Immunome Research

Prayitno et al., Immunome Res 2016, 12:2
DOI: 10.4172/1745-7580.10000120

Research Article Open Access

Tetrahydrofurane is a Component of *Annona muricata* Leaf will Induce Apoptosis Program in Cancer Cell because the Virus: A Proxy for Cancer Treatment

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Received date: June 14, 2016; **Accepted date:** August 05, 2016; **Published date:** August 15, 2016

Abstract

Introduction: The compound that allegedly derived from acetogenin has a mechanism of inhibition complex of mitochondrial. Inhibition complex of mitochondrial by acetogenin will cause a decline in the production of ATP and decrease the amount of ATP will induce apoptosis.

Aims: The aim of this study is whether *Annona muricata* (*A. muricara*) containing tetrahydrofurane that can induce apoptosis?

Method: To detection an active isolate carried out using the method of TLC. To determine the functional group terpenoids used VLC mobile phase in chloroform-ethyl acetate then used vanillin sulfuric acid. To find out more details on cluster group were characterized by FT-IR and UV-Vis spectrophotometer. Cytotoxicity test of *A. muricata* leaves extract againt HeLa cells was evaluated by MTT method which saw the level of formazan absorbantion velue.

Result: With vanillin sulfuric acid saw violet red color visible in light is a standard for terpenoid functional group. On the results of FT-IR spectrophotometry test indicated that the absorption of the lactone organic compound. From UV-Vis spectrophotometry test has high absorbance (wave length 222 and 230 nm) and may be a marker for the presence of tetrahydrofurane cluster compound. Of assay cytotoxicity obtained the value of R^2=0.9035, namely there is a correlation in welfare between viability of HeLa cells with active isolates of *A. muricata leaves*.

Conclusion: It is concluded that *A. muricata* leaves extract contain acetogenin which functional group-terpenoids, which cluster group-lactones as well as tetrahydrofurane. Cell viability of HeLa cells will progressively lower with increasing concentration of active isolates *A. muricata* leaves contains tetrahydrofurane

Keywords: Tetrahydrofurane; Lactone; Acetogenin; Apoptosis; Cancer

Abbreviations:

ATP: Adenosine Three Phosphat; TLC: Thin Layer Chromatography; VLC: Vaccum Liquid Chromatography; FT-IR: Fourier Transform-Infra Red; UV-Vis: Ultraviolet Visible; MTT:3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide; *Muricata* L: *Annonamuricata* Linn; HIV: Human Immunodeficiency Virus; DNA: Deoxyribo Nucleic Acid; PF254: Silica gel 60 PF for preparative thin layer chromatography; RPMI: Rosewell Park Memorial Institute; FBS: Fetal Bovine Serum; IC50: Half Inhibitory Concentration; SDS: Sodium Dodesil Sulfat; ELISA: enzyme Linked Immunosurbent Assay; PBS: Phosphate Buffer Saline

Introduction

Various compounds present in Annonaceae Familia, including potential anti-cancer compounds. Three compounds in *Annona muricata* Linn a potential anti-cancer namely are acetogenins, muricin and cis-annomontacin [1]. Acetogenin compounds (squamocin A, B, C, and D) and annotemoyin-1 and -2 the Annonaceae have cytotoxic effects [2], platelet aggregation inhibitor and cyclo squamosin D on seed proved showed inhibition of proinflammatory cytokines in macrophages [3], inhibitor of HIV replication [4], anti-diabetic agents (anti-hiperglikemik) and anti-oxidants [5,6], pesticide [7], and can be used in the treatment of Neisseria gonorrhea [8]. Squamocin serves as insecticides, while the ascimicin have an anti-leukemia effect [9]. Caryophyllene oxide as analgesic and anti-inflammatory activity [10],

The incidence of cancer associated with: First, there is increased expression or mutation of gene trigger cancer. Second, there is a decrease in the expression or mutation of gene cancer suppressor. Cancer trigger and suppressor gene is normal gene that has an important function in cell homeostasis. If that gene don't work, then implicated for occur of cancer. Third, the gene associated with cancer is the presence of DNA-repair enzymes. Changes in the function of these enzymes will lead to the occurrence of cancer. Fourth, the process of apoptosisis not normal as well as happen inhibition of apoptosis [11-15]. Further developments on the definition of cancer

has been suggested that incidence of cancer begins in the disorder at the level of epigenetic (methylation and/or histone modification) and continues to change at the level of genetic (mutation) [16].

