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BIOASSAY-GUIDED FRACTIONATION OF THE ACTIVE CONSTITUENT OF JUNIPERUS CHINENSIS.

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BIOASSAY-GUIDED FRACTIONATION OF THE ACTIVE CONSTITUENT OF *JUNIPERUS CHINENSIS*.

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Specially dedicated to…….

Hajjah Aishah Haji Yaakob

&

Haji Ismail Samsudin.

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BuOH:	Butanol
CCl_4:	Carbon tetrachloride
$CDCl_3$:	Deuterated chloroform
CFU/ml:	Colony forming unit per milliliter
$CHCl_3$:	Chloroform
CO_2:	Carbon dioxide
DMSO:	Dimethyl sulphoxide
EC_{50}:	Effective concentration at 50% cells reduction
EDTA:	Ethylenediaminetetraacetic acid
EtOAc:	Ethyl acetate
EtOH:	Ethanol
FCS:	Fetal calf serum
H_2O:	Water
H_2SO_4:	Sulfuric acid / Hydrogen sulfate
MEC:	Minimum effective concentration
MeOH:	Methanol
hex:	normal hexane
NCI:	National Cancer Institute
PBS:	Phosphate buffered saline
pet. ether:	petroleum ether
PMPLC:	Preparative medium pressure liquid chromatography
PTLC:	Preparative thin layer chromatography
TLC:	Thin layer chromatography

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INTAN SAFINAR ISMAIL
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Chairman: Dr. Abdul Manaf Ali.
Faculty: Food Science and Biotechnology.

Deoxypodophyllotoxin, a lignan, was afforded from the bioassay-guided fractionation of the EtOAc soluble part of the leaves and twigs of *Juniperus chinensis*. The fractionation was directed by microtitration cytotoxicity assay employing human cervical adenocarcinoma (HeLa) cell line. The activity was visible by fixing and staining the cells and comparing the number of cell reduction by the active agent with the confluent controls. A judicious combination of chromatographic techniques was adopted in purifying the active compound from the crude complex. The structure of the isolated lignan was elucidated using spectroscopic techniques including ultraviolet spectroscopy (UV), infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C-NMR), mass spectroscopy (MS), and also by comparison with the literature.

The cytotoxic concentration of deoxypodophyllotoxin which caused up to almost 100% reduction of HeLa cells was determined as 0.004 µg/ml. Cytotoxic activity of this lignan was further evaluated on different types of specific human organ tumour cell lines: KU812F (Chronic mylogeneous leukemia), TK-10 (Renal carcinoma), UACC-62 (Melanoma) as well as CEM-SS (T-cell lymphoblastic leukemia). All of the tumour cell lines studied were found to be susceptible to deoxypodophyllotoxin, nevertheless, the degree of susceptibilities was different between cell lines. Minimum effective concentration (MEC) with almost 100% reduction of the cells were observed in HeLa (0.004 µg / ml), TK-10 (0.01 µg / ml), UACC-62 (0.004 µg/ml) and CEM-SS (0.01 µg/ml). Whilst KU812F (0.04 µg/ml) inhibited only 50% the cell growth (EC_{50}). Thus, the most sensitive cell lines towards the treatment of the lignan were HeLa and UACC-62.

Antimicrobial disc diffusion assay (Bauer et al., 1966) on deoxypodophyllotoxin was carried out employing gram positive bacteria (*Bacillus megaterium, Bacillus cereus, Bacillus subtilis, Flavobacterium meningosepticum, Staphylloccus aureus, Micrococcus luteus, Chrysomonas leuteola* and *Aeromonas salmonella*), and gram negative bacteria (*Pseudomonas aeruginosa, Pseudomonas paucinobilis, Pseudomonas capacia* and *Escherichia coli*), and on yeast (*Torulopsis glabrata, Crytococcus neoformans, Saccharomyces lipolytica, Candida albicans, Candida lipolytica* and *Candida intermedia*), and also a fungi (*Aspergillus ochraceous*). The growth of most of the organisms were inhibited by

deoxypodophyllotoxin at the concentration of 10 mg/ml by producing a clearing zone with diameter ranging between 8 to 12 mm with the exception of *Pseudomonas aeruginosa, P. paucinobilis, Aeromonas salmonella* and *Candida intermedia*.

