Polyisoprenoids from Avicennia marina induces on P13k, Akt1, Mammalian target of rapamycin, Egfr, and P53 Gene Expression Using Reverse Transcription-Polymerase Chain Reaction

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Abstract

BACKGROUND: According to the Global Cancer Observatory in 2018, Asia was the first to note the incidence of colon cancer, which was 51.8% of cases of colon cancer which ranked the top three in the number of causes of death in the world. Cancer is a disease characterized by uncontrolled cell growth. Potential natural ingredient developed as chemotherapeutic agents includes from mangrove leaves. Studies reporting on the pharmacological activity of polyisoprenoid from mangrove species are still limited, therefore, it is essential to achieve the prospects, potential, and mechanisms polyisoprenoid in mangroves as a natural ingredient of pharmaceutical and medication.

AIM: The aim of the study was to investigate the inhibition activities of polyisoprenoids in mangrove plant Avicennia marina in WiDr cells induces on P13k, Akt1, mammalian target of rapamycin (mTOR), Egfr, and P53 gene expression using reverse transcription-polymerase chain reaction (RT-PCR).

MATERIAL AND METHODS: The leaves of A. marina were dried and extracted with n-hexane followed by evaporation and freeze-drying. Polyisoprenoid contents were analyzed with two-dimensional thin-layer chromatography method. Cell viability was assessed with 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyl tetrazolium bromide assay. The cycle cell was tested with flow cytometry method. The apoptotic test was determined with a double-staining method. The gene expression on P13k, Akt1, mTOR, Egfr, and P53 was analyzed by RT-PCR method.

RESULTS: The results showed that 48 h cytotoxic activity of polyisoprenoids against WiDr cells and 5-Fu (positive control) had IC50 values, 295.25 μg/mL and 17.43 μg/mL. Cell cycle analysis depicted that the inhibition of polyisoprenoid occurred in the G0-G1 phase and 5-Fu in S phase. Polyisoprenoid and 5-Fu had the same mechanism control) had IC50 values, 295.25 μg/mL and 17.43 μg/mL.

CONCLUSION: The present study confirmed that polyisoprenoids from A. marina leaves showing as chemopreventive agents for colon cancer.

Introduction

According to the Global Cancer Observatory in 2018, Asia first in the incidence of colon cancer, which is 51.8% of cases of colon cancer ranked in the top three in the number of causes of death worldwide [1]. One of the risk factors for colon cancer is related to an unhealthy diet (intake). Low fiber intake and high fat will increase the risk of colon cancer [2]. The WHO data showed in 2014 that the number of men with colon cancer was 15,985 cases, while women were 11,787 cases. The high incidence of colorectal cancer occurred in Indonesia, the death rate of men in Indonesia due to colon cancer was 10.2% (103,100 deaths, while women were 8.5% with 92,000 deaths) [3].

Cancer is a disease characterized by uncontrolled cell growth. Cancer cells may avoid apoptosis and signals that suppress growth, the ability to form new blood vessels (angiogenesis), and the ability to invade and metastasize [4].

The use of chemotherapy agents is one of the treatments for colon cancer in addition to surgery and radiation therapy. Chemotherapy agents used today generally not only to suppress the growth or proliferation of cancer cells while causing toxicity to the body but also inhibit the proliferation of normal cell division, including the bone marrow, gastrointestinal mucosa, hair follicles, and lymphocyte tissue [4]. This condition raises concerns about various side effects caused by the use of conventional chemotherapeutic agents, such as heart (cardiotoxic) disorders, nausea, diarrhea, and suppression of the immune system and the occurrence of resistance, thus increasing people’s interest in using traditional medicines [5].

Potential natural ingredient developed as chemotherapeutic agents includes from mangrove
leaves. Mangrove vegetation defined as a plant or shrub distributed in intertidal zone of tropical and subtropical region [6]. Polyisoprenoid is secondary metabolites found in mangroves, classified as dolichol and polyprenol on mangrove leaves and roots [7], [8]. So far studies reporting pharmacological activity in polyisoprenoid of mangrove species are still limited, so it is important to achieve the prospects, potential, and mechanisms polyisoprenoid in mangroves as a natural ingredient of pharmaceutical and medication [7].