Method

Preparation of active isolate and characterization of functional group of *A. muricata* L.

The detection of the active compound in this research carried out using a method of TLC. In TLC used Silica gel PF254 for silent phase and used combination of chloroform and ethyl for motion phase, in accordance with a compound that will be sought

Active isolates obtained by TLC method which graduated chloroform 100% and chloroform-ethyl acetate (93:07v/v) of leaves *A. muricata* L. until It was founds a spot in TLC. Some fractions on TLC results showing one spot then volatilized in water bath tube separated from solvent.

To find out the functional group it is used VLC mobile phases of chloroform - ethyl acetate (93:07 v/v), then with vanillin sulphuric acid and detected the colors are visible light adjust the value of Rp that is a standard for a terpenoidorganic compounds [17].

Characterization of cluster group of *A. muricata* L.

To know more detail a cluster of a compound in active isolates *A. muricata* than characterized with FT-IR and UV-Vis spectrophotometer. Using of that tools are very important in modern chemistry, moreover in characterize an organic compounds. FT-IR and UV-Vis spectrophotometry is a tool to detect a cluster of functional coumpond, identify compounds and analyze a mixture [18]. The spectrophotometer UV-Vis showed electronic energy that large enough in a molecule be analyzed, so the spectrophotometer UV-Vis more quantitative than qualitative analysis [19,20,21]. Absorption that appears at FT-IR spectrophotometer, next interpreted in Table 1.

The infrared region in a spectrum of electromagnetic waves includes waves numbers14,000 cm^{-1} until 10 cm^{-1}.The infrared region (400-10 cm^{-1}) will be useful for analyzing molecule containing atoms as heavy as an inorganic compound, but it also costs special technique better. Infra-red regions being (4,000-400 cm^{-1}) pertaining to transition vibrasion energy from the molecule of which provide information about clusters functions in that molecule. The infrared region close (12,500-4,000 cm^{-1}) that is sensitive to an overtone vibrasion [22].

On a FT-IR, a unit of the number was common used. Numbers velue of waves varies inversely against frequency or energy. Surge numbers and the wave length can be converted each other using an equation $V(cm^{-1})=1/\lambda\ (\mu m) \times 10^4$ [23]. The infrared region in a spectrum of electromagnetic waves includes wave numbers 14.000 cm^{-1} until 10 cm^{-1}. The infrared region (400-10 cm^{-1}) will be useful for analyzing molecule containing atoms as heavy as an inorganic compound, but it also costs special technique better. Infra-red regions being (4.000-400 cm^{-1}) pertaining to transition vibrasion energy from the molecule of which provide information about clusters functions in that molecule. The infrared region close (12.500-4.000 cm^{-1}) that is sensitive to an overtone vibrasion [24]. On a FT-IR, a unit of the number was common used. Numbers velue of waves varies inversely against frequency or energy. Surge numbers and the wave length can be converted each other using an equation $V\ (cm^{-1})=1/\ \lambda\ (\mu m) \times 10^4$ [22].

Absorbantion	Wave Number (cm^{-1})	Cluster Interpretation	References
1	3.397,76	O-H (hydroxil alcohol)	22
2	2.958,93	C-H (saturated alkana)	
3	2.928,07	C-H (saturated alkana)	
4	2.851,88	C-H (saturated alkana)	
5	1.870,07	C=O	
6	1.762,05	Cyclobutanon/phenolic ester	
7	1.743,72	C=O (Zenget al., 1996)	
8	1.614,49	C=C (Pradanaet al., 2015)	
9	1.465,96	H-C-H	
10	1.375,30	Carboxylate	
11	1.095,61	C-O / C-C	
12	960,59	C-H aromatic	
13	799,53	23 C-H aromatic	
14	720,44	C-H aromatic	
15	545,88	Alkil - C-H out plane,	
16	464,86	p-substitusibenzene	22

Table 1: Intepretasion of Fourier Transform Infra-Red (FT-IR) of active isolates of *A. muricata* Linn leaves.

Citotoxicity test

HeLa cells grown until confluent or reach the number needed is reached 2×10^4 density. Culture media used is RPMI 1640 because this media can support for cell growth normally and already used for HeLa cell culture [25]. In a media RPMI 1640 also added the serum of FBS.

Active Isolates resulting from VLC then tested the cytotoxicity against HeLa cell and evaluated using methods MTT assay to know the value of IC50.

