1986). The aqueous components of inflorescence *Piper betle* are potential ROS scavengers and may prevent the platelet aggregation possibly via scavenging ROS or inhibition of TXB_2 production (Lei et al., 2003).

Piperlactam S concentration-dependently prevented the copper-catalyzed oxidative modification of human low-density lipoproteins (LDL) measured through (i) the lag period, (ii) the slope of the propagation phase, (iii) the total amount of conjugated dienes formed, and (iv) the electrophoretic mobility of LDL. Fe^{2+}-induced oxidative modification of cell membrane was also significantly attenuated by piperlactam S as measured by thiobarbituric acid-reactive substances (TBARS).

Furthermore, piperlactam S effectively minimized the loss of cell viability induced by Fenton's reagent (H_2O_2/ $FeSO_4$) in cultured endothelial cells and significantly reversed H_2O_2/ $FeSO_4$-induced impairment of endothelium-dependent relaxation to acetylcholine in rat aorta.

Since the oxidative modification of LDL plays an important role in the genesis of atherosclerosis, piperlactam S may help to reduce the risk of atherosclerosis, not only by protecting LDL and membrane lipids from oxidative modification but also by reducing free radical-induced endothelial injury and/or dysfunction (Tsai et al., 2003).

1.3.6. Anti-tumor activities

In a study to find melanogenesis inhibitors from natural sources, *Piper longum* L (fruits, Piperaceae) was discovered to have an inhibitory effect on alpha-melanocyte-stimulating hormone (α-MSH)-induced melanogenesis in melanoma B16 cells. Piperlonguminine has been identified as the melanogenesis inhibitor from *P. longum* by activity-guided extraction and isolation (Min et al., 2004). A total of 112 species of edible plants from Thailand were randomly collected, and their methanol extracts were screened for in vitro anti-tumor promoting activity using the inhibition test of Epstein-Barr virus (EBV) activation in Raji cells induced by 12-O-hexadecanoylphorbol-13-acetate (HPA, 40 ng/ml). It was found that 60% of these extracts, including leaves extract of *Piper betle*, inhibited EBV activation by 30% or more at a concentration of 200 mg/ml (Murakami et al., 2000).

1.3.7. Other pharmacological activities

Piper nigrum L. fruit (black pepper) extract was found to possess growth-stimulatory activity towards cultured melanocytes. Its aqueous extract at 0.1 mg/ml was observed to cause nearly 300% stimulation of the growth of a cultured mouse melanocyte line,

melan-a, in 8 days ($p < 0.01$). Piperine (1-piperoylpiperidine), the main alkaloid from Piper nigrum fruit, also significantly stimulated melan-a cell growth. Both *Piper nigrum* extract and piperine induced morphological alterations in melana cells, with more and longer dendrites observed. The augmentation of growth by piperine was effectively inhibited by RO-31-8220, a selective protein kinase C (PKC) inhibitor, suggesting that PKC signalling is involved in its activity (Zhi et al., 1999). Except all the above, the plants of *Piper* genus have other pharmacological activities, such as hypoglycemic, reducing blood lipid, anti-fatigue, ultraviolet protection.

2. Chapter 2. The introduction of *Piper laetispicum* C.DC.

2.1. Introduction of *Piper laetispicum*

2.2. Classification

Table 4 show the the classification of *Piper laetispicum* C.DC as described in the literature.

Table 4: Classification *Piper laetispicum* C.DC.

Kingdom	Plantae
Division	Angiosperma
Class	Dicotyledonae
Order	Piperales
Family	Piperaceae
Genus	*Piper*

2.3. Vernacular names

Several vernacular name are used according to the population and the locality, such as; Shan Hu-jiao, Ye Hu-jiao, Xiao Chang-feng.

In this study, the stem was the part of the plant that was used for the inverstigations.

2.4. Botanical description

Climber's woody, to 10 m tall, dioecious. Stems drying pale brown, 2-3 mm thick, ridged, glabrous. Petiole 2-5 mm on wider side, pubescent; prophyll 2-3 mm; leaf blade oblong to ovate-oblong, rarely elliptic, (9-)12-17 × (2.7-)4-9 cm, leathery, pellucid dotted, abaxially sparsely villous, adaxially glabrous, base obliquely cordate, basal lobes usually overlapping, bilateral difference 4-5 mm, apex shortly acuminate;

veins pinnate, ca. 9 per side, apical pair arising 5-8 cm above base, next pair thickest, usually 1-1.5 cm above base, reaching middle of leaf blade, looped, others conspicuous, ± basal; reticulate veins prominent, Spikes leaf-opposed. Male spikes ca. 10 cm × 4 mm; peduncle 1-1.5 cm, glabrous; rachis pubescent; bracts broadly obovate, ca. 1.3 × 1 mm, peltate, ciliate. Stamens 2; filaments thick, ca. 1.2 mm. Female spikes ca. 10 cm at anthesis, to 15 × 1.5-2.2 cm in fruit; rachis, densely rough pubescent; bracts obovate-oblong, adnate to rachis. ca. 2 × 1.1 mm, margin free, ciliate. Ovary ovoid; stigmas 4, apex acute. Drupe subglobose, ca. 5 mm in diam., base tapered into a stalk ca. as long as fruit. Fl. Aug-Dec.

2.4.1. Geographical distribution

The plant is widely distributed in Guangdong, Guangxi, Yunnan, and Hainan Provinces of Southern China.

2.4.2. Traditional uses

As a folk medicine, this plant enjoys vast uses for invigorating circulation and reducing stasis, detumescence and analgesic (Zhonghua Herbals Editorial Committee, 1999). Besides, the aerial part of *P. laetispicum* (Hei Shagan in Dai dialect) is widely used in Dai Nationality, one of the 55 Ethnic Minorities of China, to treat epigastralgia, abdominal pain (Geng et al., 1999).

2.4.3. Pharmacology

About the pharmacological research of P. laetispicum, the conspicuous inhibition activity against Cox-1 and 5-LOP of *P. laetispicum* had been investigated in vitro (Stöhr et al., 2001). Laetispicine, the first new amide isolated from *P. laetispicum*,

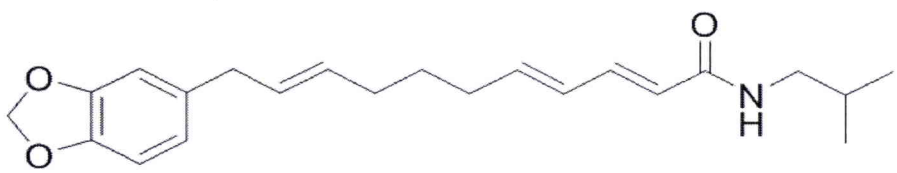
produced antinociceptive and antidepressant activities (Yao et al., 2009).

2.4.4. Phytochemistry

The phytochemistry work was proceeding under the cooperation research between the group of Prof. Shengli, Fudan University and the group of Prof. Fugang Qian, Shanghai Institute of Pharmaceutical Industry. Up to now, we have totally gotten 48 compounds from the ethyl acetate extract of *Piper laetispicum* stems (Fang et al., 2007; Pan et al., 2005). *Piper laetispicum* also contains volatile oil (Dong et al., 2007).

2.4.5. Active principle

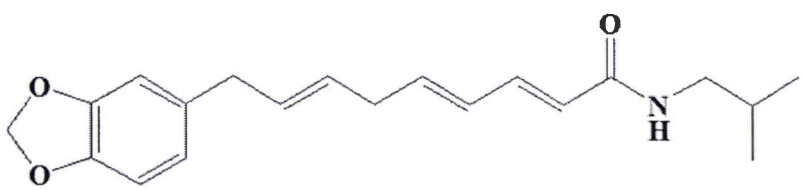
Laetispicine ($C_{22}H_{29}NO_3$)



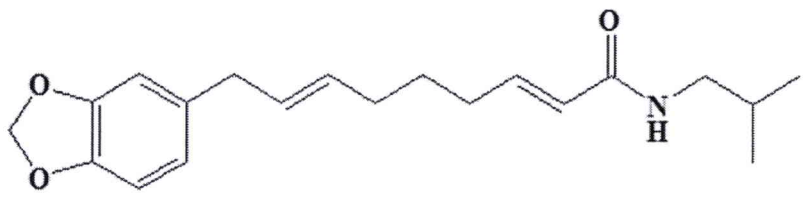
N – isobutyl - (3, 4-methylendioxyphenyl) - 2*E*, 4*E*, 9*E*-undecatrienoamide

2.4.6. Other main compounds

Leatispiamide A



Leatispiamide B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3.0. Chapter 3: The pharmacological research of *Piper laetispicum*

The research papers on pharmacological activities of *Piper laetispicum* C.DC are very few. Only in ZhongHuaBenCao, a simple introduction about the folk uses of *P. laetispicum* was mentioned. There is only 1 paper about antinociceptive and anti-inflammatory activities of *P. laetispicum* in vitro when we began to study the pharmacological activities of *P. laetispicum*. Up to now, all the pharmacological studies in vivo on *P. laetispicine* come from the group of Prof. Shengli Pan and the group of Prof. Rachid Soulimani.

Section 1

3.1. The antinociceptive and anti-inflammatory activities of *Piper laetispicum*

3.1.1. Introduction

Pain, an unpleasant perception of a nociceptive sensation, is one of the most pervasive problems in our society (Verri et al., 2006). It is also one of the major reasons patients seek medical care (Okuse, 2007). Although there is an arsenal of effective and widely used drugs, analgesics are inadequate due to the facts that the signaling mechanisms are still not fully understood (Okuse, 2007), the available analgesic drugs exert a wide range of side effects and the effects of present analgesics are either too potent or too weak (Mattison et al., 1988).

Medicinal herbs are believed to be an important source of new substances with potential therapeutic effects (Farnsworth, 1989; Blumenthal, 2000) due to their wide popular use (Almeida et al., 2001). Thus the search for new pain killers from the plant species should be a logical research strategy (Rang et al., 1998).

Generally, the plants from Piper family have anti-inflammatory and analgesic effects. In 2001, the conspicuous inhibition activity against Cox-1 and 5-LOP of 12 medicinal plants of *Piper* family, including *P. laetispicum*, had been investigated (Stöhr et al., 2001). Besides, the aerial part of *P. laetispicum* (Hei Shagan in Dai dialect) is widely used in Dai Nationality, one of the 55 Ethnic Minorities of China, to treat epigastralgia, abdominal pain (Geng et al., 1999). Considering that there has been only sparse information about *P. laetispicum*, the present studies were designed to investigate the antinociceptive and anti-inflammatory effects of the essential oils from

different organs, hydroethanol extract of stems, franctions of *P. laetispicum* crude extract and 2 individual compounds in several animal models. In addition, we also investigated the synergic action of 2 compounds with peripheral and central analgesic.

3.1.2. Material and methods

3.1.2.1. Plant materials

Piper laetispicum C. DC (Piperaceae), an endemic climbing, glabrous plant available in the southern part of China, was collected in 2006 from Hainan province, China, and was identified by Prof. Sheng-li Pan, School of Pharmacy, Fudan University, where a voucher specimen (No. 060812) of the plant material has been deposited for further reference.

3.1.2.2. Essential oils of *P. laetispicum* from roots, stems, leaves and fruits

Essential oil of roots, stems, leaves and fruits were extracted from the powdered plant materials by steam distillation in a Clevenger-type apparatus for 3 h (Craveiro et al., 1976). The percentage yields based on the dried plant were 0.18、0.19、0.51 和 3.69% (v/w).

3.1.2.3. Hydroethanol extract

The stem of *P. laetispicum* (11kg) was cut into pieces, air-dried, powdered and extracted using hydroethanol (95 %) solvent in percolator at room temperature, up to the exhaustion of the plant material (30−fold). The solvent was removed under vacuum in a rotary evaporator until yielding a dark brown colored semisolid mass (750g, yields 6.8%, w/w).

3.1.3. Fractionation

The crude extract (20g) was suspended in water and partitioned successively with petroleum ether, chloroform and ethyl acetate. Each fraction was evaporated under reduced pressure and the yields of the petroleum ether (PEF), chloroform (CHLF), ethyl acetate (EAF) and aqueous (AQF) fractions were 4 g (13.33%), 4 g (13.33%), 2 g (6.67%) and 20 g (66.67%), respectively.

3.1.4. Laetispicine and d-sesamin

The hydroethanolic extract of the stems was dissolved in water and fractionated with petroleum ether and ethyl acetate, respectively. The ethyl acetate fraction was combined, concentrated under reduced pressure to give a dark brown colored semisolid extract (60 g). Then the ethyl acetate extract was chromatographed on a silica gel column by a gradient elution using mixtures of petroleum ether and ethyl acetate (20:1-9:1-5:1-3:1-2:1-1:1) to afford six fractions. Fraction 2 was concentrated under reduced pressure to give sesamin crystallization (0.6 g). Fraction 3 was concentrated under reduced pressure and subjected to silica gel column chromatography and eluted with chloroform and methonal (50:1), to give four subfractions. Laetispicine (300 mg) was separated from subfraction 3 by repeated chromatography on silica gel column, eluted with petroleum ether and acetone.

The essential oils, crude extract, fractions and individual compounds obtained were kept in refrigerator at +4 ℃ and suspended in a solution of 2% tween80 prior to any pharmacological screening. Respective controls received only that solvent as vehicle.

3.1.5. Drugs

95% ethanol, petroleum ether, chloroform, ethyl acetate, distilled water, tween80, acetic acid, acetylsalicylic acid (ASA), morphine hydrochloride, formaldehyde, xylene and naloxone hydrochloride.

3.1.6. Animals and habituation

KM mice of either sex (18-22 g) were housed in standard environmental conditions. Food and water were available *ad libitum*. The animals were acclimatized to the laboratory for at least 7 days before testing and were used only once throughout the study. All experiments were approved by Animal Ethics Committees, Fudan University, China.

3.1.7. Pharmacological tests

3.1.7. 1. Antinociceptive tests

3.1.7.1.1. Acetic acid-induced writhing test

The method of Koster et al. (1959) was used. Essential oils, hydroethanol extract, fractions and individual compounds were orally administered to mice (n = 10) 1 h before i.p. injection of 0.6% (v/v) acetic acid (AA) at a dose of 10 ml/kg, while 2% tween80 was used as control treatment. The positive control group received 100 mg/ kg of acetylsalicylic acid (ASA). The number of writhes occurring between 5 and 25 min after acetic acid injection was recorded.

3.1.7.1.2. Formalin test

The method used in the present study was similar to that described previously by de Miranda et al. (2001) with slight modifications. Briefly, 60 min after oral administration of essential oils, hydroethanol extract, fractions and individual compounds, morphine hydrochloride (5 mg/kg, s.c.) or 2% Tween 80, 20 µl of formaldehyde 2% (v/v in distilled water) was injected subcutaneously into the plantar surface of the left hind paw of the mice. 5 min prior to this procedure, each mouse was placed in a transparent enclosure to adapt to the testing box and left freely moving and exploring (habituation). The mice were observed for 60 min after the injection of formalin, and the amount of time spent licking and biting the injected paw was recorded. The first 10 min after formalin injection is known as the early phase and the period during the following 50 min as the late phase.

3.1.7.1.3. Hot plate test

Mice were treated according to the method described by Franzotti et al. (2000). The animals were placed on a hot plate at 55±0.5 ℃. Reaction time was recorded when the animals licked their hind-paws at 30, 60, 90, 120 and 150 min after oral administration of essential oils, hydroethanol extract, fractions and individual compounds or 2% tween80. The opioid analgesic morphine chlorhydrate (5mg/kg, s.c.) was used as a positive control for the test. Only mice that showed nociceptive responses within 30 s were used and a cut-off time of 60 s was selected to prevent tissue damage, which was thus considered the maximal latency.

3.1.7.1.4. Tail immersion test

Mice were divided into groups of ten animals each. The lower 3 cm portion of the tail was immersed in a beaker of water maintained at 55 ± 0.5 ℃ (Janssen et al., 1963). The time in seconds for tail withdrawal from the water was taken as the reaction time, with a cut-off time of immersion set at 10 s. The reaction time was measured 1 h after oral administration of essential oils, hydroethanol extract, fractions and individual compounds or 2% tween80. Morphine hydrochloride (5 mg/kg) was administered subcutaneously 60 min before the test.

3.1.7.2. Anti-inflammatory activity

Xylene-induced ear edema test

The anti-inflammatory activity of extract was determined by xylene-induced ear edema test in mice. A previously described procedure was followed (Xu et al., 2005). Briefly each mouse weighing 20 − 22 g was intragastrically given single dose of a test drug or vehicle 1 h before the induction of ear edema by topical application of 0.02 ml xylene on both surfaces of the right ear. The left ear saved as a control. Mice were sacrificed by cervical dislocation 4 h after xylene application. Ear disks of 8.0mm in diameter were punched out and weighed. The extent of edema was evaluated by the weight difference between the right and the left ear disks of the same animal.

3.1.8. Statistical analysis

Data obtained were expressed as mean ± SEM and analyzed by analysis of variance (ANOVA) followed by Bonferroni's test. *P-values* less than 0.05 ($p<0.05$) were used as the significant level.

The percent of inhibition was determined using the following formula:

Inhibition (%) = 100 × [(control − experiment)/control].

3.1.9. Results

3.1.9.1. The antinociceptive effects of the essential oils in acetic acid-induced writhing test

Intraperitoneal injection of acetic acid induced 24.1 ± 2.61 (n = 10) contortions in control mice during the 15 min observation period. For the animals who received essential oil from stems, leaves, fruits and roots of *P. laetispicum* dosed at 60 mg/kg body wt., this number was 3.3 ± 1.12 (n = 10), 4.6 ± 1.09 (n = 10), 7.4 ± 2.33 (n = 10) and 13.5 ± 1.18 (n = 10), respectively (Fig. 1). For comparison, in a group of animals dosed with 100 mg/kg body wt. Aspirin (administered 60 min prior to noxious stimulation), this number was reduced to 6.1 ± 1.34 (n = 10). All the alterations induced by essential oil from stems, leaves, fruits and roots of *P. laetispicum* dosed at 60 mg/kg body wt. and by Aspirin were significant ($p<0.001$,ANOVA, Bonferroni's test).

