FLAVONOIDS CONSTITUENT ANALYSIS AND CELL CYCLE INHIBITION ACTIVITY OF ETHYLACETATE EXTRACT OF *Vernonia amygdalina* Delile. LEAVES ON LUNG CANCER CELL LINE

P.A.Z. Hasibuan^{1,*}, D. Munir², D. Pertiwi³, D. Satria³ and M.F. Lubis¹

¹Departement of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, 20155, Indonesia

²Departement of Otolaryngology Head and Neck Surgery, Faculty of Medicine, Universitas Sumatera Utara, Medan, 20155, Indonesia

³Departement of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, 20155, Indonesia

*E-mail: poppyanjelisa@usu.ac.id

ABSTRACT

Lung cancer is one type of cancer with the high incidence rate in the world, and it is very important to find the best treatment therapy to reduce the problem of lung cancer. African leaves (*Vernonia amygdalina* Delile.) have long been used as medicine in the world of health. This study evaluated the anticancer activity of ethylacetate extract (EAE) of *Vernonia amygdalina* Delile. leaves towards HTB-182 cell line. EAE was analyzed for flavonoids constituent and tested for cytotoxicity, cell cycle inhibition, apoptosis induction and inhibition of PI3KCA, EGFR and VEGFR-2 genes expression. EAE was found to contain quercetin, quercetin 3-O- α -L-arabinopyranoside and isoquercetin. EAE of *Vernonia amygdalina* Delile. leaves were found to have IC₅₀ of 10.37 ± 0.16 μ g/mL, caused accumulation in S and G₂-M phase, caused apoptosis and decreased PI3KCA, EGFR and VEGFR-2 genes expression. The results reveal that EAE of *Vernonia amygdalina* Delile. leaves have cytotoxic activities by inhibition of cell cycle. Our further study is to isolate compounds responsible for the cytotoxic activity.

Keywords: Cell Cycle, Chemical Constituents, *Vernonia amygdalina* Delile., Leaves, Ethylacetate Extract.

© RASĀYAN. All rights reserved

INTRODUCTION

Lung cancer is one of the leading types of cancer that causes cancer deaths in the world, even if the mortality rates of several types of cancer are combined.¹ In 2018 lung cancer became the type of cancer with the most number of new cases, namely 2.1 million new cases with a percentage of 11.6% of all types of cancer cases that occurred. Lung cancer is responsible for around 1.8 million cases of death or about 18.4% of the total cancer deaths in the world.² The high death due to lung cancer cases is inseparable from early diagnosis of poor cancer, genetic changes in cancer cells and limited treatment therapy, such as surgery, chemotherapy and radiotherapy.^{3,4}

Vernonia amygdalina Delile. from a family of Asteraceae come from West Africa. Phytochemical constituents such as steroidal saponins, sesquiterpene lactones, flavonoids, fatty acids have indicated some pharmacological activity such as anti-malaria, anti-inflammation, anti-tumor, anti-obesity, and other activities.⁵⁻¹⁵ The purpose of this study was to evaluate of flavonoids constituent and cell cycle inhibition activity of ethylacetate extract of *Vernonia amygdalina* Del. Leaves towards HTB-182 lung cancer cells.

EXPERIMENTAL

Preparation of Fractions

The air-dried and powdered leaves of *Vernonia amygdalina* Delile. (500 g) were repeatedly fractionated by maceration method based on previous studies.¹⁶⁻¹⁹

Flavonoids Constituent Analysis with LC-MS/MS

Analysis of flavonoids from EAE was analyzed with TSQ Quantum™ Access Max Triple Quadrupole Mass Spectrometer with mobile phase A (0.1% formic acid in water and B (0.1% formic acid in acetonitrile) with gradient method and flow rate 250 µL/minute, for the column using Hypersil GOLD aQ 50 x 2.1 mm x 1.9 µm and the time for analysis was 7.5 minute. The results were analyzed with x-Calibur software.²⁰

Cytotoxicity Activity

HTB-182 cell line (1x10⁴ cells) were grown in DMEM complete medium. After 24 hours of incubation, the medium was discharged and treated by EAE. The further procedure was followed as previously describe.^{18,21,22}

Cell Cycle Inhibition Analysis

HTB-182 cell line (1x10⁶ cells) were seeded into 6-well plate and incubated for 24 hours in incubator CO₂ 5%. for the treatment, harvested and analysis of cells with flow cytometer were followed procedure from previous study.^{19,23}