MTT assay is simple method, had several advantages so that efficient and much used in sensitive cytotoxicity test. The principle of MTT assay is spectroscopy to determine the absorbantie formazan velue. MTT will absorb into the cell and come into the respiration system of the cells in motocondria.

Working mechanism an of active enzyme in a cell motocondria is to metabolize salt tetrazolium, so a ring tetrazolium cut off by an enzyme dehydrogenase causing tetrazoliumformazan become insoluble in water but soluble in SDS 10% and saw as purple. The number of crystalline formazan formed purple will proportioned to the quantity of a living cell [26].

Ethics approval

This research has been ratified by the feasibility of conduct to be done by Research and Ethical Committee Distric Hospital of Muwardi

and Faculty of Medicine (University of SebelasMaret) led by Dr. HariWuyoso (No. 188.4/14.666/2014 and 182/UN27.06/KS/2014).

Result

Characterization of functional group of *A. muricata* L.

Of testing active isolates *A.muricata* Linn used VLC with mobile phases of chloroform-ethyl acetate (93:07 v/v), then detected with vanillin sulphuric acid, the results seen in visible light the violet red with the Rp=0.62 and 0.94 (Figure 1) and that value constituting a standard for terpenoid.

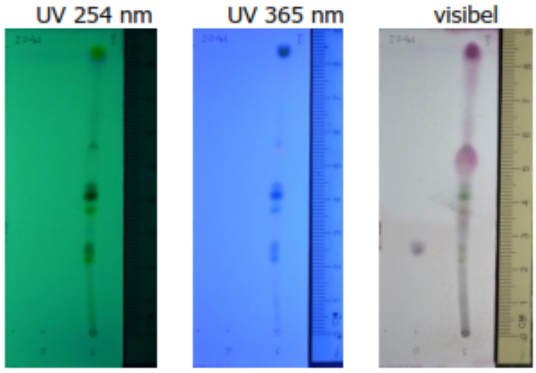

Figure 1: Results of testing of active isolates in chloroform-etil acetic on VLC showed violet red which means there are positive for terpenoid compound.

Characterization of cluster groupof *A. muricata* L.

From Figure 2 was found 16 points absorption representing 16 cluster of functional which is found in an active isolates of *A. muricata* Linn. The results of FT-IR shown by Figure 2, absorption at 3397.76 cm^{-1} wide enough which indicates the presence of a cluster of O-H alcohol, where O-H of alcohols having the unique characteristics of the form of absorption in the form of that flared at 3.600-3.300 cm^{-1}. Absorption of others are on 2928.07 cm^{-1} showing the link C-H that is asymmetric and 2851.88 cm^{-1} signifying the chain C-H symmetric, both the adjacent indicates vibrasi the chain C-H sp3. Vibrasi 1465.96 cm^{-1} vibrasi C-H are hooked by clipping vibration. On absorption 1743.72 cm^{-1} indicates absorption of a cluster of lactone. Typical absorption in the area was derived from a cluster of C=O on γ–butirolakton (ring which consists of five) suspected the lactone of acetogenin [22]. The lactone is different with ester which having absorption more long. This was confirmed by the absorption at O-C-C in 1095.61 cm^{-1} as indicating the existence of a cluster of the lactones. The alkenes had 1.680-1.600 cm^{-1} region of absorption. In Table 1, the existence of a cluster of the bond of C=C (alkenes) shown in the value of absorption 1.614. 49 cm^{-1}. This alleged was strengthened with the absorption at wave length numbers 960.59 nm, 799,53 nm and 720,44 nm that showed bending C-H aromatic [27].

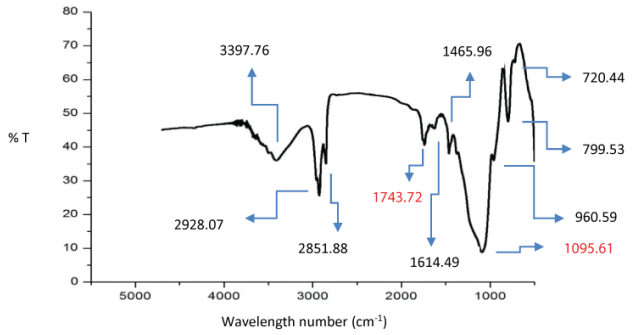

Figure 2: A graph of FourierTransform Infra-red (FT-IR) of active isolates *A. muricata*.

