UNIVERSITI PUTRA MALAYSIA

CHEMICAL CONSTITUENTS AND POSSIBLE ANTICANCER PROPERTIES OF *LAWSONIA INERMIS* AND *STROBILANTHES CRISPUS*

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By

SUSI ENDRINI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

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Chairperson: **Associate Professor Asmah Rahmat, Ph.D.**

Faculty: **Medicine and Health Sciences**

Studies on the cytotoxicity effects, antitumour components and their mechanism of action of *S. crispus* and *L. inermis* were carried out in this study. The nutrients and non-nutrients composition of *L. inermis* were determined to investigate whether the leaves are suitable for herbal tea preparation. The crude protein, ether extract, ash and moisture contents were also determined. Ash samples were further analysed to investigate water insoluble ash, water-soluble ash and alkalinity according to the International standard ISO 1576-1975 (E) and ISO 1578-1975 (E) methods, whereas acid insoluble ash was also determined. Carbohydrate content was determined following the Anthrone method. In fresh *L. inermis* leaves, the moisture content forms the bulk of tissue weight with a mean value of 69.4%. This plant was found to contain high amounts of minerals, such as potassium (29455 mg/100g), calcium (2654 mg/100g), sodium (4094 mg/100g), iron (266 mg/100g) and phosphorus (550 mg/100g). Water insoluble ash and water-soluble ash were 2 and 1.2% respectively.

The value of alkalinity of *L. inermis* at 8.6 ml acid/g and the leaves also contained 0.3% of acid insoluble ash. The extractive value of *L. inermis* leaves was 8.6%. Protein, total carbohydrate, ether extract and crude fiber of the leaves were 1.3, 0.3, 0.2 and 7.5%, respectively. The leaves also contained 0.1% caffein and 1.1% catechin. The methanolic extract of *S. crispus* displayed the strongest cytotoxic effect on colon carcinoma cell lines (Caco-2) followed by human breast cancer non-dependent hormone (MDA-MB-231), and liver cancer cell line with IC_{50}-values of 22.3, 27.2, and 29.3 μg/ml, respectively. The chloroform extract of this plant was shown to also have cytotoxic effect against Caco-2 (IC_{50}=25.1μg/ml) and human liver cancer cell lines, HepG2 (IC_{50}= 28 μg/ml). On the other hand, the chloroform extract of *L. inermis* showed a high cytotoxic effect in HepG2, followed by human breast cancer dependent-hormone (MCF-7) with IC_{50}-value of 0.3 and 24.8 μg/ml, respectively. There was no cytotoxic effect observed using methanol extract of this plant. Results from Ferric Thiocyanate (FTC) method showed the total antioxidant activity of methanol extract of *S. crispus* (MeS) was the highest (95.7%), followed by chloroform extract of *S. crispus* (CS), chloroform extract of *L. inermis* (CL) and vitamin E as control. Similar results were also obtained using the Thiobarbituric Acid (TBA) method. MeS gave the highest total antioxidant activity (82.6%), followed by CS (66.7%), CL (55.7%) and vitamin E (44.4%). Three compounds were isolated using High Performance Liquid Chromatography (HPLC) from CL., *i.e.* lawsone as a major compound, kaempferol, and quercetin. It is believed that all these compounds contributed to the cytotoxic and antioxidant activities of CL. On the other hand, the active compound of CS was isolated using Column Chromatography and analysed using Gas-Chromatography Mass Spectrophotometry (GC-MS) and identified as γ-sitosterol. This compound has cytotoxic effect against Caco-2, HepG2, and MCF-7

cell lines. The other compound that was also isolated from CS is known as stigmasterol. The essential oils of *L. inermis* and *S. crispus* obtained via hydrodistillation of fresh leaves found that the major component of *S. crispus* oil known as phytol (46.01%), an acyclyc diterpene alcohol. Other components were alpha cadinol (3.47%), tau murolol (2.49%), ledol (1.81%) and eugenol (1.08%). The essential oil of *L. inermis* also contained phytol (10.30%) and 0.31% hexahydropseudoionone and has cytotoxic effect against HepG2. It was also shown to have high antioxidant activities, whereas, the essential oil of *S. crispus* did not have any cytotoxic effect against all cell lines tested. The mechanism of action was studied by apoptosis pathway and expression of *c-myc* oncogene. Confocal laser scanning microscopy and fluorescence microscopy showed that the crude chloroform extract of *S. crispus* and *L. inermis*, essential oil of *L. inermis* and γ-sitosterol induced apoptosis in HepG2 and Caco-2 cell lines. The TUNEL assay staining revealed cells with intensely yellow fluorescence of PI-FITC. The common features were condensation of chromatin, fragmentation of DNA, and formation of apoptotic bodies. However, cells treated with 30 μg/ml of essential oil of *L. inermis* showed obvious shrunken cells with either condensed or fragmented nucleus and numerous apoptotic bodies. This was further confirmed with agarose gel electrophoresis DNA ladder analysis which showed that treated HepG2 and Caco2 cells developed multiple 180-200 bp DNA ladder fragments that are associated with apoptosis. The chloroform extract of *S. crispus* and *L. inermis*, essential oil of *L. inermis* and γ-sitosterol also suppress the expression of *c-myc* oncogene. In conclusion, *S. crispus* and *L. inermis* leaves may have potential as anticancer agents.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
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KANDUNGAN KIMIA DAN SIFAT ANTIKANSER YANG MUNGKIN DARIPADA *LAWSONIA INERMIS* DAN *STROBILANTHES CRISPUS*

