LC-MS/MS Analysis and Cytotoxic Activity of Extract and Fractions of Calophyllum Soulattri Stembark

by Nur Amalia Choironi

Submission date: 28-Mar-2023 12:29AM (UTC+0700)
Submission ID: 2048219179
File name: 4a_document.pdf (1.57M)
Word count: 3291
Character count: 17967

Indonesian Journal of Pharmacy

VOL 32 (3) 2021: 356–364 | RESEARCH ARTICLE

LC-MS/MS Analysis and Cytotoxic Activity of Extract and Fractions of *Calophyllum soulattri* Stembark

Muhamad Salman Fareza[1*], Nur Amalia Choironi[2], Sri Sutji Susilowati[1], Melina Puspita Rini[1], Vyola Festihawa[2], Irfansyah Septian Nur Fauzi[1], Esti Dyah Utami[3], Sarmoko[3]

1. Pharmaceutical Chemistry Laboratory, Department of Pharmacy, Jendral Soedirman University, Karangwangkal Purwokerto Campus, Indonesia, 53123
2. Pharmaceutical Biology Laboratory, Department of Pharmacy, Jendral Soedirman University, Karangwangkal Purwokerto Campus, Indonesia, 53123
3. Pharmacology and Clinical Pharmacy Laboratory, Department of Pharmacy, Jendral Soedirman University, Karangwangkal Purwokerto Campus, Indonesia, 53123

Info Article

Submitted: 14-02-2021
Revised: 18-07-2021
Accepted: 14-09-2021

*Corresponding author
Muhamad Salman Fareza

Email:
muhamad.fareza@unsoed.ac.id

ABSTRACT

Calophyllum soulattri (Sulatri), a plant from Clusiaceae family, has been empirically used as traditional medicine. In the present study, *C. soulattri* stem bark extract and fractions were evaluated for their toxicity against MCF-7 breast cancer cells. The chemical contents of the extract and fractions were analyzed using =liquid chromatography-mass spectrometry (LC-MS/MS). The results showed that ethanol extract and n-hexane fractions had a strong cytotoxic activity with IC_{50} values of 93.6 and 36 µg/mL, respectively. Meanwhile, as the positive control, ethyl acetate and cisplatin fractions have IC_{50} values of 233 and 16.2 µg/mL, respectively. The LC-MS/MS analysis showed that the extract and fractions contained polyporusterone A, poricoic acid D, polyporusterone F, esculentagenin, and 1-acetyl-3-(methoxy-carbonyl)-β-carboline. Therefore, *C. soulattri* stem bark extract and fractions have potential activity as an anticancer agent that can inhibit MCF-7 breast cancer cell growth.

Keywords: *Callophylum soulattri*, stem bark, LC-MS/MS analysis, MCF-7

INTRODUCTION

Cancer is one of the highest causes of death in the world. In 2020, 65.858 (16.6%) new cancer cases were reported in Indonesia (WHO, 2018). The cancer occurrence rate in Indonesia ranks 8th in Southeast Asia and the 23rd in Asia (Bray *et al.*, 2019). Natural products have attracted attention as anticancer drugs from plants such as the Calophyllum genus (Mah *et al.*, 2015). Calophyllum belongs to the Clusiaceae family, which consists of more than 200 species spread in tropical regions of Asia, East Africa, India, and Australia (Ee *et al.*, 2011). Plants of this genus are usually used traditionally for various chronic diseases such as eye infection, inflammation, leprosy, hypertension, pain, skin infection, nephritis, malaria, and tumors (Gupta and Gupta 2020).