From Figure 3, found the top of the spectrophotometer UV-Vis absorbantie at wave lengths maximum which is 210 nm, 213 nm, 216-234 nm. According to the research [28] said absorption between 220-230 nm is absorption to amines conjugated transition π→π*. Similar belief is also raised by Maryanti (2006) conjugated that regions appear on 215-230 nm absorption. This hypothetical strengthened with the ribbon absorption at infra-red spectrum in 1743.72 cm^{-1} that shows the existence of delaying vibration of C=O that conjugated by the double bond will cause absorption shifted to the regions with a wave length which is smaller than absorption of pure C=O, namely 1720-1725 cm^{-1} area [29].

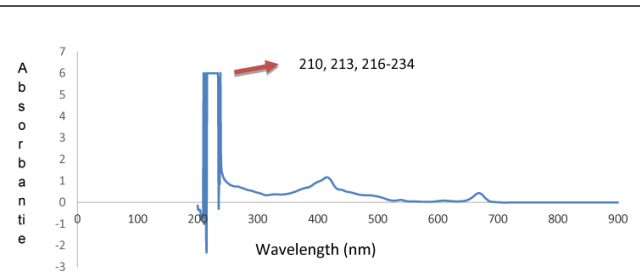

Figure 3: Charts the spectrophotometer UV-Vis active isolates *A. muricata*.

Cytotoxicity test

HeLa cell culture of ten use research model because it grows faster so as to produce more cells in a flask and as common human cells used for cell culture interests. HeLa cell as semi attached culture, because culture cells release extra-cellular matrix proteins, causing the cells stick toone another and attached to the base flask.

Cells that die are dissolved in water and remained yellow because the mitochondria of cells that die cannot perform respiration so tetrazolium ring is lost and cannot absorb the MTT reagent. The morphological characteristics of living cells are round with cell walls that glow and attached to the bottom plate due to the formation of formazan crystal (Figures 4-7). Dark dead cells and not attached to the base plate because it does not form crystals formazan, because dead cells lose their ability to maintain and provide energy for metabolic function and cell growth.

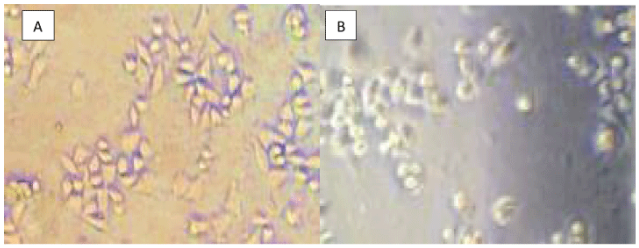

Figure 4: HeLa cells in ground of flask (A: Before treated with MTT, B: After treated with MTT).

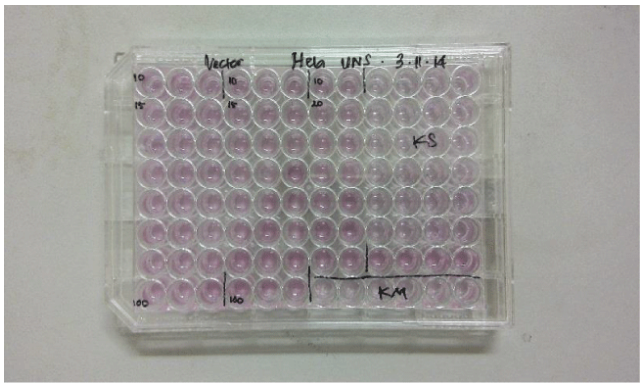

Figure 5: Mapping of HeLa cells after treated by MTT assay.

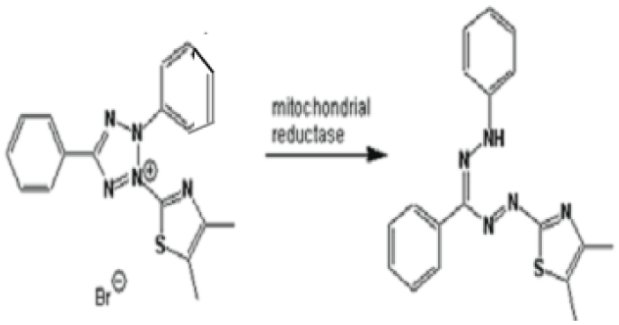

Figure 6: MTT change to formazanin mitochondria of cell [30].