Oleh

SUSI ENDRINI

April 2003

Pengerusi: **Profesor Madya Asmah Rahmat, Ph.D.**

Fakulti: **Perubatan dan Sains Kesihatan**

Dalam kajian ini, kesan sitotoksik, komponen antitumor dan mekanisme tindakan daripada *Strobilanthes crispus* dan *Lawsonia inermis* telah dikaji. Komposisi nutrien dan bukan-nutrien daripada *L.inermis* telah dilakukan bagi menentukan sama ada daun *L. inermis* adalah sesuai untuk dijadikan sebagai teh herba. Kandungan protein kasar, ekstrak eter (lemak), abu dan kelembapan telah ditentukan. Sampel abu kemudiannya digunakan untuk menentukan nilai abu tak larut air, abu larut air dan nilai alkalinya berdasarkan kaedah piawai antarabangsa ISO 1576-1975 (E) dan ISO 1578-1975 (E), abu tak larut asid juga telah ditentukan. Kandungan karbohidrat telah ditentukan mengikut kaedah Anthrone. Pada daun *L. inermis* segar, kandungan air membentuk "bulk" daripada berat tisu dengan purata nilai adalah 69.4%. Tumbuhan ini mengandungi jumlah mineral yang tinggi seperti kalsium (29455 mg/100g), kalsium (2654 mg/100g), natrium (4094mg/100g), besi (266mg/100g) dan fosforus (550mg/100g). Kandungan abu larut air dan tak larut air adalah sebanyak 2 dan 1.2%. Nilai bes daripada *L. inermis* adalah 8.6 ml asid/g dan daun ini juga mengandungi 0.3% abu tak larut asid. Nilai ekstraktif daripada daun adalah 8.6%

protein, karbohidrat total, ekstrak eter dan serat kasar daun adalah masing-masing 1.3, 0.3, 0.2 dan 7.5%. Daun ini juga mengandungi 0.1% kafein dan 1.1% katekin. Ekstrak metanol daripada *S. crispus* menunjukkan kesan sitotoksik yang paling kuat ke atas titisan sel kanser kolon (Caco-2) diikuti oleh titisan sel kanser payudara tidak bersandar hormon (MDA-MB-231) dan titisan sel kanser hepar dengan nilai IC_{50} masing-masing adalah 22.3, 27.2 dan 29.3 μg/ml. Ekstrak kloroform (yang diperolehi daripada kaedah peningkatan nilai kepolaran) daripada tumbuhan ini juga menunjukkan kesan sitotoksik ke atas Caco-2 dan Hep-G2. Di samping itu, ekstrak kloroform daripada *L. inermis* menunjukkan kesan yang sangat sitotoksik ke atas Hep-G2, diikuti dengan titisan sel kanser payudara bersandar hormon (MCF-7) dengan nilai IC_{50} masing-masing adalah 0.3 dan 24.8 μg/ml. Tiada kesan sitotoksik yang ditunjukkan oleh ekstrak methanol daripada tumbuhan ini. Hasil kajian menggunakan kaedah Ferik Tiosianat (FTC) menunjukkan aktiviti antioksidan total daripada ekstrak metanol *S. crispus* (MeS) adalah paling tinggi, diikuti dengan ekstrak kloroform *S. crispus* (CS), kloroform *L. inermis* (CL) dan vitamin E. Hasil yang serupa ditunjukkan pula dengan menggunakan kaedah Asid Tiobarbiturat (TBA). MeS mempunyai aktiviti antioksidan total paling tinggi (82.6%), diikuti oleh CS (66.7%), CL (55.7%) dan vitamin E (44.4%). Sebanyak tiga sebatian telah dipencilkan menggunakan kaedah kromatografi cecair prestasi tinggi daripada CL iaitu Lawson yang merupakan salah satu komponen utama, kamferol dan kuersetin. Diyakini ketiga-tiga sebatian ini menyumbang kepada kesan sitotoksik dan aktiviti antioksidan daripada CL. Dari sudut lain, sebatian aktif daripada CS telah pula dipencilkan dengan menggunakan kaedah kromatografi turus dan dianalisis menggunakan kromatografi gas spektrometer massa (GC-MS), dikenali sebagai gamma- sitosterol. Sebatian ini mempunyai kesan sitotoksik ke atas titisan sel kanser