Recently, it has been shown that polyisoprenoid in Nypa fruticans induced the cancer cell cycle inhibition of adenocarcinoma of the colon (COLO 320 HSR cells, cell WiDr, and LS174 cells) in G2/M phase and reduce the percentage of Bcl-2 and Bcl-xL [8]. It has been also reported that polyisoprenoid of Avicennia marina and Aerva lanata leaves has anticancer colon activity. Polyisoprenoid of A. marina has IS value of 5.195 (> 3) that is highly selective. This polyisoprenoid extract has a mechanism of inhibition of cell cycle at G0-G1 phase and apoptotic phase analysis occurs in the early apoptotic phase on the WiDr cells with flow cytometry method [9].

This study, therefore, aimed to test of biological and pharmacological activities for the treatment of colon cancer from A. marina polyisoprenoid in terms of the cycle and gene expression of P53, Egrf, P13k, Akt1, and mammalian target of rapamycin (mTOR) using the reverse transcription-polymerase chain reaction (RT-PCR) method.

### Materials and Methods

#### Plant material

Mangrove leaves of A. marina were collected the village of Lubuk Kertang, District West Brandan, Langkat, North Sumatra. The identification of A. marina has been confirmed by Herbarium Medanense and the voucher has been deposited.

#### Preparation of isolation polyisoprenoid alcohols

Powder simplicia mangrove leaves of A. marina (500 g) were macerated with a mixture of chloroform:methanol (2:1, v/v) for 48 h. Non-saponified lipid (NSL) extracts of leaves incubated of 65°C for 24 h in 86% ethanol containing KOH 2 M. NSL parts were further diluted with n-hexane and the solvent was evaporated. Then redissolved in n-hexane, a concentrated dried extract was obtained as previously reported [10].

### Isolation WiDr cells

Cell lines and cell culture conditions (WiDr cells), isolated human colon cancer cells from the large intestine of 78-year-old women were provided by the Laboratory of Parasitology collection, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. WiDr cell lines were cultured in RPMI 1640 medium, and supplement with 10% (v/v) fetal bovine serum, 1% penicillin and streptomycin, fungizone 0.5%, and in a 37°C incubator with 5% CO₂ [11].

### Cytotoxic test

Cytotoxic test conducted on colon cancer cells WiDr in this study using the 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyl tetrazolium bromide (MTT) method as previously reported [12]. WiDr colon cancer cells grown in microplate 96 wells to obtain a density of 1 × 10⁴ cells/wells. The cell was incubated in a 5% CO₂ incubator temperature at 37°C for 48 h to obtain good growth. Once it replaced with a new medium was then added extract of the test sample of each series of concentration of 5-Fu as a positive control and incubated at 37°C in 5% CO₂ incubator at 37°C for 48 h. At the end of incubation, culture media, and the sample dumped then cells were washed with phosphate buffered saline (PBS). In each of the wells was added 100 mL of culture medium (RPMI) and 10 mL MTT concentration of 5 mg/mL. The cells were incubated back for 3–6 h in a 5% CO₂ incubator at 37°C. The reaction was stopped with 10% sodium dodecyl sulphate (SDS) reagent in HCl 0.01 N. Then, the disc of 96 wells was wrapped tightly and keeps one night at room temperature. ELISA reader was used to measure the absorption at a wavelength of 595 nm [13].
(DAPI). With the fluorochrome that had the ability to interact DNA strand with bases such as PI, each cell had a different number of sets of chromosomes to give different fluorescence intensity. The more sets of chromosomes and the fluorescence intensity were higher. Apoptotic was tested by adding annexin V and PI while testing the cell cycle, PI was added. The extract was measured with a flow cytometer [14].

**Apoptotic test by double staining**

Apoptotic was detected by a double-staining method using acridine orange and ethidium bromide reagents. This method was based on DNA differences fluorescence in cells that live and die bonding acridine orange and ethidium bromide [14]. Acridine orange permeated all parts of the cell and the nucleus looked green. While ethidium bromide was intercalated with the cell membrane and the damaged nucleus was red. The color stained by ethidium bromide on dead cells more dominant compared to acridine orange so that the nucleus of dead cells was colored orange. Living cells with intact membranes had a nucleus with a uniform green color. During the process, the cells underwent apoptotic, and membrane blebbing started happening, ethidium bromide entered the cells and provided the orange color. Green fluorescence cells indicated the living cells and red fluorescence cells indicated the dead cells. Red fluorescence intact showed necrotic cells and cells that were fragmented indicated cells undergoing apoptotic. Apoptotic is programmed cell death that resulted in changes in the morphological and biochemical characteristics of cells. Stimulation of apoptotic included DNA damage, the presence of tumor necrosis factor, or absence of growth factors. Apoptotic was characterized by membrane blebbing presence without loss of membrane integrity, chromatin condensation and fragmentation, cytoplasmic compaction organelles, dilation of endoplasmic reticulum, cell volume reduction, and the formation of apoptotic bodies [15].