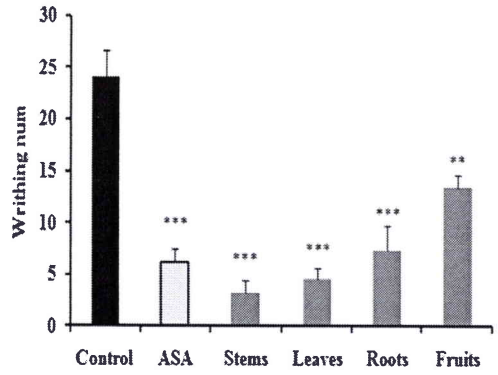


Fig. 1. Antinociceptive effects of essential oils on the acetic acid writhing test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

Antinociceptive effects induced by different doses of essential oil from stems of *P. laetispicum* on writhing test in mice are shown in Fig. 2a. Essential oil in all doses (40, 60 and 80 mg/kg) used, induced significant reduction in writhing response when compared to control as follow: 9.0 (35.22%, $P < 0.001$), 6.67 (59.63%, $P < 0.001$), and 2 (78.58%, $P < 0.001$).

Antinociceptive effects induced by different doses of essential oil from leaves of P. laetispicum on the writhing test in mice are shown in Fig. 2b. Essential oil in doses used (5～60 mg/ kg), induced significant reduction in writhing response when compared to control as follow: 14.1 (39.13%, $P < 0.001$), 7.5 (65.44%, $P < 0.001$), 7 (21.41%, $P < 0.01$), 3.8 (39.13%, $P < 0.001$), and 4.6 (65.44%, $P < 0.001$).

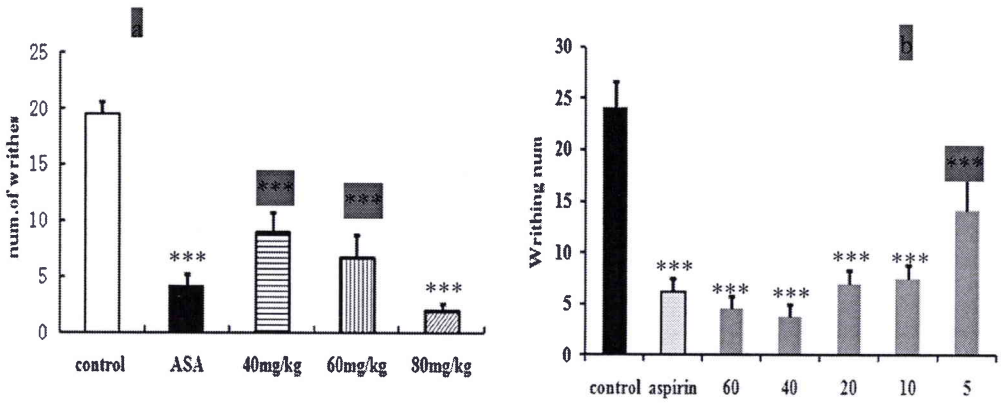


Fig. 2. Antinociceptive effects of stems and leaves essential oils on the acetic acid writhing test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.1.9.2. Antinociceptive activity of leaves of *P. laetispicum*

3.1.9.2.1. Antinociceptive in acetic acid writhing test of essential oil and total extract

Antinociceptive effects induced by different doses of total extract on the writhing test in mice are shown in Fig. 3a. Total extract in doses used (60～240 mg/kg), induced significant reduction in writhing response ($p<0.001$) when compared to control Antinociceptive effects induced by different doses of essential oil on writhing test in mice are shown in Fig. 3b. Essential oil in all doses used (20～60 mg/kg), induced significant reduction in writhing response when compared to control as follow: 7.5 (62.12 %, $P < 0.001$), 7 (64.65 %, $P < 0.001$) and 3.8 (80.81 %, $P < 0.001$). Antinociceptive effect induced by extract free from essential oil on the writhing test in mice is shown in Fig.3c. Extract without essential oil in dose of 240 mg/kg induced

significant reduction in writhing response 240 (64.85%, $P < 0.001$) when compared to vehicle-treated group.

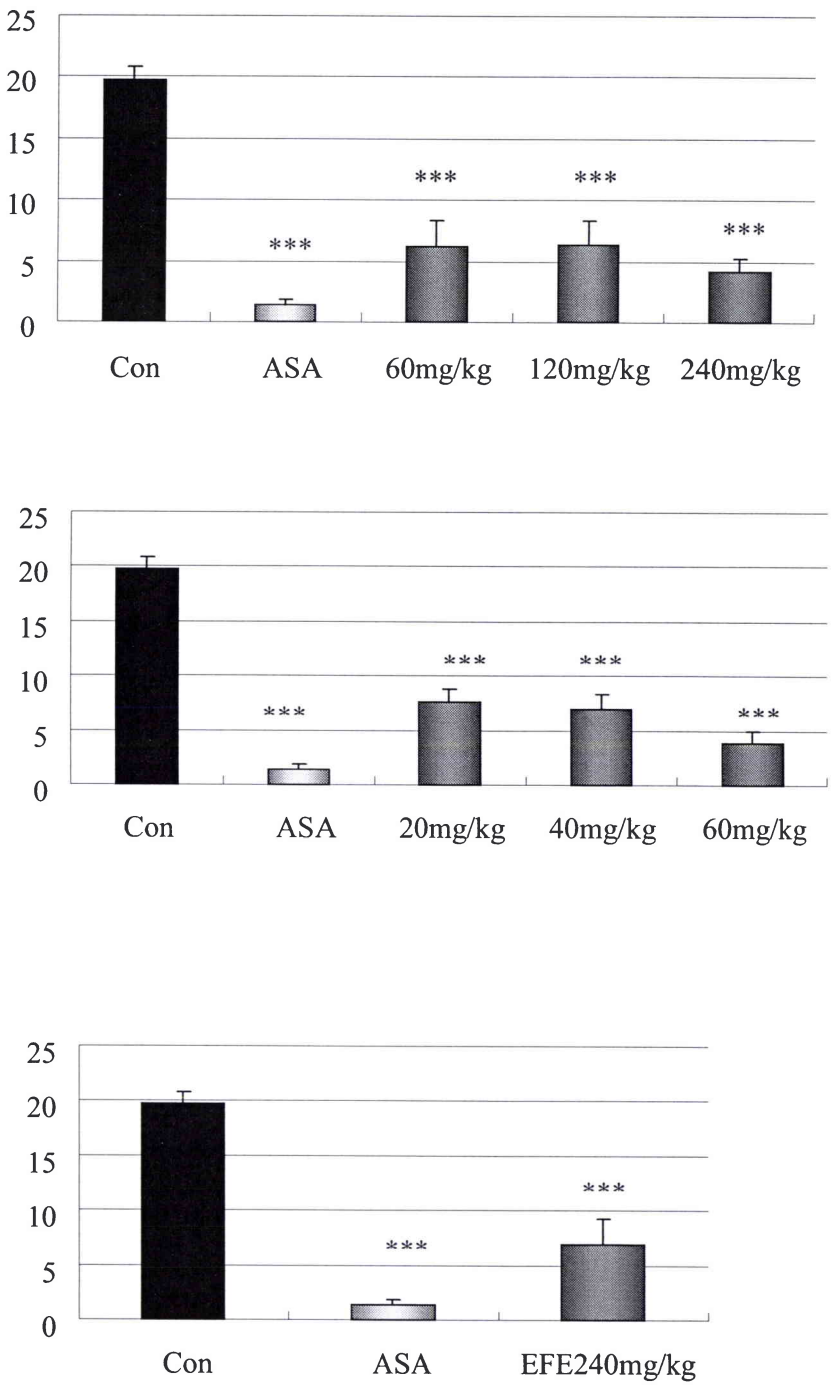
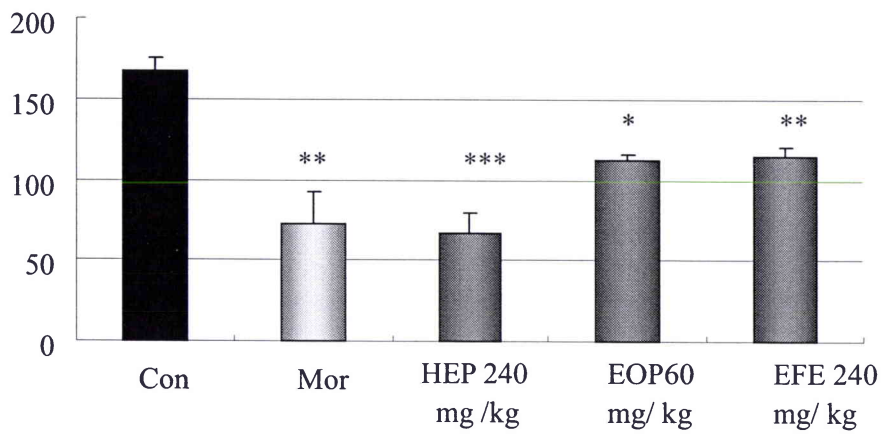


Fig. 3. Antinociceptive effects of leaves essential oil and leaves extract free from essential oil on the acetic acid writhing test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.1.9.2.2. Formalin test

Fig.4 shows the results obtained with the formalin test. During the first phase (0-10 min), the EHP (hydroethanol extract, 240 mg/ kg), EOP (essential oil, 60 mg/ kg) and EFE (extract free from essential oil, 240 mg/ kg) reduced the licking activity to 66.98 s, 112.19 s and 114.68 s, respectively. As a comparison, the administration of morphine hydrochloride (5 mg/kg, S.C.) reduced the licking time 72.97 s. In regard to the second phase (10-60 min), EHP (hydroethanol extract, 240 mg/kg), EOP (essential oil, 60 mg/kg) and EFE (extract free from essential oil, 240 mg/kg) reduced the licking activity to 76.40 s, 129 s and 253.88 s, respectively, as compared with control group.



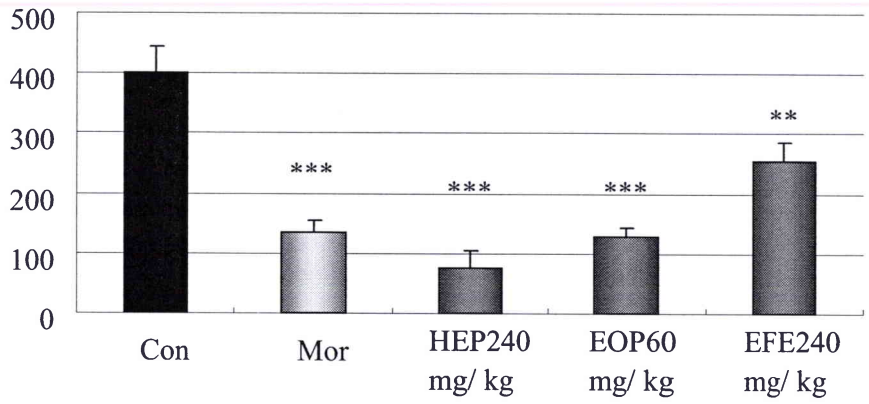


Fig. 4. Antinociceptive effects of leaves essential oil and leaves extract free from essential oil on the formalin test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.1.9.3. The antinociceptive and anti-inflammatory effects of hydroethanol extract

3.1.9.3.1 Acetic acid-induced writhing test

The results obtained with acetic acid-induced writhing test are shown in Table 5. All doses administered (60, 120, 240 and 480 mg/kg, p.o.) had a significant effect on the number of abdominal constrictions, promoting 49.11, 55.36, 73.96 and 66.03% inhibition, respectively, as compared with the control group treated with 2% tween80. Acetylsalicylic acid at dose of 100 mg/kg (p.o.) used as positive control showed 66.73% inhibition.

Table 5: Effect of hydroethanol extract of *P. laetispicum* on acetic acid induced writhing test

Group	Dose (mg/kg)	No. of writhing (per 20 min)	Inhibition (%)
Con	–	26.88 ± 2.20	–
Pl	60	13.68 ± 1.99**	49.11
	120	12.00 ± 2.30***	55.36
	240	7.00 ± 1.80***	73.96
	480	9.13 ± 2.57***	66.03
ASA	100	9.75 ± 1.87***	66.73

Values are mean ± S.E.M. $^{***}p<0.001$, significantly different from control; ANOVA followed by Bonferroni's test (n = 10). Con: control group; *Pl*: *Piper laetispicum*; ASA: Acetylsalicylic acid.

3.1.9.3.2. Formalin test

Table 6 shows the results obtained with the formalin test. During the first phase (0-10 min), the hydroethanol extract at doses of 60 and 480 mg/kg reduced the licking activity by 26.35 and 35.33%, respectively. As a comparison, the administration of morphine hydrochloride (5 mg/kg, S.C.) reduced the licking time by 73.54%. There was no significant inhibition produced at the doses of 120, 240 mg/kg, compared to control group. In regard to the second phase (10-60 min), *P. laetispicum* extract at all doses diminished the licking time by 49.50, 48.82, 46.31 and 57.63%, respectively, as compared with control group. The positive control group had an inhibition of 55.55%.

Table 6: Effect of hydroethanol extract of *P. laetispicum* on formalin test

Group	Dose (mg/kg)	0 – 10 min	Inhibition (%)	10 – 60 min	Inhibition (%)
Con	-	160.56 ± 6.87	-	372.97 ± 29.84	-
Pl	60	118.41 ± 9.53*	26.25	188.35 ± 20.62***	49.50
	120	125.61 ± 7.97	21.77	190.89 ± 19.31***	48.82
	240	143.90 ± 12.88	10.38	200.25 ± 8.79***	46.31
	480	103.83 ± 7.71**	35.33	158.03 ± 23.16***	57.63
Mor	5	42.49 ± 5.12***	73.54	165.78 ± 20.72***	55.55

Con: control group; Pl: Piper laetispicum; Mor: morphine hydrochloride.

Values are mean ± S.E.M. $^{***}p<0.001$, significantly different from control; ANOVA followed by Bonferroni's test (n = 10).

3.1.9.3.3. Hot plate test

Table 7 shows the effect of the hydroethanol extract of *P. laetispicum* on the latency time for licking and biting the paws to thermal stimulation. The extract at dose of 60 mg/kg only has the significant effect at 120min after the administration of the extract. At 120 mg/kg, a conspicuous antinociceptive activity can be seemed from 30 min to 120 min after administration. As compared with 120 mg/kg, 240 mg/kg has a later, but more intense effect and a longer time, from 60 min to 150 min after administration. 480 mg/kg has a much better activity from 30 min to 120 min. Morphine chlorhydrate has a steady and marked antinociceptive effect.

Table 7: Effect of hydroethanol extract of *Piper laetispicum* on hot plate test

Group	Dose (mg/kg)	Time after administration (min)				
		30	60	90	120	150
		Latency time(s)				
Con	-	17.97±0.62	21.38±0.79	19.25±0.51	20.14±0.85	19.12±0.78
Pl	60	25.70±1.43	29.23±4.12	33.30±3.03	40.87±4.33*	28.64±4.23
	120	38.67±4.69***	39.04±5.51*	36.34±4.72*	42.86±5.61**	35.73±4.33
	240	26.26±1.16	40.38±4.72*	43.20±4.43***	53.77±3.48***	52.17±4.38***
	480	45.24±4.01***	45.50±3.34***	35.83±3.76*	39.37±4.29*	31.29±4.67
Mor	5	52.20±2.29***	49.05±2.53***	44.63±4.39***	33.43±4.85	35.39±6.33

Con: control group; Pl: Piper laetispicum; Mor: morphine chlorhydrate.

Values are mean ± S.E.M. $^{***}p<0.001$, significantly different from control; ANOVA followed by Bonferroni's test (n=10).

3.1.9.3.4. Formalin test

3.1.9.3.4. 1.Tail immersion test

The effect of the extract on tail immersion test is shown in table 8. The extract caused an approximate inhibition of pain at the doses of 120, 240 and 480 mg/kg, and the percentage change of TWL were 57.14, 59.04 and 41.53%, respectively, which are lower than morphine hydrochloride (5 mg/kg), 134.81%. Significant at 60 mg/kg was not found.

Table 8: Effect of hydroethanol extract of *P. laetispicum* on tail immersion test

Group	Dose (mg/kg)	Pre-treatment (s)	Post-treatment (s)	Percentage of TWL (%)
Con	-	1.38 ± 0.08	1.40 ± 0.13	-
Pl	60	1.44 ± 0.06	2.10 ± 0.16	45.83
	120	1.68 ± 0.15	2.64 ± 0.16**	57.14
	240	1.66 ± 0.16	2.64 ± 0.20**	59.04
	480	1.83 ± 0.20	2.59 ± 0.10**	41.53
Mor	5	1.58 ± 0.16	3.71 ± 0.43***	134.81

Con: control group; Pl: Piper laetispicum; Mor: morphine hydrochloride.

Values are mean ± S.E.M. $^{***}p<0.001$, significantly different from control; ANOVA followed by Bonferroni's test (n = 10).

3.1.9.3.4.2. Xylene-induced ear edema test

In the xylene-induced ear edema test, the positive control drug acetylsalicylic acid at 150 mg/kg exhibited significant inhibitory effect on xylene-induced ear edema. The inhibition was up to 28.16%. *P. laetispicum* extract at 120, 240 and 480 mg/kg inhibited ear edema formation in a dose–dependent manner and the inhibition rates

were 20.53, 27.18 and 33.43%, respectively (Table 9). The potency of 240 and 480 mg/kg *P. laetispicum* extract were comparable to that of 150 mg/kg acetylsalicylic acid.

Table 9: Effect of hydroethanol extract of *P. laetispicum* on xylene-induced ear edema test

Group	Dose (mg/kg)	Ear oedema (mg)	Inhibition (%)
Con	-	7.21 ± 0.42	-
Pl	120	5.73 ± 0.44	20.53
	240	5.25 ± 0.38**	27.18
	480	4.80 ± 0.40***	33.43
ASA	150	5.18 ± 0.30**	28.16

Con: control group; Pl: Piper laetispicum; ASA: Acetylsalicylic acid.

Values are mean ± S.E.M. $^{***}p<0.001$, significantly different from control; ANOVA followed by Bonferroni's test (n = 10).

3.1.9.3.5. The antinociceptive effects of different fractions

3.1.9.3.5. 1. The acetic acid writhing test

The results of the acetic acid-induced writhing responses in mice, which indicate the analgesic activity of the PEF, CHLF and EAF, are presented in Fig. 5. It was found that all the fractions except for EAF at the given doses (120~480 mg/kg) caused a significant inhibition of the writhing responses induced by acetic acid as compared to the control, with values ranging from 42.86% to 63.19% protection. At 120 mg/kg body wt., PEF and CHLF still showed significant inhibition of the writhing responses, with inhibitions of 48.9% and 46.7%, respectively. In contrast, at a dose of 60 mg/kg body wt., only the CHLF showed any significant analgesic effect. These results taken

together indicate that HEPl possesses strong analgesic activity, with the CHLF possessing the highest analgesic activity as compared to other fractions.

