Antiproliferative activity of natural coumarins from *Calophyllum incrasaptum* M.R Henderson-Wyatt Smith against human breast cancer cells MCF

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Abstract. Objective: to evaluated the antiproliferative activity of natural coumarin from Calophyllum incrasaptum M.R Henderson-Wytt Smith against human breast cancer cells MCF-7. Methode : Coumarin from ethyl acetate fraction of C. incrasaptum M.R Henderson- Wyatt Smith was isolated by coloumn chromatographyic and structure elucidated by using spectroscopic methods and isolate compound was evaluated for their antiproliferative activities in the alamar blue assay. Result: Coumarin have antiproliferative activity against MCF-7 cancer cell lines through alamar blue assay for 4 h after treatment. Conclusions: coumarin showed good activity against cancer cell lines with IC50 value of 2.23 µg/mL.
Keywords: antiproliferative, C. incrasaptum M.R Henderson- Wyatt Smith, MCF-7

1 Introduction

Cancer is a fatal disease standing next to the cardiovascular disease. Cancer, a diverse group of diseases characterized by uncontrolled growth and spread of abnormal cells, if the spread is not controlled, it can result in death or mortality, estimate 1,660,299 new cancer cases are expected to be diagnosed in 2013, and die about 580,350 in 2013 and almost 1,600 people die per day. New cases of breast cancer in the US during 2013 is 232,340 expected in women and 2,240 in men. Deaths : an estimated 40,030 breast cancer death (39,620 women, 410 men). Risk factors breast cancer for women is bigger than men, potentially risk factor after age 18, being overweight or obese (for postmenopausal breast cancer), used of menopausal hormone therapy (combined estrogen and progestin), risk is also increased by a family history of breast cancer [1]) and the number of deaths from cancer will continue to rise, with an estimated 13.1 million people dying in 2030 [2].

Emergency and rapid spread of cancer, represent a major problem for prophylaxis and treatment cancer which becomes more difficult, and the medicines used as treatment cancer have clear limitation and unfortunately cancer projected as the primary cause of death in the future [3]. Therefore, it is necessary to discovery anticancer drugs from traditional medicine plant for curing cancer.

Some experiments have been done to develop compound which it's having anticancer activity, such as: Cao et al 1998 have isolated some of the phenyl coumarins., which it's showed cytotoxic activity against mouse leukemia P388, mouse fibrosarcoma (WEH 1640), human monocytic leukemia (HTP-1) and human lymphoblastic leukemia (MOLT4) [4].: Guilet et al., 2001[5], and Reyes-chilpa et al., 2004 have isolated coumarin derivate, costatolide, soulattrolide, & calanolide F and *C. dispar* contains coumarin of the mammea type, some of them resulted highly cytotoxic against human epidermoid carcinoma cell (KB, PC3, K562, U251[6]; Li,Y-Z et al 2010 have also isolated triterpenoid from *C. inophyllum* and their growth inhibitory effects on human leukemia HL-60 cells [7], Mousavi, S.H.(2015) have evaluated and compered of cytotoxic effect of prenyloxycoumarin and herniarinon to MCF-7 cell line [8]. Other researcher (Khasman et.al 1992) have isolated calanolide A from *C. lanigerum* var austrocoriaceum as anti-HIV. [9].

Preventing of cancer by administrating a 6-substituted coumarin derivative was patented, The substituent at the 6-position may have five or more carbon atoms such as ten or preferable fifteen carbon atom [(C-5(6-prenyloxycoumarins), C-10(6-prenyloxycoumarins), C-15(6-prenyloxycoumarins)], [10]. Preventing of cancer by using coumarin/calcone derivative was described by (Carrico-Moniz). The structure of coumarin/calcone as anticancer was saw in figure 1, 2,3,4 [9]

The structure of coumarin derivate with substituted C-5, C10 and C15 at position C-6

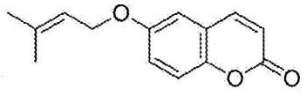

Fig 1. Structure of C-5(6-prenyloxycoumarins)

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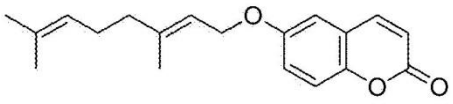