**Analysis of gene expressions in vitro with RT-PCR**

The expression of the genes was examined RT-PCR method [16]. The PCR component in 25 μL PCR Master Mix total contained 1 μL cDNA, GoTaxGreen 12.5 μL, 1 μL forward primer, 1 μL primer reverse, and 9.5 μL DNase/RNase free water. The RT-PCR was performed using iCycler (Bio-Rad) with 35 times cycle as follows: 30 s denaturation at 94°C, 30 s annealing at 48–65°C, and 30 s elongation at 72°C. The primers used for this RT-PCR was displayed in Table 1. RT-PCR product subjected to 2% agarose gel electrophoresis showed a single discrete-sized band as predicted [17].

**Results and Discussion**

**Cytotoxic test**

IC$_{50}$ value obtained from polysiprenoid in the leaves of mangrove species A. marina was 295.25 μg/mL with control positive of 5-Fu was 17.43 μg/mL (Table 2). Therefore, based on the flow and previous results on the IC$_{50}$ value, n-hexane extract was used as samples to test anticancer. The relatively high IC$_{50}$ value might be less active as anticancer because some extracts are considered to be active agent as of IC$_{50}$ values ≤100 μg/mL [16]. However, an extract value of IC$_{50}$ was 100–500 μg/mL which can be developed as anticancer with moderate classification [18]. It has been described that an extract had IC$_{50}$ >500 μg/mL, also was considered active [19]. The major polysiprenoids in A. marina leaves (95.8%) and roots (100%) were dolichol, few polyenyls in the leaves (4.2%) [10].

### Table 1: Sequences of primer used in this study

| Gene      | Sequence Amplicon | Amplican |
|-----------|-------------------|----------|
| β-actin   | 5'-TCGTCAATCTCCGCCAGCTGCTGAT-3' | 105 bp   |
| Akt       | 5'-GGAGCCGCCGAGGGACAGACTGCTGAT-3' | 240 bp   |
| P13k      | 5'-CTGGATGCTTGGCATTGCTGCTG-3' | 340 bp   |
| mTOR      | 5'-AACAAACTCATGCCCGCGCCAGCTG-3' | 110 bp   |
| PS3       | 5'-CCCATCGATCGATCCGATGATCCGATG-3' | 360 bp   |
| Egfr      | 5'-GGAGACCTGTCGAGGAGAAGCGAGAGCG-3' | 320 bp   |

mTOR: Mammalian target of rapamycin.

**Apoptotic test**

Based on the inhibition of cell cycle using PI reagent, the percentage of inhibition at each phase was obtained as shown in Figure 1 and Table 3. Table 3 shows the control group of WiDr cell accumulation in G0-G1 phase, S, and G2-M. Polysiprenoid with concentration of 1/5 IC$_{50}$ decreased the accumulation of cells in S phase and G2-M to 6.64% and 4.98%, respectively, but increased the G0-G1 phase (Table 3). The change phase might be related to the concentration. Polysiprenoid in A. alba inhibited the WiDr cell cycle in the S phase [20]. However, it can be suggested that polysiprenoid mechanism of inhibition of cell cycle occurred at G0-G1 phase. Table 3 shows 5-Fu with 1/5 concentration of IC$_{50}$ decreased in the accumulation of WiDr cells in the G2-M phase 6.42%. By contrast, the increase in the cell accumulation occurred in the G0-G1 phase, and S is 88.12 and 9.52%, respectively. However, mechanisms of cell cycle inhibition of 5-Fu indicated in the G0-G1 phase and S. Treatment with 5-Fu in cancer cells led to the accumulation of cells at the G1 phase and the beginning of the synthesis phase.