Furthermore, the addition of MTT and incubated for 4 hours, plus SDS in10% HCl. The use of 10% SDS is meant to dissolve the formazan crystals formed, so it does not appear precipitation during the reading of the ELISA Reader.

After allowed to stand overnight then read using ELISA Reader to determine the value of absorbance. The wave length used is 550 nm, the wave length is the maximum wave length to obtain measurements that are sensitive and specific [30]. The results of the observation cell by MTT assay calculated the percentage of living cells and IC50 value was counting using Microsoft Excel analysis (linear regression of log concentration) in Table 2.

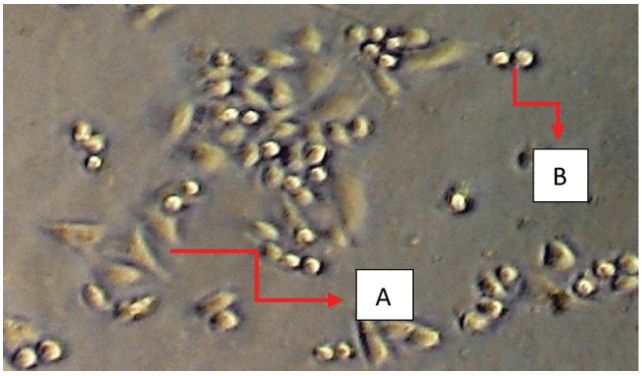

Figure 7: HeLa cells after treatment with SDS 10% (100X view) (Note: A: Life cell, B: Death cell).

No.	Concentration (µg/ml)	Absorbantion (mean)	Cell Viability (mean/%)
1	10	0.802	76.17
2	15	0.788	74.51
3	20	0.827	79.02
4	75	0.654	59.01
5	100	0.436	33.82
	Control (cell)	1.008	98.23
	Control (media)	0.144	00.00

Table 2: Absorbantion mean value of HeLa cells after treatment withmany concentration of active isolates *A. muricata Linn* (ELISA assay).

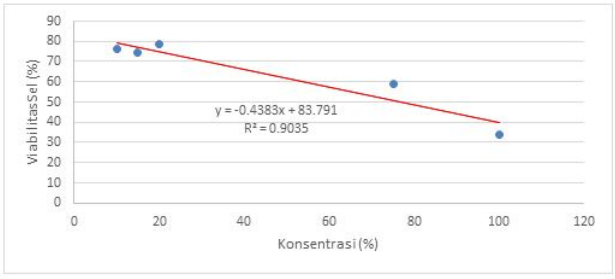

Figure 8: Graph the regression effect of the concentration of *A. muricata* with HeLa cell viability

From Figure 8 obtained value of R^2=0.9035 which is close to 1, this mean had a significant correlation between the viability of HeLa cells with active isolates of *A. muricata* Linn leaves. Equation that correlation drawed with y=0.4383 x + 83.791 (Figure 8) has a negative value, it is can be interpreted that the higher the concentration of the most active isolates of *A. muricata* Linn leaf so the lower the living HeLa cells (HeLa cell cell viability was lower). Calculated value IC50 was 77.096 ug/mL. This IC50 value was smaller than the HeLa cell

research by Witianingsih (2014), which gained value IC50 from fractionation of *A. muricata* L. for 166.32 µg/ml.

Discussion

Characterization of functional group of *A. muricata* L.

From this study it can be concluded that active isolates A.muricata Linn leaves have class of compounds terpenoids [17,31]. As it is known that the terpenoids will give red purple color when observed invisible light [32]. Compounds terpenoids can inhibit the cell cycle at the phase of mitosis (G2/M) with stabilizing threads spindle in the phase of mitosis so that the process of mitosisis not the case. Terpenoids also can trigger apoptosis through a mechanism of inhibition of the enzyme topoisomerase [33]. One group of terpenoids, namely mono terpenoid act as anti-tumor. This compound is reported to have had the chemoprevension ability in several types of cancer cells.

The mechanism of action of monoterpenoid is a way to block and suppress anti-tumor activity [34]. The example for other terpenoids group is taxol which is a diterpene compound has been used widely for the treatment of cervical cancer and breast cancer. Taxol can inhibit mitosis by disrupting micro tubule imperfect, where in the microtubule breakdown occur blockade the process of mitosis. Another term is that taxol will stabilize tubulin so as to prevent cell division [35,36].

Characterization of cluster group of *A. muricata* L.