Fig 2. Structure of C-10(6-prenyloxycoumarins)

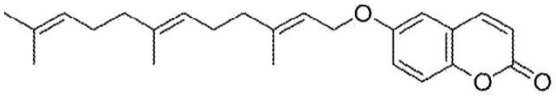

Fig 3. Structure of C-15(6-prenyloxycoumarins)

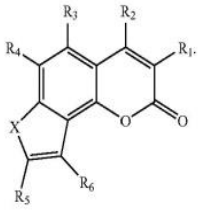

Fig 4. Structure of coumarin/chalcopne

Since coumarin showed interesting antiproliferative or anticancer activity, we decided to investigated *Calophyllum* species led to the isolation of one chemically distinct group of coumarin. In these study we are described the antiproliferative activity of the phenylcoumarin to MCF-7 cell lines from *C. incrasaptum*.

2 Materials and methods

2.1 Materials

Stem bark of *C. incrasaptum* M.R Henderson- Wyatt Smith was collected from Kalimantan Island in Indonesia. The sample was identified at the Herbarium Unit, Department of Botany Indonesian Institute of Sciences (LIPI). Solvents and chemicals used in this study are analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2 General aspects

^{1}H and ^{13}C NMR spectra were recorded on a JEOL NMR spectrometer at 500 and 125 MHz, respectively. The abbreviations s, d, and br used to describe ^{1}H spectra refer to singlet, doublet, and broad, respectively. NMR spectra were recorded in $CDCl_3$ solution on a Jeol JNM ECA 500 MHz instrument, using TMS as the internal standard. Silica gel (60–230 mesh and 230–400 mesh) were used for column chromatography and preparative TLC plates coated with silica gel (2000 micron thickness) was purchased from Merck (Darmstadt, Germany). Precoated Si gel plates SIL G/UV_{254}, 0.25 mm were used for TLC. The compounds were detected under UV light at 254 and 366 nm. Melting points were determined on an Electrothermal Fisher melting point apparatus (Scientific serial 903N0056) and are uncorrected. IR spectra were recorded on a FTIR Prestige 21 Shimadzu spectrophotometer (KBr dish) and UV spectra was taken on a Hitachi U-2000 spectrophotometer. LC-MS were recorded on Mariner Bio Spectrometric equipment with pneumatically assisted electronspray ionization (EIS) source.

3 Methods

3.1 Extraction procedure

The air-dried stems bark of *C. incrasaptum* (2.1 kg) were minced finely and shredded in an electric mill, macerated three time with MeOH (6L x72h weight of extract after solvent was removed : 400 gr), then was diluted in H_2O and partitioned with between *n*-hexane and H_2O: EtOAc and H_2O : and butanol – H_20 . The n-hexane, EtOAc and buthanol was removed under reduced pressure to give 65.8 g, 138.8 g and 609.4 g *n*-hexane, EtOAc and BuOH extract respectively.

EtOAc extract (120 gr) was separated by flash coloumn chromatography, It was eluted with a stepwise gradient of *n*-hexane followed by increasing amounts of EtOAc (20:1; 10:1; 5:1; 3:1; 1:1 ; EtOAc 100%) give 6 fractions (Fr. A1–A6), then were eluted by EtOAc:MEOH (20:1; 10:1; 5:1; 3:1 ,1:1) to give 5 fractions (Fr A7-A11). Fraction A5 was further purified several time by normal CC system on silica gel medium-pressure liquid chromatography (MPLC), eluting with *n*-hexane and 10% stepwise gradient of acetone to afford several fraction, fraction were collected and regrouped on the basis of TLC analysis. Sub fraction 68-74 was then applied to an silica column eluted with *n*-hexane – DCM (dichoromethane 50:50→0:100). Fraction 3 was futher purified by sephadex LH-20 , approximately 15 ml was collected for each fraction, collection sub fraction 33 - 47 were pooled after TLC profile analysis. We was able to pure crystal of the phenyl coumarin by recrystalyzing methode using *n*-hexane : acetone (3:1).