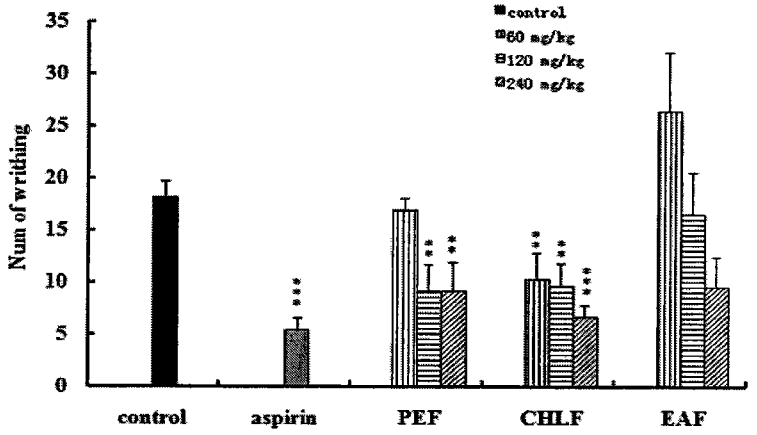


Fig. 5. Antinociceptive effects of fractions on the acetic acid writhing test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.1.9.3.5.2. The ear edema test

The PEF, CHLF and EAF (o.p.) significantly inhibited ($p<0.001$) the ear edema in the mice at the dose tested. The PEF exhibited significant anti-inflammatory activity between 120, 240 and 480 mg/kg, in a dose-dependent manner, in comparison with the control. However, a U-shaped dose–activity curve was observed for CHLF (Fig. 6). And in the EAF, a bell-shaped dose-activity curve was gotten at the given doses. All the fractions at the given doses have a significant inhibitions ($p<0.001$) in the ear edema test, except that the EAF at 120 mg/kg ($p<0.01$) and 480 mg/kg ($p<0.05$) showed mild anti-edema effects. As a positive control, Aspirin (150 mg/kg, p.o.) significantly inhibited the ear edema by 41.54%. The results demonstrate the anti-inflammatory properties of PEF, CHLF and AEF and may justify the use of this

plant for the treatment of antinociceptive and inflammatory diseases in Chinese folk and herbal medicine.

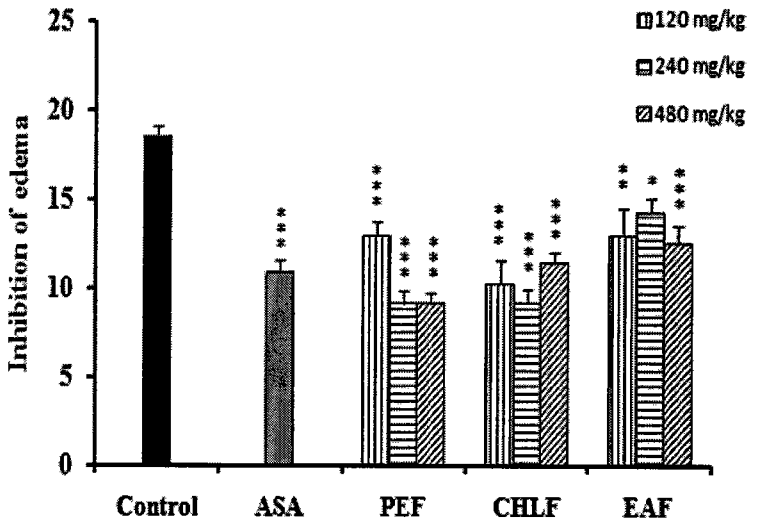


Fig. 6. Anti-inflammatory effects of fractions extract on the ear edema test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.1.9.3.6. The antinociceptive effects of individual compounds

3.1.9.3.6.1. The antinociceptive effects of laetispicine and d-sesamin in writhing test

The results obtained with acetic acid-induced writhing test are shown in Fig. 7. Similarly to acetylsalicylic acid (ASA) which decreased number of writhing by 47.20%, laetispicine also decreased this parameter. The effect was dose-dependent and, at the doses administered (10, 20, and 40 mg/kg, i.g.) it had a significant effect on the number of abdominal constrictions, promoting 50.45, 77.32, and 93.74% inhibition, respectively, as compared with the control group treated with 2% tween80,

d-sesamin, a normal ligant, also decreased the writhing number. The effect was dose-dependent and, at the doses administered (100 and 200 mg/kg, i.g.) it had a significant effect on the number of abdominal constrictions, promoting 32.5, and 73.99% inhibition, respectively, as compared with the control group treated with 2% tween80.

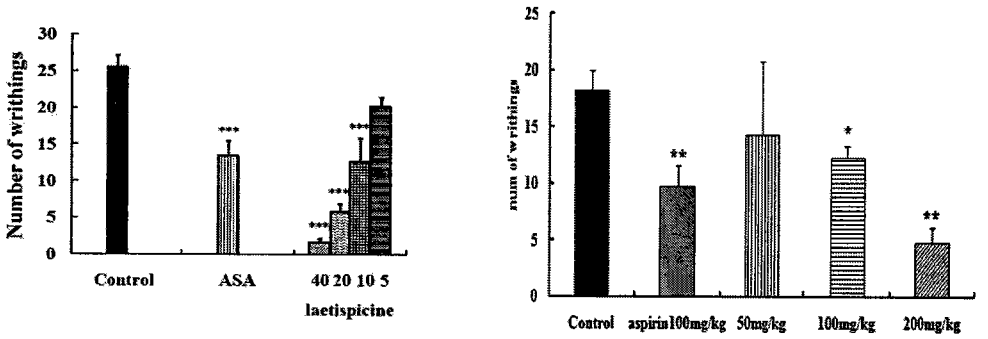


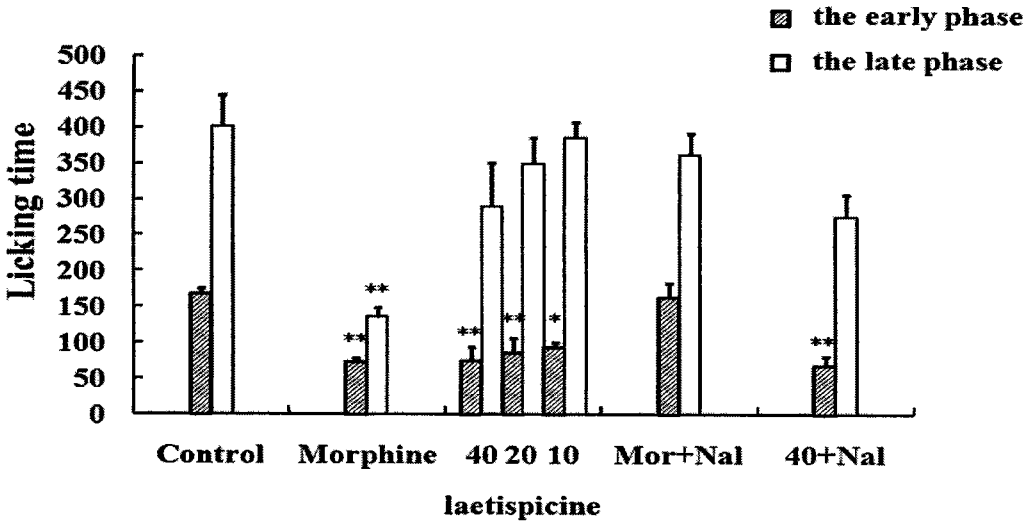
Fig. 7. Antinociceptive effects of individual compounds on the acetic acid-induced writhing test in mice.

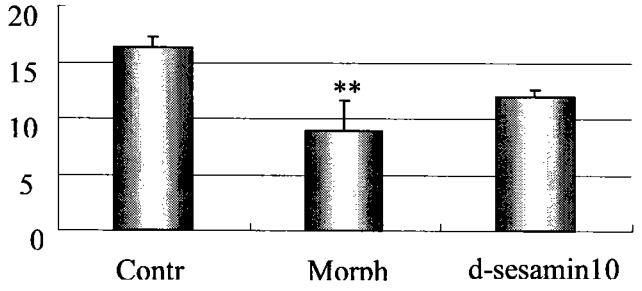
Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.1.9.3.6.2. The formalin test

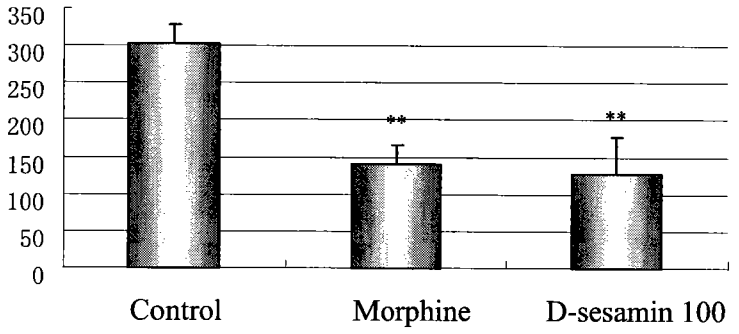
Fig. 8 shows the results obtained with the formalin test. During the first phase (0–10min), laetispicine at doses of 10, 20 and 40 mg/kg reduced the licking activity by 44.74, 49.51 and 55.86%, respectively. As a comparison, the administration of morphine hydrochloride (5 mg/kg, i.p.) reduced the licking time by 56.36%. In regard to the second phase (10–60min), laetispicine at all given doses diminished the licking time by 4.11, 13.06 and 27.63%, respectively, as compared with control group. No significant were found in the second phase. The positive control group had an inhibition of 66.23%. In order to verify the involvement of opioidergic system in the

antinociception, a groups of mice (n=10) were pre-treated with non-selective opioid receptor antagonist, naloxone (2 mg/kg, i.p.), which was injected 15 min before the administration of laetispicine (40 mg/kg, i.g.) and morphine (5 mg/kg, i.p.), and tested using the formalin test as described above. Pre-treat with naloxone however did not reverse the antinociceptive effect of laetispicine (40 mg/kg) in both phases. Pre-treat with d-sesamin at the dose of 100 mg/kg didn't induce any significant change in the first phase in formalin test, while in the second phase the administration of d-sesamin reduced the licking time by 58.26% ($p<0.01$).





d-sesamin in the first phase of formailin test



d-sesamin in the second phase of formalin test

Fig. 8. Antinociceptive effects of individual compounds on the formalin test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.1.10. Discussion

The classical animal models, including acetic acid writhing test, the formalin test, hot plate test, tail immersion test and ear edema test were used for the evaluation of the essential oils, total extract, fractions and individual compounds. The present data give pharmacological support to the validity of the analgesic effect of *Piper laetispicum*.

3.1.10.1. Antinociceptive effects of essential oils

The family Piperaceae belonging to the superorder Nymphaeiflorae, order Piperales (Dahlgren, 1980), comprises about 5 genera and 1400 species (Joly, 1977). The genera Piper and Peperomia are the most representative of the Piperaceae (700 and 600 species, respectively). Some Piper species are used in folk medicine to treat many diseases. Analysis of volatile constituents from Piperaceae species has revealed the presence of monoterpenes, sesquiterpenes and arylpropanoids (apiol, dillapiol, myristicin, safrole, sarisan,) that have shown interesting biological properties (Parmar et al., 1997; Martins et al., 1998; Moreira et al., 1998).

In our group, The essential oil compositions of *P. laetispicum* stems, leaves, fruits and roots were studied by using GC and GC/MS analyses as well as by comparison of their mass spectra and RI values with literature data (Dong et al., 2007). The results showed that all the tested organs of *P. laetispicum* contains a potent volatile oil, mainly consisting of mono- and sesqui-terpenes, which is in consistent with other report (Santos, et al., 2001)

From the present results, the essential oil from stems, leaves, fruits and roots at 60 mg/kg have significant antinociceptive activity, and the construction inhibition are 86.3%, 80.9%, 69.3%, and 44%, respectively (Fig.1).

In general, the mono- and sesqui-terpenes have anti-inflammatory and analgesic activities (Fernandez-Puntero, 1997; Galati, 2000). According to Dong, the essential oils of leaves and stems contained the most quantity sesqui-terpenes. The quantity of sesqui-terpenes in leaves is 87.67%, and in stems is 60.49%. So a detailed study was designed to test the antinociceptive effects of leaves and stems of *P. laetispicine.*

From the results showed in Fig. 2, the inhibition of writhing of leaves at 40 mg/kg was 84.23%, better than the stems, whose inhibition was 89.7% at the dose of 80 mg/kg. We concluded at least the antinociceptive effects and quantity of the sesqui-terpenes in the leaves and stems of *P. laetispicum* has a quantity- activity relationship.

In *P. laetispicum*, there are many groups of chemical compounds including amides, lignans and fatty acids. We are wondering if the sesqui-terpenes are the only group of compounds responsible to the antinociceptive effects of *P. laetispicum*. The leaves

were chosen to be the research material because essential oil leaves contained the most quantity of sesqui-terpenes (87.67%). And we have a detailed phytochemical research results about the leaves.

The results in Fig. 3 demonstrate the significant antinociceptive effect of essential oil from leaves of *P. laetispicum* at doses of 20, 40 and 60 mg/kg while the total extract (Fig.3) showed also outstanding efficacy in reduction of visceral pain at the given doses (60～240 mg/kg).

To verify if there are other compounds have the antinociceptive effects, we evaluated the total extract free from essential oil at 240 mg/kg (Fig. 3).

The data demonstrated in Fig. 3, indicated that both essential oil and the total extract free from essential oil have considerable antinociceptive activity. In writhing test, both central and peripheral analgesics can be detected but because of the lack of specificity, caution is required in interpreting the results until other tests have been performed. The formalin test was used to re-confirm the antinociceptive effects of leaves essential oil (60 mg/kg), total extract (240 mg/kg) and extract free from essential oil (240 mg/kg).

Fig. 4 showed, in the formalin test, that essential oil, total extract and total extract free from essential oil have good antinociceptive effects in the first and the second phase in the formalin test and as if there exist a synergic effect between the sesqui-terpenes and amides and/or lignans. According to Dong, the total quantity of sesqui-terpenes in the leaves essential oil is 87.67% and the phytochemical results from Fang showed the main compounds in *P. laetispicine* leaves were amide alkaloids and lignans.

Therefore, we can conclude that the presence sesqui-terpene compounds, amide alkaloids and lignans are responsible for the antinociceptive effect of leaves essential oil, and total extract free from leaves respectively.

In summary, the essential oils have good antinociceptive effects; there is an effect-activity relationship between the quantity of sesqui-terpenes and the analgesic effects; the present antinociceptive properties of *P. laetispicum leaves* could be related to presence of sesqui-terpenes, amides and lignans in this plant. Based on bibliography, no study has been conducted on interactive effects of *P. laetispicum* with these substances and its exact mechanism of action remains to be elucidated by further studies.

3.1.10.2. Antinociceptive and anti-inflammatory effects of hydroethanol extract

The present study assessed the antinociceptive and anti-inflammatory effects of the hydroethanol extract of stems from *P. laetispicum*. Four tests (chemical and thermal) were employed in evaluating the antinociceptive activity. Moreover, the anti-inflammatory activity of extract was determined by xylene-induced ear edema test in mice. The results lead us to confirm that *P. laetipicum* exerts significant analgesic and anti-inflammatory effects in the five current animal models in mice.

Acetic acid-induced writhing test is a test useful for evaluating mild analgesic non-steroidal anti-inflammatory agents, and widely used for antinociceptive screening. The abdominal writhing induced by acetic acid involves the production and release of arachidonic acid metabolites via cycloxygenase (COX) and prostaglandin

biosynthesis (Elisabetsky et al., 1995). In this context, even provided by no specific pain model, a significant and dose-related antinociceptive effect was exerted by *P. laetispicum* extract. The extract may be considered as peripherally acting as having anti-inflammatory effect.

The antinociceptive effects of *P. laetispicum* were also verified on formalin-induced spontaneous nociceptive behaviors. The formalin pain test is a very useful model of clinical pain in which the first phase seems to be due to direct chemical stimulation of nociceptors, whereas the second phase is dependent on peripheral inflammation and changes in central processing (Tjølsen et al., 1992). It is well known that drugs which act mainly centrally, such as narcotic analgesics, inhibit both phases of pain in this model while peripherally acting drugs, such as aspirin or indomethacin, only inhibit the late phase (Santos et al., 1994). Inhibition of the late phase is due to inflammation causing a release of serotonin, histamine, bradykinin and prostaglandins, which at least to some degree can cause the sensitization of the central nociceptive neurons (Verma et al., 2005). In our study, similar to some non-opiate analgesics, the hydroethanol extract of *P. laetispicum* decreased pain in both phases of the formalin test, with a greater potency in the inflammatory phase. We might suggest that the antinociceptive is likely to be mediated peripherally.

In order to evaluate the central analgesic response from the hydroethanol extract, the hot plate test (supra-spinal analgesia) and tail immersion test (spinal analgesia) were used.

In hot plate test, it was clearly demonstrated in the present study that the hydroethanol extract induced antinociception. The heat induces a cutaneous thermonociceptive effect and the stimulus integration occurs due to the stimulation of C non-mielinized fibers of slow conduction (Hendry et al., 1999). Moreover, *P. laetispicum* was less effective in causing analgesia in mice exposed to the hot plate test than acetic acid-induced writhing test and the late phase of the formalin test. This suggests that constituents present in *P. laetispicum* are particularly effective in alleviating painful inflammatory states. And this hypothesis is further strengthened by future studies.

The central analgesic effect of *P. laetispicum* extract may be reconfirmed by the results recorded in the tail immersion test. An increase in the reaction time is generally considered to be an important parameter for evaluating central antinociceptive activity (Rujjanawate et al., 2003). Indeed, the *P. laetispicum* extract showed to some extent inhibitory on the reaction time to the thermal nociceptive stimulus, while in comparison with the positive control group they were less effective.

Xylene-induced ear edema is an acute inflammation model. Ear edema may involve inflammatory mediators such as histamine, serotonin, bradykinin and prostaglandins. These mediators can induce ear edema by promoting vasodilation and increasing vascular permeability (Carlson, 1985). *P. laetispicum* extract may interfere with the actions of the above mediators to produce the anti-inflammation effect in this model.

3.1.10. 3. Antinociceptive and anti-inflammatory effects of fractions of stems

It has been suggested that the hydroethanol extract of *P. laetispicum* stems showed antinociceptive activity. In the present work, we have fractionated the HEPl and found the main analgesic fractions include PEF and CHLF. The CHLF has better antinociceptive activity than PEF. The anti-inflammatory effects were confirmed in the ear edema test. Fig. 6 showed the consistent activity with the results from acetic acid writhing test, the CHLF has the most potential anti-inflammatory activity, compared with the other fractions. The data from experiments in vivo is a little different with the results in vitro (Stöhr, 2001).

It is suggested that the observed anti-nociceptive activity might be related to its anti-inflammatory activity, which merited further studies regarding the precise site and the mechanism of action.

