ฤทธิ์ยับยั้งของสารสกัดจากเถาวัลย์เปรียงต่อเอนไซม์ไซโตโครมพี 450 ไอโซฟอร์ม 2อี1-อะนิลีน-4-ไฮดรอกซีเลสในหลอดทดลอง

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บทคัดย่อ

สารสกัดจากเถาวัลย์เปรียงมีข้อบ่งใช้สำหรับบรรเทาอาการปวดหลังส่วนล่างและอาการปวดจากข้อเข่าเสื่อม การใช้สารสกัดจากเถาวัลย์เปรียงร่วมกับยาหรือสารอื่นๆ อาจก่อให้เกิดอันตรกิริยาต่อกันโดยเฉพาะอย่างยิ่งต่อการเปลี่ยนแปลงเอนไซม์ในระบบไซโตโครม P450s วัตถุประสงค์ของการวิจัยนี้เพื่อประเมินผลของสารสกัดจากเถาวัลย์เปรียงต่อเอนไซม์ไซโตโครมพี 450 ไอโซฟอร์ม 2อี1-อะนิลีน-4-ไฮดรอกซีเลส ที่ได้จากไมโครโซมจากตับของหนูขาวใหญ่ที่เหนี่ยวนำด้วยยาฟีโนบาร์บิทอลในหลอดทดลอง โดยใส่สารสกัดจากเถาวัลย์เปรียงที่มีความเข้มข้นขนาด 0, 100, 200, 400, 600, 800, และ 1,000 ไมโครกรัมต่อมิลลิลิตรในระบบที่มี ไมโครโซม อะนิลีนไฮโดรคลอไรด์ โคแฟคเตอร์ และฟอสเฟสบับเฟอร์ บ่มที่ 37 องศาเซลเซียส 20 นาที วัดปริมาณของพาราอะมิโนฟีนอล ซึ่งเป็นผลผลิตจากการย่อยสารตั้งต้น ด้วยการทำให้เกิดสีและวัดการดูดกลืนแสง ผลการวิจัยพบว่าสารสกัดจากเถาวัลย์เปรียงมีฤทธิ์ยับยั้งการทำงานของ CYP 2อี1 อย่างมีนัยสำคัญทางสถิติโดยสัมพันธ์กับความเข้มข้นของสารสกัด และมีค่า IC_{50} เท่ากับ 321.22 ไมโครกรัมต่อมิลลิลิตร

คำสำคัญ: เถาวัลย์เปรียง CYP 2อี1 อะนิลีน-4-ไฮดรอกซีเลส

***ผู้รับผิดชอบบทความ**

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The Inhibitory Effect of the *Derris scandens* Extract on Cytochrome P450 2E1 (CYP2E1) -associated Aniline-4-Hydroxylase *in vitro*

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Abstract

The indications of the 50% ethanolic extract of *Derris scandens* (Tao-Wan-Priang) are for relieving pain in lower back pain and knee osteoarthritis. It is well established that many herbs interact pharmacokinetically with drugs by modulating the activities of cytochrome P450s enzymes. The aim of this study was to investigate the effect of the *D. scandens* extract on the activity of cytochrome P450 2E1 (CYP2E1) -associated aniline-4-hydroxylase (A4H) in the rat hepatic microsome induced by phenobarbital under *in vitro* condition. Different concentrations of the *D. scandens* extract of 0, 100, 200, 400, 600, 800, and 1,000 μg/mL were added to the reaction mixture contained microsome, aniline hydrochloride, cofactor, and phosphate buffer and incubated at 37 °C for 20 minutes. Para-aminophenol, a product of A4H was measured by colorimetric method. The results clearly showed that the *D. scandens* extract significantly inhibited CYP2E1 with a dose dependent manner and the median inhibitory concentration (IC_{50}) was 321.22 μg/mL.

