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Anti-anxiety Activity, Acute Toxicity and Cytotoxic Property of Extract of *Clausena harmandiana* (Pierre) Leaves

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Abstract

Introduction: Anxiety is one of the most common disorders of central nervous system (CNS). It is a state of excessive fear characterized by motor tension, sympathetic hyperactivity, apprehension and vigilance syndromes. During this decade, anxiety affects one-eighth of the total population of the world particularly in developed countries. Various types of herbal medicines have been used as anxiolytic drugs in different parts of the world. The purpose of present study was to evaluate anxioly tic effect of alcoholic extract of *Clausena harmandiana* (Pierre) leaves as well as its acute toxicity and cytotoxicity. **Methods**: The anti-anxiety activity was determined by light-dark exploration method in mice. Acute toxicity of *C. harmandiana* extract was evaluated in Wistar rats.The acute toxicity study was carried based on OECD guideline 420 and fixed dosage was adopted to 15,000 mg/kg. The MTT assay was carried out to evaluate the cellular toxicity activity of *C. harmandiana* on TK6 (human lymphoblasts), L929 (mouse fibroblasts) and Caco-2 (human colorectal adenocarcinoma cells). **Results:** The significant anti-anxiety activity of *C. harmandiana* extract was found when tested at 500 mg/kg. The acute toxicity suggest the oral LD_{50} of *C. harmandiana* extract is greater than 15,000 mg/kg in rats of both sexes. According to IC_{50} obtained from the MTT test, *C. harmandiana* extract was potentially toxic to TK6 cells (IC_{50} = 29.98±4.52 µg/ml) and potentially harmful to L929 (IC_{50} = 679.23±35.04 µg/ml) and Caco-2 (IC_{50} = 537.65±11.89 µg/ml) cells. **Conclusion:** Regarding results on light-dark task and acute oral toxicity tests performed in animals, it can be summarized that *C. harmandiana* extract possesses anti-anxiety property and it is safe to use. Cytotoxicity test indicates greater sensitivity of *C. harmandiana* extract to cancer cells (Coco-2) than to normal cells (L929).

Keywords : *Clausena harmandiana*, anti-anxiety, oral toxicity, cytotoxicity

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1. Introduction

Clausena harmandiana (Pierre) was first described by André Guillaumin in 1910. Its common name in Thai are “Song Faa” , “Song Faa Dong” and “Prong Faa”. This plant is commonly found in Cambodia, Loas, Vietnam and Thailand. The leaves are covered with translucent oil cells. In Thailand, it is used in folkloric medicines and as health promoting herbs. Decoction of the roots is taken as an antipyretic, antiflatulent and stomachic, and for food poisoning. It also relieves headache and bronchitis. The phytochemical analysis of the plant resulted in the identification of coumarins such as clausarin, dentatin, osthol, xanthoxyletin, nordentatin and carbazole alkaloids including heptaphylline (Wangboonskul, 1984), 2-hydroxy-3-formyl-7-methoxycarbazole and 7-methoxyheptaphylline (Chaichantipyuth, 1988) 7-hydroxyheptaphylline, claurailas A-D, girinimbrine, clausines E, K and O, 3-formly-1-hydroxy-7-methoxycarbazole, murrayanine, 7-methoxymuurraynine and lancine (Songsian, 2011).

Anxiety is one of the most common disorders of CNS among the present multiplying health problems across the world (Hyun-Sook, 2008). According to the survey done by National Comorbidity in the United States, women are twice as likely as men to be depressed and 2 to 3 times more prone to anxiety disorders (Cesar, 2009). Since most synthetic drugs are showing potential side effects, research is going across the globe to develop anxiolytics with less side effects and wild safety margins. This made the researchers to work on herbals and their related constituents to develop a potential anxiolytic. Therefore, this study was carried on *Clausena harmandiana* (Pierre) with the aim to investigate its pharmacological property on anxiolytic. Moreover, the toxicity of *C. harmandiana* was tested both in cell lines and animals.