### Table 2: IC$_{50}$ calculation from polysiprenoids against WiDr cells

| Species          | IC$_{50}$ (μg/mL) | WiDr cells (μg/mL) |
|------------------|------------------|--------------------|
| Polysiprenoid     | 295.25           |                    |
| 5-Fu             | 17.43            |                    |
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...However, the cell cycle inhibitory activity by 5-Fu depended on the type of cancer cell [21].

Table 3: Percentage accumulated in each phase of the cell cycle

| Treatment     | Concentrations (μg/mL) | This phase of the cell cycle (%) |
|---------------|------------------------|----------------------------------|
| Control cell  | -                      | G1-G0: 76.63, S: 7.22, G2-M: 17.93 |
| Polyisoprenoid| 60                     | G1-G0: 90.49, S: 6.64, G2-M: 3.98 |
| 5-Fu          | 3.6                    | G1-G0: 88.12, S: 9.52, G2-M: 6.42 |

Cell control was observed in a fluorescence microscope; it appeared that cells produced 89% green fluorescence (Figure 1). In contrast to this observation, polyisoprenoid only occupied 45% fluorescence orange (dead cells), compared to the positive control (5-Fu). Polyisoprenoid produced 40% orange fluorescence indicated that polyisoprenoid had a position similar to the first line therapy cancer colon (5-Fu) (Figure 2).

**Gene expression**

RT-PCR is a single DNA synthesis method of using mRNA as template. DNA strand that has been formed to cDNA was then amplified by PCR [20]. In this test, WiDr cells treated with the test compounds polyisoprenoid with a concentration of 10 μM. Measurement of the expression of P13k, Akt1, mTOR, P53, and Egfr using RT-PCR method. Furthermore, the electrophoresis of the gene test results to produce band was depicted in Figure 4.

The PCR amplification of selected genes along with internal standard of β-action was displayed in Figures 3-4. Polyisoprenoid and 5-Fu downregulated the expression of P13k. Activation of P13k has been described in human cancer. This condition occurred due to amplification, overexpression, or of mutations in regulatory subunit P110 or P85. Amplification of 3q26 chromosomal region, which contained the gene which encodes the PIK3CA p110α catalytic subunit of P13k, occurs in 40% of ovarian [22] and 50% of cervical carcinoma [23]. Somatic mutations of this gene have also been detected in several types of cancer and resulted in increased activity of P13k kinase P13k mutant relative to the wild type. P85 mutations in the regulatory subunit have also been detected [24], [25]. Since any changes in individual components resulted in the activation pathway, this study therefore suggested that activation pathway is one of the most common molecular changes in cancer.

The test results of gene expression were shown in Figure 3, polyisoprenoid and 5-Fu decreased the expression of Akt1. Akt1 gene provided instructions for making a protein called kinase Akt1. This protein is found in many types of cells throughout the body, where it plays an important role in many signaling pathways. For example, Akt1 kinase regulates the growth and cell division (proliferation), a process in which cells mature to perform specific functions (differentiation), and cell survival. Kinase Akt1 also help control apoptotic, which is the self-destruction of cells when to be damaged or no longer needed. Signaling involving kinase Akt1 appears to be important for the normal development and function of the nervous system. The present study supported the role of the kinase Akt1 to cell communication in the cell between nerve cells (neurons), neuron survival, and memory formation. Akt1 gene belongs to a class known as oncogenes. When mutated, oncogenes potentially cause normal cells to be cancerous [24].

Figure 4 shows that polyisoprenoid and 5-Fu decreased the expression mTOR. mTOR pathway is involved in the synthesis of proteins by regulating the intake of amino acids, tRNA, and translation initiation. 21 amino acids play a role in mTOR that affects the rate of protein synthesis. mTOR pathway is divided into two distinct protein complexes, namely, TORC1 and TORC2. TORC1 plays a critical role in determining cell size while TORC2 involves in regulating cell shape and actin cytoskeleton [26], [27].
As depicted in Figure 4, polyisoprenoid and 5-Fu upregulated the expression P53. P53 is a tumor suppressor protein that acts as a cell cycle regulator. P53 protein plays an important role in response to cellular stress, such as exposure to carcinogens [28]. This protein would inhibit the proliferation of abnormal cells that have been initiated carcinogens to prevent the development of neoplasms. Protein inactivity can lead to cancer malignancy is malignant [29]. Besides functioning to regulate cell proliferation, p53 also regulates apoptosis, inhibits angiogenesis, and regulates DNA repair. In cancer commonly mutated p53 activity [30]. Most of the p53 mutation is the case which is missense mutation. Mutations can be in the form of degradation of p53, the loss of ability of p53 to induce cell cycle arrest or apoptosis, and lose affinity binding of p53 to DNA damaged [31]. Saponins, flavonoids, polyphenols, and physalin [32] play a role in the inhibition of cancer cells. Saponin compounds inhibit the formation of Bcl-2 expressed too high, induces caspase-3 protein expressed is too low, increase the expression of p53, and may also trigger the G1 cell cycle arrest [33].

