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

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ABSTRACT

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

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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>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>Size (bp)</th>
<th>Temp (°C)</th>
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<tbody>
<tr>
<td>VEGFR-2</td>
<td>5'-GTGTCAAAATCCCTGCGAAGTA -3’</td>
<td>280</td>
<td>55.5</td>
</tr>
<tr>
<td></td>
<td>5'-GAAATGGGATTGTAAGGATG -3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>5'-CAACATCTCCGAAAGCA-3’</td>
<td>660</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>5'-CGGAACCTTGGCGACTAT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3KCA</td>
<td>5'-GGACAATCGCAATTCAG-3’</td>
<td>300</td>
<td>53.5</td>
</tr>
<tr>
<td></td>
<td>5'-TGGTGCTTTGATCCTGCTG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-GCTCCTCTGTAGCGCAAGT-3’</td>
<td>105</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>5'-TCGTCATACTTCTGCTGTGAT-3’</td>
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</tbody>
</table>

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>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Molecule Structure</th>
<th>Molecule Weight</th>
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<tbody>
<tr>
<td>1</td>
<td>Quercetin</td>
<td>C_{15}H_{10}O_{7}</td>
<td>302.2360</td>
</tr>
<tr>
<td>2</td>
<td>Quercetin 3-O-α-L-arabinopyranoside</td>
<td>C_{20}H_{18}O_{11}</td>
<td>434.3530</td>
</tr>
<tr>
<td>3</td>
<td>Isoquercetin</td>
<td>C_{21}H_{20}O_{12}</td>
<td>464.0955</td>
</tr>
</tbody>
</table>

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
Inhibitory Concentration 50% (IC\textsubscript{50})

The IC\textsubscript{50} value of EAE on HTB-182 cells was 10.37 ± 0.16 µg/mL. The cytotoxicity estimate of a natural product is related to the content of active compounds in these plants including \textit{Vernonia amygdalina} Delile. Flavonoids are estimated as active compounds.\textsuperscript{27} This fact was to indicate that EAF can inhibit cell growth that S and G\textsubscript{2}/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.\textsuperscript{28-31}

Analysis of Cell Cycle

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

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 study, 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 ug/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 µg/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 µg/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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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**

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**REFERENCES**