Caco-2 dan Hep-G2. Minyak pati daripada *L. inermis* dan *S. crispus* juga telah di ekstrak dan komponen utama kedua-duanya dikenali sebagai fitol iaitu alkohol diterpena asiklik. Komponen-komponen lain dari minyak pati *S. crispus* adalah alfa-kadinol (3.47%), tau-murolol (2.49%), ledol (1.81%) dan eugenol (1.08%). Manakala minyak pati *L. inermis* mengandungi 0.31% heksahidropseudoionona dan mempunyai kesan sitotoksik ke atas titisan sel kanser Hep-G2. Ianya juga mempunyai aktiviti antioksidan yang tinggi, manakala minyak pati *S. crispus* pula tidak menunjukkan kesan sitotoksik ke atas sebarang titisan sel kanser yang telah digunakan, meskipun ianya juga mempunyai aktiviti antioksidan yang tinggi. Mikroskop "Scanning Laser Confocal" dan mikroskop pendaflour menunjukkan bahwa ekstrak kloroform kasar daripada *S. crispus* dan *L. inermis*, minyak pati *L. inermis* dan γ-sitosterol mengaruh apoptosis pada titisan sel kanser HepG2 dan Caco-2. Pewarnaan asai TUNEL mendedahkan sel dengan pendafluor berwarna kuning daripada Propidium Iodida-Fluoresens Isotiosianat (PI-FITC). Ciri-ciri yang biasa terjadi pada apoptosis adalah kondensasi kromatin, fragmentasi DNA dan pembentukan badan apoptotik. Bagaimanapun, sel-sel yang diberi perlakuan dengan 30 μg/ml minyak pati daripada *L. inermis* menunjukkan pengecutan sel yang ketara diikuti dengan kondensasi dan fragmentasi nukleus serta terbentuknya badan-badan apoptotik. Analisa "DNA-ladder" elektroforesis gel agarosa daripada sel HepG2 dan Caco-2 yang telah diberi perlakuan menunjukkan terdapatnya fragmen-fragmen DNA di antara 180-200pb yang berkaitan dengan apoptosis. Ekstrak kloroform daripada *S. crispus* dan *L. inermis*, minyak pati daripada *L. inermis* dan γ-sitosterol boleh menindas pengekspresan onkogen *c-myc*. Kesimpulannya, *S. crispus* dan *L. inermis* berpotensi sebagai agen antikanser.

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I certify that an Examination Committee met on 3rd April 2003 to conduct the final examination of Susi Endrini on her Doctor of Philosophy thesis entitled "Chemical Constituents and Possible Anticancer Properties of *Lawsonia inermis* and *Strobilanthes crispus*" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (HigherDegree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy.

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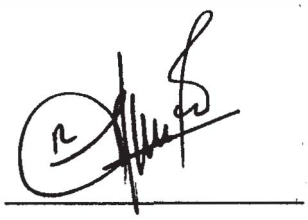
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowleged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



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CDK	Cyclin Dependent Kinase
APC	Anaphase-Promoting Complex
SPF	S-Phase Promoting Factor
DNA	Deoxyribose Nucleic Acid
VHL	Von Hipplel-Lindau syndrome
FAP	Familial Adenomatous Polyposis
HCC	Hepatocellular Carcinoma
AFP	Alfa-Feto Protein
TACE	Transcaetheter Arterial Chemoembolization
PEI	Percutaneous Ethanol Injection
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
RDA	Recommended Daily Allowance
FTC	Ferric Thiocyanate
TBA	Thiobarbituric Acid
GC-MS	Gas Chromatography- Mass Spectrophotometry
IR	Infra Red
HPLC	High Performance Liquid Chromatography
MRNA	messenger Ribose Nucleic Acid
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction