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PENGASINGAN SEBATIAN AKTIF BERPANDUKAN PENCERAKINAN BIOLOGI TERHADAP *JUNIPERUS CHINENSIS*.

oleh
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Deoksipodofilotoksin, sejenis lignan, yang telah berjaya diasingkan hasil daripada pemeringkatan bahagian larut EtOAc daun dan batang *Juniperus chinensis* berpandukan kepada biocerakinan berasaskan kepada pencerakinan kesitotoksikan mikrotitratan menggunakan jujukan sel karsinoma serviks (HeLa). Aktiviti kesitotosikan dapat dilihat melalui proses pelekatan dan pewarnaan sel, dan seterusnya membandingkan pengurangan sel oleh deoksipodofilotoksin berbanding dengan kawalan. Pelbagai kombinasi teknik kromatografi telah digunakan untuk menulenkan lignan ini. Strukturnya telah dikenalpasti menggunakan berbagai teknik spektroskopik seperti spektroskopi ultralembayung (UV), spektroskopi inframerah (IR), spektroskopi resonans magnet nukleus proton-1 dan karbon-13 (^{1}H dan ^{13}C-NMR), spektroskopi jisim serta perbandingan dengan data kajian terdahulu.

Kepekatan deoksipodofilotoksin yang menyebabkan pengurangan 100% sel HeLa adalah 0.004 µg/ml. Aktiviti kesitotosikan lignan ini seterusnya telah diuji ke

atas pelbagai jujukan sel kanser yang lain iaitu KU812F (leukemia kronik), TK-10 (karsinoma renal), UACC-62 (melanoma), CEM-SS (T-cell leukemia lymphoblastic) dan MCF-7 (karsinoma payudara). Deoksipodofilotoksin didapati aktif terhadap kesemua jujukan sel tersebut tetapi pada darjah yang berbeza-beza. Kepekatan berkesan minima (MEC) yang menyebabkan pengurangan hampir 100% sel dilihat pada HeLa (0.004 μg/ml), TK-10 (0.01μg/ml), CEM-SS (0.01 μg/ml) dan UACC-62 (0.004 μg/ml). Kepekatan berkesan yang merencat pertumbuhan sel sebanyak 50% (EC_{50}) adalah KU812F (0.04 μg/ml). Kesimpulannya, HeLa dan UACC-62 merupakan jujukan-jujukan sel yang paling sensitif terhadap deoksipodofilotoksin.

Cerakinan antimikrob juga telah dilakukan berasaskan teknik perebakan cakera (Bauer et al., 1966). Mikroorganisme yang telah digunakan bagi tujuan cerakinan ini adalah bakteria gram positif (*Bacillus megaterium, Bacillus cereus, Bacillus subtilis, Flavobacterium meningosepticum, Staphylloccus aureus, Micrococcus luteus, Chrysomonas leuteola* dan *Aeromonas salmonella*), bakteria gram negatif (*Pseudomonas aeruginosa, Pseudomonas paucinobilis, Pseudomonas capacia* dan *Escherichia coli*), yis (*Torulopsis glabrata, Crytococcus neoformans, Saccharomyces lipolytica, Candida albicans, Candida lipolytica* dan *Candida intermedia*) dan juga kulat (*Aspergillus ochraceous*). Deoksipodofilotoksin pada kepekatan 10 mg/ml telah berjaya merencat pertumbuhan kebanyakan organisme yang digunakan dengan julat diameter di antara 8 hingga 12 milimeter kecuali terhadap *Pseudomonas aeruginosa, P. paucinobilis, Aeromonas salmonella* dan *Candida intermedia*.