3.1.10.4. Antinociceptive effects of individual compounds

In the present experiments, the antinociceptive effects of two individual compounds from the CHLF and EAF were evaluated in the acetic acid writhing test and formalin test.The results of the acetic acid induced writhing test showed that a potent antinociceptive activity in laetispicine (Fig. 7). The effect of laetispicine (10 mg/kg, 50.45% inhibition) was comparable to that of ASA (100 mg/kg, 47.2% inhibition). And d-sesamin at 100 mg/kg and 200 mg/kg can produce significant antinociceptive activity. Acetic acid-induced writhing test is a test useful for evaluating mild analgesic non-steroidal anti-inflammatory agents, and widely used for antinociceptive screening

(Gene et al. 1998). The abdominal writhing induced by acetic acid involves the production and release of arachidonic acid metabolites via cycloxygenase (COX) and prostaglandin biosynthesis (Elisabetsky et al. 1995). Therefore, the result of the acetic acid-induced writhing suggests that the mechanism of action of antinociception of laetispicine and d-sesamin may be linked partly to the cyclo-oxygenase enzymes.

The formalin pain test is a very useful model of clinical pain in which the first phase seems to be due to direct chemical stimulation of nociceptors, whereas the second phase is dependent on peripheral inflammation and changes in central processing (Tjølsen et al. 1992). In Fig. 8, laetispicine significantly inhibited the first phase of paw licking in this study. However, d-sesamin didn't change this parameter. It is well known that drugs which act mainly centrally, such as narcotic analgesics, inhibit both phases of pain in this model while peripherally acting drugs, such as aspirin or indomethacin, only inhibit the late phase (Santos et al. 1994). The early phase is said to be probably a direct result of stimulation of nociceptors in the paw and said to reflect centrally mediated pain while the late phase is said to be due to inflammation occurring following the release of serotonin, histamine, bradykinnin, and prostaglandins (Tjølsen et al. 1992). In our study, the result of the formalin test may therefore suggest that laetispicine possess central antinociceptive effect in acute nociceptive model but weak peripheral antinociceptive properties.

Naloxone cannot reverse the antinociceptive effect of laetispicine in both phases, and this may also suggest that central opioid receptors are not involved in the antinociceptive action of laetispicine. In contrast to laetispicine, d-sesamin only has a significant effect in the second phase, which indicated that d-sesamin acts mainly peripherally.

Section 2: The antidepressant activity of *Piper laetispicum*

3.2.1. Introduction

Depressive disorders, including major depression and dysthymia, are serious disabling illnesses (Williams et al., 2000). Approximately one in five persons is affected by a mood disorder at some point (Kessler et al., 1994). Although significant progress has been made in the research work of the depression, current treatments for depression are inadequate for many individuals, and progress in understanding the neurobiology of depression is slow (Nestler et al., 2002).

Medicinal plants have played a crucial role in world health and the use of natural products with therapeutic properties is as ancient as human civilization (Rates, 2001). According to the World Health Organization (WHO), about 65～80% of the world's population in developing countries depends essentially on plants for their primary health care (Calixto, 2005). It has been estimated that one of every three Americans has used herbal remedies (Brevoort, 1998). Spending on herbal products in the United Kingdom is over £ 40 m a year (Vickers and Zollman, 1999) and in Germany, France, Italy, there also exist some appropriate guidelines for registration of herbal medicinal

preparation (Calixto, 2000). With the growing interest in and use of natural products in the world, as well as with the urgent need of advent of effective, low-cost, well tolerated antidepressants, many plants have been reported to have antidepressant activity and can be effective therapeutic alternatives for treatment of depression (Carlo, et al., 2001).

Piper is a large genus of herbs, including more than 1000 species widely distributed throughout the tropical and subtropical regions of the world. A large number of experimental and clinical studies indicate that *piper* species has wide ranging pharmacological and biological activities. Among *Piper* species, one of the most studied is *Piper methysticum*, also known as kavakava. It has been well documented that kava extracts have a wide variety of biological activities, including antidepressant effects. Some isolated amides from *Piper* genus have been reported to possess a broad spectrum of biological activities. Results from previous studies demonstrated that piperine, the first amide isolated from Piper species, showed antidepressant-like activity (Lee et al., 2005; Li et al., 2007). And piplartine from *P. berculatum* possessed anxiolytic and antidepressant effects in mice (Cícero Bezerra Felipe et al., 2007).

Piper laetispicum C.DC. (Piperaceae), popularly known in folk as Xiao Chang-feng, Shan Hu-jiao, Ye Hu-jiao, is an endemic climbing, glabrous plant available in the southern part of China. Based on the antidepressant effects of *Piper* genus, we postulated that *P. laetispicum* extract and some fractions might have antidepressant activity and sought evidence of this effect experimentally. This study was undertaken

to evaluate the influence of hydroethanol extract, the fractions, laetispicine and the derivatives of laetispicine on the duration of immobility in the forced swimming test (FST) and in the tail suspension test (TST). The mechanism underlying the antidepressant action of *P. laetispicum* was studied as well.

3.2.2. Material and methods

3.2.2.1. Plant, hydrethanol extract, franctions and laetispicine

Piper laetispicum C. DC (Piperaceae), an endemic climbing, glabrous plant available in the southern part of China, was collected in 2006 from Hainan province, China, and was identified by Prof. Sheng-li Pan, School of Pharmacy, Fudan University, where a voucher specimen (No. 060812) of the plant material has been deposited for further reference.

3.2.2.1.1. Hydroethanol extract

The stem of *P. laetispicum* (11kg) was cut into pieces, air-dried, powdered and extracted using hydroethanol (95 %) solvent in percolator at room temperature, up to the exhaustion of the plant material (30−fold). The solvent was removed under vacuum in a rotary evaporator until yielding a dark brown colored semisolid mass (HEPl) (750g, yields 6.8%, w/w).

3.2.2.1.2. Fractionation

The crude extract (20g) was suspended in water and partitioned successively with petroleum ether, chloroform and ethyl acetate. Each fraction was evaporated under reduced pressure and the yields of the petroleum ether (PEF), chloroform (CHLF), ethyl acetate (EAF) and aqueous (AQF) fractions were 4 g (13.33%), 4 g (13.33%), 2

g (6.67%) and 20 g (66.67%), respectively.

3.2.2.1.3. Laetispicine

The hydroethanolic extract of the stems was dissolved in water and fractionated with petroleum ether and ethyl acetate, respectively. The ethyl acetate fraction was combined, concentrated under reduced pressure to give a dark brown colored semisolid extract (60 g). Then the ethyl acetate extract was chromatographed on a silica gel column by a gradient elution using mixtures of petroleum ether and ethyl acetate (20:1-9:1-5:1-3:1-2:1-1:1) to afford six fractions. Fraction 3 was concentrated under reduced pressure and subjected to silica gel column chromatography and eluted with chloroform and methanol (50:1), to give four subfractions. Laetispicine (300 mg) was separated from subfraction 3 by repeated chromatography on silica gel column, eluted with petroleum ether and acetone.

3.2.2.1.4. The derivatives of laetispicine

The derivatives of laetispicine were presented by group of Prof. Jingkang Shen, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Hydroethanol extract (HEPl), fractions (PEF, CHLF, EAF and AQF), laetispicine and derivatives of laetispicine obtained were kept in refrigerator at +4 ℃ and suspended in a solution of 2% tween80 prior to any pharmacological screening. Respective controls received only that solvent as vehicle.

3.2.2.2. Drugs

95% ethanol, petroleum ether, chloroform, ethyl acetate, distilled water, tween80, clomipramine, imipramine hydrochloride, L-arginine, naloxone hydrochloride and pcpa (4-Chloro-DL-phenylalanine)

3.2.2.3. Animals and habituation

Male KM mice (18-22 g) were housed in standard environmental conditions. Food and water were available *ad libitum*. The animals were acclimatized to the laboratory for at least 7 days before testing in the forced swimming and open field tests. The mice were used only once throughout the study. All experiments were approved by Animal Ethics Committees, Fudan University, China.

Swiss albino male mice (OF1) aged 9 weeks at the receipt from the breeder company (Charles River, France) and weighing 40–45 g were used in the tail suspension test. The animals were housed under a 12-h light: 12-h dark schedule (lights on starting at 8:00 p.m.) with water and food ad libitum (SD Dietex-France). Animal rooms were at a constant temperature of 21±2 °C and relative humidity of 55±10%. All animal procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

3.2.2.4. Pharmacological tests

3.2.2.4.1. Forced swimming test (FST)

The forced swimming test adopted here is a modification of the method described by Porsolt et al. (1977). Briefly, mice were individually forced to swim for 15 min in glass cylinders (height: 20 cm, diameter: 14 cm), containing 10 cm of water at 25 ℃,

which is a pre-test, and then mice were removed and dried before being returned to cages. Twenty-four hours later, mice were placed in the cylinders again for a 6-min test in the same system depicted above. The duration of immobility was recorded during the last 4 min of the 6-min testing period. Groups of 10 mice were treated with vehicle (10 ml/kg, p.o.), drug-treated groups (10 ml/kg, p.o.), clomipramine (50 mg/kg, i.p.) and imipramine (30 mg/kg, i.p.), 1 h after the administration the mice were submitted to TST.

3.2.2.4. 2. Tail suspension test (TST)

The tail suspension test (TST) was performed according to the method described by Steru et al. (1985) with slight modifications. Briefly, the mice were individually suspended by the tail from a metal rod using adhesive tape. The rod was fixed 50cm above the surface of a table. The total duration of immobility was measured during 5min. 'Immobility' was defined as when they hung passively and were completely motionless. Groups of 10 mice were treated with vehicle (10 ml/kg, p.o.), drug-treated groups (10 ml/kg, p.o.), and imipramine (30 mg/kg, i.p.), 1 h after the administration the mice were submitted to TST.

3.2.2.5. Preliminary mechanisms of actions

3.2.2.5.1. Serotonergic depletion

In order to investigate the possible contribution of the serotonergic system to the effect of different fractions of *P. laetispicum* and laetispicine in the FST, mice were pretreated with p-chlorophenylalanine (PCPA). PCPA is known to reduce the concentration of brain serotonin by inhibiting its biosynthesis. In the present

experiments the mice were injected i.p. either with vehicle (control group) or with PCPA. PCPA was administratered at the dose of 100 mg/kg once daily for 4 consecutive days. After the last injection of PCPA, mice were treated with 2% tween 80, different franctions of *P. laetispicum* or laetispicine and tested in forced swimming test or tail suspension test 60 min later.

3.2.2.5.2. Opioid system

To investigate the participation of the opioid system in the laetispicine effect in the FST, animals were pre-treated with naloxone (1 mg/kg, i.p., a non-selective opioid receptor antagonist) 15 min before administration of laetispicine (10 mg/kg, p.o.) or 2% tween80 (10 ml/kg, p.o.); a further 60 min elapsed before the animals were tested in the FST.

3.2.2.5.3. NO pathway

In a separate series of experiments, we also investigated the possible participation of the L-arginine-nitric oxide pathway in the anti-immobility effects of the extract. To this end, mice were pre-treated with L-arginine (750 mg/kg, i.p., a precursor of nitric oxide, a dose that produced no effect in the FST), and after 15 min they received laetispicine (10 mg/kg, p.o.) or 2% Tween 80 (10 ml/kg, p.o.) and were tested in the FST 60 min later as described above.

3.2.2.5.4. Open field test

In order to detect any association of immobility in the FST and TST with changes in motor activity, the locomotor behaviours of animals treated with hydroethanol extract, activity fractions, laetispicine and bioactivity derivatives were tested in an open field.

The method used in the present study was similar to that described previously by Sun-Hee Kim et al. (2005). Briefly, each mouse was placed in the center of the open-field apparatus, and the locomotor activity was assessed immediately before the FST. The open-field apparatus was a square, 40 cm in every side, which was demarcated into 16 equal areas. The score locomotion (number of line crossings within 5 min) and rearing frequencies (number of times an animal stood on its hind legs) were recorded. In this experiment, the animals received the same drugs and doses as those used when measuring immobility. The open-field apparatus was washed with deodorant solution and dried before each behavioral test to eliminate possible odor clues left by previous subjects. Experiments were performed in a dark room, and the apparatus was illuminated by a 60 W bulb positioned 1 m above the center of the circle.

3.2.2.6. Statistical analyses

Data obtained were expressed as mean ± SEM and analyzed by analysis of variance (ANOVA) followed by Bonferroni's test. *P-values* less than 0.05 ($p<0.05$) were used as the significant level.

3.2.2.7. Results

3.2.2.7.1. Antidepressant effects of hydroethanol extract

3.2.2.7.1.1. The forced swimming test

Fig. 9a illustrates the effect of HEPl on the duration of immobility time in the FST model. The acute administration of HEPl at the doses of 120, 240 and 480 mg/ kg significantly decreased the immobility time to 147.795 ± 9.61 s ($p < 0.01$), 133.789 ±

4.58 s ($p < 0.001$), and 138.789 ± 8.94 s ($p < 0.001$), respectively, as compared to control group of 179.675 ± 6.34 s. Imipramine (30 mg/ kg i.p.) also showed a significant reduction of immobility time (128.014 ± 10.47 s, $p < 0.001$), compared to vehicle treated animals. No significant change in immobility time was observed for HEPl treated mice at 60 mg/kg.

The effects of administration with HEPl once daily for 7 days were evaluated in FST in mice (Fig. 9b). HEPl at the doses of 120, 240 and 480 mg/kg reduced the duration of immobility in forced swimming test, resulting in a 22.1%, 35.3% and 32% immobility reduction compared with the control group, respectively (Fig. 9b). In the forced swimming test, the effect of HEPl at dose of 240 mg/ kg was similar to those observed for the classical antidepressant imipramine (30 mg/ kg, i.p.). The percentage of inhibition for imipramine was 42% in forced swimming test.

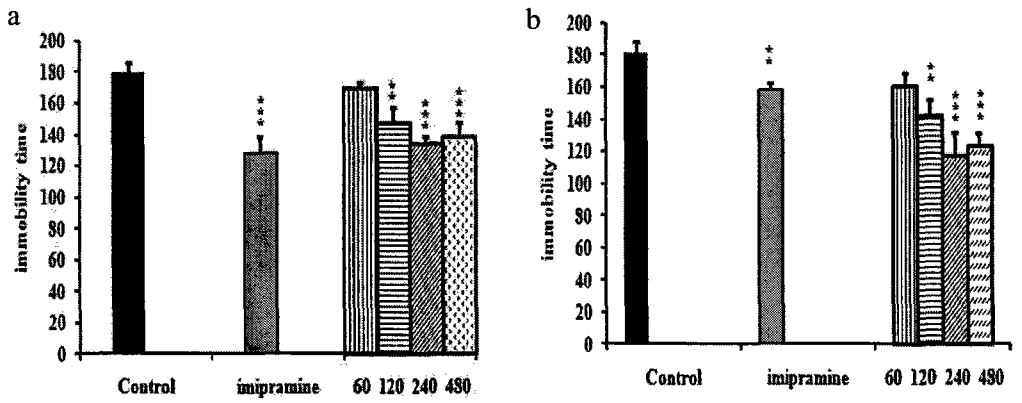


Fig. 9: Antidepressant effects of hydroethanol extract on the forced swimming test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.2.2.7.1.2. Open field test

In order to determine whether HEPl really has an antidepressant-like action, we have to find out whether HEPl has significant action on the central nervous system. In this study, HEPl at 60, 120, 240 and 480 mg/kg and imipramine at 30 mg/kg, which dramatically reduced immobility time in the FST in mice, produced no significant difference in rearing and crossing in the open field behavioral test, compared with control as shown in Fig. 10.

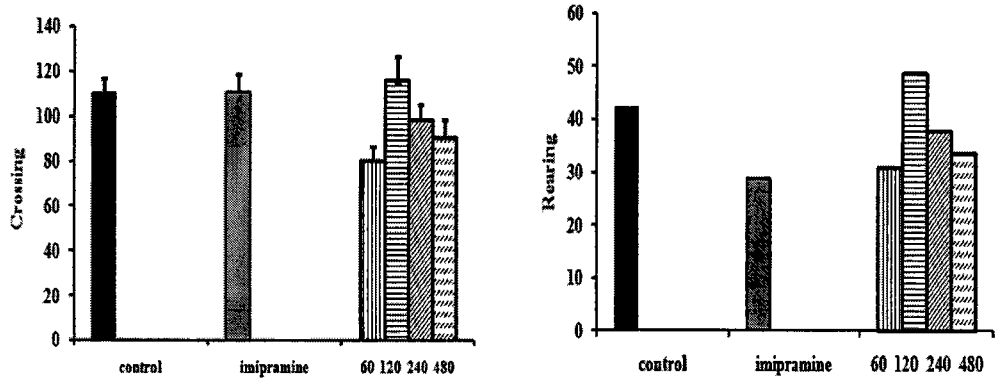


Fig.10. Locomotor effects of hydroethanol extract on the open field test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.2.2.7.2. Antidepressant effect of fractions

3.2.2.7.2.1. The forced swimming test

The effects of the oral administration of the fractions of *P. laetispicum* (PEF, CHLF, EAF and AQF) in the immobility time in the FST were shown in Fig. 11. Oral administration of 120, 240 and 480 mg/kg of AQF didn't produce any significant change on the immobility time in mice to the FST. However, oral treatment with doses of 120, 240 and 480 mg/kg of PEF, CHLF and EAF reduced significantly the

immobility time, in comparison to control group receiving the vehicle and submitted to FST. Imipramine (30 mg/kg, i.p.) showed a significant reduction of immobility time (p<0.001), compared to vehicle treated animals (Fig. 11).

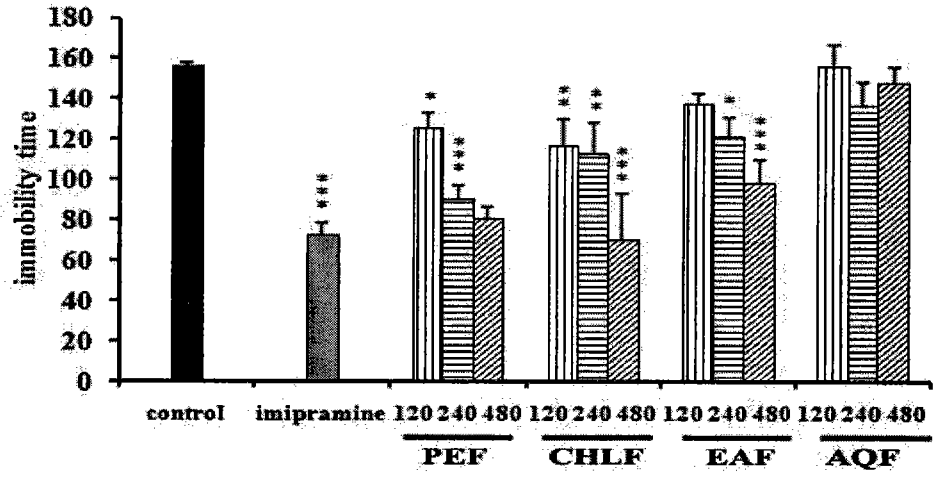


Fig. 11: Antidepressant effects of PEF, CHLF, EAF and AQF on the forced swimming test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.2.2.7.2.2. The open field test

Fig. 12 shows the results obtained with open field test. The treatment animals with PEF, CHLF, and EAF showed no differences compared with control animals in 5-min open field test at the dose range used in the present study. The administration of imipramine in the FST did not cause any significant change in the number of crossing and rearing in the open field test.