Keywords: *Derris scandens*, CYP2E1, Aniline-4-hydroxylase

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Introduction

Derris scandens (Roxb.) Benth, or Jewel Vine, known as Tao-Wan-Priang in Thai, is an evergreen climbing shrub with branched stems up to 20 meters long growing from a taproot. This woody vine is growing throughout Southeast Asia. It is the well-known Asian medicinal plant. The stem of *D. Scandens* has been used as the active ingredient in the Thai traditional medicine recipes for pain treatment, for example of osteoarthritis, joint diseases, musculoskeletal diseases, rheumatic diseases, muscle tension, etc[1]. The major active constituents of *D. scandens* stem extracts are benzyls and isoflavones, including genistein, coumarins, scandinone, scandenin, prenylated isoflavones, and isoflavone[2, 3]. The leaf and root extracts of *D. scandens* showed anti-inflammatory activity on carrageenan-induced paw edema in rats[4].

The clinical trials showed that the 50% ethanol extract of *D. scandens* resulted in better effective treatment, fewer side effects, and non-toxic effects when compared with a nonsteroidal anti-inflammatory drug (NSAIDs) such as diclofenac[5] and naproxen[6]. The major adverse events were gastrointestinal symptoms. The adverse events (AEs) of *D. scandens* showed no different relative risk with NSAIDs[7].

From the Thailand National List of Essential Medicines (2016), the *D. scandens* extract is a drug developed from Thai medicinal plants. One formulated capsule contains 400 mg of the 50% ethanol extract from the stem of *D. scandens.* Its indications are relieving pain in lower back pain and knee osteoarthritis[8]. Low back pain and knee osteoarthritis are frequently found in the elderly. The increasing of prevalence tendency is also found. Anti-inflammatory drugs, such as NSAIDs, are given to treat patients. However, the adverse effects of anti-inflammatory drugs are reported such as irritation and ulcers of the gastric and intestine system. The Thai Ministry of Public Health has the policy to support the research and development of herbal plants to be processed into high-quality goods and promote the use of the Thai herbs[9]. Therefore, the treatment effect of *D. scandens* is to replace or coadminister with other analgesic drugs by physicians and patients themselves.

Herbal and other natural remedies could affect the disposition of conventional pharmaceuticals through inhibition of human cytochrome P450 (CYP). The potential interaction of medicinal plants with clinically used drugs

is a major safety concern, especially for drugs with narrow therapeutic index (e.g. warfarin and theophylline) and may lead to treatment failure or life threatening adverse reactions[10, 11]. The data of the study suggested that the *D. scandens* extract contained constituents altering the activities of major drug metabolizing enzyme whose route of activity is mainly via cytochrome P450 systems. *D. scandens* extract showed the potent inhibitory activity against CYP1A2, weak potent inhibitory activity against CYP2C9, and did not affect CYP3A4 activity[12].

Cytochrome P450 2E1 has received a great deal of attention in recent years because of its vital role in the activation of many low molecular weight hepatotoxic chemicals such as ethanol, benzene, CCl_4, paracetamol, nitrosamines, pyridine and cancer suspect agents[13-16]. It is well established that CYP2E enzymes are mainly involved in mutagen and carcinogen metabolism[11, 17]. Aniline-4-hydroxylase (A4H) has been known as a member of mixed-function oxidases belonging to P450 2E1 gene family. Jeong *et al*. reported that the protective effects of 18*β*-glycyrrhetinic acid against the carbon tetrachloride-induced hepatotoxicity may be due to its ability to block the bioactivation of carbon tetrachloride, primarily by inhibiting the expression and activity of P450 2E1, and its free radical scavenging effects[18]. In this respect; the aim of this study was to determine the effect of the *D. scandens* extract on the activity of rat hepatic CYP2E1-associated A4H *in vitro*.

Materials and Methods

1. The Extract, Drug and Chemical Reagents

The commercial “GPO Thao-Wan-Priang Capsules”, 50% ethanolic extract from the stem of *D. scandens* produced by the Government Pharmaceutical Organization of Thailand was used. Aniline hydrochloride, glucose-6-phosphate, nicotinamide, and para-aminophenol were obtained from Sigma-aldrich Co Ltd. All other chemicals and solvents were of the highest grade commercially available.