Calophyllum soulattri, also known as Sulatri, is one of the endemic Calophyllum species in Indonesia. Traditionally, aqueous extract of the stem bark or leaf is used to treat inflammation and rheumatic (Tanjung *et al.*, 2018). Besides, ethanol extract of *C. soulattri* stem bark has strong antioxidant activity with an IC_{50} value of 6.82 mg/L (Septiana *et al.*, 2018). In addition, *C. soulattri* stem bark extract and fractions inhibited various tested bacteria with inhibition diameter value of 8-12 mm (Khan *et al.*, 2002). The essential oil of *C. soulattri* stem bark has cytotoxic activity against some cancer cells such as SNU-1 (gastric carcinoma), NCl-H23 (lung adenocarcinoma), Hep G2 (hepatocellular carcinoma), K562 (leukemia), and Sk-MEL-28 (melanoma) with IC_{50} values ranging from 21.8 to 33.3 µg/mL (Mah *et. al.*, 2013).

A previous phytochemical study showed that the C. *soulattri* stem bark contained xanthones, coumarin, phloroglucinol, steroids, and arylbenzofuran with strong cytotoxic properties against some cancer cells (Mah *et al.*, 2012; Lim *et al.*, 2017; Tanjung *et al.*, 2018). To the best of our knowledge, the LC-MS/MS analysis and antiproliferative effect of extract and fraction of *C. soulattri* stem bark on MCF-7 still have not been reported. Thus, in the present study, we aimed to

analyze the chemical compound from *C. soulattri* stem bark extract and fractions which have the potential as a cytotoxic agent against MCF-7 breast cancer cells.

MATERIALS AND METHOD

The stem bark of *C. soulattri* was collected from Pernasidi village, Cilongok subdistrict, Banyumas regency, Central Java, Indonesia and was identified at the Plant Taxonomy Laboratory of Biology Faculty, Jenderal Soedirman University (code 1703106). Distilled methanol, ethyl acetate, and *n*-hexane with technical grade were used for extraction and fractionation. Cisplatin (Sigma Aldrich, USA), penicillin-streptomycin (Gibco Thermo, Uppsala, Sweden), dimethyl sulfoxide (Merck, Germany), phosphate-buffered saline (Uppsala, Sweden), Reagent PrestoBlue™ Cell Viability (Thermo Fischer Scientific, Uppsala, Sweden), RPMI medium (Gibco Thermo, Uppsala, Sweden), Fetal Bovine Serum (Gibco Thermo, Uppsala, Sweden), Trypsin-EDTA (Gibco Thermo, Uppsala, Sweden), and Trypan Blue (Gibco Thermo, Uppsala, Sweden) were used for the cytotoxicity test.

Extraction and Fractionation

The air-dried stem bark powder of *C. soulattri* (2kg) powder was macerated in methanol for 3x24 h and then evaporated under reduced pressure to give a dark brown residue (130g). The methanol extract was redissolved in methanol-water (9:1) and partitioned through liquid-liquid extraction using *n*-hexane and ethyl acetate at a ratio of 1:1 to obtain hexane and ethyl acetate fractions. The fractions were evaporated to dryness under vacuum to obtain n-hexane (22.3g) and ethyl acetate (44.0g) extracts.

Component Analysis by LC-MS/MS

The chemical compositions of methanol, *n*-hexane and ethyl acetate extracts were analyzed using LC-MS/MS Waters Xevo G2-XSQTof equipped with an electrospray ionization interface (ESI) and mass detector. The extracts were injected (1µL) into a C8 column (2.1 x 11mm, 1.7µm, acquity uplc BEH). The solvents used were water-formic acid 0.1% (A) and acetonitrile-formic acid 0.1% (B). The mobile phase composition was 5% B for 1min, 5-100% B over 11min, and 100% B for 2min at a flow rate of 0.3mL/min. MS analysis was performed in positive ionization mode between *m/z* 50 and 1200and the operating parameters were optimized as follows: ESI ionization type, acquisition time 0-16min, high CE ramp start: 10.00-40.00eV, Low CE 6.00eV, collision gas, cone volume 30V, collision energy 6.00eV, desolvation temperature 500°C, desolvation gas flow 1000L/h, Cone gas flow 50L/h.