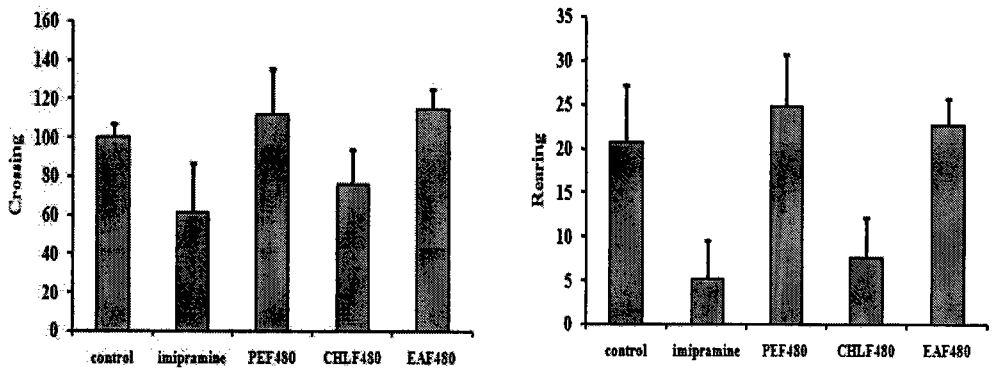


Fig.12: Locomotor effects of PEF, CHLF, and EAF on the open field test in mice. Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.2.2.7.2.3. Effect of pre-treatment with PCPA on the antidepressant effect of PEF, CHLF and EAF in the FST

The results in Fig. 13 show that PCPA alone (100 mg/kg, once a day, for 4 consecutive days) did not modify the immobility time, while pre-treatment of mice with PCPA didn't block the reduction in the immobility time elicited by PEF and EAF (480 mg/kg, o.p.) in the forced swimming test, but at least partially blocked the reduction in the immobility time elicited by CHLF at the dose 480 mg/ kg ($p<0.05$).

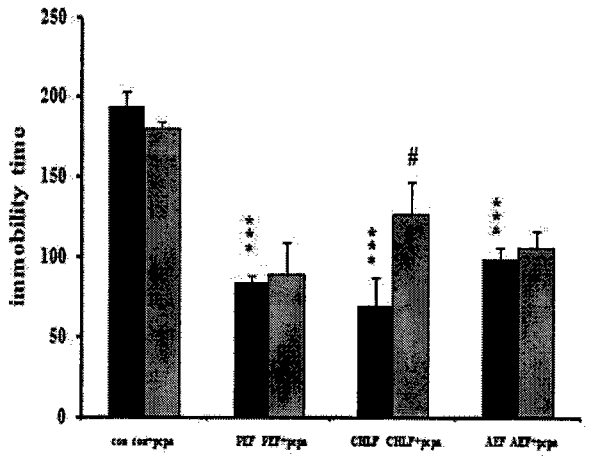


Fig. 13: Effect of pretreatment of mice with PCPA on PEF, CHLF, and EAF-elicited decrease in the forced swimming test.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; $^{\#\#}p<0.01$, significantly different from drug-treated group, ANOVA followed by Bonferroni's test (n = 10).

3.2.2.7.3. The antidepressant effects of laetispicine

3.2.2.7.3.1. The forced swimming test

In the forced swimming test (Fig. 14), the treatment with doses of 5, 10, 20 and 40 mg/kg of laetispicine significantly and dose-dependently decreased the immobility time ($p<0.001$), in comparison to control group receiving the vehicle and the inhibitions were 38.18, 39.79, 58.77 and 67.28%, respectively. The animals with clomipramine (50 mg/kg, i.p.) had a 50.29% inhibition.

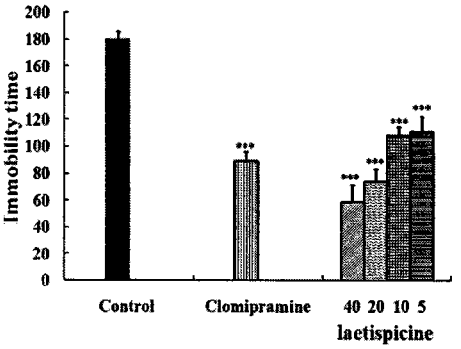


Fig. 14: Antidepressant effects of laetispicine on the forced swimming test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.2.2.7.3.2. The tail suspension test

As shown in Fig. 15, immobility time in the TST was significantly reduced after treatment with 10 and 20 mg/kg of laetispicine, whose activity are better than the positive control imipramine (30 mg/kg, i.p.), indicating a significant antidepressant-like effect. The decrease in immobility time of laetispicine at 5 mg/ kg showed no significant activity in the test.

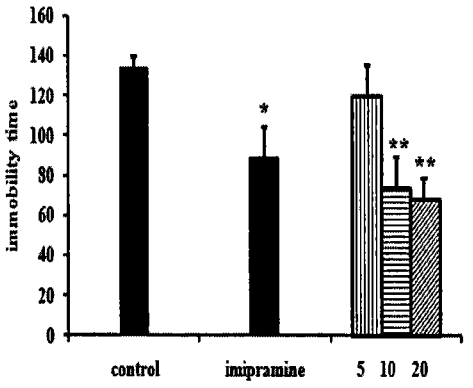


Fig. 15: Antidepressant effects of laetispicine on the tail suspension test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.2.2.7.3.3. The open field test

Table 10 shows the results of laetispicine obtained with open field test. The treatment animals with laetispicine showed no differences compared with control animals in 5-min open field test at the dose range used in the present study. The administration of clomipramine in the FST did not cause any significant change in the number of crossing and rearing in the open field test. The Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

Table 10: Antidepressant effects of laetispicine on the open field test in mice

Dose (mg/kg)	Crossing	Rearing
Control	110.5±6.32	42.43±4.45
Clomipramine	98.4±7.04	37.72±9.69
5	136.0±5.06	53.53±8.78
10	131.5±5.19	61.03±6.93
20	113.1±11.23	29.24±7.65
40	132.5±7.17	47.43±7.48

3.2.2.7.3.4. Effect of pre-treatment with PCPA on the antidepressant effect of laetispicine in the FST

The results in Fig. 16 show that PCPA alone (100 mg/ kg, once a day, for 4 consecutive days) did not modify the immobility time, and pre-treatment of mice with PCPA didn't blocked the reduction in the immobility time elicited by laetispicine (10 mg/ kg, o.p.) in the tail suspension test ($P<0.01$).

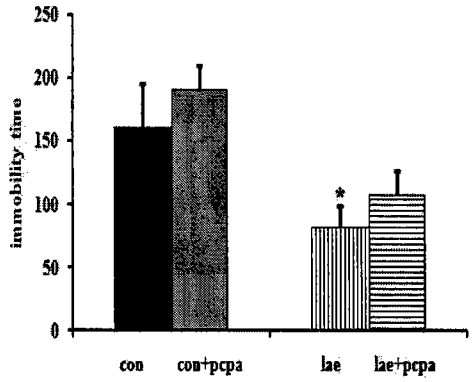


Fig.16. Effect of pretreatment of mice with PCPA on laetispicine-elicited decrease in the forced swimming test.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; $^{\#\#}p<0.01$, significantly different from drug-treated group, ANOVA followed by Bonferroni's test (n = 10).

3.2.2.7.3.5. Opioid system and NO pathway

The pre-treatment of animals with naloxone (1 mg/kg, i.p.) did not significantly affect laetispicine effect in the FST. However, the pre-treatment of mice with L-arginine (750 mg/kg, i.p.) blocked the effect of laetispicine in the forced swimming test. (Fig. 17).

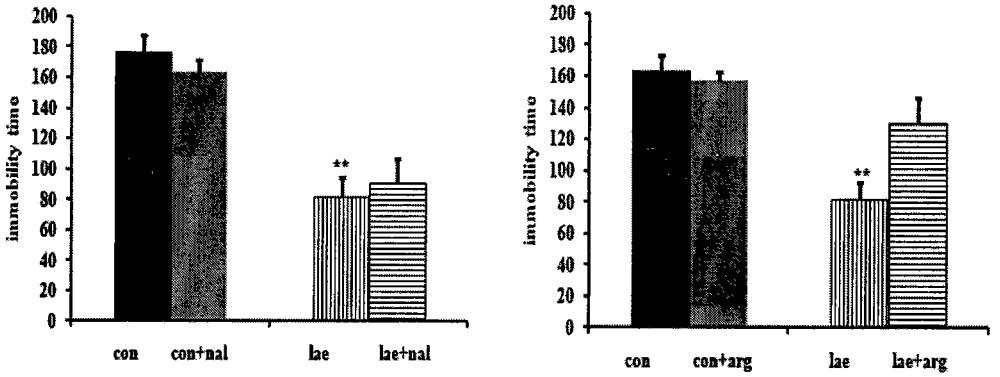


Fig.17. Effect of pretreatment of mice with naloxone or L-arginine on laetispicine-elicited decrease in the forced swimming test.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; $^{\#\#}p<0.01$, significantly different from drug-treated group, ANOVA followed by Bonferroni's test (n = 10).

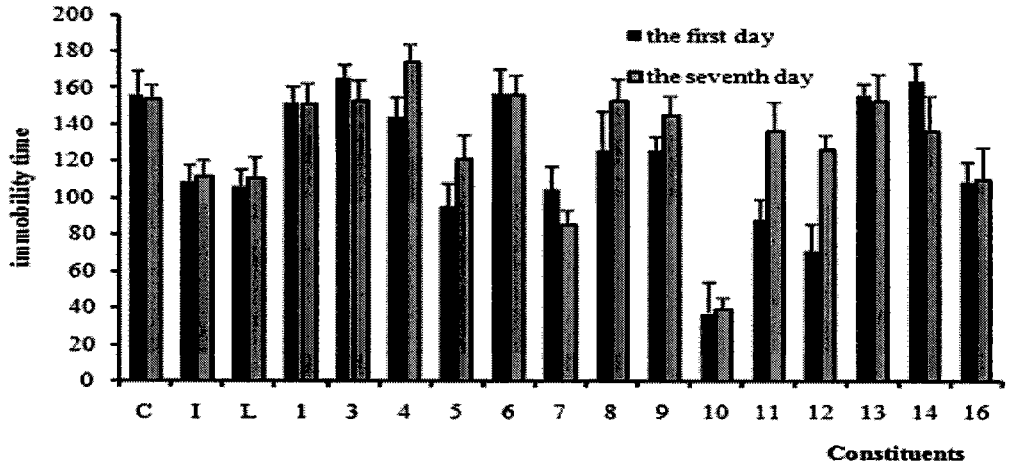
3.2.2.7.4. The antidepressant effects of derivatives of laetispicine

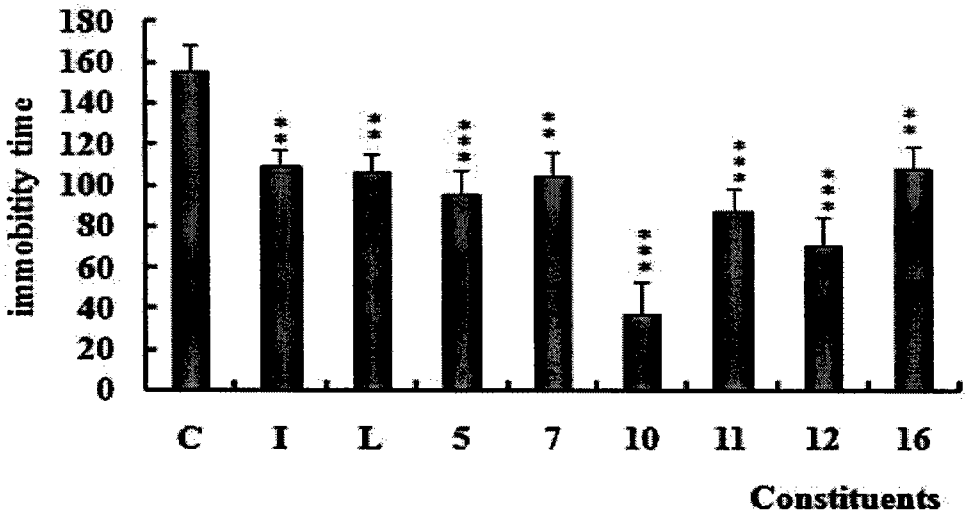
3.2.2.7.4.1. The antidepressant effect of derivatives in FST

Fig. 18 shows the effect of administration of derivatives of laetispicine (10 mg/kg, p.o.), laetispicine (10 mg/kg) and clomipramine (50 mg/kg, i.p.) in the forced swimming test. Post hoc analysis revealed that ysy-5, ysy-7, ysy-10, ysy-16, laetispicine at doses of 10 mg/kg, and clomipramine, at dose of 50 mg/ kg, led to a significant reduction in time spent immobile compared to the control group, respectively.

After the acute administration with the derivatives and laetispicine, ysy-5, ysy-10, ysy-11, ysy-12 ($p<0.001$) and ysy-7, ysy-16 and laetispicine ($p<0.01$) resulted a significant decrease in the immobility time.

The effects of administration with ysy-5, ysy-7, ysy-10, ysy-11, ysy-12, ysy-16 and laetispicine once daily for 7 days were evaluated in FST in mice. Ysy-7 and ysy-10 at the dose of 10 mg/kg reduced the duration of immobility in the FST, resulting in a 43.04%, 68.35% immobility reduction compared with the control group, respectively ($p<0.001$). Morever, the same doses of ysy-16 and laetispicine also significantly decreased immobility with a respective percent reduction of 30.38%, 29.80% ($p<0.01$); and ysy-5 resulted in 18.99% immobility reduction ($p<0.05$).





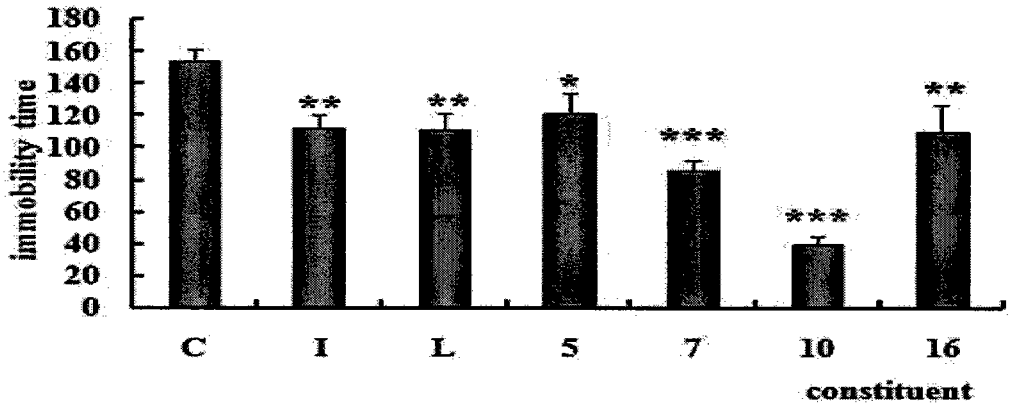


Fig.18. Antidepressant effects of derivatives of laetispicine on the FST in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.2.2.7.4.2. Open field test

The locomotor effects of the activity derivatives of laetispicine were evaluated in the open field test. Table 1 shows the results obtained with open field test. The treatment animals with ysy-5, ysy-7, ysy-10 and ysy-16 showed no differences compared with control animals in 5-min open field test at the dose of 10 mg/ kg used in the present study. The administration of clomipramine in the FST did not cause any significant change in the number of crossing and rearing in the open field test (data not shown).

3.2.2.7.5. Discussion

In this series experiments, forced swimming test and tail suspension test were used to evaluate the antidepressant effects. Both FST and TST are widely used to screen new antidepressant drugs (Porsolt et al., 1977, 1978, 1979; Steru et al., 1985). These tests are quite sensitive and relatively specific to all major classes of antidepressant drugs including tricyclics, 5-HT-specific reuptake inhibitors, MAO inhibitors, and atypical

(Porsolt et al., 1977; Steru et al., 1985; Detke et al., 1995). In FST, mice are forced to swim in a restricted space from which they cannot escape, and are induced to a characteristic behavior of immobility. This behavior, reflecting a state of despair, is reduced by several agents which are therapeutically effective in human depression. The TST also induces a state of despair in animals like that in FST. This immobility, referred to as behavioral despair in animals, is claimed to reproduce a condition similar to human depression (Steru et al., 1985; Willner, 1984).

In FST and TST, false-positive results can be obtained with certain drugs, in particular psychomotor stimulants, which decrease immobility time by stimulating locomotor activity (Bourin et al., 2001).

The present study provides behavioral evidence for the antidepressant-like activities of HEPl. In our preliminary studies, acute HEPl administration showed a significant activity to reduce the immobility time at doses of 120, 240 and 480 mg/kg in forced swimming test in mice. Considering that clinical antidepressant effects often appear after chronic treatment, HEPl was administrated orally for 7 consecutive days for the investigation of the antidepressant-like property in mice in FST. In this model, HEPl produced a dramatic inhibition of the duration of immobility, with a profile comparable to that observed for the classical antidepressant drug imipramine. Though the antidepressant action of HEPl was less potent than imipramine based on the given data, the effect of HEPl, as well as other herbal medicine, is slow, mild and lasting, without (or with lower) undesirable side-effects; these are advantages over the classical antidepressants.

As changes in the duration of immobility could also result from effects on locomotor activity caused by central nervous system stimulants, the mice were tested in the open field test just before FST. The results showed that HEPl, at doses that produced an antidepressant-like effect, did not significantly change locomotor behaviour. Therefore, HEPl appears to produce a specific antidepressant-like behavioral effect.