3.2 Preparation of Cancer Cells Line

Cancer cells line used in this study is breast carcinoma (MFC-7), and the cell line was cultured in DME Medium with FBS 10%. These cell was cultured at 37°C with moisture content of 95% and 5% CO_2 for 3 days until the cell cultures undergo confluence 60-70%. After that the old media removed, replaced with new medium and incubated again for 24 hours. Culture cells then washed with PBS 1-2 times as much and was suspended using trypsin-EDTA solution. Cells that have been suspended added with new.

3.3 In vtro antiproliferative assay

Antiproliferative assay was used by Alamar Blue method in triplicate against human breast carcinoma (MFC-7) cell lines. The MCF culture cells line of 100 µL was supplemented with 10 µL of pure test compound (05; 1 and 1.5 ppm). It was then incubated for 24 hours at a 37°C.

Colourring process was done by adding Alamar blue solution for 4 hours. The colour intensity of the cell line was measured by using ELISA plate reader at 560 nm (excitation) and 590 nm (emission) wavelength. Percent viability is calculated as in equation 1. While IC_{50} of the active extract was calculated by linear regression analysis between percent survival showed in equation 2. Alamar Blue was used to color the sample, and the IC_{50} of the pure compound (Alamar Blue Assay, U.S. Patent No.5,501,959). [12]

$$\% \ Viability = \frac{OD\ (cell+sample)-OD\ (negative\ control}{OD\ (cells)\ -\ OD\ (negative\ control)} \ x\ 100$$

4 Result and Discussion

4.1 Results of Elucidation structure

Phenylcoumarin was isolated via column chromatography (CC) as an yellow amourphous from an EtOAC extract of steam bark of *C incrasaptum* M.R Henderson- Wytt Smith, mp 184-185ºC. The UV absorbtion of phenylcoumarin characterized a 5,7,10 threeoxygenated coumarin, and give a positive test with a vaniline/H_2SO_4 reagent, an intense deep blue spot develops over 24 hour period. LC-MS revealed the molecular formula to be $C_{21}H_{18}O_5$ (*m/z* 351,40 $[M+1]^+$), by ESIMS analysis, i,e., the molecule had threeteen degrees of unsaturation. The UV spectrum exhibited two maxima at λ_{max} 256 and 319 nm were indicative of a dimethylchromanone derivative, whilst the FTIR spectra showed important peaks explaining the bending, stretching, and double-bond absorptions of the coumarin. The C-H stretching of absorption for CH_3 occurred at wavelengths of 2937 cm^{-1}. One alkane peaks were observed showing the bending absorption for CH_3 (v_{max} 1332 cm^{-1}). spectrum indicated absorption bands v_{max} 1732 cm^{-1} attributed to non-conjugated carbonyl group (C=O), and v_{max} 1691 cm^{-1} as conjugated C=O carbonyl groups, v_{max} 1597-1332 (aromatic C=C) and v_{max} 1153 cm^{-1} (C-O), and mono substituted benzene ring at v_{max} 767 and 702 cm^{-1} (Figure 5).

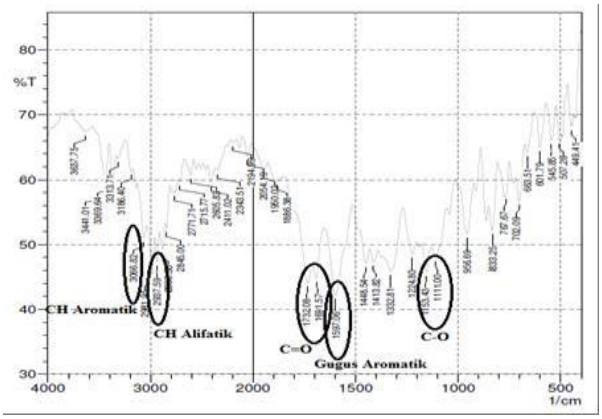

Fig 5. IR spectrum of isolate compound

Determination of structure was performed usinga NMR device. The 1-NMR spectrum of isolaed compound (phenyl coumarin) is evidenced by the presence of two proton singlet (δ_H 6.07, 1H,s, H-3) and δH 6.73, 1H,s,H-6) and group of signal belonging to five aromatic proton of the phenyl group [(δ7.34-7.40, *m* : δ_H = 7,34 (1H, *m*), 2 protons at δ_H 7.40 (2H, *m*), and 2 protons at δ_H 7.38 (2H, *m*)]. The presence of a singlet-shaped signal at δH 3.14 ppm (3H,s, H-13) shows the proton of one methoxy group. Addition signal included these of dimethylchromanone ring (δ_H 4.28, 1H, *m*, H-8, and δ_H 2.55, 1H, *m*,H-9 : δ_H 1.52, 3H, *d, J* = 6.5 Hz, H-11 and δ_H 1.18, 3H, *d, J* = 7.1 Hz, H-12) (Figure 6).