Cytotoxic Activity

Cytotoxic activity was tested using resazurin assays with colorimetric reagent PrestoBlue (Boncler *et al.*, 2014). The MCF-7 breast cell lines were obtained from the Central Laboratory of Padjajaran University, West Java, Indonesia, and were maintained in the incubator at 37°C with 5% CO_2. MCF-7 cells were seeded into 96 well plates with a density of 17,000 cells/well. Next, each extract of *C. soulattri* stem bark was dissolved using DMSO and diluted using media to obtain eight concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.63, and 7.81µg/mL. Microtube and 96 well plates were labeled according to dilution concentrations and treatments (standard/sample). Media was disposed of on each well and each 100µL sample and positive control of cisplatin was moved on to each well as per label. The plate was then incubated for 48 hours. Media of each well was added with a mixture of 10µL PrestoBlue reagent and 90µL RPMI media. The plate was incubated for 2h until the color changed. The absorbance was measured at 570nm using a multimode reader. The test for all samples and positive control were performed in triplicate.

RESULTS AND DISCUSSION

Identification of Chemical Content

Two kilograms powder of air-dried *C. soulattri* stem bark extraction resulted in 130g dark brown methanol extract (6.50% yield). This yield was lower than the yield of methanol extract of *Sulatri* stem bark maceration by Tanjung *et al* (2018) which was 21.54%. The methanol extract was fractioned with *n*-hexane solvent, followed by ethyl acetate solvent. The *n*-hexane fraction obtained was 22.3g (yield 1.11%), while the ethyl acetate fraction was 44.0g (yield 2.20%). The methanol extract, *n*-hexane, and ethyl acetate fractions were then analyzed for their chemical content using LC-MS/MS (Figure 1). Further MS analysis of extract and fractions conducted to five major compounds: polyporusterone A **(1)**, polyporusterone F **(2)**, poricoic acid D **(3)**, esculentagenin **(4)**, and 1-acetyl-3-(methoxy-carbonyl)-β-carboline **(5)** (Figure 2 and Table I).