2. Experimental Animals

Adult male Wistar rats (180-200 g) were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. Rats were housed in the Faculty of Science, Rangsit University, Thailand, under standard environmental conditions of 22 $\pm$ 1 °C, 60-70% humidity, and 12 h light and 12 h dark cycle. All animals had free access to water and standard pellet laboratory animal diet and acclimatized for at least 1 week before use. Before experiments began, the animals were deprived of food for 12 h. The

experiment was conducted in accordance with the Care of Laboratory Animals and Ethical Guidelines of the National Research Council of Thailand. The experiment protocol was submitted and approved for ethical considerations by the Rangsit University Animal Ethics Committee (ID RSEC02/2558).

3. Microsomal Preparation

The CYP enzymes were induced in rats by intraperitoneal injection of phenobarbital (75 mg/kg BW/day) for 7 days. Rats were starved overnight and sacrificed by cervical dislocation. The entire liver was perfused with ice-cold 0.9% NaCl in a short time to eliminate any possible effects due to diurnal variation and rinsed in cold 0.15 M Tris-KCl buffer (pH 7.4). Liver homogenates were prepared in ice-cold 0.15 M Tris-KCl buffer (pH 7.4) and centrifuged at 10,000 rpm for 20 min at 4 °C. The microsomal fraction (pellet) was precipitated by centrifuging the supernatant at 100,000 g for 60 min at 4 °C and stored at -70 °C until assay of A4H[19]. Microsomal protein was determined by the method of Lowry[20] using bovine serum albumin (BSA) as a standard at 660 nm. A standard curve of 0 to 100 µg/mL BSA was also constructed and was used for calculation of protein amounts in microsomes.

4. Determination of CYP2E1-associated Aniline-4-hydroxylase

The reaction mixture contained phosphate buffer (100 mM, pH 7.4), NADP (0.5 µM), glucose-6-phosphate (10 µM), nicotinamide (50 µM), $MgCl_2$ (25 µM), aniline hydrochloride (5 µM), rat hepatic microsome (6-8 mg protein) and the various final concentrations (0, 100, 200, 400, 600, 800, and 1,000 µg/mL) of the *D. scandens* extract dissolved in distilled water were added to the reaction mixture in a final volume of 1.0 mL. The reaction mixture was incubated for 20 min at 37 °C. The reaction was then stopped by the addition of 0.5 mL 20% trichloro acetic acid (TCA). The contents were then mixed, centrifuged at 3,000 rpm for 10 min. The supernatant (0.5 mL) was treated with 0.25 mL of 10 % Na_2CO_3 solution and 0.5 mL of 2% phenol in 0.2 N NaOH, mixed and placed in an incubator at 37 °C for 20 min. p-Aminophenol formed during the enzyme action reacts with phenol in the alkaline medium to form a blue colored product, which was measured at 630 nm. A standard curve of 0 to 100 µM p-aminophenol was constructed and was used for calculation of activity of A4H in microsome[21, 22]. The experiment was performed in triplicates.

5. Data Analysis

The experimental data were expressed as mean with their standard error of means (SEM, triplicates). Results were reported as specific activity of A4H in the presence of the *D. scandens* extract in a unit of μM of p-aminophenol produced/hr/mg microsomal protein compared with basal activity. A basal activity represents the specific activity observed in the absence of the *D. scandens* extract.

The degree of A4H inhibition was calculated as the percentage of inhibition using the formula;

$$\frac{(a-b)\times 100}{a}$$

a = Specific activity in the absence of the *D. scandens* extract

b = Specific activity in the presence of the *D. scandens* extract

Median Inhibitory Concentration (IC_{50}) Determination

A least-squares linear regression analysis of the log concentration–response curves allowed the calculation of the concentration that produced 50% of inhibition (IC_{50}).

6. Statistical Analysis

The significances of different among the various treated groups and the control group were analyzed by one-way analysis of variance (ANOVA) followed by Post Hoc LSD test using the Statistics Package for Social Sciences (IBM SPSS statistic 21) program for windows. A *P value*<0.05 was considered statistically significant.