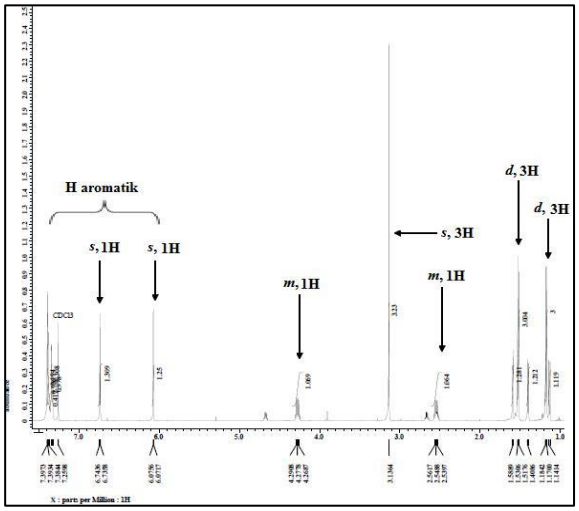

Fig 6. ^{1}H-NMR spectrum of isolate compound

The ^{13}C-NMR data shows 21 peaks of carbon atoms, nine C quartener atoms (including two carbonyl downfield signals at δ 159.43 and 191.65), seven C-H aromatic, two aliphatic protons (δ_H 1.52, *m,* H-8 & δ_H 2.55, *m*, H-9), the presence of two methyl groups (CH_3) is supported by ^{13}C-NMR data with a carbon chemical shift of 19.65 and 10.49 ppm and one methoxy group at 62.77 ppm (Figure 7).

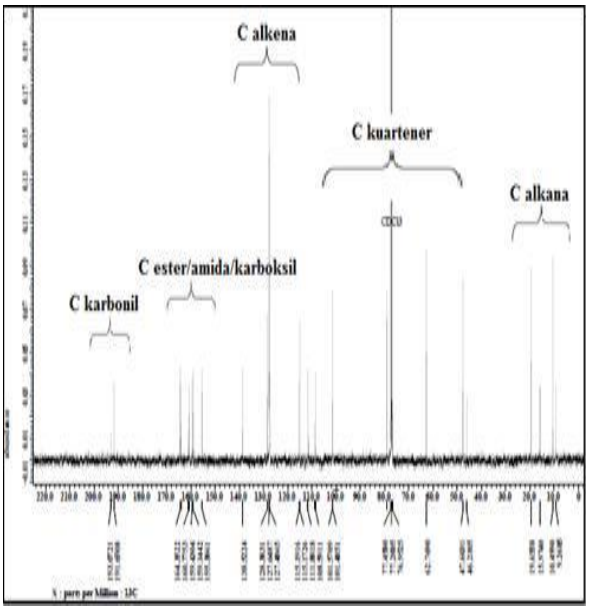

Fig 7. ^{13}C-NMR spectrum of isolate compound

This phenyl coumarin compoun have a methoxy sustituted at C-5(singlet methyl signal at δ_H 3.14 ppm). The NMR signals observed were in accordance with the presence of coumarin nucleus, with the benzenoid ring (δ_C 159.43, 115.17, 155.38, 108.59, 160.77, 101,48, 164.35, 111.88 and 159.14) an unsubtituted phenyl ring (138.12, 128.38, 127.49 and 127.65) one a methoxyl group (δ_C = 62.77) and a cromanone ring (δ_C = 191.5, 78.99, 47.68, 19.65 and 10.49). A correlation from the phenyl C1' (δ 138.2) to H-3 confirmed that a phenyl group was attached to C-4 as in isolated compound.