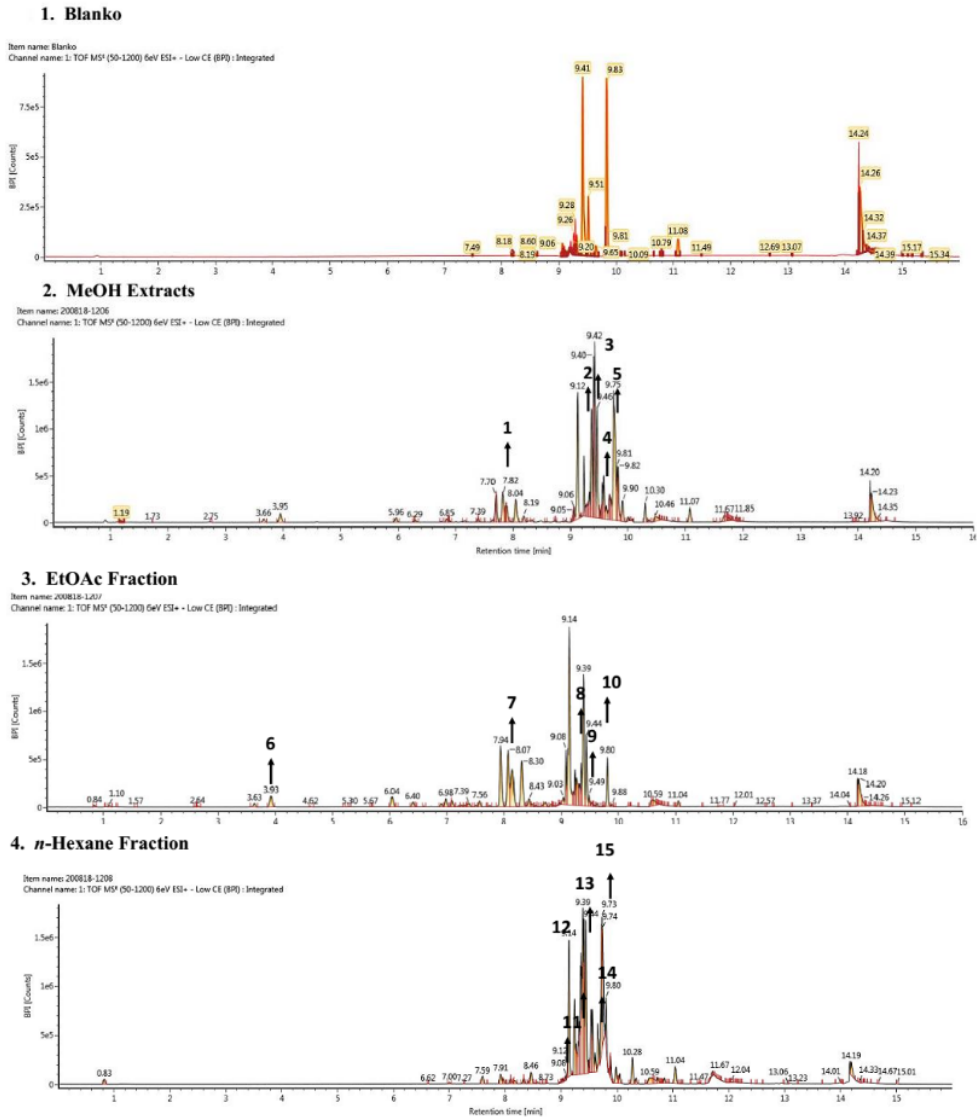

Figure 1. Liquid chromatography profiles of (1) blank, (2) methanol extract, (3) ethyl acetic fraction, and (4) *n*-hexane fraction

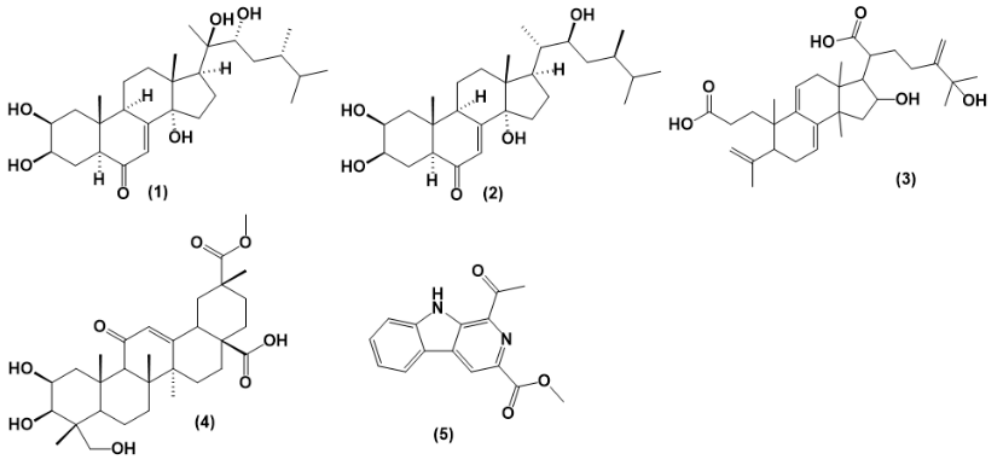

Figure 2. Chemical structures of compounds: **(1)** polyporusterone A, **(2)** polyporusterone F, **(3)** poricoic acid D, **(4)** esculentagenin, **(5)** 1-acetyl-3-(methoxy-carbonyl)-β-carboline