Figure 4 shows that polyisoprenoid and 5-Fu downregulated the expression of Egfr. Some reports indicate that an increase in copy number of Egfr genes or gene mutations that are responsible for signaling streams is an important determinant of response or resistance to anti-Egfr antibody [34]. This study analyzed the impact of KRAS mutations on the clinical activity of anti-Egfr targeted treatment. The underlying hypothesis is that most of KRAS mutations cause a gain of function activates Ras pathways/MAPK. As the activated signal transduction at the level of KRAS proteins, inhibition upstream by the Egfr-targeted agents becomes ineffective [34].

β-actin was widely used as an internal control in the analysis of gene expression because it is a housekeeping gene, the gene continuously expressed for a living organism. Housekeeping genes have stable expression levels in various tissues during development stage [35].

Table 4 summarizes the gene expression ratio to internal standard from polyisoprenoid extract.

### Table 4: Gene expression ratio of polyisoprenoid extract

| Gene | Treatment groups | Ratio standard ± SE |
|------|------------------|--------------------|
| P13k | Control cell | 1 ± 0.00* |
| Polyisoprenoid | 0.65 ± 0.05** |
| 5-Fu | 0.95 ± 0.008** |
| Akt1 | Control cell | 1 ± 0.00** |
| Polyisoprenoid | 0.70 ± 0.011** |
| 5-Fu | 0.95 ± 0.002** |
| mTOR | Control cell | 1 ± 0.00** |
| Polyisoprenoid | 0.75 ± 0.008** |
| 5-Fu | 0.72 ± 0.005** |
| P53 | Control cell | 1 ± 0.00** |
| Polyisoprenoid | 2.45 ± 0.01** |
| 5-Fu | 2.27 ± 0.01** |
| Egfr | Control cell | 1 ± 0.00** |
| Polyisoprenoid | 0.04 ± 0.01** |
| 5-Fu | 0.04 ± 0.01** |

*Significantly different with the normal group (control cell) p<0.05, **Significantly different from the positive control group (5-Fu) p<0.05, there is a significant difference with the polyisoprenoid group. #p=0.05 was no significant difference in 5-Fu group. mTOR: Mammalian target of rapamycin.

The lowest expression of Egfr was in polyisoprenoid which showed that the effect was not significantly different from that of 5-fu but significantly was different from cell control (Table 4). The polyisoprenoid and 5-Fu downregulated gene expression of P13k, Akt, mTOR, and Egfr by contrast upregulated P53 gene expression.

### Conclusion

Polyisoprenoid from A. marina inhibited the cell cycle cancer phase G0-G1 phase and phase S. Polyisoprenoid and 5-Fu improved WiDr cell apoptotic in the early phase of apoptotic with a double-staining method. Polyisoprenoids downregulated the P13k,
Akt1, mTOR, and Egfr gene expression, and however, upregulated P53 gene expression. The present study confirmed that polyisoprenoids from A. marina leaves showing as chemopreventive agents for colon cancer.

References

1. IARC. Estimated Cancer Incidence, Mortality, and Prevalence Worldwide In 2012. Geneva: International Agency for Research on Cancer, World Health Organization; 2019. Available from: http://www.globocan.iarc.fr/pages/fact_sheets_cancer.aspx. [Last accessed on 2019 Jun 04].

2. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, et al. Globocan 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11. Lyon, France: International Agency for Research on Cancer; 2013. https://doi.org/10.1002/jicd.29210

3. IARC. The High Incidence of Colorectal Cancer in the Country. Geneva: World Health Organization; 2014. http://www.who.int/mediacentre/factsheets/fs297/en. [Last accessed on 2019 Jun 05].