Observation of Apoptosis

HTB-182 cell line (1 x 10⁵ cells/well) were seeded into 24-well plate on coverslips and incubated for 24 hours. After that, the cells were treated and incubated for 24 hours. The 10µL acridine orange-ethidium bromide was added and incubated for 15 minutes and inspected under the confocal microscope.²⁴

Expression of PI3KCA, EGFR and VEGFR-2

HTB-182 cell line (1x10⁶ cells) were seeded into 6-well plate and incubated for 24 hours. After that, the cells were treated with EAE 2.5 µg/mL and then incubated for 24 hours. Isolation RNA and cDNA synthesis procedures were adopted from the reported literature.²⁵⁻²⁶ The gene expression of PI3KCA, EGFR and VEGFR-2 were determined by RT-PCR. The oligonucleotide primers for PI3KCA, Akt-1, Akt-2 and beta-actin were shown on Table-1.

Table-1: Primers Sequences, Size Product and Annealing Temperature

Gene		Primer Sequences	Size (bp)	Temp (°C)
VEGFR-2	F	5'- GTGTCAGAATCCCTGCGAAGTA -3'	280	55.5
	R	5'- GAAATGGGATTGGTAAGGATG -3'		
EGFR	F	5'-CAACATCTCCGAAAGCCA-3'	660	58
	R	5'-CGGAACCTTGGGCGACTAT-3'		
PI3KCA	F	5'-GGACAATCGCCAATTTCAG-3'	300	53.5
	R	5'-TGGTGGTGCTTTGATCTG-3'		
β-actin	F	5'-GCTCCTCCTGAGCGCAAGT-3'	105	58
	R	5'-TCGTCATACTCCTGCTTGCTGAT-3'		

RESULTS AND DISCUSSION

Flavonoids Constituent of EAE

Flavonoids constituent analysis from EAE was determined to obtain the information of flavonoids contain in EAE with LC-MS/MS. The result was given in Fig.-1 and Table-2.

Table-2: Flavonoids Constituent of EAE

No.	Compound	Molecule Structure	Molecule Weight
1	Quercetin	C ₁₅ H ₁₀ O ₇	302.2360
2	Quercetin 3-O-α-L-arabinopyranoside	C ₂₀ H ₁₈ O ₁₁	434.3530
3	Isoquercetin	C ₂₁ H ₂₀ O ₁₂	464.0955

The results were showed that EAE contains some flavonoids such as quercetin, quercetin 3-O-α-L-arabinopyranoside and isoquercetin. The profile was predicted based on molecule weight and fragmentation pattern. Predictions were analyzed based on a percentage of similarity between the target compound and library on the computer used software.²⁰

E:\DATA RAW\...F_Usu_Afirtaa_3

RT: 0.00 - 7.50 SM: 3B

Fig.-1: Chemicals Constituent of EAE with LC-MS/MS

Inhibitory Concentration 50% (IC₅₀)

The IC₅₀ value of EAE on HTB-182 cells was 10.37 ± 0.16 $\mu\text{g/mL}$. The cytotoxicity estimate of a natural product is related to the content of active compounds in these plants including *Vernonia amygdalina* Delile. Flavonoids are estimated as active compounds.²⁷ This fact was to indicate that EAF can inhibit cell growth that S and G₂/M phase on cell cycle. The isolated polyphenols from plants including kaemferol, quercetin, anthocyanins, coumarin acid, and ellagic acid were shown to inhibit the growth (inhibit cell cycle and induce apoptosis) of human breast (MCF-7), oral (KB, Cal-27), colon (HT-29, HCT-116), and prostate (LNCaP, DU-145) tumor cell lines.²⁸⁻³¹

Analysis of Cell Cycle

The effect of EAE is given in Fig.-2. Whereas treatment of EAE 2.5 $\mu\text{g/mL}$ caused cell accumulation at S and G₂/M phase (16.19% and 19.74%) and for the control cell (13.9% and 14.78%).

Fig.-2: Percentage of Cell Cycle Phase of HTB-182 Cells. (a) Control Cell, (b) EAE 2.5 $\mu\text{g/mL}$

Apoptosis

Apoptosis can be defined as a process of programmed cell death and this process depends on the biochemical mechanism of the cell. In this study²⁴, Figure-3 showed EAE was induced apoptosis. Apoptosis HTB-182 cells were observed used a confocal microscope. HTB-182 cells were treated with EAE with a concentration 2.5 $\mu\text{g/mL}$.