The antidepressant effects of PEF, CHLF, EAF and AQF were evaluated in the forced swimming test in mice to find the responsible fraction(s) for the antidepressant activity of HEPl. The results showed that the acute administration with AQF did not alter the parameters in FST (Fig. 3); the PEF, CHLF and EAF at the given doses have good antidepressant activities in a dose-dependent manner. Compared with the control group, PEF and CHLF at the doses of 480 mg/kg ($p<0.001$) have the similar effect with positive control-imipramine at 30 mg/kg ($P<0.001$), while the antidepressant effect of EAF ($p<0.01$) is little weaker than imipramine. The activity at dose of 120 mg/kg in EAF was not found. However, 120 mg/kg orally administration of PEF and CHLF created significant decreases in the immobility time ($p<0.05$ and $p<0.01$, respectively). All the three fractions' antidepressant–like effect was not associated with the locomotor effects. And the number of readings was decrease (Fig. 4) by the mice treated by imipramine and CHLF. Antidepressants reduced immobility time at doses that either do not change or even decrease motor behavioral in the open tests (Kirby and Lucki, 1997). The mechanisms of these three fractions were evaluated (Fig. 5). In the present study, prior administration of PCPA (an inhibitor of serotonin synthesis) didn't reduce the antidepressant-like action caused by PET and EAF in the

FST, but it blocked the antidepressant activity caused by CHLF. These findings suggested that a serotonergic mechanism may not be involved in the antidepressant-like effect of PEF and EAF. However, serotonergic system takes part in, at least partially, the mechanism of action of CHLF in anti-immobility time activity.

For finding the compounds responsible to the antidepressant activity, the first and main amid alkaloid from *P. laetispicum* were evaluated in FST and TST.

In the present work, we demonstrated that laetispicine, a bioactive amide isolated from *P. laetispicum*, was effective in producing antidepressant effect when assessed in the forced swimming, tail suspension and open field test.

The results presented here show, to our knowledge for the first time, that laetispicine given orally is effective in producing significant antidepressant-like effects, when assessed in FST and in TST. The antidepressant-like effect of laetispicine in FST and TST was stronger than that of clomipramine and imiparimine, used as a standard antidepressant in a dose of 50 mg/ kg in FST and 30 mg/ kg in TST. The antidepressant effect of laetispicine at 5 mg/kg was not found in TST. The reasons might be related to the different strain of the mice. In FST and TST, false-positive results can be obtainedwith certain drugs, in particular psychomotor stimulants, which decrease immobility time by stimulating locomotor activity (Bourin et al., 2001). The anti-immobility effect of laetispicine seems not to be associated with any motor effects, since mice treated with laetispicine did not exhibit increased ambulation when tested in amotor activity meter (Table 1). This indicates that a psychostimulant effect

is not responsible for the decrease in the immobility elicited by laetispicine in both FST and TST. The antidepressant-like effect of laetispicine is specific.

The precise mechanisms by which laetispicine produced antidepressant-like effect are not completely understood.

The serotonergic system has been recognised as playing an important role in mood disorders and specifically in the aetiology of depression and pain, and drugs acting on serotonergic system have been implicated in the treatment of depressive disorders and pain (Risch and Nemeroff, 1992; MacFarlane, 1997; Millan, 1999; Fürst, 1999). According to our group study, laetispicine has also the antinociceptive effects. Thus, the possible involvement of the serotonergic system in the antidepressant-like effect of laetispicine was investigated. In the present study, prior administration of PCPA (an inhibitor of serotonin synthesis), at a dose known to inhibit serotonin synthesis (Pini et al., 1996), didn't reduced the antidepressant-like action caused by laetispicine in the TST. This finding suggested that a serotonergic mechanism may not be involved in the antidepressant-like effect of laetispicine. And our data also demonstrated that the activation of the opioid system seem unlikely to be involved in the antidepressant-like effect of laetispicine in the FST, while the L-arginine-nitric oxide pathway might be partially involved in the antidepressant effect of laetispicine.

In summary, results from the present study show that HEPl, PEF, CHLF, EAF and laetispicine exert an antidepressant activity in FST and TST in mice. Our results point out to the potential use of fractions or laetispicine as an antidepressant drug. However, the antidepressant activity of other compounds from *P. laetispicum* and the possible

mechanisms deserve further attention.

A series of laetispicine derivatives were synthesized and evaluated as potential antidepressants. The compounds were evaluated in FST to reveal potential antidepressant activity and the existence of undesirable side effects. Several derivatives were more active than imipramine. On the basis of its activity in FST, ysy-7, ysy-10 and ysy-16 have the potential for further development. The structure-activity relationship of derivatives of laetispicine can provide important information to decrease the toxicity and improve the effects of the natural products.

Section 3

3.3. The anxiolytic activity of *Piper laetispicum*

3.3.1. Introduction

Anxiety disorders are probably the most common of the psychiatric disorders, with a lifetime prevalence of about 15 to 20% in the general population (Rosenbaum and Gelenberg, 2001). Anxiety is a very diffuse mental condition characterised by an unpleasant feeling of fear and apprehension with no identifiable source.

Anxiety can be experienced in a number of ways, including behaviourally, affectively, physically and cognitively. Behaviourally, anxiety manifests as fight or flight responses, help seeking and excessive dependence on others. Emotionally, anxiety can produce a dysphoric state of arousal, such as during a panic attack, which is exceedingly unpleasant. Physically, a person may experience cardiac (e.g. chest tightness and palpitations), neurological (e.g. paresthesias and tremulousness), gastrointestinal (e.g. diarrhoea and nausea) or respiratory (e.g. hyperventilation and dyspnoea) symptoms. Cognitively, a person may engage in catastrophic thinking, hypervigilance, apprehension, rumination and worry (American Psychiatric Association, 1994).

A broad range of non-pharmacological treatments is available for specific anxiety disorders. For example, systematic desensitisation is effective in greatly reducing or eliminating phobias (McGlynn, 1994). However, the treatment of choice for anxiety and anxiety disorders, especially by family physicians, is pharmacological. Drug

treatment has been proven effective and is a lot less labour intensive than psychosocial therapies. Many anxiety disorders respond well to antidepressants [especially the selective serotonin reuptake inhibitors (SSRIs), venlafaxine, tricyclic antidepressants and monoamine oxidase (MAO) inhibitors] and anti-anxiety agents (especially benzodiazepines) (Janicak et al., 1991; Barrett and Rapaport, 2000). From the early 1960s, the benzodiazepines became the most frequently prescribed anti-anxiety agents. Other benzodiazepines typically prescribed for anxiety disorders include chlorazepate, chlordiazepoxide, clonazepam, diazepam, halazepam, lorazepam, oxazepam and prazepam. The common adverse effects of benzodiazepines include sedation, impaired cognition and ataxia. Their behavioural adverse effects include irritability, depression, hyperactivity, aggression and disinhibition (Van Der Bijl and Roelofse, 1991; Bond, 1998). Other limitations include their potential for abuse (because of their rapid absorption and onset of action), tolerance, dependence, rebound after discontinuation and the likelihood of increasing the intoxicating potency of alcohol. Nonbenzodiazepine anxiolytics, such as buspirone, have an efficacy similar to benzodiazepines but have fewer serious adverse effects and less risk of a fatal consequence due to overdose (Janicak et al., 1991). Other agents with anxiolytic properties, such as the SSRIs and venlafaxine, may cause some forms of sexual dysfunction, which is the cause of much morbidity and often leads to patient noncompliance (Janicak et al., 1991).

As at present the etiologic factors responsible for anxiety and tension are not expected to decrease, there is a need for new anxiolytic drugs with less potential to induce

adverse reactions.

Among the natural products that are considered to be efficacious in the management of anxiety and stress, *Piper methysticum* is one of the most famous medicinal plants. *Piper methysticum* (kava kava) is a perennial plant native to the Pacific Island region, and has been used ceremonially for thousands of years. Traditionally, a beverage is prepared, and then drunk before the evening meal. Indigenous methods of mastication of the kava root have given way to grinding or pounding the plant substance, which is then mixed with water or coconut milk. A water infusion of kava is prepared from a powder or macerate of the dried root and rhizome, where much of its biological activity is found. The claimed therapeutic effects of the herb have made it in many parts of the world a popular nonprescription treatment for alleviating mild to moderate cases of nervous anxiety, stress, insomnia, restlessness and muscle fatigue (Singh, 1997; Blumenthal et al., 1998). In some countries – Germany for instance – it is also available by physician's prescription. The active constituents that are responsible for the pharmacological activity of kava are kavapyrones or kavalactones.

Piperine is the first amide isolated from *Piper* species (*Piper longum and Piper nigrum*), and displays anxiolytic activities (Wattanathorn et al., 2008). Piplartine, an amide alkaloid isolated from the roots of *Piper tuberculatum*, was reported to have anxiolytic effect (Cícero Bezerra Felipe, F., et al., 2007). Based on the above information, this series of experiments are designed to verify the anxiolytic effect of ethyl acetate extract and laetispicine in several classical animal models. In addition, the mechanism of action of the extract and laetispicine were studied.

3.3.2. Material and Methods

3.3.2.1. Ethyl acetate extract and laetispicine from *Piper laetispicum*

Piper laetispicum C. DC. (Piperaceae), an endemic climbing, glabrous plant available in the southern part of China, was collected in 2007 from Hainan province, China, and was identified by Prof. Sheng-li Pan, School of Pharmacy, Fudan University, where a voucher specimen (No. 060812) of the plant material has been deposited for further reference.

3.3.2.2. Fractionation

The crude extract (20g) was suspended in water and partitioned successively with petroleum ether, and ethyl acetate. The ethyl acetate fraction was combined and evaporated under reduced pressure. The yield of the ethyl acetate fraction was 6 g (30%).

3.3.2.3. Laetispicine

The hydroethanolic extract of the stems was dissolved in water and fractionated with petroleum ether and ethyl acetate, respectively. The ethyl acetate fraction was combined, concentrated under reduced pressure to give a dark brown colored semisolid extract (60 g). Then the ethyl acetate extract was chromatographed on a silica gel column by a gradient elution using mixtures of petroleum ether and ethyl acetate (20:1-9:1-5:1-3:1-2:1-1:1) to afford six fractions. Fraction 3 was concentrated under reduced pressure and subjected to silica gel column chromatography and eluted with chloroform and methanol (50:1), to give four subfractions. Laetispicine (300 mg) was separated from subfraction 3 by repeated chromatography on silica gel column,

eluted with petroleum ether and acetone.

Ethyl acetate extract and laetispicine obtained were kept in refrigerator at +4 ℃ and suspended in a solution of 2% tween80 prior to any pharmacological screening. Respective controls received only that solvent as vehicle.

3.3.2.4. Drugs

95% ethanol, petroleum ether, ethyl acetate, distilled water, tween80, diazepam and flumazenil

3.3.2.5. Animals and habituation

Swiss albino male mice (OF1) aged 9 weeks at the receipt from the breeder company (Charles River, France) and weighing 40–45 g, were used. The animals were housed under a 12-h light: 12-h dark schedule (lights on starting at 8:00 p.m.) with water and food ad libitum (SD Dietex-France). Animal rooms were at a constant temperature of 21±2 °C and relative humidity of 55±10%. All animal procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/ 609 / EEC).

3.3.2.6. Pharmacological tests

3.3.2.6.1. Light/dark choice test

The light/dark choice test was performed according to previously described methods with minor modifications (Crawley and Goodwin, 1980; Anseloni et al., 1995; Bailey et al., 2007). The Light/ dark box apparatus consists of two compartments (light/ dark, surface ratio 3:2), divided into 15 squares: 9×9 cm. The dark box (black PVC,

27×18×29 cm) is illuminated by a dim red light and is divided into six squares. The lit box (white PVC, 27×27×29 cm) is illuminated by a white light located 1.50 m above the device and is divided into 9 squares. The two compartments communicate by means of a small door (7×7 cm). All the experiments were carried out in a dark room and after each test the light/dark box was cleaned with a 10% ethanol solution. Each test took 5 min. At the beginning of the test, each animal was placed in the middle of lit box with its head facing the door of the dark box. The amount of time spent in the lit box, the number of entries into the lit box (all four feet) were recorded. Drugs were orally administered 60 min before testing (vehicle-treated group, ethyl acetate extract-treated groups, laetispicine-treated groups) or intraperitoneal administered 30 min before testing (diazepam-treated group and flumazenil-treated group). Ten animals per group were used for each drug. All doses were administered in a volume of 10 ml/kg. Mice were randomly allocated to the following groups: vehicle control (2% Tween 80), ethyl acetate extract (120 mg/kg, 240 mg/kg in 2% Tween 80), Laetispicine (5 mg/kg, 10 mg/kg, and 20 mg/kg in 2% Tween 80), diazepam (2 mg/kg in saline), flumazenil (5 mg/kg in saline). Flumazenil was applied at dose 5 mg/kg administered in combination with ethyl acetate extract at 240 mg/kg (group: 240+Flu), laetispicine at 10 mg/kg (group: 10+Flu) and diazepam at 2 mg/kg (group: Dzp+ Flu)

3.3.2.6.2. Elevated plus maze test

The elevated plus-maze test was performed as described (Holmes et al., 2002). All parts of the apparatus were made of dark polyvinyl plastic. The maze was elevated to a height of 50 cm and had two open (30 × 5 cm) and two closed arms (30×5×15 cm),

arranged in a manner that the arms of the same type were opposite to each other and connected by an open central square (5×5 cm). To prevent mice from falling of the open arms, a rim (2.5 mm high and 8 mm deep) surrounded the perimeter of the open arms. At the beginning of the 5-min test session, mice naive to the apparatus and with no previous drug treatment were placed individually in the central square of the maze, facing one of the closed arms. An entry in the arm was counted when the animal placed all four paws into the arm. The total number of visits to the open arms, the total number of visits to the closed arms and the cumulative time spent in the open arms were recorded. The results were expressed in number of entries into closed arms, percentage of time spent in the open arms and percentage of entries into open arms. After each test, the apparatus was cleaned with a 10% ethanol solution. Drugs were orally administered 60 min before testing (vehicle-treated group, ethyl acetate extract-treated groups, laetispicine-treated groups) or intraperitoneal administered 30 min before testing (diazepam-treated group). Ten animals per group were used for each drug. All doses were administered in a volume of 10 ml/kg. Mice were randomly allocated to the following groups: vehicle control, laetispicine (5, 10 and 20 mg/ kg), diazepam (2 mg/ kg).

3.3.2.6.3. Hole-board test

The hole-board apparatus consisted of gray Perspex panels (40×40 cm, 5 cm thick) with 16 equidistant holes, each 3 cm in diameter, on the floor. Photocells below the surface of the hole measured the number of head dips. The board was positioned 15 cm above a table. Mice were randomly allocated to the following groups: control (2%

Tween 80, p.o.), diazepam (2 mg/ kg, i.p.), and laetispicine (5, 10 and 20 mg/kg, p.o.). Each mouse was individually placed on the center of the board facing away from the observer and allowed to freely roam about the apparatus. Diazepam and laetispicine were administered 30 and 60 min before the test, respectively. The number of head dips on the hole-board was counted for 5 min (Takeda et al., 1998; Wei et al., 2007; Silva et al., 2007). After each trial, the floor of the apparatuswas wiped with 10% methanol to remove traces of previous paths. The test sessions were recorded with a camera mounted vertically above the hole-board.

3.3.2.6.4. Open field test

The open field was a 400×400 mm gray plastic arena with 300 mm high walls surrounding the field. Thin black stripes were painted across the floor dividing it into 16 quadratic blocks. The open field instrument was cleaned after each test session to prevent the next mouse from being influenced by the odors deposited in the urine and feces of the previous mouse. The mouse was placed in the center of arena and an observer manually quantified the mouse's spontaneous ambulatory locomotion for 5 min. During this period, the number of squares crossed and the number of rearings were measured.

3.3.3. Statistical analysis

Data obtained were expressed as mean ± S.E.M and analyzed by analysis of variance (ANOVA) followed by Bonferroni's test. *P-values* less than 0.05 ($p<0.05$) were used as the significant level.

3.3.4. Results

3.3.4.1. The anxiolytic effects of ethyl acetate extract

3.3.4.1.1. The light/dark test

The *Piper laetispicum* ethyl acetate extract increased significantly the time in the light box, at a dose of 240 mg/ kg, whereas the effect at 120 mg/ kg were not significantly different from the control group, treated with vehicle (Fig. 1b). Compared with the control group, the transitions at doses of 120 and 240 mg/ kg were not significantly changed (Fig. 19a). The positive group (Diazepam 2 mg/ kg, i.p.) significantly increased the transitions and prolonged the time in lit box. To verify if the anxiolytic effect of ethyl acetate extract was due to an interaction with BZD receptors, we pre-treated mice with Flumazenil at the dose of 5 mg/kg. The results reported in Fig. 1 demonstrated that the anxiolytic effect of the ethyl acetate extract is not blocked by the administration of BZD receptors antagonist, Flumazenil (5 mg/ kg, i.p.).

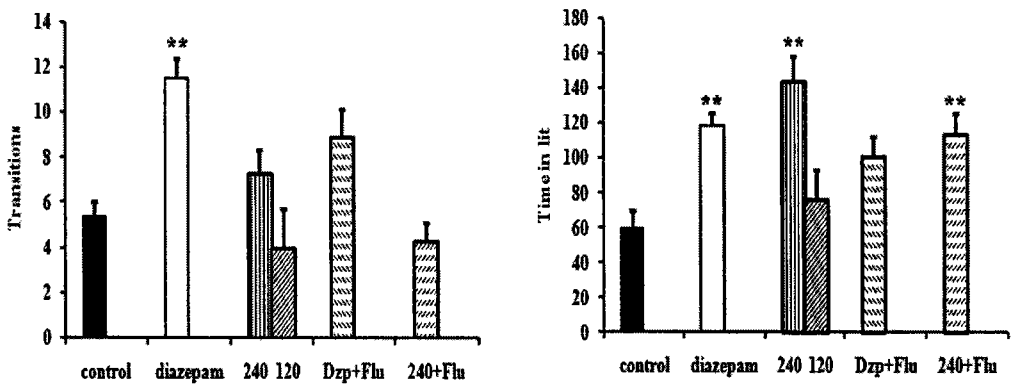


Fig. 19: Anxiolytic effects of ethyl acetate extract on the light/dark test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10). Dzp: diazepam; Flu: flumazenil.

3.3.4.1.2. The elevated plus maze

The behavioral effects of ethyl acetate extract or diazepam on mice behavior in the elevated plus-maze are summarized in Fig. 20. A single ethyl acetate extract treatment at 240 mg/ kg produced an anxiolytic-like effect in the percent of open time parameter ($p< 0.05$). As shown in the vehicle-treated group, mice typically avoided spending time in or entering open arms (Fig. 20b, 20c). Vehicle-treated mice remained in open arms for 12.4 ± 5.21 s, whereas ethyl acetate extract-treated mice (240 mg/ kg) spent significantly more time (36.3 ± 13.9 s) in open arms ($P < 0.05$). However, ethyl acetate extract-treated mice (240 mg/ kg) didn't make significantly entries into open arms than vehicle-treated mice (Fig. 20c). In addition, no significant change was observed in terms of the time spent or number entries at 120 mg/ kg. Moreover, the diazepam-treated (2 mg/ kg, i.p.) group significantly increased time spend and number entries in open arms than the vehicle-treated group ($P < 0.05$).