CHAPTER I

INTRODUCTION

Plant Natural Products

The natural world was once the sole provider of all medicinal agents. Today, the plant kingdom still provides a wide array of natural products with diverse chemical structures and variety of biological activities. Natural products contribute over 50% of all drugs in clinical use and higher plant derived drugs represent 25% of the total available drugs (Balandrin et al., 1993). Fansworth (1977) stated that there are about 250,000 to 500,000 species of higher plants alone from which pharmacological screening could be carried out as they are untapped reservoir, only awaiting to be investigated. Among the plants are the ferns and their allies which are about 10,000 species and consist of the gymnosperms which represents a small group of some 700 species in 65 genera. The most dominant group of plants found on land and man's principal source of healing plants is the angiosperms, which comprises of at least 250,000 species in 10,500 genera in 300 families. One-quarter of this group are monocotyledons and the rest are dicotyledons (Thomson, 1978).

Malaysia offers a biodiverse plant resource of some 15,000 species of higher plants. Located near the equator, the country is endowed with the tropical rain forest

which is known to provide some 1,300 species of the whole plants which have been recognized for their medicinal properties (Burkill, 1966). About 1,000 plant species have undergone simple chemical screenings, but fewer have been subjected to the chemical and pharmaceutical studies (Goh et al., 1993). However, much intensive studies are being carried out on the Malaysian plants. Numerous cytotoxic compounds have been isolated including 5-hydroxy-7-methoxyflavone or tectochrysin obtained from *Fissistigma latifolium* (Jubri et al., 1995) and two new podophyllotoxin derivatives from *Casearia clarkei* (Shaari & Waterman, 1995).

The search for health beneficial agents from natural sources has been a crucial quest of mankind since prehistoric time. This search, especially for potential anticancer agents could be traced back at least to the Ebers Papyrus in 1550 B.C. (Kingston et al., 1990). A documentary evidence of the quest is a written text in which forty plants are recommended including barley, flax, absinth, coriander, fig, onion, garlic, dates, juniper and grapes (Cordell et al., 1993). During the 1880's, the active principles of a number of plant drugs were isolated and it was realized that the clinical effects of drugs such as opium, cinchona, and ipecacuanha could be attributed to the chemical compounds morphine, quinine and emetine, respectively (Lewis and Elvin-Lewis, 1977). Moreover, in 1975, about 20,525 different species of plants were screened for animal antitumour activity (Fansworth and Bingel, 1977).

However, the scientific search of medicinal natural products started only recently with the investigations by Hartwell and coworkers (1951) on the application of podophyllotoxin from *Podophyllum peltatum L.* and its derivatives, as anticancer agents. Indeed, the modern era of drug development from plants is greatly explored with the discovery of drugs such as vincristine, vinblastine, adriamycin, mitomycin, anthramycin, taxol and other natural products (Kingston et al.,1990).

Active Components of Medicinal Plants

The healing value of the curative herbal drugs from plant origin is due to the presence of active chemical principle(s) producing a physiological effect. Many of the active agents are highly complex involving many functionalities in their structures, and their exact chemical nature occasionally is still unknown. Others have been isolated, purified and synthesized.

Compounds of known structure isolated from higher plants surveyed in 1975, were mostly from the plant groups of monocots, dicots and gymnosperms with dicot plants contributing the largest number of natural product compounds. According to the survey, about 325 higher plants have relevant potential use as drugs. The majority of biologically active plant principles were alkaloids (73/325), followed by sesquiterpenes (47/325), diterpenes (26/325), triterpene saponins (22/325), triterpene

aglycones (26/325), flavonoids (18/325), coumarins and quinones (15/325 each), sterols (17/325) and monoterpenes (13/325) (Fansworth and Bingel, 1977).