PI3KCA, EGFR and VEGFR-2 Genes Expression

RT-PCR method was used to evaluated PI3KCA, EGFR and VEGFR-2 gene expression in HTB-182 cells after the treatment with EAE 2.5 $\mu\text{g/mL}$. EAE showed a significant down-regulatory effect on the expression of PI3KCA (0.54 ± 0.02); EGFR (0.62 ± 0.01), and VEGFR-2 (0.74 ± 0.02) after treatment of EAE at 2.5 $\mu\text{g/mL}$. The inhibition of EAE towards PI3KCA, EGFR and VEGFR-2 genes expression in HTB-182 are given in Fig.-4.

PI3KCA is one of kinase protein which regulates the development of cancer through PI3K/Akt/mTOR pathway and quercetin can reduce the expression of serin/threonine kinase, Akt, mTOR, MAPK, ERK1/2, JNK proteins.³² EGFR and VEGFR-2 are a transmembrane receptor that plays an important role in endothelial cell development and is thought to mediate the key effect of the endothelial-specific mitogen VEGF on cell proliferation and permeability.³³ Therefore, the majority of VEGFR-2 actions are related to angiogenesis. VEGFR-2 receptors and VEGFR-2 mRNA are largely expressed in breast cancer.³⁴

Fig.-3: Apoptosis Analysis. HTB-182 Cells. (a) Control Cells; (b) EAE 2.5 µg/mL (↓ apoptosis Cells).

Fig.-4: Gene Expression after Treatment with EAE. (a) β -actin Expression; (b) PI3KCA Expression; (c) EGFR Expression; (d) VEGFR-2 Expression

CONCLUSION

The results reveal that ethylacetate extract of *Vernonia amygdalina* Delile. leaves contain quercetine and its derivates compounds and effective as anticancer towards HTB-182 cell lines by several mechanisms such as cell cycle inhibition, apoptosis induction and decrease PI3KCA, EGFR and VEGFR-2 genes expression.

ACKNOWLEDGEMENT

This research was funding by Universitas Sumatera Utara through Non PNBUSU funding 2019 research grant No. 4167/UN.5.1.R/PPM/2019 (01 April 2019).

REFERENCES

1. C.P. Andika, N. Fariz, A. Sita, Z. Jamal, S. Elisna, *CDK-2324*, **42**, 833(2015).
2. World Health Organization, *International Agency for Research on Cancer*, pp. 1-3 (2018), DOI:10.1002/ijc.31937
3. C. Wei, W. Jun, L. Sulai, W. Shaoqiang, C. Yuanda, Z. Wolong, *Journal of Experimental and Clinical Cancer Research*, **35**, 1(2016), DOI:10.1186/s13046-016-0357-4
4. H. Jayoung, J. Paiboon, A. Olufunmilola, K. Dongwo, *International Journal of Environmental Research and Public Health*, **15**, 1(2018), DOI:10.3390/ijerph15071509
5. H. Ohigashi, M. Jisaka, T. Takagaki, H. Nozaki, *Agricultural and Biological Chemistry*, **55**, 1201(1991), DOI:10.1080/00021369.1991.10870699
6. G.O. Igile, W. Oleszek, M. Jurzysta, S. Burda, *Journal of Agricultural and Food Chemistry*, **22**, 2445(1994), DOI:10.1021/jf00047a015
7. A. Sinisi, E. Millan, S.M. Abay, A. Habluetzel. *Journal of Natural Products*, **78**, 1618(2015), DOI:10.1021/acs.jnatprod.5b00179
8. G.O. Igile, W. Oleszek, M. Jurzysta, R. Aquino, *Journal of Natural Products*, **589**, 1438(1995), DOI:10.1021/np50123a016
9. M. Jisaka, H. Ohigashi, T. Takagaki, H. Nozaki, *Tetrahedron*, **48**, 625(1992), DOI:10.1016/S0040-4020(01)88123-0

