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TITLE: ANTIPROLIFERATIVE ACTIVITY OF *QUERCUS INFECTORIA* GALLS EXTRACTS TOWARDS HELA AND CAOV-3 CANCER CELLS AND ITS APOPTOSIS INDUCING ACTIVITY ON SELECTED CANCER CELL LINES

by

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ABSTRAK

Quercus infectoria adalah satu tumbuhan oak yang boleh didapati di Asia Minor termasuk Greece dan Iran. Projek ini dijalankan untuk menguji aktiviti antiproliferasi ektrask galls dari *Quercus infectoria* secara *in vitro.* Tiga ektrask dari pelarut aqua menggunakan kaedah rendaman dan sokhlet, satu ektrask dari pelarut ethanol dengan keadah rendaman, dan satu ektrask dari pelarut methanol menggunakan keadah rendaman diguna dalam projek ini. Kesemuaan lima ektrask kasar dirawat ke titisan sel kanser HeLa dan Caov-3. Kesemuaan ektrask menunjuk IC_{50} kurang daripada 100 µg/ml. Aktiviti antiproliferasi yang paling berkesan ialah ekstrak kasar etanol terhadap titisan sel HeLa dengan IC_{50} 2.82 ug/ml. Titisan sel HeLa (titisan sel yang paling potensi telah dirawat dengan ekstrak kasar ethanol selama 24, 48, dan 72 jam untuk perwarnaan dengan dye Hoescht 33258 untuk mangaji perubahan morfologi pada membran sel dan nuklues yang menrupakan kesan apoptotilk. Kesimpulannya, ekstrak dari Manjakani mengandungi kompound aktif yang berpontensi bertindak sebagai agen antikanser.

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ABSTRACT

Quercus infectoria is an oak tree available in Asia Minor include Greece and Iran. The project is conducted to evaluate the antiproliferative activity of *Quercus infectoria* Olivier galls' extracts *in vitro*. Three extracts from aqueous crude extract by soaking and soxhlet method, one extract from ethanol crude extract by soaking method and one extract from methanol extract by soaking method were used. All the five crude extracts were used to treat HeLa and Caov-3 cancer cells line to screen for their antiproliferative activity. All the extracts shown to have IC_{50} less than 100 μg/ml. Among them, the highest antiproliferative activity of IC_{50} 2.82 μg/ml was shown by the ethanol crude extract treated HeLa cancer cell lines. HeLa cells (most potent cell lines) was then treaed with ethanol crude extract for 24, 48 and 72 hours and stain with dye Hoescht stain 33258 stain to demonstrate the morphological changes occur in cell and nuclear membrane as a result of apoptosis. In conclusion, *Quercus infectoria* galls' extracts contain potentially active compounds that act as anticancer agent.

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LIST OF SYMBOLS

%	percentage
cm2	Centimeter square
cm	centimeter
mm	Millimeter
nm	nanometer
um	micrometer
ml	Milliliter
ul	Microliter
g	Gram
mg	Milligram
ug/ml	Microgram per mililiter
mg/ml	Miligram per mililiter
V	Volt
Hz	Hertz
W	Watt
v/v	Volume per volume
w/v	Weight per volume
M	Molar
®	Registered trademark
=	Equals to
rpm	Relative centrifugal force

LIST OF ABBREVIATIONS

CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonuclei acid
EDTA	Ethyenediaminetetra acetic acid
FBS	Fetal bovine serum
FDA	Food and drug administration
GCMS	Gas-liquid chromatography-mass sprectometry
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HPV	Human pappiloma virus
MRSA	Methicilin-resistant staphylococcus aurens
NaCl	Sodium chloride
NaH_2CO_3	Sodium bicarbonate
Na_2PO_4	Sodium hydrophosphate
NCI	National Cancer Institute
OD	Optical density
PBS	Phosphate buffered saline
PGE2	Prostagladin E2
QI	*Quercus infectoria*
rpm	Round per minutes
SEM	Scanning electron microscope
SME	Standard mean error
TEM	Transmission electron microscope
UK	United Kingdom
US	United State
WHO	World Health Organization