Table I. *m/z* profiles of the identified five major compounds

Peak	t_R (min)	Formula	Observed *m/z*	Neutral mass (Da)	Adduct	Mass error	Identification
MeOH extract							
1	7.82	$C_{30}H_{26}O$	403.2038	402.1984	+ H	-1.9	-
2	9.36	$C_{28}H_{46}O_6$	501.3156	478.3294	+ Na	3.2	Polyporusterone A
3	9.43	$C_{31}H_{46}O_6$	537.3168	514.3294	+ Na	-1.8	Poricoic acid D
4	9.68	$C_{28}H_{46}O_5$	485.3218	462.3345	+ Na	-1.9	Polyporusterone F
5	9.76	$C_{29}H_{48}O_5$	499.3388	476.3502	+ Na	-0.6	-
EtOAc fraction							
6	3.93	$C_{15}H_{12}N_2O_3$	291.0748	268.0848	+ Na	0.8	β-carboline derivative
7	7.94	$C_{30}H_{26}O$	403.2040	402.1984	+ H	-1.7	-
8	9.10	$C_{31}H_{46}O_8$	547.3255	546.3193	+ H	-1.1	Esculentagenin
9	9.36	$C_{28}H_{46}O_6$	501.3165	478.3294	+ Na	-2.2	Polyporusterone A
10	9.43	$C_{31}H_{46}O_6$	537.3167	514.3294	+ Na	-1.9	Poricoic acid D
***n*-hexane fraction**							
11	9.11	$C_{31}H_{46}O_8$	547.3251	546.3193	+ H	-1.5	Esculentagenin
12	9.35	$C_{28}H_{46}O_6$	501.3167	478.3294	+ Na	-2.0	Polyporusterone A
13	9.41	$C_{28}H_{46}O_5$	537.3164	514.3294	+ Na	-2.2	Poricoic acid D
14	9.67	$C_{31}H_{46}O_6$	485.3214	462.3345	+ Na	2.3	Polyporusterone F
15	9.74	$C_{29}H_{48}O_5$	499.3384	476.3502	+ Na	-0.9	-

Liquid chromatography analysis of methanol extract showed five main peaks at 7.82, 9.36, 9.43, 9.68, and 9.76min retention times. Each was then fragmented and resulted in five fragmentation spectra with candidate mass (*m/z*) of 403.2038, 501.3156, 537.3168, 485.3218, and 499.3388. Based on the internal spectra database search, the compounds polyporusterone A, poricoic acid D, and polyporusterone F were identified at retention times 9.36, 9.43, and 9.68min, respectively. Polyporusterone A showed fragmentation ion at *m/z* 156.0769 ($C_8H_{12}O_3$), 2018.1010 ($C_{12}H_{17}O_3$), and 391.2469 ($C_{23}H_{35}O_5$) (Figure 3).

