ORIGINAL ARTICLE

Study on biological activities of *Mansao hymenaea* (DC.) A. Gentry leaf extracts

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Abstract

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Petroleum ether extract (1) and ethanolic extract (2) of garlic vine leaves [*Mansoa hymenaea* (DC.) A. Gentry] were investigated for biological activities using the following tests : brine shrimp toxicity test, pediculicidal activity (*in vitro*) test, cytotoxicity test against COR L-23 (large cell lung carcinoma) using the SRB assay, antimicrobial activity and antioxidant activity by the DPPH radical scavenging assay. The cytotoxic activity against brine shrimp and headlice as well as the EC_{50} of antioxidant activity of 1 were higher than those of 2. However cytotoxicity of 1 against lung cancer cell line was less than that of 2 (IC_{50} = 35.39 and 6.44 μg/ml, respectively). Antifungal activities against three fungi (*Tricophyton rubrum*, *T. mentographytes*, *Microsporum gypseum*) of 1 were higher than those of 2 but 2 possesed higher antibacterial activity against *Staphylococcus* spp than 1.

Key words : garlic vine, *Mansoa hymenaea,* pediculicidal, brine shrimp, cytotoxicity, antimicrobial, antioxidant

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การศึกษาฤทธิ์ทางชีวภาพของสารสกัดจากใบกระเทียมเถา [*Mansoa hymenaea* (DC.) A. Gentry]

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การศึกษาฤทธิ์ทางชีวภาพของสารสกัดใบกระเทียมเถา [*Mansoa hymenaea* (DC.) A. Gentry] ที่สกัดด้วยปิโตรเลียมอีเทอร์ และเอทธานอล โดยการทดสอบ ฤทธิ์ฆ่าไรน้ำ ฤทธิ์ฆ่าเหา ฤทธิ์เป็นพิษต่อเซลล์มะเร็งปอด (COR L23) ใช้วิธี SRB assay ฤทธิ์ต้านเชื้อแบคทีเรีย และฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH assay ผลการทดลองพบว่าสารสกัดชั้นปิโตรเลียมอีเทอร์แสดง ฤทธิ์ฆ่าไรน้ำ เหา และฤทธิ์ต้านอนุมูลอิสระได้ดีกว่าชั้นเอทานอล ผลของความเป็นพิษต่อเซลล์ปอดของสารสกัดชั้นปิโตรเลียมอีเธอร์ฆ่าเซลล์มะเร็งปอดได้น้อยกว่า สารสกัดชั้นเอทานอล (IC_{50} = 35.39 และ 6.44 มคก./มล. ตามลำดับ) สารสกัดชั้นปิโตรเลียมอีเธอร์แสดงฤทธิ์ฆ่าเชื้อรา 3 ชนิด (*Trichophyton rubrum, T. mentographytes, Microsporum gypseum*) ดีกว่าสารสกัดชั้นเอทานอล แต่สารสกัดชั้นเอทานอลแสดงฤทธิ์ฆ่าเชื้อแบคทีเรียชนิด *Staphyllococcus* spp. ดีกว่าสารสกัดชั้นปิโตรเลียมอีเทอร์

ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์ คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Garlic vine (*Mansoa hymenaea,* synonym names: *Cydista acquinoctalis, Mansoa alliacea, Pachyptera hymenaea* and *Pseudocalymna alliaceum*) is a woody vine. The leaves of this vine, when crushed smell nearly the same as crushed garlic (*Allium sativum*). *Mansoa hymenaea* is a native plant of Brazil and Peru (Thetburanatham, 1987). It is an ornamental plant in Thailand because of its attractive large pink flowers. In South Africa it is used traditionally to treat rheumatoid arthritis and as a muscle relaxant (Luna *et al*., 1984). Previous research on this plant has shown that its wood contains a naphoquinone compound (lapachone). The methanolic extract of *Mansoa hymenaea* wood had cytotoxic activity against V-79 cells (colon cancer cell line) (IC_{50} = 60 μg/ml) (Itokawa *et al.,* 1992). The dichloromethane and methanolic extracts of *Mansoa hymenaea* leaves showed antifungal activity against *Trichophyton mentagrophytes* and *Microsporum gypseum* (10 mg/disc) (Freixa *et al.,* 1998). Surprisingly, little research has been reported on this plant worldwide. Studies on the leaves of this plant should be further carried out. This plant grows very well in most the parts of Thailand, and in the case of containing bioactive compounds, it may be of commercial use as a medicine.

Material and Methods

1. Plant material

The leaves of *Mansoa hymenaea* were collected from Hat Yai, Songkhla, Thailand, in July, 2002. The herbarium specimen of the plant was compared with the herbarium in Southern Center of Thai Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University.