4. McDonald GT. Inhibition of phosphatidylinositol 3-kinase promotes tumor cell resistance to chemotherapeutic agents via a mechanism involving delay in cell cycle progression. Exp Cell Res. 2010;316(19):3197-206. https://doi.org/10.1016/j.yexcr.2010.08.007

5. Kumijansiti R, Hamid SI, dan Rahmawati K. Efek sitotoksik in vitro dari ekstrak buah mahkota dewa (P helaria macrocarpa) terhadap kultur sel kanker mieloma. Media Eksakta. 2008;7(1):48-54. https://doi.org/10.23869/bphbjr.17.2011114

6. Akter R, Uddin SJ, Grice ID, Tiralongo E. Cytotoxic activity of Ethanol Extracts Plant Sala (Cynometra carioleifera Blume). J Nat Med. 2017;78:18-31. https://doi.org/10.1007/s11010-016-0154-x

7. Niranjana R, Gayathri R, Mol SN, Sugawara T, Hirata T, Miyashita K, et al. Carotenoids modulate the hallmir control of cancer cells. J Funct Foods. 2015;18:968-85. https://doi.org/10.1016/j.jff.2014.10.017

8. Sari PD, Basyuni M, Hasibuan AP. The inhibition of polyisoprenoids from NYPA fruticans leaves on cyclooxygenase 2 expression of widr colon cancer cells. Asians J Pharm Clin Res. 2018;11(8):154-7. https://doi.org/10.22159/ajpcr.2018. v118.26098

9. Illian ND, Basyuni M, Hasibuan AP. Polyisoprenoids from Avicennia marina induces several genes in WiDr cells. Malar J. 2010;9:49. https://doi.org/10.1186/1475-2875-9-49

10. QRohman et al. Polyisoprenoids from Avicennia marina induces several genes in WiDr cells.

11. Chevalley S, Coste A, Lopez A, Pipy B, Valentin A. Flow cytometry for the evaluation of anti-plasmodial activity of drugs on Plasmodium falciparum gametocytes. Malar J. 2010;9:49. https://doi.org/10.1186/1475-2875-9-49

12.开放获取马奇德医学生物科学。2020年4月20日;8(A):146-152.

13. Haryato H, Muhtadi M, Indrayudha P, Azizah T, Suhendi A. Chemoprevention Research Centre. The Protocol of Apoptotic Test Double Staining Method. Yogyakarta: Cancer Chemoprevention Research Centre; 2013. p. 1-4. Available from: http://www.ccrc.farmasi.ugm.ac.id/en/?page_id=240. [Last accessed on 2019 Jun 06].

14. Zhang Y, Guan XY, Dong B, Zhao M, Wu JH, Tian XY, et al. Expression of MMP-9 and WAVE3 in colorectal cancer and its relationship to clinicopathological features. J Cancer Res Clin Oncol. 2011;138(12):2035-44. https://doi.org/10.1007/s00432-012-1274-3

15. Sudjadi S. Bioteknologi Kesehatan. Protokol Penggunaan RTPCR. Yogyakarta: Kanisius. 2008. p. 142.

16. Cancer Chemoprevention Research Centre. The Protocol of Apoptotic Test Double Staining Method. Yogyakarta: Cancer Chemoprevention Research Centre; 2013. p. 1-4. Available from: http://www.ccrc.farmasi.ugm.ac.id/en/?page_id=240. [Last accessed on 2019 Jun 06].

17. Weerarpeeyakul N, Nonpunya A, Barustux S, Thitmelaroch T, Sripriandilukh B. Evaluation of the anticancer potential of six herbs against a hepatoma cell line. Chin Med. 2012;7(1):15. https://doi.org/10.1186/1749-8546-7-15

18. Machana S, Weerarpeeyakul N, Barusux S, Nonpunya A, Sripriandilukh B, Thitmelaroch T. Cytotoxic and apoptotic effects of six herbal plants against the human hepatocarcinoma (HepG2) cell line. Chin Med. 2011;6(1):39. https://doi.org/10.1186/1749-8546-6-39

19. Patapathy S, Jena BK, Antitumor and growth effector screen of leaf extracts of selected mangroves of Bhitar ranki, Odisha. Int J Technol Enhanc Emerg Eng Res. 2013;1(4):25-30. http://www.globocan.iarc.fr/pages/fact_sheets_cancer.aspx.