CHAPTER I

INTRODUCTION

The use of natural products with therapeutic properties is as ancient as human civilisation and, for a long time, mineral, plant and animal products were the main sources of drugs (Hernandez-Ceruelos *et al.*, 2002). In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Schwartsmann *et al.*, 2002). However, the potential use of plants as a source of new drugs is still poorly explored. Of the estimated 250,000 - 500,000 plant species, only a small percentage has been investigated phytochemically and an even smaller percentage has been properly studied in terms of their pharmacological properties (Rates, 2001)

Lawsonia inermis Linn (Henna) is a plant, which grows wild in abandoned areas (Muhammad and Mustafa, 1994) and commonly known as 'inai' in Sumatra and Malaysia or 'Pachar kuku' in Java. This plant is a known worldwide as a cosmestic agent used to stain hair, skin and nails (Hanna *et al.*, 1998). In India and Pakistan, henna is widely used by both men and women for coloring of the nails, fingers, hands, and hair. However, it is not only relevant to cosmetics. Henna also was reported to have anti tuberculostatic activity (Sharma, 1990). Shihata *et al.*, (1978) showed that henna extracts possess hypotensive, intestinal antispasmodic, and uterine sedative effects. Alcoholic extracts of henna leaves showed mild anti bacterial activity against *Micrococcus pyrogenes* var *Aureus* and *Eschericia coli* (Kritikar and Basu, 1981). The leaves also used in the manufacture of perfumed oils

and as a tanning agent (Uphof, 1968). According to Burkhill (1966), the leaves are used in wounds, ulcers, cough, bronchitis, lumbago, rheumatalgia, inflammations, diarrhoea, dysentry, leucoderma, scabies, boils, anaemia, haemorrhages, fever, falling of hair and greyness of hair (Vaidyaratnam, 1995). Poultices of the leaf are said to remedy various types of tumours (Hartwell, 1971).

In Malaysia, the leaf decoction is used after childbirth, and for beri-beri, rheumatism, skin disorders, stomach disorders, and venereal disease. In Indonesia, leaves are used for jaundice, leprosy, and scurfy affections (Perry, 1980). Mixed with the poisonous *Plumbago*, it is said to be an abortifacient. A tea of the leaves is said to be taken to prevent obesity, and an ointment made of very young fruit has been suggested for the treatment of itch. Elsewhere, the plant is used for amebiasis and headache (Leung, 1980). Cambodians drink a root decoction as a diuretic. In Arabic medicine, the bark decoction is used for jaundice and nervous symptoms (Uphof, 1968). In the Philippines, the shrub is used as an antiherpetic.

On the other hand, *Strobilanthes crispus* ZII 109 (L) Bremek or *Saricocalix crispus* ZII 109 (L) Bremek (Acanthaceae) plant is native to countries from Madagascar to Indonesia (Sunarto, 1977) and was first authored by Thomas Anderson (1832-1870) who classified the plant under Spermatophyta (Flowering plants and Gymnosperma) (Brummit and Powell, 1992).

Strobilanthes (cone-head) was named from the combination of 'strobilos' which means a pine-cone, and 'anthos' which means flower (Plowden, 1968). *Crispus* means to be phyllostachyus or spike-like leaf ('phyllo' means leaf, and

'stachyus' means spike) (Jackson, 1960). The conjunction of the names leads to the meaningful definition of the plant physical. It is commonly known as daun picah beling in Jakarta or enyoh kilo, kecibeling or kejibeling in Java (Sunarto, 1977).

This bush-like plant can be found on riverbanks or abandoned fields while some Javanese use this plant as fence. The leaves are oblong-lanceolate rather obtuse and shallowly crenate-crispate (Backer & Bakhiuzen, 1965). The top surface of the leaves is darker green in colour and less rough as compared to underside (Sunarto, 1977). The leaves covered with short hairs whereas the flowers are short, dense and panicled spikes (Backer and Bakhuizen, 1965). This plant has many cystoliths of calcium carbonate, and an infusion is mildly alkaline (Perry & Metzger, 1980).

A study in Indonesia found that an infusion of the dried leaves of *S.crispus* has been used as antidiabetic, diuretic, antilytic and laxative (Sunarto, 1977). A recent study (Kusumoto *et al.,* 1992) indicated that the water extract of *S.crispus* contained compounds with very high binding affinity to protein molecules that bind the active site of reverse transcriptase. It inhibits the proliferation of retrovirus; an agent in viral disease such as acquired immune deficiency syndrome (AIDS) and Adult T-cell Leukemia.

In this study, the anticarcinogenic effect of *S. crispus* and *L. inermis* were investigated. The active compounds were extracted and determinations of their mechanism of action were done, through the suppression of *c-myc* gene expression and apoptosis pathway. Concurrently, nutrient composition of *L. inermis* was also