2. Materials and Methods

Preparation of *Clausena harmandiana* ethanolic extract

The fresh leaves of *C. harmandiana* were thoroughly washed and dried in hot air oven at temperature 50°C for two days. The dried samples were ground into powder and subjected for 95% ethanol extract using percolation at room temperature. The extract was filtered and the

filtrate was concentrated on rotary evaporator under reduced pressure. The extract was kept at -20°C until utilisation.

Determination of anti-anxiety activity of *Clausena harmandiana* extract by light-dark exploration method

Thirty Swiss male albino mice weighing about 20 gm each were used for this study. They were purchased from the National Animal Centre (Mahidol University, Salaya Campus, Nakhon Pathom 73170, Thailand). The animals were acclimatized for at least 1 week in a 12-hour light/ dark cycle with free access to standard food and water. According to the requirement of the National Act on the Use of Experimental Animals (Thailand), the protocols of the animal experiments were approved by the Animal Ethics Committee of Thailand Institute of Scientific and Technological Research (TISTR) (ID#T-56005, Sept 30, 2011). For experimentations, they were randomly divided into five groups with 6 per group. Each group was received corticosterone in drinking water for 16 days to induce anxiety-like symptoms. On 17th day, each group of mice was orally administered as the followings. Group 1: served as control received 20%w/v of acacia in water (acacia solution), Group 2: served as positive control received 30mg/kg Phenobarbital in acacia solution, Groups 3, 4 and 5 served as treatment groups received *C. harmandiana* extract at 250, 500 and 750 mg/kg, respectively. After an hour of respective treatment, mice were individually subjected for anxiety paradigms using the light-dark task test for 10 min. At the beginning of the task, the mouse was placed in a far corner of the dark chamber, facing the light compartment. The latency to enter and the time spent in the light chamber with all four paws were recorded (in second). The number of entries into and the number of rears in the light and dark chamber were noted. The number of locomotor activities in each compartment was alsocounted by infrared radiation sensor mounted in each compartment of the light-dark box. The entire data was illustrated as Mean ± SD values. Statistical analysis for comparison between control *vs* treated groups was done by one-way ANOVA. The level of statistical significant consider was $p<0.01$.

Dertermination of acute oral toxicity of *Clausena harmandiana* extract in rats

The acute oral toxicity test of *C. harmandiana* extract was performed as per Organisation for Economic Co-operation and Development (OECD) guidelines No.420. Briefly, 20 healthy young adult Wistar rats of both sexes (ten of each sex) were administered in a single dose of *C harmandiana* extract at 15,000 mg/kg and designed as the treatment group. Animals were fasted 18 hr prior to dosing. The other 10 rats (ten of each sex) were un-treated and served as control group. The treated rats were immediately observed for any toxic signs (ill-health or beha vioural changes) and deaths during the first 4 h and daily thereafter for a total of 14 days.

Determination of cytotoxic property of *Clausena harmandiana* extract by MTT assay

The cytotoxic activity of *C. harmandiana* extract was carried using the MTT [3-(4, 5-Dime thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay on 3 cell lines including TK6 (human lymphoblasts), L929 (mouse fibroblasts) and Caco-2 (human colorectal adenocarcinoma cells). All cell lines were p urchased from the American Type Culture Collection (ATCC), USA. Cells were grown in DMEM medium supplied with 10% fetal bovine serum (FBS) at 37°C in humidified atmosphere containing 5% CO_2. For experimentation, the overnight cultures of TK6, L929 and Caco-2 cells were trypsinised using 0.25% trypsin-EDTA and the cells density was adjusted to 1×10^5 cells/ml with fresh media (RPMI for TK6 cell line and DMEM for L929 and Caco-2 cell lines). 200 µl of cell suspension of each cell line were loaded onto a 96-well plate. *C. harmandiana* extract was prepared for cell treatments at various concentrations (10, 25, 100, 250 and 500 mg/ml for Caco-2 cells; 300, 350, 400, 450 and 500 mg/ml for L929 cells; 1.25, 2.5, 5.0, 50 and 500 mg\ml for TK6 cells) whereas concurrent vehicle control was included. The treatments were performed for 24 hours and in triplicates. By the end of treatment time, media-containing test sample were removed and cells were washed with 1x HBSS before