Long-distance 3J and 2J (HMBC) proton H 6,07 ppm on C-3 has been proved by H-correlation at δH 6.07 with C-4a (δC 108,59), C-2 (δC 155,43) and C-1 '(δC 138.52), this proves the position of H 6.07 ppm at C-3. The presence of two doublet-shaped signals at δH 1.52 ppm (3H, *d J*, 6.5 Hz) and 1.18 ppm (3H, *d J* 7,1 Hz) show protons of two methyl groups. HMBC data for proton correlation of methoxy group (δH = 3.14 ppm) with carbon C-5 (δC 160,77) assured the position of methoxy on C-5.

Phenyl group in C-4 positions is evidenced by the correlation of proton aromatics δ_H 7.38 ppm with carbon at δ_C 155.38 ppm, and proton correlation δ_H 6.07 ppm (H3) with carbon at δ_C 138.52 ppm (Figure 8). HMBC correlation of isolate compound was explained in figure 8.

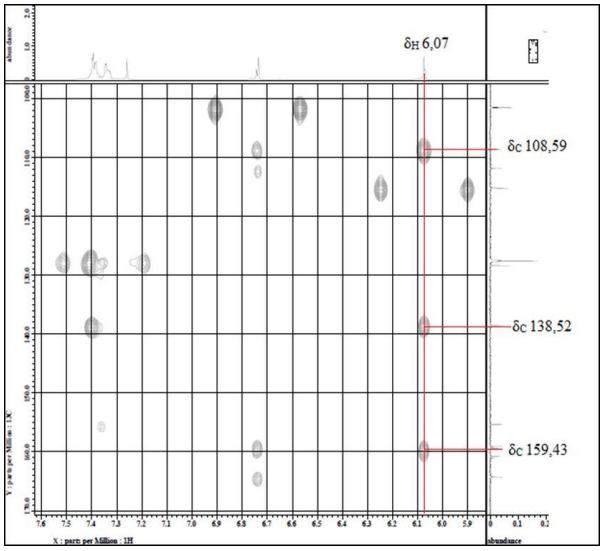

Fig 8. HMBC spectrum of isolate compound

All NMR 1D and 2D data was reported in Figure 9 and Table 1.

Table 1. Data NMR 1D and 2D and COSY chemical shift (ppm) of phenyl coumarin

No	C	H-NMR (δH) ppm	Cosy
1	0		
2	159.43	-	
3	115.17	6.07 (1H,*s*)	-
4	155.38	-	-
4a	108.59	-	-
5	160.77	-	-
6	101.48	6.73 (1H,*s*)	-
6a	164.35	-	-
7	0	-	-
8	78,99	4.28 (1H, *m,J* =6.5 Hz)	H-8 - H9
9	47.68	2.55 (1H, *m,J* =6,5 Hz)	H-9- H-8
10	191.65	-	-
10a	111.88	-	
10b	159.14	-	-
11	19.65	1.52 (3H, *d, J* =6.5 Hz)	H11-H8
12	10.49	1.18 (3H,*d. J* = 7.1 Hz)	H12-H-9
1'	138.2	-	-
2'	128.38	7.38 (1H, *m*)	H-2'-H-3'
3'	127.65	7.40 (1H,*m*)	H-2'-H-4'
4'	127.49	7,34 (1H,*m*)	H-3'–H5'
5'	127.65	7.40 (1H,*m*)	H-4'. -6'
6'	128.38	7.38 (1H, *m*)	H-6'-H-5'
OCH_3	62.77	3.14 (3H, s)	

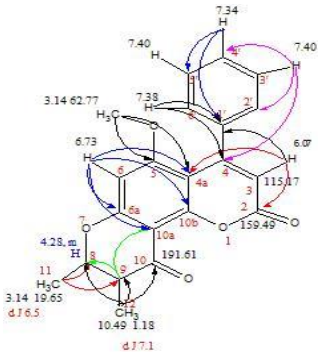

Fig 9. HMBC correlation of of isolate compound

This paper report the crystal structure of one isolated compound from *C. incrasaptum* as phenyl coumarin (Figure 10).

