ACAP

제8회 부산 아시아태평양액티브에이징 컨퍼런스

The 8th Busan Active Aging Conference in Asia Pacific

국민밀착형 항노화발전 방안

Citizens' Orientation to Anti-Aging

Venue BEXCO, BUSAN, KOREA 한국, 부산, 벡스코

Date 2013. 06. 27. ~ 06. 28.

주 최 Host Organization: 부산광역시, 아시아태평양액티브에이징컨소시움
Busan Metropolitan City, Active Aging Consortium in Asia Pacific

주 관 Organization: 항노화기술개발사업단, (사)노인생활과학연구소
Research Center for Anti-Aging Technology Development
Research Institute of Science for the Better Living of Elderly

후 원 Sponsors: 부산대학교 PUSAN NATIONAL UNIVERSITY, AABC Asian Aging Business Center, University of Hawaii Manoa, Universitas Indonesia Centre for Ageing Studies

[A24]

Title	**THE ANTICANCER FROM LEAF EXTRACT OF ANNONA MURICATA AGAINTS HeLa CERVICAL CANCER CELL LINE**
Name	Lili Indrawati[1], Tri Budi W Rahardjo[1], Dinni Agustin[1], Sofy Meilany[2], M Wien Winarno[3]
Affiliation	1 Centre for Ageing Studies University of Indonesia, 2 Institute of Human Virology and Cancer Biology the University of Indonesia, 3 Center for Biomedical and Basic Technology of Health, Minister of Health of Republic of Indonesia
Introduction	Cervical cancer continues to be a widespread public health problem in women worldwide especially in developing country like Indonesia. The data from thirteen pathology centers in Indonesia describes that cervical cancer stands the first-ranked among all cancer. The soursop (*Annona muricata*) is a traditional medicinal plant which is empirically by the people of Indonesia are used for anti-inflammatory and anti-tumor. This study aims to determine the cytotoxic effects from extracts of leaves of soursop and fraction results in cancer cells HeLa.
Method	The research was carried out by extraction using ethanol and extraction using water. Cytotoxic test performed by the method of MTT assay. The parameters obtained from the cytotoxic test was IC50 values, ie values that produce inhibitory concentrations of cancer cells by 50%.
Result	The results showed that the ethanol extracts of leaves of the soursop has a cytotoxic activity with IC50 values of 1,787µg / mL, while aquos extracts and onfusion of the leaves have cytotoxic activity with IC50 values of 1,781 µg / mL and 1,51 µg/mL respectively. All that values is better than that obtained from 5-Fluorouracil with IC50 values of 1,82 µg/mL.
Conclusion	Ethanol and water extracts, and infusion of leaves of the soursop has a cytotoxic activity with IC50 values less than IC50 of 5-Fluorouracil.
Keywords	cervical cancer, HeLa cell line, soursop (*Annona muricata*), cytotoxic

In Vitro Anticancer Activity of Leaves Extracts from *Annona muricata* againts HeLa Cervical Cancer Cell Line

Lili Indrawati[1], Tri Budi W Rahardjo[1], Sofy Meilany[2], M Wien Winarno[3]

[1]Centre for Ageing Studies University of Indonesia, [2] Institute of Human Virology and Cancer Biology the University of Indonesia, [3]Center for Biomedical and Basic Technology of Health, Minister of Health of Republic of Indonesia

Abstract

Cervical cancer continues to be a widespread public health problem in women worldwide especially in developing country like Indonesia. The data from thirteen pathology centers in Indonesia describes that cervical cancer stands the first-ranked among all cancer. The soursop (*Annona muricata*) is a traditional medicinal plant which is empirically used by the people of Indonesia for anti-inflammation and anti-tumor. This study aims to determine the cytotoxic effects from extracts of leaves of soursop in cervical cancer cells HeLa.
Method: The research was carried out by extraction using ethanol and water. Cytotoxic test performed by the method of MTT assay. The parameters obtained from the cytotoxic test was IC50 values, ie concentrations that produce inhibitory of cancer cells by 50%.
Result: The results showed that the ethanol extracts of soursop leaves has a cytotoxic activity with IC50 values of 1,787µg / mL, while aquos extracts the leaves has cytotoxic activity with IC50 values of 1,781 µg / mL. All that values is better than that obtained from 5-Fluorouracil with IC50 values of 1,82 µg/mL.
Conclusion: Ethanol and water extracts of soursop leaves has a cytotoxic activity with IC50 values less than IC50 of 5-Fluorouracil.