2. Extraction

The leaves were dried at 50°C , and the dried leaves (350 g) were macerated for 3-5 days with petroleum ether (3 × 500 ml) and ethanol (3 × 500 ml) The organic solvents were evaporated to dryness under reduced pressure.

3. Biological activities assay

3.1 Brine shrimp toxicity assay

The leaf extracts were first dissolved in

DMSO (10 mg/ml). These samples were diluted with artificial sea water to afford concentrations in the range of 0.1-1.0 mg/ml. Each dilution was tested in triplicate for brine shrimp toxicity test (Meyer *et al*., 1982). The LD_{50} values were obtained by probit analysis using the SPSS for Windows version 10.

3.2 *In vitro* assay for pediculicidal activity

Headlice were collected from school girls suffering from this headlice were used in this assay. The headlice were stored in a bottle with its lid punched with small holes until required for the assay. The petroleum ether extract (1) and ethanol extract (2) were prepared in a carbopol gel base at six different concentrations (10, 20, 30, 40, 50, 60 % w/w in 100 mg gel base). One gram of this gel preparation was spread onto glass slides (2.5 × 7.5 cm) and 10-15 headlice were put on the gel of extracts, and the percentage of dead headlice calculated at half hour intervals for 3 hours, and compared with hair gel base used as control. LD_{50} values were calculated by the prism program.

3.3 *In vitro* assay for cytotoxic activity

(1) Human cell lines

Large cell lung carcinoma (COR-L23) cell lines were used. COR-L23 cells were established and kindly provided by Dr. P. Twentyman and Dr. P. Rabbitts of MRC Clinical Oncology & Radiotherapeutics Unit, Cambridge, UK. They were cultured in RPMI 1640 medium supplemented with 10% heated inactivated foetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 g/ml streptomycin. The cells were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity (Keawpradub *et al.,* 1997; Keawpradub *et al*, 1999). According to their growth profiles, the optimal plating densities of each cell line were determined as 1×10^3 cells/well to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number was analysed by the SRB assay. In the assay, cells were washed with magnesium - and calcium- free phosphate buffered saline (PBS) (Oxoid Ltd.). PBS was decanted and cells were detached with 0.025% of 1ml trypsin-EDTA (Sigma Chem Co.) and PBS was added to a final volume of 5 ml. The cell pellet obtained by centrifugation (1000 g , 5 min) was resuspended in 10 ml of RPMI medium to make a single cell suspension and viable cells were counted by the trypan blue exclusion method using a haemocytometer. The cell suspension was then diluted with medium to give a final cell concentration of 1×10^3 cells/well. One hundred l/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24 h the cells were treated with the leaf extracts. The leaf extract (1 and 2) and vincristine sulphate were initially dissolved in DMSO. Vincristine sulphate (Sigma, Lot No. 34H0447) was used as a positive control. Both of the leaf extracts were diluted in the medium to produce 8 concentrations, and 100 ml/well of each concentration was added to the plates (6 replicates) to obtain final concentration of 0.1, 0.5, 1, 5, 10, 25, 50, 100 µg/ml for extract and 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 100 nM for vinblastine sulphate. The final mixture used for treating the cells contained not more than 1% of the solvent, the same as in solvent control wells. The plates were incubated for exposure times of 72 hours. At the end of each exposure time, the medium was removed, the wells were washed with medium, and then 200 ml of fresh medium added. The plates were incubated for a recovery period of 6 days and the cell number was analysed by the SRB assay.

(2) Sulphorhodamine B (SRB) assay

The antiproliferative assay, SRB (sulphorhodamine B) used to assess growth inhibition, was performed according to method of Skehan *et al* (1990). This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB (Skehan *et al.,*1990). In the assay, cells were fixed by layering 100 ml of ice-cold 40% trichloroacetic acid (TCA, Aldrich Chemical Co.) on top of the growth medium. Cells were incubated at 4°C for 1 h., after the plates were washed five times with cold water, excess water drained off and the plates left to dry in air. SRB stain (50 ml; 0.4% in 1% acetic acid) (Sigma Chem Co., St.Louis, U.S.A.) was added to each well and

left in contact with the cells for 30 minutes. The cells were then washed with 1% acetic acid, rinsing 4 times until only dye adhering to the cells was left. The plates were air dried and 100 µl of 10 mM Tris base [tris (hydroxy methyl) aminomethane, pH 10.5] (Sigma Chem Co., St.Louis, U.S.A.) added to each well to solubilise the dye. The plates were shaken gently for 20 minutes on a gyratory shaker. The absorbance (OD) of each well (6 replicates) was read on a SLT 340 ATTC plate reader (SLT Labinstrument, Australia) at 492 nm as an indication of cell number. Cell survival was measured as the percentage absorbance compared to the control (non-treated cells). The IC_{50} values were calculated from the Prism program obtained by plotting the percentage of survival *versus* the concentrations. According to National Cancer Institute guidelines (Boyd, 1997), extracts with IC50 values < 20 µg / ml were considered active.

