ejpmr, 2016,3(1), 46-49

SJIF Impact Factor 2.026

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 3294-3211

EJPMR

ANTI-CANCER ACTIVITY OF AQUILARIA MALACENSIS LEAVES ON HUMAN CERVICAL CANCER CELLS

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Article Received on 05/11/2015 **Article Revised on 26/11/2015** **Article Accepted on 16/12/2015**

ABSTRACT

Background: Recent studies have shown that anti-oxidant and herbal derivatives may be effective against prevailing high risk human papilomavirus infection but these leads are yet to prove their efficacy in preclinical and subsequent clinical studies. Aquilaria malacensis belongs to the, locally known as gaharu are reported to contain more than a dozen chemical constituents The stem of Aquilaria sinensis, another species of Aquilaria, reported to possess anti-cancer activity Moreover, the pesence of glycoside moieties like saponins, antraquinones, steroidal glycosides and flavonoids could inhibit tumor growth. **Methods:** The leaves of *Aquilaria malacensis* were extracted and fractionated to get fractionn-hexane, ethylacetate and etanol-aquadest. Cytotoxic activity of every fraction was assayed with MTT assay in HeLa cell culture. Phytochemical assay was done to explore chemical constituent in every fraction. **Results:** Fraction ethylacetate and n-hexane had the same potential to inhibit cervical cancer growth from concentration 15,63 µg/ml to 125 µg/ml. In the concentration more than 125 µg/ml, The potention to inhibit cervical cancer cell growth increased for fraction n-hexane but the potention decreased for fraction ethylacetate. The inhibitory concentration 50% (IC 50) for fraction n-hexane was 163 µg/ml. **Conclusion:** Our study therefore demonstrates presence of anticancer in gaharu leaves that can be further exploited as a potential anticancer.

KEYWORD: Aquilaria- Fraction – HeLa Cell- Anti cancer.

BACKGROUND

For many centuries, plants have been a rich source of therapeutic agents and provided basis for several synthetic drugs. Despite great development of organic synthesis, currently 75% of prescribed drugs worldwide are derived from plant sources, showing that plant species are still an important source of new drugs for diseases that continue to lack a cure, such as cancer.[1]

Cancer is a major problem of public health in many other parts of the world, including Indonesia. Cervical cancer is the principal cause of cancer related mortality in women of the developing countries that contribute more than 85% of global disease burden.[2] Persistent infection with high risk human papilomavirus, most notably of the type 16 and 18, is an essential prerequisite for the development of cervical cancer, resulting in dysregulation of host cell cycle by targeting pivotal cell cycle proteins p53 and pRB by their viral gene products E6 & E7,respectively.[3-7] Constitutive expression of high risk human papilomavirus E6 & E7 oncogene is mainly dependent on the availability of host cell transcrition factors that act upon viral promoter and enhancer region. Activator protein-1 (AP-1), a heterodimer of a group of structurally and functionally related members of the Jun and Fos family is one of the transcription factors that are essential required for viral oncogen expression.[8] Mutational inactivation of AP-1 cis-acting site within the high risk human papiloma virus upstream regulatory region (URR) that facilitates AP-1 interactions revealed a complete loss of transcriptional activity of the E6/E7 promoter and showed a key role of AP-1 in HPV-mediated carcinogenesis.[9] Though human papilomavirus-mediated mechanism of cervical carcinogenesis is now well defined, anti-human papilomavirus therapeutics for elimination of human papilomavirus infections are yet to become a clinical reality and conventional physical ablation of human papilomavirus-infected lession in precancer stage are in clinical practice. Currently, there is no human papiloma virus specific therapy for treatment cervical cancer. Recent studies have shown that anti-oxidant and herbal derivatives may be effective against prevailing high risk human papilomavirus infection but these leads are yet to prove their efficacy in preclinical and subsequent clinical studies.[10-13]

Aquilaria malacensis belongs to the family Thymelaeaceae, locally known as gaharu. *Aquilaria malacensis* distributed in Indonesia, Malaysia,

Philippines and Burma. Gaharu leaves are reported to possess antidiabetic, antiinflamatory, antioxidant, antibacterial, and antiviral activities.[14-16] The methanolic extract of leaves gaharu is reported to contain more than a dozen chemical constituents belongs to flavonoids, alkaloids, terpenoids and glycosides class of secondary metabolites that can be extracted.[14] The stem of *Aquilaria sinensis*, another species of Aquilaria, reported to possess anti-cancer activity.[17] Moreover, the pesence of glycoside moieties like saponins, antraquinones, steroidal glycosides and flavonoids could inhibit tumor growth.[18]

In view of absence of anti-human papilomavirus therapeutic for prevention and treatment of crvical cancer, in the present study, we examined leaves of *Aquilaria malacensis* for presence of anti-cancer against human cervical cancer cells, HeLa that harbours high risk human papilomavirus type 18.