CHAPTER 1:

PREFACE

1.1: INTRODUCTION

Over the years, the interest in research on natural products has increased in the discovery of more efficient drugs for cancer treatment (Calixto, 2000; Rates; 2001; Philipson, 2001). The lack of modern inexpensive medicines for needy population tend to increase the run for cheap alternatives, that have rather doubtful efficiency, promising miracles without side effects (Calixto, 2000; Taylor & Staden, 2001). Side effect and high cost of conventional treatments as well as their therapeutic limitation have revive researches for plant based medicines (Eun & Pezzuto, 2002).

Consumption of a large amount of plants have shown to decrease the risk of cancer. Anticancer drugs based on natural products have continue to be an active area of research throughout the world (Spainhour, 2005). Extract and compound from plants are potential source for anticancer agent. (Cordell *et al.*, 1993).

In this research, a white oak, *Quercus infectoria* Olivier (QI) is chosen for screening potential anticancer agent. QI is a small oak, indigenous to Greece and Iran (Umachigi, 2008). The leaves of Q. *infectoria* is ovate-oblong, sinuate-dentatte, very smooth, and deciduous (Daniel, 2005).

QI is a small tree or shrub, growing to four to six feet tall. Its stem is crooked and its leaves on short petioles with a few short mucronate teeth on each side (Henrietta, 2009). The commercially available product of QI's nut-galls (*Gallae officinorum*) are

produced by the *Cynips gallae tinctoriae* on the QI.

Hippocrates have employed the nut-gall as an astringent, both internally and externally. Over the years, Native Americans have used white oak acorns as food staple and the inner bark as an ingredients in cough medicine, as tonic, as expectorant, and for treatment for rheumatism, bleeding hemorrhoids, diarrhea, dysentery and wounds. White oak was listed in *United State Pharmacopecia* from 1820 until 1916, and in *National Formulary* from 1916 to 1936. (Foster, 1998).

Figure 1.1: *Quercus infectoria* galls

(Source: http://www.dehlvi.com/dynamic-images/ingradient/Quercus-infectoria.jpg)

1.2: GENERAL: *Quercus infectoria* Olivier

1.2.1: Classification of *Quercus infectoria* Olivier

Kingdom: Plantae (Plants)

Sub kingdom: Tracheobionta (Vascular plants)

Subdivision: Spermatophyta (Seed plants)

Division: Magnoliophyta (Flowering plants)

Class: Magnoliopsida (Dicotyledons)

Subclass: Hamamelididae

Order: Fagales

Family: Fagaceae (Beech family)

Genus: Quercus L. (oak)

Species: *Quercus infectoria* Olivier (aleppo oak)

(NRCS, 2009)

1.2.2: Classical and Common Name

ayurvedic: Maajuphalaka (Bhaavaprakaasha), maayaaphala *uniani:* Maajuphal; Maazu (Persian), Maaphala

Siddha: Mochakai, Mashikkai

English: Oak Galls, Aleepo galls, Mecca galls

Malay: Manjakani

(Foster, 1998)

Quercus is the classical name for oak tree, represented by over 400 species. Two basic groups are recognized in North America, the white oak group (sub-genus Leucobalanus) and the red or black oak group (sub-genus erythrobalanus). (Foster, 1998)

The QI galls are excrescences on the *Quercus infectoria*, which is stimulated by the reaction between plant hormones and powerful growth regulating chemicals

produced by insects or mites (Townsend, 1998). It is the result of the puncture of the bark of young twigs by the female gall-wasp, *Cynips Gallae-tinctoriae*, who lays eggs inside (Cook, 1869). It is used in commercial and as medicine. The young larvae that hatches from the eggs feeds upon the tissues of the oak and secretes in its mouth a peculiar fluids, which is capable of stimulates the trees cells to rapidly divide and result an abnormal development, the gall.