3.4 Antimicrobial activity

(1) Microorganisms

The tested organisms used were bacteria and fungi. The bacteria used were four Gram positive bacteria (*Staphylococcus aureus* ATCC 25923*, Staphylococcus epidermidis, Bacillus subtilis, Streptococcus faecalis)* and six Gram negative bacteria (*Escherichia coli* ATCC 25922*, Salmonella typhimurium, Salmonella typhi, Shigella flexneri, Shigella sonnei, Pseudomonas aeruginosa)*. These bacteria samples were provided by the Department of Pathology, Faculty of Medicine, Prince of Songkla University. Fungi included one yeast : (*Candida albicans)* and three dermatophytes (*Trichophyton rubrum, Trichophyton mentagrophytes* and *Microsporum gypseum)*. These were provided by the Department of Microbiology, Faculty of Science, Prince of Songkla University.

(2) Media

The media were the Mueller Hinton agar (MHA) (Difco, Becton Dickinson and Company, Spark, USA) for bacteria, and Sabouraud Dextrose agar (Difco, Becton Dickinson and Company, Spark, USA) for fungi.

(3) Reference antibiotics

Tetracycline (30 µg/disc) (Fluka, Fluka Chemie GmbH, Switzerland), and clotrimazole (Sigma, Sigma-Aldrich Chemie GmbH, Germany) were used as positive controls.

(4) Antimicrobial Assay (Preliminary - susceptibility testing)

The agar diffusion method (Barry and Thornsberry, 1991) was used to screen for antimicrobial activities of the petroleum ether (1) and ethanolic (2) extracts of *M. hymenaea*. Sterile discs (6 mm) were impregnated with 10 µl of reconstituted crude extracts (concentration 500 mg/ml in DMSO) and placed on the surface of Mueller Hinton agar (MHA) for bacteria and Sabouraud dextrose agar (SDA) for fungi dispersion plates inoculated with the microbes. Each extract was tested in triplicates. Control discs contained 10 µl dimethyl sulfoxide (DMSO) and standard antibiotics used as positive controls were composed of tetracycline (30 µg/disc) for bacteria and clotrimazole (5 µg/disc) for fungi. Agar plate containing bacteria and yeast was incubated at 37 °C for 24 h and 30 °C for 48 h, respectively. Dermatophyte fungi were incubated at 30 °C for 7 days. Inhibition zones were recorded as the diameter of growth-free zones, including the diameter of the disc, in millimetres at the end of the incubation period.

(5) Antimicrobial Assay [Determination of Minimal Inhibitory Concentration (MIC)]

The agar dilution method (Sahm and Washington II, 1991; Shadomy and Pealler 1991) was used to determine the minimal inhibitory concentration (MIC) : the minimal concentration completely inhibited the growth of the microorganisms of the petroleum ether extract and the ethanolic extract, using tetracycline and clotrimazole as positive controls. The bacteria were grown overnight in MHA, and the yeast and dermatophyte fungi were grown on SDA slant for 2 days and 10 days, respectively. Inoculates of 10^3-10^4 CFU were spotted with micropipette on agar supplemented with the extract or antibiotic at con-

centrations ranging from 9.8-5000 µg/ml for the leaf extract, 0.12-16 µg/ml for tetracycline and 0.002-0.12 µg/ml for clotrimazole by two fold serial dilution. Agar plate containing bacteria and yeast was incubated at 37 °C for 24 h and 30 °C for 48 h, respectively. Dermatophyte fungi were incubated at 30 °C for 7 days.

3.5 Antioxidant activity

The following assay procedure was modified from that described by Yamasaki *et al.,* (1994). Samples for testing were dissolved in ethanol to obtain a high concentration of 200 µg/ml. Each sample was further diluted for at least 5 concentrations (two-fold dilutions). Each concentration was tested in triplicate. A portion of sample solution (500 µl) was mixed with an equal volume of 6×10^{-5} M DPPH (in absolute ethanol) and allowed to stand at room temperature for 30 min. The absorbance (A) was then measured at 520 nm. BHT (butylated hydroxytoluene), a well known synthetic antioxidant, was tested in the same system as a positive standard. The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The results were expressed as percentage inhibition, $\%\text{inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100$. EC_{50} value (effective concentration of sample required to scavenge DPPH radical by 50%) was obtained by linear regression analysis of dose-response curve plots of % inhibition *versus* concentration.