Keywords: cervical cancer, HeLa cell line, soursop (*Annona muricata*), cytotoxic

Background

Non Communicable Diseases are the leading cause of death in the world, responsible for 63% of the 57 million deaths that occurred in 2008. Cancers is one of the major cause of these deaths (WHO, 2011a). The global burden of cancer increases each day. Presently, there are more than 22 million cases in the world with more than half in developing countries (Am´egbor *et al*., 2011).

In developing countries cancer is the second leading cause of death. The burden of cancer is rising in economically developing countries (Harrington *et al*., 2005; Jemal, *et al*., 2011). The number of new cancer cases is rising annually in Indonesia during the last two decades. The exact incidence and prevalence of cancer are not known because there are no population registry in Indonesia. However data collected from hospitals in several regions indicate that cancer incidence increased by 2-8% per year during the last decade (Ministry of Health, 2006; Tjindarbumi and Mangunkusumo, 2002).

The global burden of cancer continues to increase largely because of the aging and growth of the world population alongside an increasing adoption of cancer-causing behaviors, particularly smoking, in economically developing countries. Cancer survival tends to be poorer in developing countries, most likely because of a combination of a late stage at diagnosis and limited access to timely and standard treatment (Jemal *et al*., 2011)

Cervical cancer continues to be a widespread public health problem in women throughout the world, especially in developing country like Indonesia. Cancer in Indonesia is positioned as the fifth of cause of death, due to increasing number of cancer patients year by year by the increasing life expectancy of Indonesian women. Cancer of the cervix also appears as the most frequent cancer among female, and its position was the first rank, followed by ovary and uterine cancer. These cancers are included in the ten most frequent cancers in Indonesia. The data from thirteen pathology centers in Indonesia shows that cervical cancer stands the first-ranked among all cancer (23.43% from 10 most common cancers among men and women; 31.0% from 10 most common cancers among women) (Aziz, 2009; Nuranna *et al*., 2012).

Despite substantial progress in the development of anticancer therapies, the incidence of cancer is still increasing worldwide. The majority of neoplasms have defects in the apoptotic process (Hotchkiss *et al*., 2009). Nevertheless, many types of cancers have the mechanism to avoid apoptosis induced by anticancer drugs. This causes the need for combination therapy (Psahoulia, *et al*., 2007). The intervention of multistage carcinogenesis by regulating intracellular

signaling pathways may give molecular basis of chemoprevention with a wide variety of dietary phytochemicals (Khan, *et al*., 2007).

Currently, chemoprevention by the use of naturally occurring dietary substances is considered as a practical approach to decrease the ever-increasing incidence of cancer (Khan, *et al*., 2007). Also there is an awakening of interest in herbal product at global level and the conventional medicine is now starting to accept the use of botanicals once they are scientifically validated (Gilani and Atta-ur-Rahman, 2005). It is becoming more acceptable that ethnopharmacology cannot be separated from human nutrition and the conservation of the biodiversity that constitutes its resource base (Heywood, 2011).

Members of family Annonaceae have been investigated as potential sources of biologically active Annonaceous acetogenins, some of which demonstrated a powerful anti-tumor activities (Yu, 1999). Currently, 34 acetogenins have been identified in the leaves of *A. muricata* (Champy *et al*., 2004). The cytotoxicity of acetogenins has been known to be stronger in tumorous than in normal cells (Garcia-Aguirre *et al*., 2008). The primary site of action of the acetogenins is complex I of the electron transport chain in mitochondria (Gupta *et al*., 2011). Study in mice showed that annonacin inhibited the normal growth of the lung tumors during two-week period (Taylor, 2002).