Results and Discussion

The effect of the *D. scandens* extract on CYP2E1-associated A4H activity was shown in Table 1. The 50% ethanolic extract of *D. scandens* produced a dose-dependent inhibiting effect on A4H activity *in vitro* compared with the control group. The extracts treatment at a dose of 100 to 1,000 μg/mL caused a statistically significant decrease in the activity of A4H with the percentage of inhibition at 13.2 $\pm$ 2.02 to 78.4 $\pm$ 5.91% in rat hepatic microsome. Figure 1 showed the inhibition of A4H activity by the 50% ethanolic extract of *D. scandens*. Concentration needed for 50% inhibition (IC_{50}) of A4H was 321.22 μg/mL. The *D. scandens* extract showed a significantly dose dependent inhibition of CYP2E1-associated A4H from rat hepatic microsome induced by phenobarbital. Recent data from Nooin *et al*. (2017) reported that the 98% ethanolic extract of

D. scandens inhibited CYP2E1 activity in human liver microsome and IC_{50} was 150.67 ± 7.51 mg/mL[23]. Patil and Magdum reported that the ethanolic extracts of *Euphorbia hirta* L, *Euphorbia tirucalli* Linn, *Euphorbia nerrifolia* Linn were showing the potent inhibitory activity on phenobarbitone induced aniline hydroxylase enzyme *in vitro* with IC_{50} at 310.45, 381.35, and 481.85 µg/mL, respectively. This *E. hirta* L extract was found to have the significant activity against the chemically induced tumor[24].

The major active constituents of *D. scandens* stem extracts are benzyls and isoflavones, including genistein, coumarins, scandinone, scandenin, prenylated isoflavones, and isoflavone[2, 3]. The phytochemical contents of the ethanolic extract of *D. scandens* including the phenolic, flavonoid, tannin and alkaloid compound were also reported[23]. Further research is needed to investigate which of constituent that inhibits CYP2E1-associated A4H.

Table 1 Inhibitory Effect of the *D. scandens* extract on rat hepatic microsomal CYP2E1-associated A4H *in vitro*. (Values are mean + SEM of triplicates)

D. scandens extract (µg/mL)	Activity of aniline-4-hydroxylase (µM of p-aminophenol produced/hr/mg microsomal protein)	% inhibition
0	28.4 ± 2.20	0
100	24.7 ± 2.23	13.2 ± 1.17**
200	21.7 ± 1.99	23.7 ± 1.12**
400	15.4 ± 3.21*	46.9 ± 7.25**
600	10.6 ± 2.87**	63.8 ± 7.39**
800	7.2 ± 1.86**	75.4 ± 4.68**
1000	6.3 ± 1.32**	78.4 ± 3.00**

. *$p \leq 0.05$, **$p \leq 0.01$ compare to the absence of the *D. scandens* extract