The growth of the gall continues only as long as the egg or larva lives or reach maturity and passes into a chrysalis, a fully developed gall-wasp emerges and escapes into air through a hole it bored with its mandible inside or the gall.

For medicinal use, galls are collected before the insect escape. Those galls from which the insect has escaped are commonly larger, lighter colored, and less astringent. They are termed white galls. The galls that are gathered before the insect has escaped include black or blue nut-galls (*Gallae nigra; Seu caeruleae*); green nut-galls (*Gallae virides*). These are called by the natives as "*yerli*". They vary from the size of a pea to that of a hazel-nut, and have a grayish color. Externally they are frequently tuberculated, but the surface of the tubercles and the intervening spaces is usually smooth. Their texture is compact, but fragile. They have no odor, but a styptic and powerful astringent taste (Henrietta, 2009).

Figure 1.2: *Quercus infectoria* galls (manjakani) (Source: Pin et al., 2006)

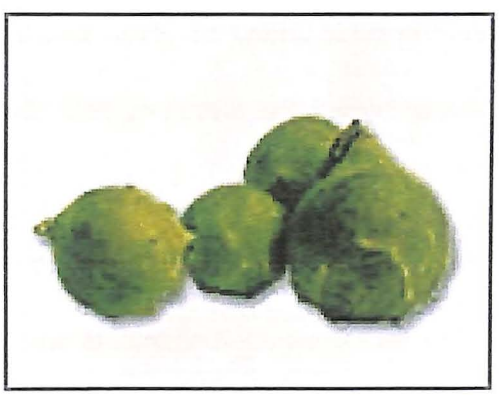

Figure 1.3: Green nut-galls or *gallae virides (Source:* http://tjh.sg/Ing_QuereusInfectoria.html)

The galls extract contains Tannic acids and Gallic acids, which are powerful astringents (Pin *et al.,* 2006). Oak galls contain 50-70% tannins (gallotannins). It also contains Gallic acids (2-4%), ellagic acids, nyctanthic acid and rubric acid; gum, starch, sugar and essential oil. Amentoflavone hexamethyl ether, iso-crytomerin and beta-sitosterol also been isolated (Khare, 2004). The main constituents found in the galls of QI were tannin (50-70%) and small amounts of free Gallic acid and ellagic acid (Dar & Ikram, 1976, Wart & Kumar, 2001 and Kokate, 1994). The antioxidants present in plants may contribute to the anti-carcinogenic effect, and other such as flavanols have been able to inhibit cell proliferation *in vitro* (Scalbert *et al.*, 2005). The presence of flavanols was studied recently as non-timber products from several species of Quercus (Gonclaves *et al.*, 2008). Furthermore, the Gallic acids presents in oak galls is can endogenous product in plants that possesses anti carcinogenic activity (Shahrzard *et al.*, 2001).

Rohana *et al* (2004) reported that the QI galls aqueous extract showed high potential in skin whitening and antioxidant properties as the extract inhibited the super oxide and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities, and tyrosinase activities . Aqueous extract of QI galls was reported to have high hydrolysable tannin content which inhibits the lethality of the *Naja kaouthia* (Thai cobra) venom (Pithayanukul et al., 2004). The hydrolysable tannins including tannic acid and Gallic acid are powerful astringent that are prescribed in diarrhea. The extract of QI galls also shown high antimicrobial activity against *Escherichia coli* (*E. coli*) O157:H7 (Voravuthikunchai et al., 2004). The scientific studies of aqueous extract of QI galls have revealed its potential to provide an alternative for modern medicinal products as well as cosmetics and skin care products. However, no previous study was

done on the antiproliferative activity of *Quercus infectoria* Olivier galls extract.