Acetogenin compound has a characteristic form of the lactone group at one end so that characterization using infrared spectrophotometry can help uncover [23]. To detect the presence of acetogenin further alleged used FT-IR measuring the absorption of infrared radiation in wave numbers 4.000-400 cm^{-1}.

FT-IR can be used to detect and analyze the functional groups of compounds mixture [37]. There two kinds of vibration that is stretching and bending, stretching vibration is arrhythmic movement along the axis so that the distance between atoms bond to be increased or decreased. Bending vibration can occur due to changes in bond angles between the bonds on an atom [22]. Referring to Pradana et al. (2005), the interpretation made by the absorption maximum wave length of 500 cm^{-1}, until only taken 15 points uptake that meets the standard table range FT-IR wave numbers. In the interpretation output wave numbers FT-IR encountered aromatic C-H3 pieces (numbers 12-14, Table 1) were also found in the general structure acetogenin (Figure 2).

Exodus chart UV-Vis spectrophotometer (Figure 3) corroborate previous allegations of FT-IR of the existence of derivatives acetogenin, it is characterized by the existence of groups that have similar shapes ketolactone aromatic C-Hin the wave length range 216-234 nm [38]. According absorptionat a wave length of 222 and 230 nm indicates ketolactone group derived from the double bond C=C, C=O, and C-O single bond, which also shows the transition of electrons in a molecular orbital $n \rightarrow \pi^*$ [22]. Absorption at a wave length of 230 nm of Figure 3 shows the presence of tetrahydrofuran group derived from C-O-C bond which is then followed by an alkyl group as a substituent which forms a cyclic structure such as this cyclopentana. This absorbtional so shows the transition of electrons in a molecular orbital $\rightarrow \sigma^*$. According to Ferras [37], the UV-Vis test results on high absorbance (wave length 222 and 230 nm) may be a marker for the presence of cluster ketolactone and tetrahydrofuran.

Referring to these results, the presence of the compound at a wave length group is allegedly owned by derivatives acetogenin namely rollidecin, rollitacinorrollinacin [38]. Compound allegedly derived from acetogenin have a mechanism of inhibition of mitochondrial complex by among others the occurrence of DNA methylation, a mechanism it will interfere with the electron transfer process. Inhibition of mitochondrial complex by acetogenin will cause a decline in the production of ATP. Decrease the amount of ATP will induce apoptosis [39,40].

Cytotoxicoty test

Confluent cell said when the cell is already attached and meet growing container cover culture surface area of culture flask [41]. Nature HeLa cells are semi attached so as to require trypsin to release the interactions between the molecules of the surface glycoproteins and proteoglycans tissue flasks until the cells cannot be attached at the bottom of culture flask [42]. For the first trypsin been given PBS, which serves to remove serum in RPMI1640 medium because this serum can inhibit the action of trypsin [43]. HeLa cell morphology looks somewhat tapered shape due to the inherent tissue at the base ofthe flask, whiledulytrypsinshownare roundbecause it is notattached to the base tissue flask.

MTT Assay method has several advantages, namely simple, efficient and sensitive that is widely used in cytotoxic test. Principle of MTT Assay is to determine the value of the absorbance spectroscopy formazan. MTT will absorbed into the cells and into the mitochondria of cells in the respiratory system

IC50<100 pg/mL has been categorized as a compound anti-proliferatif. Velue of IC50 between isolates compared to fraction are much smaller, this occurs because the compound contained in the fractionis still common, has not reached the most active compound. HeLa cell inhibition mechanism can occur through three mechanisms, among there are cell cycle arrest (cessation of cell cycle), cell cycle delay (inhibition of cell cycle) or apoptosis mechanism [44],

Mechanism of soursop leaf ethanolic extract in inhibiting proliferation and induction of apoptosis through two mechanisms: (1) p53 dependent pathways; (2) the other path that is independent of p53. Possible alternative stabilization pathways and activation of p53 by soursop leaf extract, including through (1) a direct path, and (2) indirectly. Indirect mechanisms, namely (1) through direct induction of DNA damage by the target base pairs, thymidylate synthase enzyme, topoisomerase, microtubules in the mitotic process; (2) modification of the oncogenic protein of high risk HPVE6; and (3) the active in gradient soursop leaf serves as a proteasome inhibitor [45].