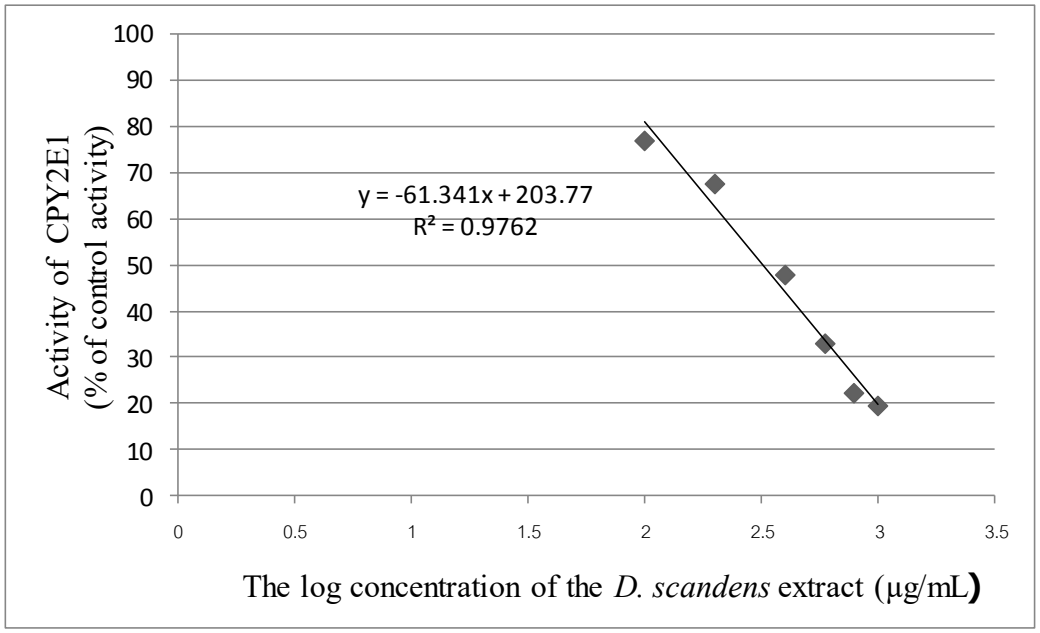

Figure 1. Concentration–response curve for the activity of the *D. scandens* extract on rat hepatic microsomal CYP2E1-associated A4H *in vitro*

The *D. scandens* extract showed the potent inhibitory activity against CYP1A2, weak potent inhibitory activity against CYP2C9, and did not affect CYP3A4 activity[12]. The result from these studies indicates that *D. scandens* extract drug might have a potential not only to inhibit and/or induce the metabolism of certain co-administered drugs but also influence the development of toxicity and carcinogenesis. CYP1A and CYP2E enzymes are mainly involved in metabolic activation of toxic substances[15, 17]. As the results of this metabolic activation, organ toxicity, mutagenesis and carcinogenesis may be observed. Inhibition of CYP1A2 and CYP2E1 results in decreased amounts of reactive metabolites formation. This may, in turn, further decrease potentiate the risk of organ toxicity, mutagenesis and malignant due to and other toxic chemicals metabolized by CYP1A2 and CYP2E1[15, 25]. It is well known that a number of non-toxic herbs are having activities like membrane-stabilizing, hepatoprotective, and anti-oxidation related with CYP2E1 inhibitory effects. Aliyu *et al*. reported that an aqueous extract of *Cochlospermum planchonii* rhizomes showed the hepatoprotective effect against CCl_4-treated rats from liver damage[26]. Its plausible hepatoprotective mechanisms may be by inhibiting cytochrome P-450 monooxygenases aminopyrine-N-demethylase and aniline hydroxylase. The protective effects of 18β-glycyrrhetinic acid against the carbon tetrachloride-induced hepatotoxicity may be due to its

ability to block the bioactivation of carbon tetrachloride, primarily by inhibiting the expression and activity of P450 2E1, and its free radical scavenging effects[18]. These are also observed with *Terminalia belerica Roxb.*[27], *Urtica urens*[28], *Polygonum bistorta* Linn., and tannic acid[29]. Many organosulfur compounds, such as diallyl sulfide from garlic, are the potent inhibitors of CYP2E1; this may provide an explanation for garlic's chemopreventive effects[11].

CYP2E1 isoform is inducible not only by various chemical agents such as ethanol, benzene, CCl_4, paracetamol (acetaminophen), nitrosamines, pyridine and cancer suspect agents[13-16], but also by fasting and diabetes induction. CYP2E1 may provide a biochemical basis for the increased incidence of occult liver disease and certain cancers noted in obese individuals[30]. Chlormethiazole (CMZ), a CYP2E1 inhibitor prevents hepatic carcinogenesis induced by diethylnitrosamine in alcohol-fed rats[31]. Indications of the *D. scandens* extract are relieving pain in lower back pain and knee osteoarthritis. Recently, the synergistic analgesic interaction between the *D. scandens* extract drug, and paracetamol in the acetic acid-induced abdominal constriction in a mouse model was reported[32]. The result of the present work indicated that the *D. scandens* extract showed a significantly dose dependent inhibition of CYP2E1-associated A4H. The *D. scandens* extract might cause herb–drug interaction through selective inhibition of CYP2E1. Future studies will attempt to further elucidate its mode of actions and also its interaction in clinical uses and the effects of the *D. scandens* extract on phase II xenobiotic metabolizing enzymes are needed to investigate further.

Conclusion

In conclusion, the *D. scandens* extract showed a dose dependent inhibition of CYP2E1-associated A4H *in vitro* with IC_{50} at 321.22 µg/mL. This may, in turn, further decrease potentiate the risk of organ toxicity, mutagenesis and malignant due to and other toxic chemicals metabolized by CYP2E1.

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