The ethanol extract of the galls of *Q. infectoria* have a high potential as antibacterial agent against methicillin-resistant *Staphylococcus aurens* (MRSA) (Voravuthikunchai *et al,* 2007). Galls of *Q. infectoria* possess pleiotropic therapeutic activities, with particular efficacy against inflammatory disease. Oral administration of gall extract significantly inhibited carrageenan, histamine, serotonin and prostagladin E2 (PGE2) induced paw edemas The extract also inhibit various functions of macrophages and neutrophiles relevant to inflammatory responses (Kaur *et al.,* 2004).

Tannins are polyphenols with astringent taste. Two groups of tannins, namely hydrolyzable tannin and condensed tannin are identified in plants (Daniel, 2005). Hydrolyzable tannins are soluble in water, contain simple phenolic acids esterified with one or more sugars molecules and are hydrolyzed by dilute acids. The condensed tannins are insoluble in water, which on treatment of acids, are capable of yielding complex products of unknown composition called "tannin reds" or "Phlobaphenes".

The hydrolyzable tannins are abundant in leaves while the condensed tannins are abundant in wood. Usually a single species contains only one of these groups (Cai *et al.,* 2005)

1.2.4: Ethnopharmacology Study:

Traditional used medicinal plants have recently received the attention of the pharmaceutical and scientific communities (Taylor *et al.,* 2001). Ethnopharmacology was defined as the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by human. The purpose of this field of study was the validation of traditional preparation either through the isolation of active substances or through pharmacological findings on indigenous drug preparation (Holmstedt & Bruhn,

1995).

Gall of *Quercus infectoria* (QI), or better known as Manjakani, is originated from Western Asia and Southern Europe. The galls of QI have been used for centuries traditional medicines in Asian countries for treating inflammatory disease (Council of Scientific and Industrial Research (CSIR), 1995). Majuphal, a widely known plant in Indian traditional medicine has been used as dental powder and in the treatment of toothache and gingivitis. (Chopra *et al.*, 1956 and Hwang *et al.*, 2000). The galls of QI have also been pharmacologically documented to possess astringent, anti-tremorine, local anesthetic (Hussein *et al.*, 2000), antiviral (Fatima *et al.*, 2001), antibacterial, antifungal, (Digraki *et al.*, 1999), larvicidal (Redwane *et al.*, 2002) and anti-inflammatory (Kaur *et al,.* 2008) activities. The ethnopharmacological studies can contribute greatly to modern medicine and leads to discovery of novel useful drugs.

1.3: HYPOTHESIS

Researches had done proved that QI exhibited antimicrobial and antiviral activity. The QI galls contain a large amount of antioxidant such as tannin, ellagitannin. The antioxidant properties are useful to prevent cell damages that eventually may leads to cancer. The extracts of QI galls maybe used as cancer preventive agent and antiproliferative agents.

The QI galls were extracted using two methods: soxhlet and soaking, with three different solvents, namely ethanol, methanol and distilled water. The extracts are tested *in vitro* against HeLa (cervical cancer) and Caov3 (ovarian cancer) cell lines. The antiproliferative activities are evaluated using methylene blue assay. The assay is easy to handle, low in cost and gives minimum erratum (Hasmah, 2006). The extracts are in a concentration that has antiproliferative effect but it does not distract *in vitro* metabolite

function system has the potential to become anticancer agent (Ng Yen, 2002). According to Wilson (1986), 50% of Inhibitory Concentration (IC_{50}) less than 100μg/ml is used as criterion to determine significant biological activity of an extract. However, the concentration less than 20 μg/ml is preferred in order to classify biological activity of natural product from plants (Suffness & Pezzuto, 1991).

The determination of IC_{50} for each type of extract using graph. This method can be very helpful in order to understand in depth the potential of biological extract used (Cordell *et al.*, 1993). The best IC_{50} is the lowest concentration can to inhibit 50% of the maximum possible inhibitory response prior to proceeding with Hoescht Stain. Hoescht 33258 stain is used to show DNA fragmentation in nucleus of dying cell (Eric & Huseyin , 2003).