METHODS

Materials

The human papilomavirus 18 positive human cervical cancer cell line, HeLa was obtained from The American Type Culture Collection (ATCC). Roswell Park Memorial Institute (RPMI) 1640 media, fetal bovine serum (FBS), 3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicilin-streptomicin solution and all other reagents were of analytical grade and purchased from Sigma.

Collection of Plant Material and Identification

The leaves of *Aquilaria malacensis* were collected from Gaharu Plantation in Gandus District, Palembang, South Sumatera Province, Indonesia, in the month of June-July, identified and authenticated by the Indonesia Science Institute (LIPI). The collected plant material was made free from foreign organic matter.

Extraction and Fractination

Dried gaharu leaves (750 grams) were extracted with ethanol under refluxing (3 hours x 2 L), followed by removal of the solvent in vacuum, to yield a dried ethanol extract (168,3 grams). The ethanol extract were suspended in aquadest and extracted successively with n-hexane and ethylacetat. It was got fraction n-hexane (15,8 grams), ethilacetat (50,5 grams) and fraction residu (ethanol-aquadest) (102 grams).

Cell Culture

HeLa cells were cultured in RPMI 1640 media supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and maintained at 37°C in humidified atmosphere of 5% CO_2. Fraction (5 mg) was suspended in DMSO (25 µL) and RPMI 1640-FBS (25 µL), the concentration of fraction was 100.000 µg/ml. Fraction 100.000 µg/ml (22 µL) was added 4378 µL RPMI 1640 – FBS, the concentration of fraction was 500 µg/ml. It was made serial doses 500, 250, 125, 62,5, 31,25 and 15,63 µg/ml.

MTT Assay

The cells ($1x10^3$) seeded in 96-well plate and grown overnight, were treated with serial concentration of fraction for 24 hours, 48 hours and 72 hours. Two hours prior to treatment duration, cultures were supplemented with MTT. After the incubation at 37°C, the cells were lysed with lysis buffer containing 50% of dimethyl formamide and 20% SDS and absorbance was measured at 570 nm using microplate reader (Biorad). The percentage of cell viability was calculated using the following formula: Percentage cell viability = (OD of the experiment sampel – OD of the control of media)/ OD of Control of Cell) x 100%. Inhibition concentration 50% (IC 50) was measured by probit analysis using SPSS 16.

Phytochemical Analysis

Specimen from each fraction was examined to check the presence of bioactive phytochemicals. Thin layer chromatography (TLC) GF_{254} was used as stationary phase. Solvent n-hexan: ethylacetate: formiat acid (6: 4: 0, 2) was used to examine flavonoid. Solvent Chloroform: metanol: water (64: 50: 1) was used to examine saponin. Solvent n-hexan: etylacetate (93: 7) was used to examine terpen. Solvent Toluene: etylacetate: dietylamine (7: 2: 1) was used to examine alkaloid. Solvent: Etylacetate: formiat acid: toluene: water (6: 1, 5: 3: 0, 5) was used to examine fenolik. After that, Citroborat was sprayed to TLC to examine flavonoid. Lieberman-Bourchart was sprayed to TLC to examine saponin. Anisaldehide-Sulphat acid was sprayed to TLC to examine terpen. Dragendorf was sprayed to TLC to examine alkaloid. $FeCl_3$ was sprayed to TLC to examine fenolic.

RESULT

Comparative Analysis of *Aquilaria malacensis* Fractions for Growth Inhibitory Activity Against Cervical Cancer Cells

All fractions of *Aquilaria malacensis* were examined for cell-growth inhibitory properties on HeLa cells. Cells treated with seril concentration of test sampel were examined by MTT assay fo cell viability.

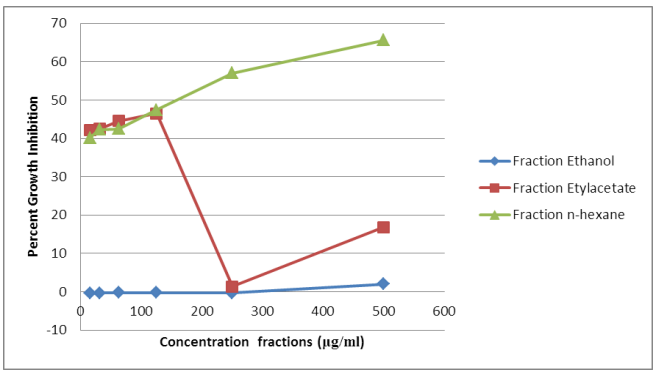

Figure 1. Dose-dependent effect of *Aquilaria malacensis* Fractions on the viability of human cervical cancer.