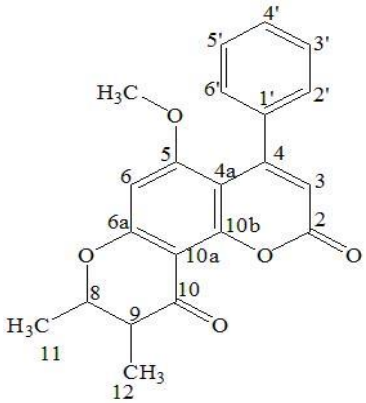

Fig 10. Chemical structure of phenyl coumarin

Hormon oestrogen has the crucial role in development of the breast cancer, the most frequent malignant disease in women, therefore many therapies are designed to block

this activity. Cinnamoyl-coumarin derivatives were especially effective in eostrogen-dependent cancer, such as breast (MCF7) cancer cell line. The phenylcoumarin in these paper have anticancer activity too, becauce coumarin are proven to posses a wide range of biological activitied , e. i as anticancer.

4.2 Results of cell viability

Inhibition of cell viability caused by prenyl coumarins including phenylcoumarin from *C. incrasaptum* was examined using Alamar Blue assay. The cytotoxic effect of pure test compound (phenyl coumarin) against MCF-7 breast cancer cells was determined with alamar blue assay at range of 0-1.5 ppm after 24 h of treatment period. The cell viability can be qualitatively assessed by the color intensity of alamar blue. The intensity of the alamar blue dye increased when the concentration of pure test compound decreased. The results are graphically represented in Figure 11`. Cell viability (%) was observed to be conversely proportioned to the concentration of the pure test compound, these results showed that these phenylcoumarin compound indicated decreased cell viability (that treatments showed growth inhibition) cel MCF7 responded in a dose-dependent. The pure test compound showed effective cytotoxic effect with the IC_{50} value of 2,6 ppm in MCF-7 breast cancer cells

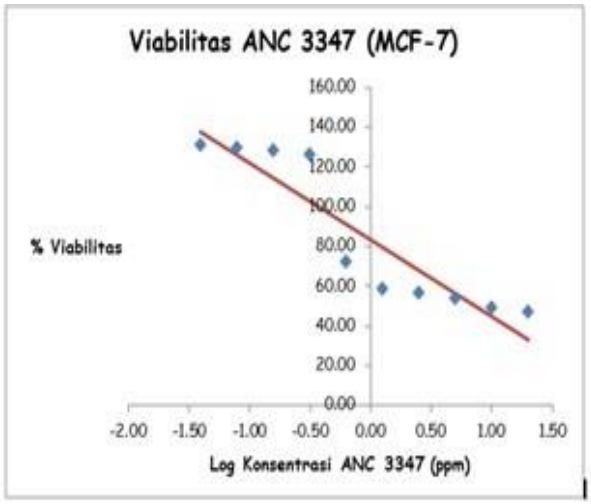

Fig 11. Cytotoxic effects of phenylcoumarin on MCF7 cells. Relationship between log concentration with % viabilirty

lines obtained from regression : Y = -54.04 Log X + 68.80 with R = 0.90 IC_{50} = 2,23 µg/mL
IC_{50} values in (µg/mL), IC_{50} < 4 µg/mL is higest cytotoxic,
IC_{50} 4-30 µg/mL considered cytotoxic and in active, IC_{50}=> 40 µg/mL

4.3 Discussion

According to the results, we treated the MCF7 cell line with different concentrations of phenylcoumarin for 24 h. The results indicated phenylcoumarin induced cell death in concentration –dependent manner.

Phenyl coumarin is a group of compounds which have shown to psses preventive and therapeutic effects on breast cancer.

5 Conclusions

Using a combination of NMR techniques (^{1}H and ^{13}C NMR, HMQC, HMBC and COSY) and IR spectroscopy combined with DEPT calculations, it has been possible to determine the structure of phenyl coumarin (Figure 10)

Phenylcoumarin exerts antiproliferative effect in breast carcinoma cell line, and can be considered for furher mechanistic evaluation in human its biosafety and anticancer effect.

The results demonstrated that treatmen of MCF7 cells with phenylcoumarin results in a significant decrease in cell viabilty, IC_{50} values against MCF7 is 2.23 µg/mL.

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