The mechanism of inhibition of proliferation proteins can be caused by damage to the target DNA base pairs, this mechanism is initiated by the occurrence of DNA methylation (Figure 9). DNA methylation involves a DNA methyltransferase enzymethatis responsible for the addition of cluster of methil. In eukaryote, the addition of a methyl group on the cytosine bases in the chromosomal DNA molecules cause cytosine bases transformed into5-metilcytosin [46].

DNA methylation patterns are not random, but rather limited to cytosin in sequence 5'-CG-3'. Methylation is able to suppress the activity of genes. Experiments were carried out through the methylated and non-methylated genes are inserted into cells through cloning shows the measurement results in which the gene expression level of gene expression does not occur if the methylated DNA sequence. In addition, methylation patterns in DNA testing showed that the

chromosomal location of genes that are active in the area that is not methylated, for example, which is the expression of a human gene that is common in the non-methylated CpG- islands [46].

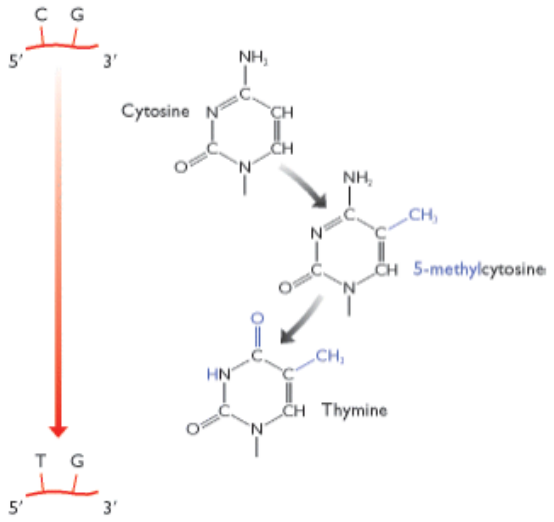

Figure 9: The process of DNA methylation.

Conclusion

It is concluded that *A. muricata* leaves extract contain acetogenin which functional group-terpenoids, which cluster group-lactones as well as tetrahydrofurane. Cell viability of HeLa cells will progressively lower with increasing concentration of active isolates *A. muricata* leaves contains tetrahydrofurane.

Concept for Publication

Need to know that in our institutions there were 100 more a professor, but the findings of research results was lowed a not until one title/1professor/1 year. Therefore I really want to increase this figure. Therefore I want that the results of research can be published in Journal of Biomedical Science that famous.

In this research I want to tell about herbal to manage illness in Protein Conformational Disorders (PCDs), including cancer. And it can be cure by herb compounds, like *AnnonaMuricata* Linn.

My big hope to continue to improve my role in order to help develop the science of knowledge especially the field of medicine and particularly cancer management who continue to grow very fast.

Availability of Data and Material

All the study materials easily available with reservations and is available in Parasitology Laboratory of Gajah Mada University and Central Laboratory of Sebelas Maret University. Similarly, the research data has been documented in the research logbooks.

Funding

Higher Education Competitive Research Project Ministry of Education and Culture Republic of Indonesia for Grand Featured Research Universities 2013. No. 165a/UN27.11/PN/2013

Author Contribution

AP performed the study, made a protocol of the laboratory, prepared data analysis, collected data, contributed to the drafting, wrote and revising of the paper. ANA, VSD, IA, MH, MSF and AYE provide laboratory work, generated extracts, performed cell culture experiments. OPA provide as a designer.

Acknowledgements

We acknowledge the Higher Education Competitive Research Project Ministry of Education and Culture Republic of Indonesia for Grand Featured Research Universities 2013, LPPT of Gajah Mada University and special thanks to Prof. Rafik Karsidi as the rector of Sebelas Maret University Surakarta Indonesia, Prof Sulistyo as the Chairman of the Institute for Research and Community Service, Prof Hartono as the dean of Faculty of Medicine of Sebelas Maret University Surakarta Indonesia and special thanks to Prof Wihaskoro Sosroseno and Prof Suhartono Taat Putra for inspiration to write this article.

Author Information

I was a teaching staff as additional activities with a dentist in at a hospital and university researchers about tropical diseases including cancer.

My research activities starts on the scope of local in 1991, then progressed to the sphere of national in 1997 and continue to grow to the international research in 2001. Some grand research often got both national and international. My publication there are about 30 title at the national level and 10 title on the international level including those that was published in Biomed Central Group namely Journal of Carcinogenesis in 2006 (Journal of Carcinogenesis 5: 13. 2006).

My ID of an author scopus 14122272000 with H-index 1 (1 document-12 citation)

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