Ethanol extracts of leaves of the soursop has a cytotoxic activity toward breast cancer cells T47D with IC50 values of 17.149 µg / mL. The results of the four fractions obtained by fractionation and the ethyl acetate fraction were the fraction that has the best cytotoxic activity with IC50 values of 30.112 µg / mL. Apoptosis assay results showed that the ethyl acetate fraction were able to induce apoptosis of cells (Prasasti, 2012).

This study aims to determine cytotoxic activity of *A. muricata* leaves extract on cervical cancer cell lines by conducting *in vitro* investigation. It will also provide people about evidence-base-research information on the potential usefulness of traditional medicine that sometimes constitutes the only affordable source of health care.

MATERIALS AND METHODS

A. Collection of plant material. The plant was collected from Bogor, Indonesia. The collection was made in July 2012. The plant was identified by Research Center for Biology- Cibinong Science Center (CSC) Indonesia as those of *Annona muricata* Linn. (family: Annonaceae). The material taken is soursop leaf.

B. Preparation Simplicia. The leaves are washed with running water to remove dirt or dust attached to the leaf, then dried with oven at 60 ± 1ºC, to ensure that no damage occurs an unstable compound by heating. Simplicia soursop leaf then powdered using by grinder.

C. Preparation of *Annona muricata* leaf ethanol extract

Soursop leaf powder of 250 g performed maceration using ethanol solvent during 3x24 hours. Once filtered, the filtrate was evaporated to obtain ethanol extract of leaves of soursop. The selection method is chosen in addition to being easy, simple and expected to reduce the risk of damage to the content of the compounds so it is a suitable method used in the study. The extract obtained was 52.21 g which is mean the rendemen about 20,88%.

Preparation of *Annona muricata* leaf aqueous extract

A. muricata fresh leaves were air-dried at room temperature. The air-dried leaves of the plant was milled into fine powder in a Waring commercial blender. The powdered leaf was macerated in distilled water and extracted twice, on each occasion with distilled water at room temperature for 48 h (with occasional shaking). The resulting aqueous extract finally yields 10 % of aqueous leaf extract of *A. muricata* (AME). Without any further purification, the aqueous extract thus obtained was refrigerated and subsequently used in this study.

D. Preparation of stock solutions of test material

The soursop leaf ethanol extract was weighed 5 g, followed by retrieval of DMSO (Dimethyl sulfoxide) to 5 ml (stock solution concentration of 1g/ml). 10mg/ml soursop leaf aqueous extract was prepared by dilution of the stock with sterile DMSO and stored as stock solutions for subsequent use in research. Cytotoxic concentration of extract to a test carried out by using the dilution medium. 5 Fluorourasil concentrations obtained by dilution with medium. As a control solvent, used 2% DMSO (v/v), ie the highest concentration of DMSO in the test compound.

Cell Culture

HeLa cell line was acquired from Institute of Human Virology and Cancer Biology (IHVCB) Univerisity of Indonesia in the presence of 100 U/ml of penicillin and 100 µg/ml of streptomycin. Cells were incubated at 37°C with 95% air and 5% CO_2 . All cells were maintained below passage 20 and used in experiments during the linear phase of growth.

Cytotoxic test with soursop leaf extract on HeLa cervical cancer cells by MTT assay

MTT assay was performed to assess the cytotoxicity of the plant extracts. Cells were cultured in 96-well microtiter plates. Cells were treated with varying concentrations of *A*. *muricata* leaf extracts for 24 h and incubated. To each well, 20 µl of MTT was added. After the addition of MTT, the plates were incubated for 3 h in a dark chamber. Then 100 µl of DMSO was added to dissolve the formazan crystals (100 µl of DMSO replaces the 100 µl of the culture media in each well). The absorbance was taken at 595 nm using the ELISA reader. The following formula was used to calculate the percent of inhibition:

Inhibition (%) = $(1 - OD_s /OD) \times 100$

where OD_s = Optical density of the sample and OD = Optical density of the control

RESULTS AND DISCUSSION

Cytotoxicity test is a qualitative and quantitative tests to determine how cell death. The method used to see cytotoxic effects of extracts of leaves of the soursop on HeLa cervical cancer cells is the MTT assay. The principle of the MTT assay is a spectroscopic method is by determining the absorbance value of formazan. MTT will be absorbed into the cell and entered into the system of cell respiration in mitochondria. The action of the enzyme active mitochondria in cells was metabolize tetrazolium salts, resulting in termination of tetrazolium ring by dehydrogenase enzymes which lead to tetrazolium formazan transformed into water-insoluble but soluble in SDS 10% and the purple coloured. Formazan formed is colored purple will be proportionate to the number of living cells.

Cells that die dissolved in water and remain yellow because the mitochondria of cells that die are not respiration tetrazolium ring is disconnected so it can not reduce MTT reagent to formazan and the color is still yellow. The observations made by microscopic showed that the number of formazan formed in control wells with media more than the formazan formed in the wells treated test compound. This suggests that the treatment of extract of leaves of the soursop on HeLa breast cancer cells can lead to death. Cells that are dead will not be affected by the MTT reagent. Characteristic morphology of living cells is round with a protected cell wall that shines and stuck to the bottom plate, while the dark-colored cells that die and are not attached to the base plate. After addition of MTT and incubated for 4 hours of diving, added SDS in 10% HCl. The reason the use of SDS 10% as it can dissolve the formazan crystals and the results of MTT reaction did not cause precipitation. After settling for 4 nights, then used an ELISA reader to determine absorbance values. 595nm wavelength is used because it is the maximum wavelength in order to obtain a sensitive and specific measurements. Absorbance value of each test compound can be seen in Table 1. The results of Table 1 showed that the higher of the concentration of test compound, is the lower absorbance values. This may imply that the test compound has a potency in inhibiting or killing the HeLa cells. The stronger intensity of the color purple is obtained the greater the absorbance.

Absorbance data obtained, is used to calculate IC50 values. IC50 value indicates the value of concentration that can inhibit proliferation of HeLa cancer cells by 50%. IC50 value of ethanol extract is 1,787µg/mL which is indicates that the concentration 1,787µg/mL, ethanol extract inhibit proliferation of HeLa cancer cells by 50%. while aquos extracts the leaves has

cytotoxic activity with IC50 values of 1,781 µg / mL. All that values is better than that obtained from 5-Fluorouracil with IC50 values of 1,82 µg/mL.

Table 1. The mean absorbance, percentage inhibition of HeLa cells and IC50 values from ethanol and aquos extract of leaves of *A.muricata*

Test Materials	Concentration	Mean absorbance	Living Cells	Retardation	IC50
	(ug/mL)		(%)	(%)	(ug/mL)
Ethanol extract	250	0,316	1,58	98,42	1,787
of *A. muricata*	125	0,244	1,41	98,59	
	62,5	0,540	2,07	97,93	
	31,3	1,166	3,48	96,52	
	15,6	2,165	5,70	94,30	
	7,8	2,176	5,71	94,29	
Water extract	250	2,097	-0,85	100,85	1,781
of *A. Muricata*	125	2,303	1,55	98,45	
	62,5	2,395	2,59	97,41	
	31,3	2,496	3,86	96,14	
	15,6	2,540	4,33	95,67	
	7,8	2,535	4,21	95,79	
5 Fluorouracil	250	1,959	-0,27	100,27	1,82
	125	2,163	1,45	98,55	
	62,5	2,292	2,53	97,47	
	31,3	2,508	4,43	95,57	
	15,6	2,513	4,44	95,56	
	7,8	2,529	4,53	95,47	

CONCLUSIONS Ethanol extracts of soursop leaves has a cytotoxic activity with IC50 values of 1,787µg / mL, while aquos extracts the leaves has cytotoxic activity with IC50 values of 1,781 µg / mL. All that values is better than that obtained from 5-Fluorouracil with IC50 values of 1,82 µg/mL. Ethanol and water extracts of soursop leaves has a cytotoxic activity with IC50 values less than IC50 of 5-Fluorouracil.