adding 200 µl of MTT (1.25 mg/ml^{-1}) to each well. Cells were further incubated in dark at 37ºC in humidified incubator containing 5% CO_2 for 3 h. At the end of 3 h incubation time, MTT-containing media was discarded by pipetting. The crystals fomazans formed in cells were washed once with 1x HBSS and then dissolved by adding 200 µl of DMSO. The absorbance value was measured at 570 nm by micro-plate reader system. The Cytotoxicity criteria from MTT assay were used to calculate the percentage viability of the cells using following equation**:** .

$$\% \text{ Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

A graph of absorbance (Y-axis) plotted against sample concentrations (X-axis) was constructed. The cytotoxicity of *C. harmandiana* extract was presented as 50% inhibitory concentration (IC_{50}), the concentration of test sample required to reduce the absorbance to half (50%) that of the control.

3. Results and Discussion

Anti-anxiety activity of *Clausena harmandiana* extract

The effect of *C. harmandiana* extract on anxiety-like behavior in the light and dark chamber compared with the control group (un-treated rats) and the positive control group (phenobarbital treatment) was demonstrated in Table 1.

Light-dark task is widely employed paradigm for screening of anxiolytics. It is carved in such a way to observe trend of rodents to analyse a novel environment when confronted with aversive properties of brightly illuminated area. Anxiolytics tend to increase the time spent in light, reduce the latency to enter light arena and increase the numer of crossings between two compartments. The shorter latency to enter and the longer duration of time spent in the light

compartment indicates the higer tendency to be sedative effect. Our results as demonstrated In Table 1. treatment of *C. harmandiana* at 500 mg/kg as well as phenobarbital (30 mg/kg) significantly decreased the latency of emerging into the light compartment. On the other hand, the time spent in light chamber, the number of entries in light and dark chambers and the numbers of rears in the light chamber happened to be significantly increased in these two groups of mice. The results of light-dark task test indicate that 500 mg/kg of *C. harmandiana* possesses the sedative effect and at the higher activity than standard drug (phenobarbital 30 mg/kg) considering on the time spent in light chamber and latency to enter the light chamber parameters.

Table 1 Anti-anxiety activity of *Clausena harmandiana* extract on rats by using the Light-dark task test

Group	N	No. of entries in chamber		No. of rear in chamber		No. of locomotor activities		Time spent in light chamber (sec.)	Latency to enter the light chamber (sec.)
		Light	Dark	Light	Dark	Light	Dark		
Control	6	7.6±2.8	8.4±2.7	6.0±2.2	28.6±8.6	7.8±3.2	43.2±20.1	95.4±34.5	505.6±34.3
Phenobarbital (30 mg/kg in10%acasia)	6	22.5±2.3[a]	23.5±2.3[a]	12.2±2.8	27.7±5.9	36.0±5.6[a]	7.6±2.8[b]	202.0±20.0[b]	398.7±20.9[b]
Clausena harmandiana 250 mg/kg (in10% acasia)	6	12.5±1.8	13.3±1.7	10.7±3.7	33.3±5.1	16.5±5.5	68.5±8.4	114.8±21.2	453.7±20.7
Clausena harmandiana 500 mg/kg (in10% acasia)	6	13.8±1.4	714.4±1.5	15.4±0.9[a]	30.8±7.7	27.0±6.0[b]	70.4±6.6	219.8±26.8[b]	378.2±26.2[b]
Clausena harmandiana 750 mg/kg (in10% acasia)	6	11.2±2.1	12.3±2.2	12.8±3.2	26.0±4.8	22.2±24.5[a]	57.8±9.4	187.0±36.4	422.2±235.5

All value are given in mean±SEM, [a]$p< 0.01$, [b]$p< 0.05$ as compare with the control group.