10. O. Quasie, Y.M. Zhang, H.J. Zhang, J. Luo, *Phytochemistry Letters*, **15**, 16(2016), DOI:10.1016/j.phytol.2015.11.002
11. P. Erasto, D.S. Grierson, A.J. Afolayan, *Food Chemistry*, **45**, 195(2007), DOI:10.1016/j.foodchem.2006.12.013
12. E.U. Eyong, I.J. Atangwho, E.D. Oku, M.A. Agiang, *African Journal of Biotechnology*, **10**, 10258(2011)
13. A.A. Adedapo, A.T. Otesile, K.O. Soetan, *Pharmaceutical Biology*, **45**, 564(2008), DOI:10.1080/13880200701498978
14. C.A. Egedigwe, C.E. Eijike, I.I. Ijeh, G.I. Onwuka, *African Journal of Traditional, Complementary and Alternative Medicine*, **13**, 89(2016), DOI:10.1080/13880209.2016.1216135
15. I.J. Atangwho, E.E. Edet, D.E. Uti, A.U. Obi, M.Z. Asmawi, *Saudi Journal of Biological Sciences*, **19**, 385(2012), DOI:10.1016/j.sjbs.2012.05.003
16. D. Satria, M. Furqan, S. Hadisahputra, Rosidah, *International Journal of Pharmacy and Pharmaceutical Sciences*, **7**, 73(2015).
17. R. Anggraeni, S. Hadisahputra, J. Silalahi, D. Satria, *International Journal of PharmTech Research*, **6**, 2032-2035 (2014).
18. P.A.Z. Hasibuan, C. Jessy, D. Satria. *International Journal of Pharmacy and Pharmaceutical Sciences*, **7**, 155(2015).
19. U. Harahap, P.A.Z. Hasibuan, P. Sitorus, N. Arfian, D. Satria, *Asian Pacific Journal of Cancer Prevention*, **19(2)**, 565(2018), DOI:10.22034/APJCP.2018.19.2.565
20. Y. Tine, Y. Yang, F. Renucci, J. Costa, A. Wele, J. Paolini. *Natural Product Communications*, **12(12)**, 1865(2017), DOI:10.1177/1934578X1701201213
21. Rosidah, P.A.Z. Hasibuan, G. Haro, P. Masri, D. Satria, *Asian Journal of Pharmaceutical and Clinical Research*, **11(1)**, 33(2018), DOI:10.22159/ajpcr2018.v11s1.26560
22. D. Satria, J. Silalahi, G. Haro, S. Ilyas, P.A.Z. Hasibuan, *Asian Pacific Journal of Cancer Prevention*, **18(2)**, 399(2017), DOI:10.22034/APJCP.2017.18.2.399
23. D. Satria, J. Silalahi, G. Haro, S. Ilyas, P.A.Z. Hasibuan, *Rasayan Journal of Chemistry*, **12(2)**, 803(2019), DOI:10.31788/RJC.2019.1225180
24. M.F. Lubis, P.A.Z. Hasibuan, U. Harahap, *Asian Journal of Pharmaceutical Research*, **7(4)**, 12(2019), DOI:10.22270/ajprd.v7i4.533
25. N. Auliafendry, Rosidah, Yuandani, S. Suryani, D. Satria, *Open Access Macedonian Journal of Medical Sciences*, **7(1)**, 24(2019), DOI:10.3889/oamjs.2019.017
26. A. Dalimunthe, P.A.Z. Hasibuan, D. Satria, *Open Access Macedonian Journal of Medical Sciences*, **7(9)**, 1422(2019), DOI:10.3889/oamjs.2019.317
27. A. Dalimunthe, P.A.Z. Hasibuan, D. Satria, *Asian Journal of Pharmaceutical and Clinical Research*, **10(11)**, 404(2017), DOI:10.22159/ajpcr.2017.v10i11.20204
28. A. Zurynn, L. Anna, S.M. Barbara, K.W. Anna, G. Maciej, *International Journal of Oncology*, **48**, 2521(2016), DOI:10.3892/ijo.2016.3444
29. V.R. Yadav, P. Sahdeo, S. Bokyoung, K. Ramaswamy, B.A. Bharat, *Toxins*, **2**, 2428(2010)
30. C.E. Elson, S.G. Yu, *The Journal of Nutrition*, **124**, 607(1994), DOI:10.3390/toxins2102428
31. G.J. Kelloff, C.W. Boone, J.A. Crowell, V.E. Steele, R.A. Lubet, L.A. Doody, W.F. Malone, *Journal of Cellular Biochemistry*, **26**, 1(1996), DOI:10.1002/jcb.240630703
32. H. Liao, X. Bao, J. Zhu, J. Qu, Y. Zhen, *Chemico-Biological Interactions*, **242**, 91(2015), DOI:10.1016/j.cbi.2015.09.022
33. Y. Safdari, M. Khalil, M.A. Ebrahizmadeh, Y. Yazdani, S. Farajnia, *Pharmacological Research*, **93**, 1(2015), DOI:10.1016/j.phrs.2014.12.004
34. N. Ferrara, H.P. Gerber, J. LeCounter, *Nature Medicine*, **9**, 669(2003), DOI:10.1038/nm0603-669

[RJC-5625/2020]