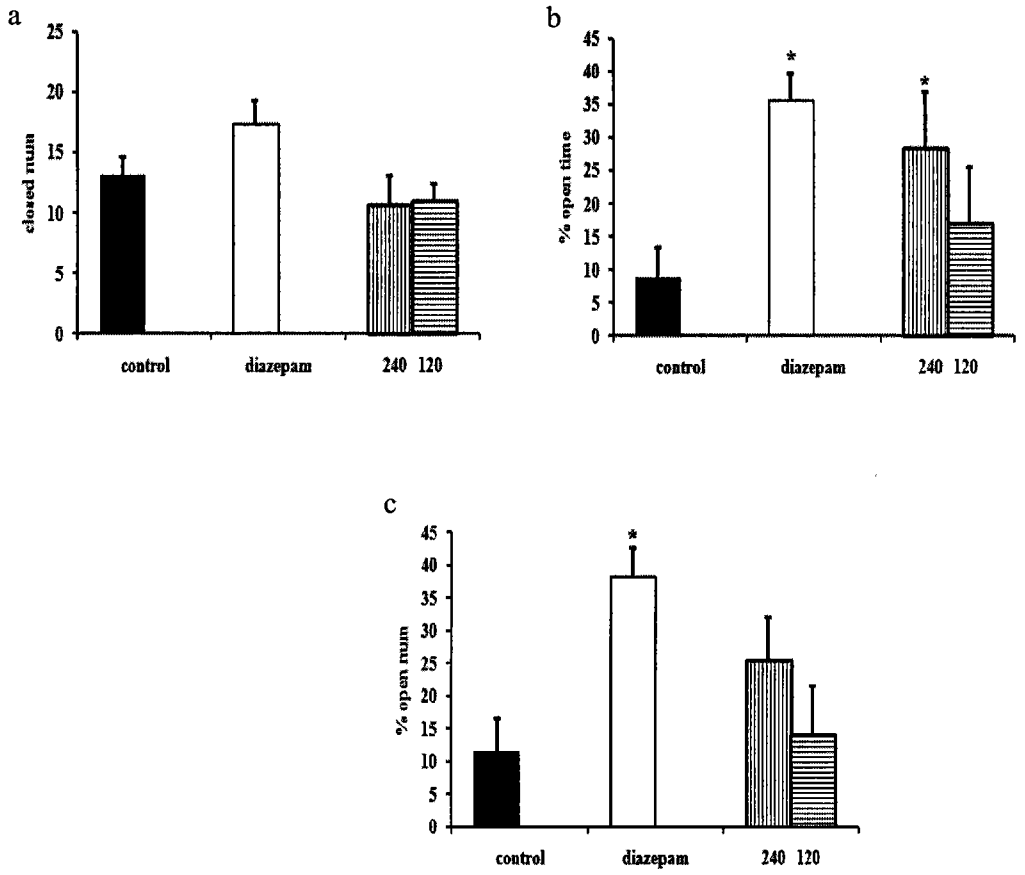


Fig. 20: Anxiolytic effects of ethyl acetate extract on elevated plus maze test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.3.4.1.3. Open field test

The mice were administrated 120, 240 mg/kg of ethyl acetate extract. The number of crossed squares and the number of rearings by the group treated with ethyl acetate extract were not significantly different compared to the control group (data not shown) indicating that ethyl acetate extract did not cause any difference in spontaneous activity or locomotion compared with the control mice.

3.3.4.2. The anxiolytic effects of laetispicine

3.3.4.2.1. The light/dark test

Results of the light/dark test are shown in Fig. 21. Comparisons between the vehicle control group and experimental groups (Bonferroni's test) indicated that the administration of diazepam at 2 mg/ kg significantly increased ($p<0.01$) the number of transitions between the two compartments and the time spent by mice in the light area. Laetispicine, at the doses of 10 mg/kg, significantly increased the number of transitions between the two compartments and the time spent in the light area and (both $P< 0.01$), while the significant effects at the doses of 5 mg/ kg and 20 mg/ kg were not found. These data point out to an inverted U-shaped activity of the total extract. Finally, to verify if the anxiolytic effect of laetispicine was due to an interaction with BZD receptors, we pre-treated mice with Flumazenil at the dose of 5 mg/ kg. The data reported in Fig. 21 demonstrate that the anxiolytic activity of laetispicine was not inhibited by the BZD antagonist.

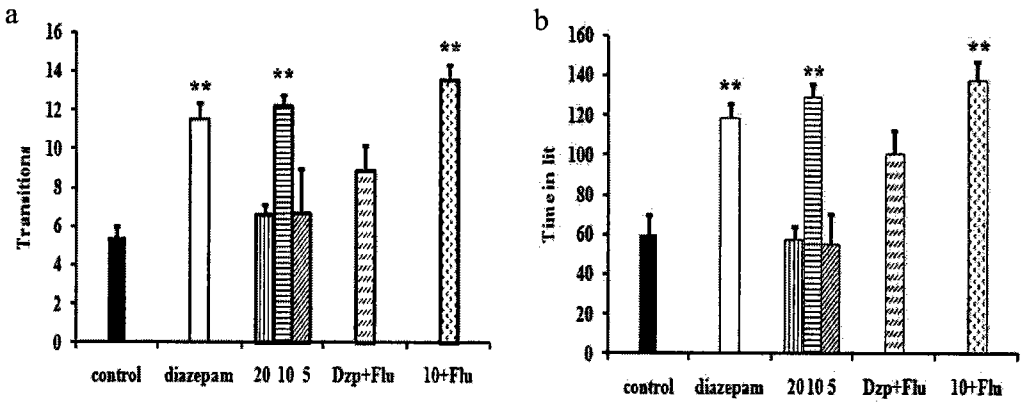


Fig. 21: Effects of laetispicine on the light/dark test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10). Dzp: diazepam; Flu: flumazenil.

3.3.4.2.2. The elevated plus maze

The results are shown in Fig. 22. Both laetispicine (10 mg/ kg) and diazepam (2 mg/ kg) resulted in a significant increase in entries into open arms and the percentage of time ($P < 0.05$ and $P < 0.01$), compared with the control group. However, some differences were observed between the two drugs, e.g., diazepam increased open arm entries and total arm entries at the same time while laetispicine had no such effect.

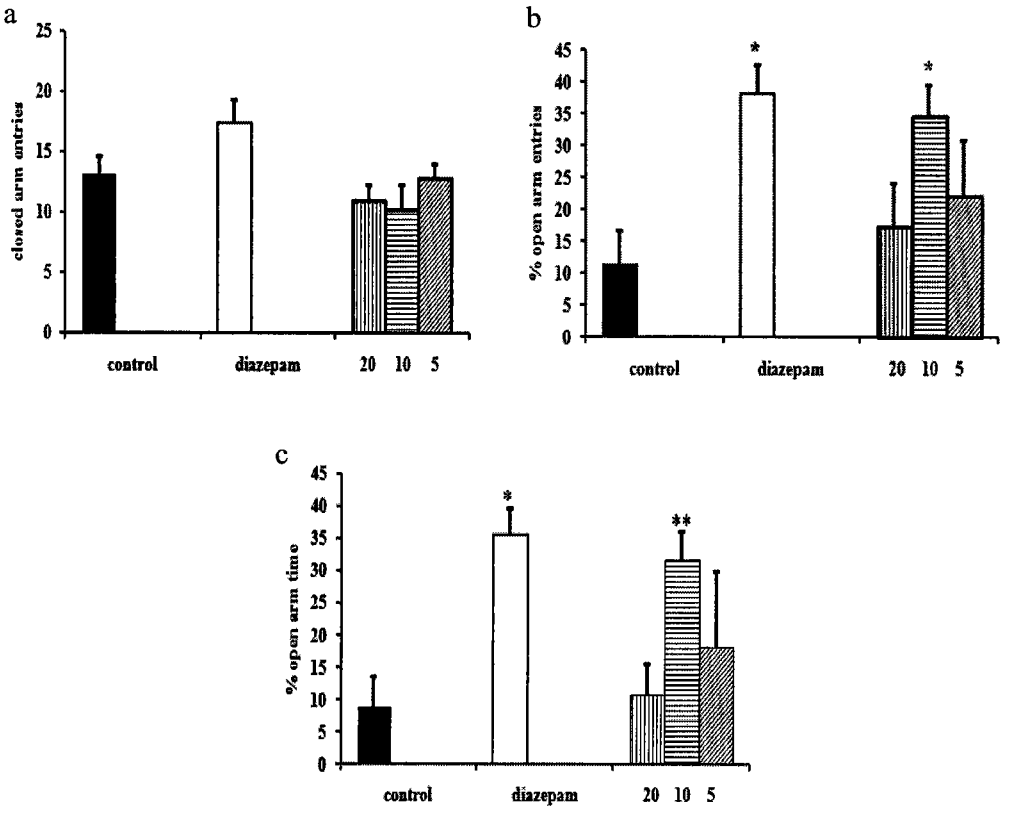


Fig. 22: Effects of laetispicine on the elevated plus maze test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.3.4.2.3. The hole-board test

The data are summarized in Fig. 23. Laetispicine (10 mg/kg) significantly increased head-dip counts ($p < 0.01$), whose effect was better than diazepam (2.0 mg/kg, $p<0.05$).

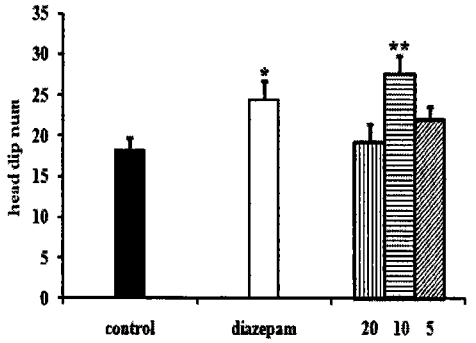


Fig. 23: Effects of laetispicine on the hole-board test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.3.4.2.4. The open field test

To determine whether a possible stimulatory effect of laetispicine modified exploratory behavior, we performed a spontaneous locomotor activity test. However, laetispicine (5, 10 and 20 mg/ kg) produced no significant changes in the number of crossed squares and the number of rearings versus the vehicle control group (Fig. 24).

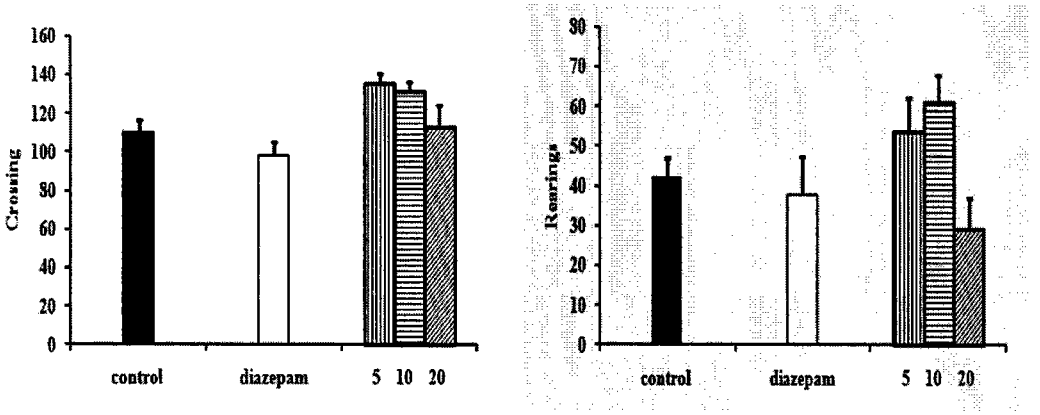


Fig. 24: Effects of laetispicine on the open field test in mice.

Values are mean ± S.E.M. $^{**}p<0.01$, significantly different from vehicle-treated control; ANOVA followed by Bonferroni's test (n = 10).

3.3.5. Discussion

A number of animal models of anxiety have been reported for evaluating new

compounds with potential anxiolytic action, and for experimentally reproducing the effects of drugs with clinical efficacy for treatment of generalized or panic anxiety (File, 1990). They also have been used to investigate the pathophysiological mechanisms underlying such emotional disorders.

In this work, the light/dark test, elevated plus maze test and hole-board test were used to verify the anxiolytic activities of ethyl acetate extract and laetispicine from *P. laetispicum*. And open field locomotion test was performed to evaluate the locomotion of ethyl acetate extract and laetispicine. It was demonstrated that the administrations of the ethyl acetate extract and laetispicine in mice were able to induce anxiolytic effects, without modifying significantly the spontaneous motor activity. For ethyl acetate extract, only the anxiolytic effects at doses of 120 and 240 mg/kg were evaluated, because of the toxicity effect at the dose of 500 mg/kg (see Chapter 4).

The light/dark choice procedure is a typical conditioned conflict situation based on the natural tendencies of mice to avoid a bright-lit enclosure and to escape from a novel environment when forced into it (Crawley, 1981; Misslin, 1989). The light/dark box is widely used for rodents as a model for screening anxiolytic or anxiogenic drugs, based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of rodents in response to mild stressors, that is, a novel environment and light. In the present study, the transitions and the time in lit were recorded. However, it has been reported that simply the measurement of the time spent in the light area, but not the number of transfers, is the most consistent and

useful parameter for assessing an anxiolytic action (Young and Johnson, 1991).

By the light/dark model of anxiety we demonstrated the ability of the ethyl acetate extract (240 mg/kg) to counteract anxiety in mice, submitted to an aversive stimolous (light). The fact that the anxiolytic effect was not blocked by the injection of a BZD antagonist, flumazenil, suggests the BZD receptor system didn't involved in the anxiolytic effect of *Piper laetispicum* ethyl acetate extract.

Fig. 3 showed that laetispicine (10 mg/ kg) could increase both the time in the light area and transitions between the light and dark box, suggesting that laetispicine possesses anxiolytic properties. But the anxiolytic effects of laetispicine at 5mg/ kg and 20mg/ kg were not found.

The anxiolytic effect was also evidenced through the elevated plus maze test. The elevated plus-maze is a well-established animal model for testing anxiolytic drugs (Dawson and Tricklebank, 1995; Kulkarni and Reddy, 1996). The elevated plus maze represents a forced exploration, i.e., no non-stressed situation, since all parts of the situation are novel (Lister, 1990; Belzung and Le Pape, 1994). This is a frequently used animal model of anxiety due to the use of unconditioned spontaneous behavior (Rodgers and Dalvi, 1997). In this test, the percentages of entries into open arms and of time spent in open arms have generally been used as indices of anxiety. Generally, an anxiolytic agent can increase the frequency of entries into open arms and increased the time spent in open arms of the elevated plus maze.

In the present study, single administration of ethyl acetate extract prolonged the time spent in the open arms, but didn't increase the number of entries into open arms (Fig.

2).

Fig. 4 showed that laetispicine at 10 mg/ kg could increase the time in the light area and the number of entries into the open arms, suggesting again that laetispicine possesses anxiolytic properties. At the same time, the entries of closed arms was not affected by the administered of laetispicine.

The hole-board test, which was first introduced by Boissier and Simon (1962, 1964), provides a simple method for measuring the response of an animal to an unfamiliar environment and is widely used to assess emotionality, anxiety and/or responses to stress in animals. Takeda et al. (1998) showed that head-dipping behavior was sensitive to changes in the emotional state of the animal, and suggested that the expression of an anxiolytic state in animals may be reflected by an increase in head-dipping behavior.

In the present study, laetispicine at dose of 10 mg/ kg increased head - dip counts. These results indicate that laetispicine has a significant anxiolytic effect in this paradigm. On the other hand, the unaltered result of the open field test for the ethyl acetate treated group and laetispicine treated group indicated that both ethyl acetate extract and laetispicine caused neither a stimulating nor sedating effect at the doses tested in this study.

Based on the results of our behavioral experiments, ethyl acetate extract and laetispicine did not alter spontaneous behavior, exerted significant anxiolytic effect at the chosen dosage regimen and the anxiolytic effects were not involved by the BDZ receptor.

The dose – response curve of laetispicine was bell-shaped in three animal tests in our work. It has been reported that some non-classic anxiolytic compounds, such as 5-HT3 receptor antagonists and 5-HT4 receptor antagonists (Vasar et al., 1993; Silvestre et al., 1996), also have a bell-shaped dose–response curve in some animal tests. These phenomena suggest that (1) anxiety may be a nervous disorder mediated by multiple neuronal pathways in the central nervous system, and (2) different models of anxiety test predominately represent activities of certain neuronal systems. Therefore, under certain circumstances, some drugs cannot express their dose-dependent effects in anxiolytic studies.

Several reports existed on compounds isolated from the ethyl acetate extract of *Piper laetispicum* stem bark including amides, ligants and fatty acids (Fang et al., 2007; Pan et al., 2005). Of these constituents, amide alkaloids are the main constituents in *Piper* genus. In this class of compounds, piperine and piplartine were known to have good anxiolytic activity (Cícero Bezerra Felipe, F., et al., 2007; Wattanathorn et al., 2008). Laetispicine is one of the main constituents in the ethyl acetate extract of *P. laetispicine.* The photochemical results in our group showed that laetispicine is a specific pure compound, only found in *Piper laetispicum* within medicinal plants of piper genus checked in our group. From the results we have gotten, at least, laetispicine is partially responsible to the anxiolytic activity of ethyl acetate extract.

From the present experiments we can conclude that both ethyl acetate extract and laetispicine have anxiolytic effects without changing the locomotor behavior, but the anxiolytic activity of ethyl acetate extract is weak. Furthermore, as far as regards the

mechanism of action, it is noteworthy that the effect of ethyl acetate extract and laetispicine were not blocked by an antagonist of BZD receptors. The present study demonstrated for the first time that ethyl acetate extract and laetispicine have anxiolytic effects, while the mechanisms of actions were still unclear.

3.3.6. Perspectives

Pain, depression and anxiety symptoms are highly prevalent conditions.

Depression and painful symptoms commonly occur together. The prevalences of pain in depressed cohorts and depression in pain cohorts are higher than when these conditions are individually examined. When pain is moderate to severe, impairs function, and/or is refractory to treatment, it is associated with more depressive symptoms and worse depression outcomes (e.g., lower quality of life, decreased work function, and increased health care utilization). Similarly, depression in patients with pain is associated with more pain complaints and greater impairment. Depression and pain share biological pathways and neurotransmitters, which has implications for the treatment of both concurrently.