Results and Discussion

The percent yield of ethanolic extraction (26.6%w/w) was more than that of petroleum ether extraction (3.2% w/w). Petroleum ether extract showed higher activity in the brine shrimp toxicity test with an IC_{50} of 268 µg/ml, compared to 428 µg/ml for the ethanol extract. Antioxidant activity of petroleum ether extract was three times higher than of that of the ethanolic extract (EC_{50}=19.0 and 65.7 µg/ml, respectively) and the antioxidant activity of petroleum ether extract was comparable to that of BHT (16 µg/ml). Similarly, those of the pediculicidal and antimicrobial activities of the petroleum ether extracts were higher than those of the ethanolic extract. LD_{50} of petroleum ether extract on pediculicidal activity assay at one, two and three hours was 0.32, 0.01 and 0.01 g/g of gel respectively, whereas those of the ethanolic extract were 0.43, 0.32 and 0.26 g/g of gel, respectively. The ethanolic extract showed high cytotoxic activity against the lung cancer cell line (IC_{50} 6.4 µg/ml), but the petroleum ether extract exhibited less cytotoxic activity (35.5 µg/ml) based on NCI definition [IC_{50} value > 20 µg/ml (Boyde, 1997)]. It is of interest to note that in a previous report, the methanolic extract from the wood of *M. hymenaea* showed cytotoxic activity against colon cancer (Moroshi, 1992). However, both of the organic solvent extracts showed negative results against all gram negative bacteria and some positive bacteria (*Strephylococcus faecalis*) but showed high antifungal activity (Table 1), especially against three fungal species (*Trichophyton rubrum, T. mentagrophytes, Microsporum gypseum),* with MIC less than 20 µg/ml (Table 2). These results show that *M. hymenaea* leaves, which have an odor like garlic, exhibit antimicrobial and cytotoxicity activities similar to garlic (Imwidthaya *et al.,* 1978; Siegers *et al.,* 1999). These results are consistant with previous studies, indicating that dichloromethane extracts of the leaves were active against *Mycosporum gypsum* and *Trichophyllum mentagrophytes* at concentration 10 mg/disc. These reports, however, did not report the MIC values for the extracts (Freixa *et al*., 1998). The petroleum ether extracts showed potent antifungal activity, especially against *Tricophyton rubrum,* with an inhibition zone comparable to that of clotrimazone (49.5 mm and 47.2 mm., respectively) (Table 1). Surprisingly, MIC of the crude petroleum extract was similar to that of pure clotrimazole, suggesting that isolation of the responsible active constituent from the crude extract might yield a powerful antifungal agent. Future investigations should be focused on the separation, isolation and elucidation of potent antifungal compounds from petroleum ether extract. The ethanolic extract should be investigated for cytotoxicity in different types of

Table1. Inhibition zone of antimicrobial activities of petroleum ether and ethanolic extracts from the leaves of *M. hymenaea.* and antibiotics

Strains	Inhibition zone (mm.)			
	Pet. ether ext.	ethanol ext.	tetracycline	clotrimazole
S. aureus ATCC 25923	7.4	10.4	28.5	-[b]
S. epidermidis	7.0	13.4	13.4	-
B. subtilis	7.2	7.2	18.9	-
S. faecalis	-[a]	-[a]	12.1	-
E. coli ATCC 25922	-	-	23.3	-
S. typhimurium	-	-	23.8	-
S. typhi	-	-	21.2	-
S. flexneri	-	-	10.1	-
S. sonnei	-	-	-[a]	-
P. aeruginosa	-	-	8.7	-
C. albicans	7.3	7.2	-[b]	24.5
T. rubrum	49.5	37.5	-	47.2
T. mentographytes	40.0	12.9	-	44.1
M. gypseum	20.2	12.6	-	42.8

Note : a = No inhibition zone, b= Not tested, concentration of extracts = 5 mg/disc

Table 2. Diameter length of clear zone (mm) and MIC (mg/ml) of extracts on microorganism (n=3)

Microorganism	Petroleum ether extract	Ethanolic extract
	MIC (mg/ml)	MIC (mg/ml)
Staphylococcus aureus (ATCC25923)	5	5
Bacillus subtilis	2.5	*5*
Staphylococcus epidermidis	5	0.156
Candida albicans	>5	5
Trichophyton rubrum	0.0195	0.078
T. mentagrophytes	≤0.00976	0.156
Microsporum gypseum	0.0195	0.078

cancer cell lines and compared with the normal cell and isolated anticancer compounds.

Conclusion

The results obtained from this study indicated that the petroleum extracts of *Mansao hymenaea* leaves showed high antifungal activity, and should contain active constituents against dermatophyte fungi. These constituents may also be valuable in treatment of headlice infestation. It is likely that its ethanolic extract showed high cytotoxic activity against lung cancer, so anticancer compounds should be isolated from this extract.

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