References

Am´egbor, K. Darre, T., Ay´ena, K.D., Padaro, E., Tengu´e, K., Abalo, A. and Napo-Koura, G. 2011. Cancers in Togo from1984 to 2008: Epidemiological and Pathological Aspects of 5251 Cases. Journal of Cancer Epidemiology. Volume 2011, Article ID 319872, 7 pages. doi:10.1155/2011/319872

Aziz, M. F. 2009. Gynecological cancer in Indonesia. *J Gynecol Oncol* Vol. 20, No. 1:8-10, March DOI:10.3802/jgo.2009.20.1.8

Champy P, Ho¨glinger GU, Fe´ger J, *et al*. 2004. Annonacin, a lipophilic inhibitor of mitochondrial complex I, induces nigral and striatal neurodegeneration in rats: possible relevance for atypical parkinsonism in Guadeloupe. *J Neurochem* 88:63– 69.

Gupta A., Pandey S., Shah D.R., Yadav J.S., Seth N.R. 2011. Annonaceous Acetogenins: The Unrevealed Area for Cytotoxic and Pesticidal Activities. *Systematic Reviews in Pharmacy* Vol 2 Issue 2.

Harrington, L., Bristow, R.G., Hill, R.P., and Tannock, I.F., 2005. Introduction to Cancer Biology. In: The basic ***science*** of oncology (Eds. Tannock, I.F., Hill, R.P., Bristow, R.G., Harrington, L.). The McGraw-Hill Companies, Inc.

Hotchkiss, R.S., Strasser, A., McDunn, J.E. and Swanson, P.E. 2009. Cell Death. *N Engl J Med* 361:1570-83.

Heywood, V.H. 2011. Ethnopharmacology, food production, nutrition and biodiversity conservation: Towards a sustainable future for indigenous peoples. *Journal of Ethnopharmacology* 137 1– 15.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D. 2011. Global Cancer Statistics., *CA CANCER J CLIN* 61:69–90

Khan, N., Afaq, F. and Mukhtar, H. 2007. Apoptosis by dietary factors: the suicide solution for delaying cancer growth. *Carcinogenesis* vol.28 no.2 pp.233–239.

Ministry of Health. 2006. *The National Cancer Control Guidelines*, Jakarta, Ministry of Health of Indonesia.

Nuranna, L. Aziz, M. F. Cornain, S., Purwoto, G., Purbadi, S., Budiningsih, S., *et al*. 2012. Cervical cancer prevention program in Jakarta, Indonesia: See and Treat model in developing country. *J Gynecol Oncol* Vol. 23, No. 3:147-152.

Psahoulia, F.H., Drosopoulos, K.G., Doubravska, L. *et al*. 2007. Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts. *Mol Cancer Ther* 6:2591-2599.

Prasasti, E., Rachmani, N., Suhesti, T.S., Aditiyono, R.W., 2012. The Breast of Anticancer from Leaf Extract of Annona *muricata* against Cell Line in T47D. *International Journal of Applied Science and Technology. Vol. 2 No. 1*.

Taylor, L. 2002. Technical Data Report for Annona muricata *Annona muricata***.** Preprinted from Herbal Secrets of the Rainforest, 2nd edition. Published and copyrighted by Sage Press, Inc.

Tjindarbumi, D., Mangunkusumo, R. 2002. Cancer in Indonesia, Present and Future. *Jpn J Clin Oncol*; 32 (Suplemen 1) S17-21.

World Health Organization - *NCD Country Profiles* , 2011a.

Yu DQ. Recent works on anti-tumor constituent from Annonaceae plants in China. *Pure Appl. Chem*., Vol. 71, No. 6, pp. 1119±1122, 1999.