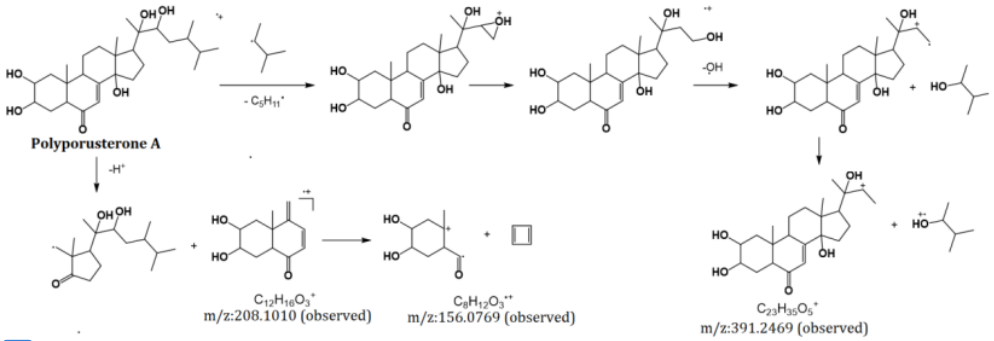

Figure 3. The proposed fragmentation pattern for polyporusterone A

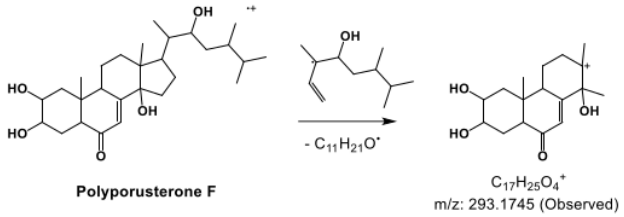

Figure 4. The proposed fragmentation pattern for polyporusterone F

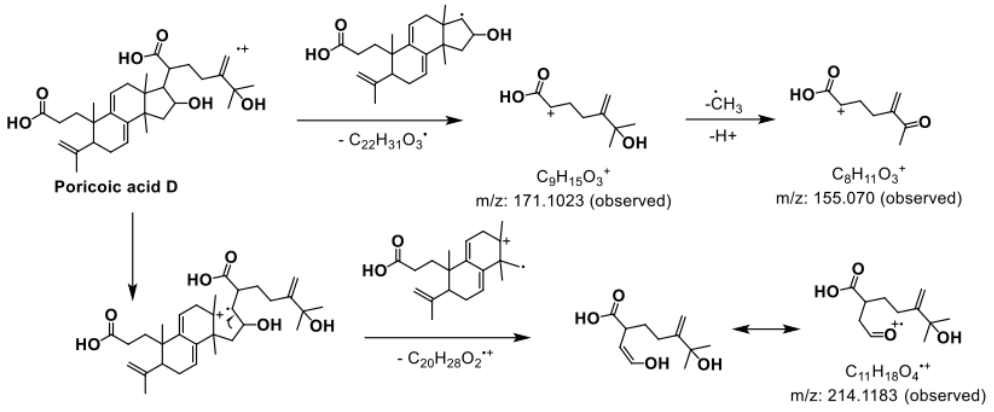

Figure 5. The proposed fragmentation pattern for poricoic acid D

Polyporusterone F fragmentation produced ion mass at *m/z* 293.1745 for ion-molecule $C_{17}H_{25}O_4^{+}$ (Figure 4). Meanwhile, poricoic acid showed fragmented ions at *m/z* 171.1023 ($C_9H_{15}O_3$), 155.070 ($C_8H_{11}O_3$), and 214.1183 ($C_{11}H_{18}O_4$) (Figure 5).

Liquid chromatography analysis of *n*-hexane fraction showed five main peaks at 9.11, 9.35, 9.41, 9.67, and 9.74min retention times. Each peak was fragmented and resulted in five fragmentation spectra with candidate mass (*m/z*) of 547.3251, 501.3167, 537.3614, 485.3214, and 499.3384.

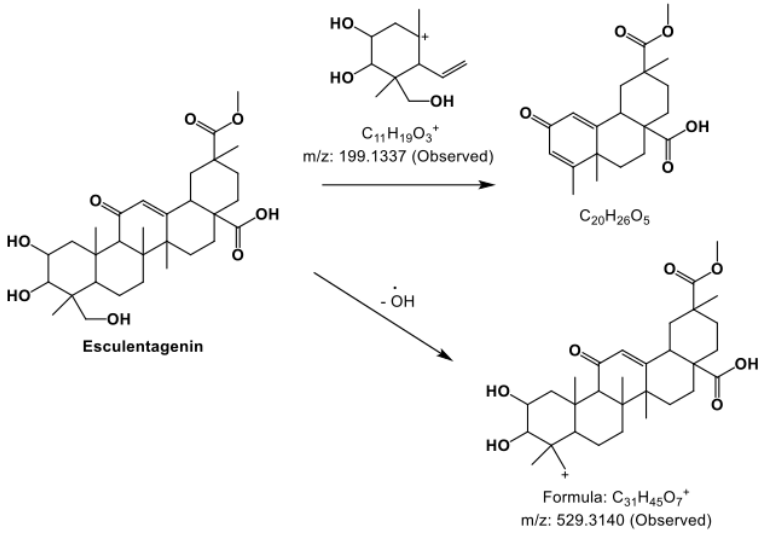

Figure 6. The proposed fragmentation pattern for esculentagenin

Based on the internal spectra database, the compounds esculentagenin, polyporusterone A, polyporusterone F, and poricoic acid D were identified at retention times 9.11, 9.35. 9.41 and 9.67min, respectively. Esculentagenin when fragmented showed two ion peaks at *m/z* 199.1337 and 529.3140 for ion-molecules $C_{11}H_{19}O_3^+$ and $C_{31}H_{43}O_7^+$, respectively (Figure 6). A previous report showed that the n-hexane fraction of *C. soulattri* stem bark contained β-sterol compound (Mah *et al.*, 2012).