1.4: RESEARCH OBJECTIVE:

1. The aims of this research are:
2. To determine the potential of *Quercus infectoria a*s an antiproliferative agent towards HeLa and Caov-3 cancer cell lines.
3. To determine the morphological changes through DNA fragmentation event in HeLa or Caov-3 cancer cells based on their antiproliferative activity.

CHAPTER 2:

LITERATURE REVIEW

2.1: CANCER

The normal cell in our body has the potential to become cancer cell (Weinberg, 1996). Cancer might be thought of as a disease characterized by a deregulated cellular growth. There are over 100 different types of cancer, and each is classified by the types of cell that is initially affected (Medical News Today, 2009)

Cancer can harm the body when damaged cells divide uncontrollably to form lumps or masses of tissue called tumors (except in the case of leukemia when cancer prohibits normal blood function by abnormal cell division in the blood stream). Tumors can grow and interfere with digestive, nervous, and circulatory systems and dispates hormones that alter body function. Cancer is the ultimate result of cells that grow uncontrollably. Normal cells in the body follow an orderly path of growth, division and death. Programmed cell death is called apoptosis, and when this process is interfered, cancer begins to form. Unlike normal cells, cancer cells do not experience programmatic cell death but growth and divide continuously (Medical News Today, 2009)

Cancer is clinically treated by surgery, radiotherapy and chemotherapy. After surgical ablation of progressive cancer, however, metastasized tumor cells continue to progress and making cancer treatment difficult (Fidler & Kripke, 1977). Anticancer drugs and radioactive rays mostly damage DNA or suppress DNA duplication to kill

tumor cells from growing rapidly (Kligerman, 1973). They also affect normal cells to cause serious adverse effects (bone marrow function inhibition, nausea, vomiting and alopecia and others). More effective anticancer drugs with high selectivity against only malignant cells and with ability to repress tumor metastasis are desired (Demirtas *et al.*, 2009)

In 2007, cancer have caused death about 7.6 million people worldwide (WHO, 2006). In 2006, Peninsular Malaysia have a total of 21,773 cancer cases registered in the National Cancer Registry. It comprised of 9,974 males and 11,799 females (National Cancer Registry (NCR), 2006). The different between benign and malignant tumor are shown in Table 2.1. Malignant tumors are also known as cancer.

Table 2.1: Differences between benign and malignant tumor

(Source: Azimahtol, 1998)

Benign	**Malignant**
It is not classified as cancer	It is classified as cancer
Do not need life-long treatment	Need life-long treatment
The tumor is capsulate and can be remove	Tumor can be remove if early stage
Rarely recur	Can recur and metastasis
Slow tumor growth rate	Fast tumor growth rate

2.1.1: Cervical Cancer

Cervical cancer was once the leading causes of deaths among women in United States (CDC, 2009). Cervical cancer refers to cancer that forms in cervical tissue, which is the organ connecting the uterus and vagina. Cervical cancer is almost always caused by Human papilloma virus (HPV) infection.

According to National Cancer Institute, the estimated new cases of cervical cancer in United State alone is 11,270 in 2009 and deaths cause by cervical cancer is estimated to be 4070 cases. In US alone, an estimated over 2 billion US dollar is spent on the treatment of cervical cancer per year (Brown *et al.*, 2001). The cervical cancer was the third most common cancer among women. There were a total of 1,074 cases in 2006 registered with National Cancer Registry (NCR, 2006). In Peninsular Malaysia, cervical cancer accounts for 12.9% of all cancers in women (NCR, 2006).

When the cancer is detected at early stage, the survival rate is close to 100%. The prognosis for invasive cervical cancer depends on the stage of which the cancer is detected. The stage of a cancer is a measure of how far the cancer has progressed, and the tissue it invaded.

The earliest stage of cervical cancer, more than 90% women survive at least 5 years after diagnosis. while for late stage of cervical cancer, the prognosis is significantly worse. Twenty percent (20%) or less women with stage IV cervical cancer survive 5 years (CancerHelp UK, 2009a).