20. Illian ND, Basyuni M, Hasibuan AP, Sumpardi S. Anticancer activity of polyisoprenoids from Avicennia Alba Blume. J Nat Med. 2019;18(3):1477-87. https://doi.org/10.4103/pm.pm_201_18

21. Liu HC, Chen GG, Vlantis AC, Leung BC, Tong, MC, van Hasselt CA. 5-fluorouracil mediates apoptosis and G1/S arrest in laryngeal squamous cell carcinoma via a p53-independent pathway. Cancer J. 2006;12(6):482-93. https://doi.org/10.1007/10959-004-00008

22. Lim YJ, Rhee JC, Bae YM, Chun WJ. Celecoxib attenuates PIK3CA is implicated as an oncogene in ovarian cancer. Nat Genet. 1999;21(1):99-102. https://doi.org/10.1038/504217a0

23. Shyestesh L, Lu Y, Kuo WL, Baldocchi R, Godfrey T, Collins C, et al. PIK3CA is implicated as an oncogene in ovarian cancer. Nat Genet. 1999;21(1):99-102. https://doi.org/10.1038/504217a0

24. Ma J, Sawai H, Ochi N, Matsuo Y, Xu D, Yamasuda A, et al. PTEN regulates angiogenesis through PIK3/Akt/VEGF signaling pathway in human pancreatic cancer cells. Mol Cell Biochem. 2009;331(1-2):161-71. https://doi.org/10.1007/s11010-009-0154-x

25. Jiménez C, Portela RA, Mellado M, Rodríguez-Frade JM, Collard J, Serrano A, et al. Role of the PI3K regulatory subunit in the control of actin organization and cell migration. J Cell Biol. 2000;151(2):249-62. https://doi.org/10.1083/jcb.151.2.249

26. Foster DA. Phospholipid acid signaling to mTOR: Signals for
the survival of human cancer cells. Biochim Biophys Acta. 2009;1791(9):949-55. PMid:19264150

28. Luo, Cai Y, Peng Z, Liu T, Yang S. Chemical composition and in vitro evaluation of the cytotoxic and antioxidant activities of supercritical carbon dioxide extracts of pitaya (Dragon fruit) peel. Chem Cent J. 2014;8(1):1. https://doi.org/10.1186/1752-153x-8-1 PMid:24386928

29. Wang S, Gu Y, Zebell SG, Anderson LK, Wang W, Mohan R, et al. A noncanonical role for the CKI-RB-E2F cell-cycle signaling pathway in plant effector-triggered immunity. Cell Host Microbe. 2014;16(6):787-94. https://doi.org/10.1016/j.chom.2014.10.005 PMid:25455564

30. Zhu J, Jiang J, Zhou W, Chen X. The potential tumor suppressor p73 differentially regulates cellular p53 target genes. Cancer Res. 1998;58(22):5061-5. PMid:9823311

31. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell. 1992;69(7):1237-45. https://doi.org/10.1016/0092-8674(92)90644-r PMid:1535557

32. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. Nature. 1991;351(6326):453-6. https://doi.org/10.1038/351453a0 PMid:2046748

33. Nakamura S, Katoh E, Koshikawa T, Yatabe Y, Nagasaka T, Ishida H, et al. Clinicopathologic study of nasal T/NK-cell lymphoma among the Japanese. Pathol Int. 1997;47(1):38-53. https://doi.org/10.1111/j.1440-1827.1997.tb04433.x PMid:9051691

34. Raju B, Mehrotra R, Oijordsbakken G, Al-Sharabi AK, Vasstrand EN, Ibrahim SO. Expression of p53, cyclin D1 and Ki-67 in pre-malignant and malignant oral lesions: Association with clinicopathological parameters. Anticancer Res. 2005;25(6C):4699-706. PMid:16334163

35. Yoon D, Wang Y, Stapleford K, Wiesmuller L, Chen J. P53 inhibits strand exchange and replication fork regression promoted by human Rad51. J Mol Biol. 2004;336(3):639-54. https://doi.org/10.1016/j.jmb.2003.12.050 PMid:15095978