Liquid chromatography analysis of ethyl acetate fraction showed five main peaks at retention times of 3.93, 7.94, 9.10, 9.36, and 9.43 minutes. Each peak was then fragmented and resulted in five fragmentation spectra with candidate mass (*m/z*) 291.0748, 403.2040, 547.3255, 501.3165, and 537.3167. Based on the internal spectra database, the compounds 1-acetyl-3-(methoxy-carbonyl)-β-carboline, esculentagenin, polyporusterone A, and poricoic acid D were identified at 3.93, 9.10, 9.36, and 9.43min retention times, respectively. Radical ion-molecule at m/z 139.0244 ($C_{10}H_5N$) was estimated from the fragmentation of β-carboline derivatives compound (Figure 7). Previous studies reported that ethyl acetate extract of *C. soulattri* stem bark contained prenylated benzofuran-3-ones and soulatro coumarin compounds (Tanjung *et al.*, 2018; Abbas *et al.*, 2011). All important fragment ions of identified compounds (Table II.)

Evaluation of Cytotoxic Activity

The cytotoxic properties of *C. soulattri* stem bark extract and fractions were tested at concentrations ranging from 7.81 to 1000µL/mL. Cisplatin was used as the positive control. Cisplatin is one of the chemotherapy agents used as an adjuvant, neoadjuvant, or combination in breast cancer (Baek *et al.*, 2020). Treatments using *C. soulattri* stem bark extract and fraction altered MCF-7 cell morphology. Increased concentration of extract or fraction generally causes damage to MCF-7 cell morphology. MCF-7 cells started exhibiting damaged morphology at concentration 62.5 µg/mL on the treatment of methanol extract, at concentration 15.6 µg/mL on the treatment *n*-hexane extract, and 125 µg/mL on the treatment of ethyl acetate fraction of *C. soulattri* stem bark. Cisplatin's positive control seems to induce damage to the MCF-7 cells with treatment at 31.3µM (Figure 8).

The cytotoxic test result showed that *n*-hexane fraction possessed the strongest cytotoxic property than any other extract and fraction with an IC_{50} value of 35.96µg/mL. The IC_{50} values of methanol extract and ethyl acetate fraction obtained were 93.6 and 233µg/mL, respectively.

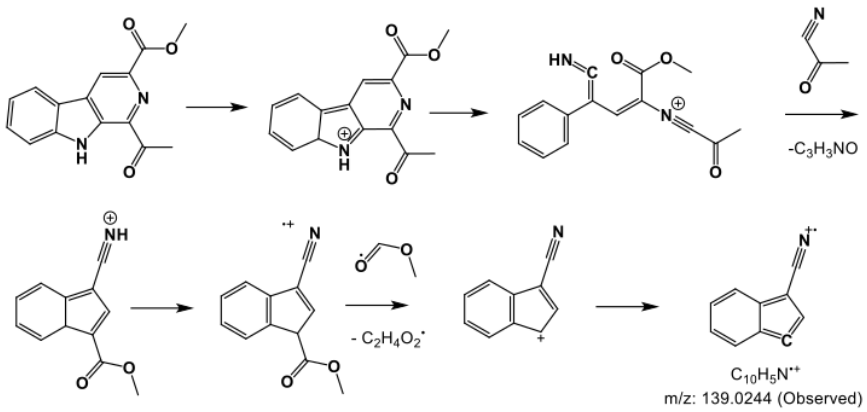

Figure 7. The proposed fragmentation pattern for 1-acetyl-3-(methoxy-carbonyl)-β-carboline