Acute oral toxicity *Clausena harmandiana* extract

The evaluation of toxic property of *C. harmandiana* is crucial when considering public health protection because exposure to plant extract can result in undesirable effects on consumers. Hence, in this study the acute oral toxicity of *C. harmandiana* leave extract was investigated in rats. Oral administration of crude *C. harmandiana* extract at the highest dose of 15,000 mg/kg resulted in no mortalities or evidence of adverse effects, implying that *C. harmandiana* in non-toxic. Throughout 14 days of the treatment no changes in behavioral pattern, clinical sign and body weight of rats in both control and treatment groups. Also there were no any significant elevations observed in the biochemical analysis of the blood serum (BUN,

creatinine, AST and ALT). Further, gross pathological examination revealed normal architecture and no significant adverse effects observed on the kidney, liver, heart, lung, spleen, intestines, ovary and testis.

Cytotoxicity of *Clausena harmandiana* extract

The anti-proliferative activity of *C. harmandiana* extract was determined using MTT cell viability assay, where the IC_{50} value was used as a parameter of cytotoxicity. Treatments of *C. harmandiana* extract at various concentrations in TK6, L929 and Caco-2 for 24 h resulted in toxic effect in a dose-dependent manner (data did not show). Moreover, the MTT results obtained from 3-independent experiments indicated among these 3 cell lines used in this study, TK6 cell line was most sensitive to *C. harmandiana* extract. The IC_{50} (inhibitory concentration inhibited cell growth by 50%) values of *C. harmandiana* extract in TK6, L929 and Caco-2 were illustrated in Table 2.

According to the classification of the cytotoxicity for natural ingredients (Gad Shayne, 1999), the *C. harmandiana* extract could be categorized as potentially toxic substance (10 µg/ml < IC_{50} < 100 µg/mL) to TK6 cells and as potentially harmful (100 µg/mL < IC_{50} < 1000 µg/ml) to L929 and Caco-2 cell lines.

Table 2. The growth inhibitory effects-IC50 values (µg/ml) of *C harmandiana* extract on TK6, L929 and Caco-2 cell lines by MTT assay after 24 h exposure.

Cell line	*IC_{50} (µg/mL)
TK6 (human lymphoblast cells)	29.98 ± 4.52
L929 (mouse fibroblast cells)	679.23 ± 35.04
Caco-2 (human colorectal adenocarcinoma cells)	537.65 ±11.89

* Values are mean ± SD. Data obtained from 3 independent experiments.

Conclusion

The findings in this study suggest that the *Clausena harmandiana* extract possess anti- anxiety activity when tested at 500 mg/kg. Its sedative effect is greater than the standard group (phenobarbital 30 mg/kg). The non-toxic nature of *C. harmandiana* is evident from the acute oral toxicity test when tested up to maximum dose at 15,000 mg/kg in rats. During an observation period of 14 days, *C. harmandiana* did not reveal any toxic signs and deaths. Also, there were no any significant elevations observed in the biochemical analysis of the blood serum as well as significant adverse effects on gross pathological findings at necropsy. The cytotoxicity test indicate the potentially toxic property to TK6 cells and potentially harmful to L929 and Caco-2 cells. Our study generates new and updated information on biological activity of *C. harmandiana* leaves that has not yet published before. The fruitful

results obtained in this study particularly on anti-anxiety and non-acute toxic in animals will promote and strengthen utilization of this plant. Further pharmacological and chemical investigations may be required to elucidate the exact mechanism of action of this *C. harmandiana* extract and to identify the active principles responsible for such effects.

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