Juniperus

Besides random collection of plant materials, targeted collection based on chemotaxanomic relationship and the ethnomedical information is normally used in current search for bioactive compounds. In general, juniper has an extensive history as a folk medicine, primarily as diuretic and carminative, useful in dropsy and renal affections. It continues to be widely employed as flavor, notably in gin, and as one of the perfume ingredients (Chandler et al., 1986). In ancient times the berries of the *Juniperus* were swallowed to cause abortion, hence was named 'bastard killer'. Moreover, this particular species was chosen for this investigation when it showed a promising effect in the screening of fourteen plants (Table 9).

Juniperus is one of the chief genera in the family of Cupressaceae beside *Callistris, Widdringtonia, Thuja, Libocedrus, Cupressus,* and *Chamaecyparis*. The plant is prostrate to upright in the pyramid-like shape with resinous and incense smell wood and a pale reddish-brown, scaling off bark. Leaves are needle-like or scale-like opposite or in three and in some species they may be spirally arranged in juvenile forms, they are closely placed and glaucous green with slight tone of blue. *Juniperus* which

is commonly known as cedar consists of 40 species of aromatic, terebinthinate, and small or large bushy shrubs of Cupressaceae.

Juniper tree is a communal plant in the North and West Himalaya and it grows to an elevation of 5000 feet. The tree spread across widely in the cool and temperate regions of the world but attain their maximum development in the Mediterranean region, the North Atlantic Island and Eastern North America. In Asia this shrub is found mostly in Caucasus where it reaches to 12,000 feet in height, the Caspian districts, Siberia, China and Japan.

Juniperus chinensis or Chinese juniper also known as evergreen blue pine (conifer) is a mutual ornamental growth originated from China and Japan. The leaves are tiny and set very closely on the twigs. The juvenile leaves are needlelike and spiny, while the adult leaves are scale-like. It was introduced to neighboring South-East Asian countries primarily as an ornamental plant (Corner, 1988). Three major compound groups; flavones, lignans and terpenes were successfully extracted from the leaves (Lee et al., 1995). Biological studies have indicated antitumor, antibacterial, antifungal, abortificient, antiinsectant, antifertility, antiplatelet, vasorelaxing and antiviral activities of this species (Ali et al., 1996).

Isolation of the Active Compound from *J. chinensis*

In this study, the leaves at the height of 4 feet of *J. chinensis* were collected for the determination of cytotoxic material upon the human tumor cells. In many investigations the bioassay-directed fractionation of crude plant extract is widely utilized in order to obtain the biologically active constituent(s). The success or the failure of studies with bioactive factors depends exclusively on whether one succeeds in the isolation (Hostettmann et al., 1991). In the course of fractionating the desired plant crude, a combination of judicious chromatographic methods were employed.

During the National Cancer Institute (NCI) screening of 35,000 plant species (1960-1986), a number of *in vivo* and *in vitro* methods have been used in assessing the bioactivities from plants. Recently, the *in vitro* protocols using established panel of cell lines displayed the potential of replacing whole animal studies in the preliminary screening (Suffness & Douros, 1982). *In vitro* cytotoxicity is an activity that is consistent with antitumor activity which can assist in deciding the type of materials to be subjected to fractionation procedures. In fact, *in vitro* assay systems are less time consuming, inexpensive and require only a small amount of samples which are ideal in fast directing the purification of a crude complex.

Objective of Investigation

Although many compounds of both natural and synthetic origin proved to have good activities experimentally, only a small number have been proven useful in the clinic. Therefore, there is a continuing need for active compounds with novel structures and mechanisms of action. Thus, the possible prospect of discovering novel compound(s) of natural origin with an antitumor inhibitory property has encouraged the accomplishment of this current study on *Juniperus chinensis*. Hence, the major purpose of this study is to isolate the active compound(s) from *Juniperus chinensis* plant using bioassay-guided fractionation utilizing an established mammalian HeLa cell line as the key assay guide. After acquiring the bioactive compound(s), other cell lines; KU812F (Chronic mylogeneous leukemia), TK-10 (Renal cancer), UACC-62 (Melanoma), and CEM-SS (T-cell lymphoblastic leukemia) were utilized as *in vitro* models for further evaluation of the cytotoxic activity on organ specific cultures.