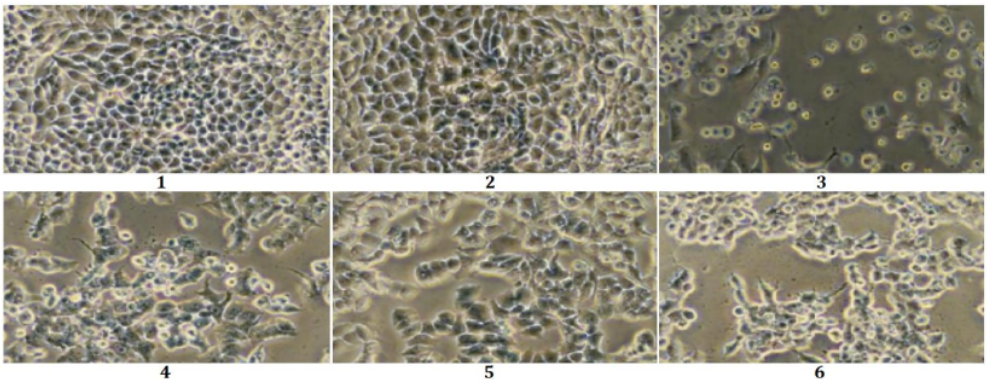

Figure 8. Morphology of MCF-7 cells after treatments with: (1) media+cells (2) DMSO (3) cisplatin 31.3µM (4) methanol extract 62.5µg/mL (5) *n*-hexane fraction 15.6µg/mL (6) ethyl acetic fraction 125µg/mL

Table II. Important MS/MS fragment ions of the identified five major compounds

Compound	MS/MS Fragmentation observed (*m/z*)
Polypurusterone A	156.0769, 208.1010, 391.2469
Polyporusterone F	293.1745
Poricoic acid D	155.070, 171.1023, 214.1183
Esculentagenin	199.1337, 529.3140
1-acetyl-3-(methoxy-carbonyl)-β-carboline	139.0244

The IC_{50} values of both extract and fraction were compared to the IC_{50} value of cisplatin as the positive control which was 53.48µM or 16.17µg/mL. Based on the IC_{50} value, methanol extract and *n*-hexane fraction have a strong cytotoxic property. An extract is classified to have a strong cytotoxic property when it has IC_{50} value <100µg/mL and moderate when it has IC_{50} value 100-1000µg/mL against cancer cells (Nurani *et al.*, 2020). The compounds expected to present

cytotoxic activity against MCF-7 cancer cell-derived from methanol extract and hexane fraction on *soulattri* stem bark are poricoic acid D and poiporusteron F. Isomer of poricoic acid D, which is poricoic acid G, is reported to have cytotoxic properties on leukemia cell HL-60 with an IC_{50} value of 39.3nM (Ukiya *et al.*, 2002). Poliporusteron F isolated from *Polyporus umbellatus* fruit was reported to have cytotoxic properties to inhibit proliferation of L-1210 leukemia cells with an IC_{50} value of 36µg/mL (Ohsawa *et al.*, 1992). Poliporusteron A compound isolated from *Polyporus umbellatus* fruit has anti-hemolytic activity (protects red blood cells from lysis because of free radicals) with an IC_{50} value of 50µg/mL (Sekiya *et al.*, 2005).

CONCLUSION

n-hexane extract showed the strongest cytotoxic property among methanol extract and ethyl acetate fraction in inhibiting MCF-7 cancer cell growth with IC_{50} 35.96 µg/mL. Poricoic acid D and poiporusteron F compound are expected to present cytotoxic properties. Further research related to the isolation of the active compound may be conducted in order to develop a new anticancer agent.

ACKNOWLEDGMENT

We would like to express our sincere thanks to LPPM UNSOED for funding support (Competition Scheme BLU Grant No.159/UN23.14/PN.01.00/2019).

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