Major depressive disorder (MDD) affects approximately 120 million people every year (or approximately 5.8% of men and 9.5% of women; Anxiety disorders are second only to MDD as the most commonly diagnosed psychiatric conditions in primary care. About 7% of men and 12% of women suffer from an anxiety disorder in any year. 'Anxious depression' or 'co-thymia' are terms used to diagnose the presentation of non-psychotic depression that co-occurs with anxiety, and the 12-month prevalence of generalised anxiety disorder (GAD) is about 3%. Comorbidity between depressive and anxious disorders commonly occurs. Approximately 60–80% of patients with GAD will suffer from a mood disorder within their lifetime, and MDD and GAD have been observed to share common familial, genetic and biochemical traits.

In fact, in our research, laetispicine is the emphasis. The activities of laetispicine were evaluated totally in 7 animal models and the results showed significantly effects on these models in vivo. In addition, the mechanisms of laetispicine in BZD receptor, opioid system, No pathway and 5-HT system were studied. Only one positive result was obtained in No pathway. The effects of laetispicine in 5-HT, NE and DA in vitro were tested by Dr. Wang before in our group, which means the negative effects. With all the results obtained from our group, we have to say the data disappointed us. While these negative data also means a huge hope. Why? Because of the side effects of current agents, new drugs on pain, depression and anxiety are in urgent need. Let's take antidepressants as an example, all available antidepressant medications are based on serendipitous discoveries of the clinical efficacy of two classes of antidepressants more than 50 years ago. These tricycle and monoamine oxidase inhibitor antidepressants were subsequently found to promote serotonin or noradrenaline function in the brain. Some newer agents are more specific but have the same core mechanisms of action in promoting these monoamine neurotransmitters. This is unfortunate, because only ~50% of individuals with depression show full remission in response to these mechanisms. This review summarizes the obstacles that have hindered the development of non-monoamine-based antidepressants, and provides a progress report on some of the most promising current strategies. So, maybe laetispicine can give us a big surprise in sooner future.

Accordingly, we suggest that the further mechanisms research might focus on:

1. GABA and NMDA receptors

The role for the amino acid neurotransmitter y-aminobutyric acid (GABA) in mood disorders was first proposed 15 years ago, based on the clinical observation that valproic acid, a GABA agonist, was effective in the treatment of bipolar disorder. Since then, considerable data, both preclinical and clinical, have accumulated supporting the involvement of GABA in mood disorders. The relationship between GABA and pain was published in *Nature* by Dr. Luc Jasmin.

N-methyl-D-aspartate (NMDA) receptor antagonists have therapeutic potential in numerous CNS disorders ranging from acute neurodegeneration (e.g. stroke and trauma), chronic neurodegeneration (e.g. Parkinson's disease, Alzheimer's disease, Huntington's disease, ALS) to symptomatic treatment (e.g. epilepsy, Parkinson's disease, drug dependence, depression, anxiety and chronic pain).

2. Antioxidant activities

Oxidative stress is involved in many acute and chronic diseases including cancer, cardiovascular disorders and neurodegenerative diseases. Oxidative free radicals increase during chronic depression and are reported to play an important role in the pathogenesis of depression. In 2005, Hovatta et al. first described a relationship between oxidative stress and the level of anxiety. In summary, the GABA, NMDA and the antioxidant activities should be the future mechanisms research emphasis of laetispicine.

4. Chapter 4. The toxicity research of *Piper laetispicum*

4.1. Introduction

Since ancient times, people have used plants as medicines. Recent trends have shown a dramatic rise in the world in use of complementary and integrative medicine approaches to health. Forty-two percent of Americans reported using alternative therapies in 1997; 40% for treatment of chronic illness and 60% for disease prevention. Despite this rapid growth, there is limited evidence about the effectiveness and toxicity of alternative medicine.

Extracts of *Piper methysticum*, or kava, have been consumed in the South Pacific Islands for centuries without any reported serious side effects (Lebot et al., 1992; Moulds and Malani, 2003). Kava and kava-derived products are generally considered as very safe. While in 2002, the German health authorities banned kava extract containing products based on the suspicion of a potential liver toxicity, as derived from adverse effect reports (Schmidt et al., 2002).

Even though *Piper laetispicum* is found to have antinociceptive, antidepressant and anxiolytic activities, to our knowledge its toxicity has been not studied. Therefore, the present study was designed to assess the acute and chronic oral toxicity effects of EAF extract of *P. laetispicum* stems in mice.

4.2. Materials and methods

Piper laetispicum C. DC. (Piperaceae), an endemic climbing, glabrous plant available in the southern part of China, was collected in 2007 from Hainan province, China, and was identified by Prof. Sheng-li Pan, School of Pharmacy, Fudan University, where a voucher specimen (No. 060812) of the plant material has been deposited for further reference.

4.2.1. Ethyl acetate extract

The crude extract (20g) was suspended in water and partitioned successively with petroleum ether, and then ethyl acetate. The ethyl acetate fraction (EAF) was combined and evaporated under reduced pressure. The yield of the EAF was 6 g (30%). EAF of the stems was stored at +4 ◦C until further use.

4.2.2. Animals and habitation

KM mice of either sex (18-22 g) were housed in standard environmental conditions. Food and water were available *ad libitum*. The animals were acclimatized to the laboratory for at least 7 days before the acute toxicity and were used only once throughout the study. All experiments were approved by Animal Ethics Committees, Fudan University, China.

Swiss albino mice (OF1) aged 9 weeks at the receipt from the breeder company (Charles River, France) and weighing 40 – 45 g were used in chronic toxicity research. The animals were housed under a 12-h light: 12-h dark schedule (lights on starting at 8:00 p.m.) with water and food ad libitum (SD Dietex-France). Animal rooms were at a constant temperature of 21±2 °C and relative humidity of 55 ± 10%. All animal

procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/ 609/ EEC).

4.2.3. Toxicity studies

The two toxicity studies were performed.

4.2.3.1. Acute toxicity study in mice

Healthy KM mice of both sexes, weighing ± 25 g, were randomly divided into groups of 10 animals. They were deprived of food, but not water 15 h prior to the administration of the test suspension. The control group received 2% Tween 80 (vehicle) administered orally by gavage or intraperitoneally. The EAF extract of *P. laetispicine* stems suspended in 2% Tween 80 was administered orally at doses of 200, 400, 800, 1200, 1800, 2000, and 2200 mg/kg; or intraperitoneally at doses of 50, 200, 400, 500, 600, and 800 mg/ kg. Next, the general behaviour of the mice was observed at times 0, 15, 30, 60, 120, and 240 min after the treatment. The animals were observed for morbidity and mortality once a day, for up 7 days, with food and water *ad libitum*. The number of survivors after the 7-day period was noted.

The toxicological effect was assessed on the basis of mortality, which was expressed as the median lethal dose (LD_{50}) (Miller and Tainter, 1944).

4.2.3.2. Chronic toxicity study in mice

OF1 mice were divided into 5 groups of 10 animals. One group served as control, and received 2% Tween 80 at dose of 10 ml/ kg. The remaining groups received four dose levels of the EAF of *P. laetispicum* stems (50, 250, or 500 mg/ kg) suspended in 2 % of Tween 80, administered orally by gavage daily for a period of 90 days. The results

for the animals treated with 50, 250 and 500 mg/ kg of the EAF extract of *P. laetispicum* stems were compared to the control group. Body weight was measured weekly, and the animals were observed daily for signs of abnormalities throughout the study. At the end of a 90-day period, the animals were deprived of food for 15 h and then sacrificed by decapitation. Their selected organs were carefully dissected and removed for weighing and macroscopic examination and the blood were collected for biochemical and haematological examinations. The brain was also preserved for the further research. Histopathological analyses were done only for the animals treated with the 500 mg/kg dose.

4.2.3.3. Organ weights

The organs of all the animals were examined macroscopically. The positions, shapes, sizes, and colours of the internal organs were visually observed for signs of gross lesions. Heart, lungs, stomach, liver, kidneys were collected and weighed. These organs plus the esophagus, small and large intestine were macroscopically examined, fixed in 10% formalin, and preserved in 70% ethyl alcohol. For the 500 mg/ kg EAF extract, tissue slides were prepared and stained with haematoxylin and eosin for microscopic examination.

4.2.4. Behavioral tests

4.2.4.1. Open field test

In order to detect any association of toxicity effects in the chronic administration of drugs with changes in motor activity, the activities of animals treated with EAF were tested in an open field. General motor activity was assessed on a circular open-field

platform (50cm diameter×27cm high walls), with the floor divided into 36 squares delimiting peripheral, medium and central circular areas. Mice were placed individually into the open-field and after 1 min of habituation, during a 6-min period, the number of squares crossed with the four paws were used to measure locomotor activity. The number of rearing was also considered.

4.2.4.1. Y-maze spontaneous alternation

Immediate working memory performance was assessed on EAF, by recording spontaneous alternation behaviour in a Y-maze (Hughes, 2004). The maze was made of black painted wood. Each arm was 25 cm long, 14cm high, 5 cm wide and positioned at equal angles. Mice were placed at the end of one arm and allowed to move freely through the maze during a 5-min session. The series of arm entries were recorded. An arm entry was considered to be completed when the four paws of the mouse were completely placed in the arm. Alternation was defined as triplet of explored arms and counted only if the mouse entry into the three arms of maze, without revisiting the first arm at the third visit. The percentage of spontaneous alternation was calculated as the ratio of successful overlapping alternations by the total possible triplets (defined as the total number of arm entries minus 2) multiplied by 100.

4.3. Statistical analyses

Data obtained were expressed as mean ± SEM and analyzed by analysis of variance (ANOVA) followed by Bonferroni's test. *P-values* less than 0.05 ($p< 0.05$) were used as the significant level.

The percent of inhibition was determined using the following formula:

$$Inhibition\ (\%) = 100 \times [(control\ -\ experiment)/\ control].$$

4.4. Results

4.4.1. Acute toxicity in mice

The effects of oral and intraperitoneal treatment of the EAF extract of *P. laetispicum* on mortality and LD_{50} values evaluated. Behavioural changes began 20min after the administration, but were reversible before 24 h. The values of LD_{50} were 1530.0 and 538.8 mg/ kg for oral and intraperitoneal administration of the EAF extract, respectively. No significant difference in body weight gain of the surviving animals was noted between the control and any of the treated groups over the period of observation (results not shown).

4.4.2. Repeated-dose oral toxicity study in mice

4.4.2.1. Body weight

The gain in body weight as a function of time (weeks) of the mice is shown in Fig. 25. In whole periods of treatment, there were no significant differences in body weight gain between the drug-treated groups and the vehicle group.

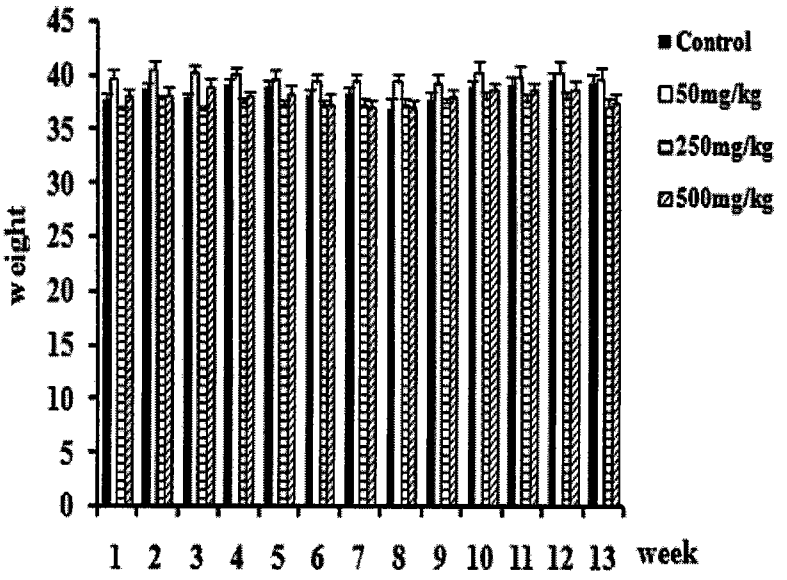


Fig. 25: Effect of EAF of P. laetispicum on the body weight.

Values are mean ± S.E.M. $^{***}p<0.001$, significantly different from control; ANOVA followed by Bonferroni's test (n = 10).

4.4.2.2. Organ weight

Table 11 shows the effects of the EAF extract on the weight of some vital body organs in mice. There is no significant change in the weights of spleen, lung, heart and kidney compared to the control group. However, there was a significant increase in mean liver weight (500 mg/kg; $p < 0.01$) compared to the control group (2% Tween 80).

Table 11 Effect of EAF extract of *P. laetispicum* stems on the weights of organs of mice

Organ	Control	50mg/kg	250mg/kg	500mg/kg
Spleen(g)	0.089	0.091	0.085	0.084
Lung(g)	0.360	0.384	0.362	0.389
Heart(g)	0.211	0.189	0.180	0.230
Liver(g)	1.943	2.071	2.003	2.469**
Kidney(g)	0.392	0.373	0.381	0.369

Values are mean ± S.E.M. $^{***}p<0.001$, significantly different from control; ANOVA followed by Bonferroni's test (n = 10).

4.4.2.3. Open-field test

No significant difference between the treatment groups and controls was seen after 90 days treatment for ambulatory behaviour in the OFT (results not shown).

4.4.2.4. Y-maze

The chronic administration of EAF in mice did not impair mice special memories compared with the vehicle-treated group.

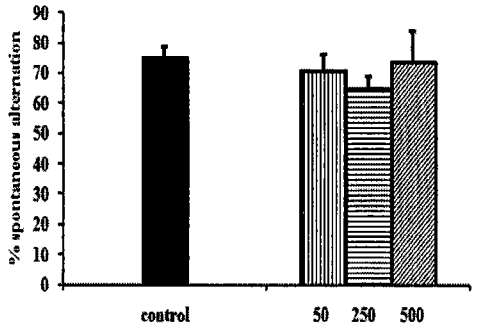


Fig. 26: Effect of EAF of P. laetispicum on Y-maze.

Values are mean ± S.E.M. $^{***}p<0.001$, significantly different from control; ANOVA followed by Bonferroni's test (n = 10).

4.5. Discussion

Herbal medicine is gaining popularity in the world. Herbal remedies are often believed to be harmless because they are "natural," and are commonly used for self-medication without supervision. This increase in popularity and the scarcity of scientific studies on their safety and efficacy have raised concerns regarding toxicity and adverse effects of these remedies (Saad et al., 2006). These products contain bioactive principles with the potential to cause adverse effects (Bent and Ko, 2004). Investigation of the acute toxicity is the first step in the toxicological investigations of an unknown substance. The index of the acute toxicity is the LD_{50}.

The results of the present study indicated that the toxicity of the EAF extract of *P. laetispicum* stems is low. During the 7-day period of acute toxicity evaluation, some signs of toxicity were observed, but they were all quickly reversible. The high LD_{50} values of the EAF extract administered by the oral and intraperitoneal routes (LD_{50} = 1530.0 and LD50 = 538.0, respectively) show its low acute toxicity. Substances with

an LD_{50} value of 1000 mg/kg by the oral route are regarded as being safe or of low toxicity (Clarke and Clarke, 1977). The difference observed between the LD_{50} values of the oral and intraperitoneal routes may be explained by the low bioavailability of any component(s) that might cause toxicity, either because of poor absorption from the gastrointestinal tract, or as a result of a high first-pass effect and rapid metabolism to non-toxic metabolites. The high LD_{50} values obtained indicate that the extract can be administered with a high degree of safety.

In the repeated-dose oral toxicity study in mice given the EAF extract of *P. laetispicum* stems at doses of 50, 250 and 500 mg/kg for 90 days, there was no change in animal behavior, while the mortality at the dose of 500mg/kg was 50% after 90 days treatment. Changes in body and internal organ weights can indicate adverse side effects. Generally, weight loss is a simple and sensitive index of toxicity after exposure to toxic substance (Raza et al., 2002; Teo et al., 2002). The results showed no significant differences in body weight gain. It is noted that there is a significant increase in the weight of liver at the dose of 500 mg/kg after 90 days administration. This liver increase might be the main reason for the 50% mortality in this group. Kidney and liver toxicity has been reported following the use of phytotherapeutic products (Corns, 2003; Hilaly et al., 2004; Isnard et al., 2004; Saad et al., 2006). The famous kava was banned till now by USA because of the Liver toxicity. So, according to the present preliminary results, the biochemical evaluationsand histopathological analysis related to liver parameter should be the future research emphasis.

In conclusion, the lowtoxicity of the EAF of *P. laetispicum* stems, evidenced by high

LD_{50} values, suggests a wide margin of safety for therapeutic doses. In the repeated-dose oral toxicity study, 50% mortality at 500 mg/kg group were observed, a significant increase in liver weight in the same group were seen. There were no other remarkable effects in mice. These toxicity studies suggest that the target organ of EAF extract could be liver and the further detailed research about the biochemical and histopathological evaluation should be concentrated on.

4.6. General conclusions

This thesis includes two parts, the pharmacology evaluation and the toxicity study on *Piper laetispicum*. From the results obtained, we can conclude that the results give more idea about the further study on Piper laetispicum, laetispicine and the related derivatives.

- The antinociception, antidepressant and anxiolytic researches show that:

-The essential oils have good antinociceptive effects, the sesqui-terpenes compounds are the main compounds responsible for the activity. While the extract free from the essential oil, with amids alkaloids and lignant as the main compounds, also show a significant antinociceptive activity. There exists a synergic analgesic effect between the sesqui-terpenes and amides and/or ligants.

- Both the total extract and fractions show strong peripheral analgesic activity.

- Leatispicine and d-sesamin are the two main compounds from the organic fractions of *P. laetispicum* extract. Laetispicine has a dose-dependent central antinociceptive activity without the involvement of the opioid receptor, but d-sesamin acts mainly peripherally.

- HEPl produces a specific antidepressant-like behavioral effect without drug tolerance after sub-chronic administration.

- Serotonergic system takes part in the mechanism of action of CHLF in anti-immobility time activity, but not PEF and EAF.

- The opioid system and serotonergic mechanism seem unlikely to be involved in the antidepressant-like effect of laetispicine, while the L-arginine-nitric oxide pathway might be partially involved in the antidepressant effect of laetispicine.

- Among the derivatives, ysy-7, ysy-10 and ysy-16 have the potential for further development. And these data give us some information about the structure and activity relationship.

-The mechanism of actions of extract, fractions, laetispicine and derivatives deserve further research.

-The anxiolytic activity of AEF is weak.

-The dose response curve of laetispicine in the three animal models is bell-shaped, which has a relationship with its mechanism of action.

-BZD receptor isn't involved in the mechanism of actions of EAF and laetispicine.

And from the toxicity researches it is found that:

-Toxicity in EAF is weak.

- Liver might be the target organ in the chronic administration.

- Chronic administration doesn't affect the locomotor activity and memory ability.

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