Result shown in figure 1 demonstrate presence of growth inhibitory property in fraction ethanol, ethylacetate and n-hexane whereas fraction ethanol had minor growth promoting activity. Fraction ethylacetate and n-hexane had the same potential to inhibit cervical cancer growth from concentration 15,63 µg/ml to 125 µg/ml. In the concentration more than 125 µg/ml, The potention to inhibit cervical cancer cell growth increased for fraction n-hexane but the potention decreased for fraction ethylacetate. The inhibitory concentration 50% (IC 50) for fraction n-hexane was 163 µg/ml.

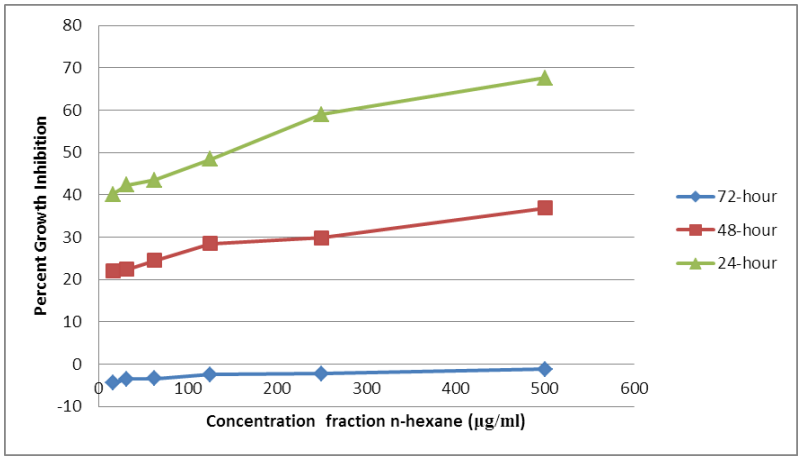

Figure 2. HeLa cells incubated with increasing concentration of fraction n-hexane were examined for cell viability at 24 hour, 48 hour and 72 hour.

Result shown in figure 2, cells were treated with increasing concentration of fraction n-hexane for 24 hour, 48 hour and 72 hour to examine the dose kinetics and time course of growth inhibition. Fraction n-hexane showed highest dose-dependent inhibition at 24 hour. A longer incubation of treated cultures resulted in partial recovery of growth inhibition at 48 hour and complete recovery by 72 hour.

Analysis of Fractions of *Aquilaria malacensis* for Presence of Bioactive Phytochemicals

Table 1. Bioactive Phytochemicals in Fractions.

No.	Phytochemical	Fr. Ethanol	Fr. Ethylacetate	Fr. n-hexane
1	Flavonoid	+++	++	+
2	Terpenoid	+	++	+++
3	Fenolic	+++	++	+
4	Saponin	+	+	+
5	Alcaloid	++	++	+++

Table 1 shown the main bioactive phytochemicals in fraction ethanol were flavonoid and fenolic. Bioactive phytochemiclas in fraction ethylacetate were flavonoid, terpenoid, fenolic and alcaloid. Terpenoid and alcaloid were the main bioactive phytochemicals in fraction n-hexane.

DISCUSSION

A dose-dependent cytotoxic activity observed in fractions of gaharu demonstrates a potential therapeutic utility of this medicinal plant against cervical cancer. The inhibitory effect of fraction n-hexane was maximal at 24 hour but declined thereafter. These observations suggest that active principle might get metabolized or get inactivated during culture process and is no more available to impose growth inhibitory.

Interestingly, comparative analysis of fractionated leaf extracts in our experiments revealed cytotoxic activity that specifically resolved in n-hexane. As a general notion, HeLa cells are much more resistant than many cell lines. Therefore, further studies are warranted on a panel of cell lines to verify the anti cancer potential of fraction n-hexane. These observations suggest that active principle might get metabolized or get inactivated during culture process and is more available to impose growth inhibitory effect. However, these assumptions need further investigation.

The study showed presence of high levels of terpenoid and alcaloid in fraction of n-hexane. Terpenoid has been extensively reported effects against tumor cells, which exhibit the ability to suppress the growth of cancer cells by inducing tumor cell differentiation and apoptosis and inhibiting tumor angiogenesis, invasion and metastasis.[19]

CONCLUSIONS

Our study therefore demonstrates presence of anticancer in gaharu leaves that can be further exploited as a potential anticancer.

ACKNOWLEDGMENTS

This study was supported by research foundation from Sriwijaya University, Palembang, Indonesia.

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