PHYTOCHEMICALS ANALYSIS OF *Baccaurea motleyana* Mull. Arg. EXTRACTS AND ANTIPROLIFERATION EFFECT AGAINST PANC-1 CELL THROUGH p53 AND Bcl-2 EXPRESSIONS

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**ABSTRACT**

*Baccaurea motleyana* Mull. Arg (Baccaurea) is a tall plant whose fruit is consumed as food in Indonesia. This study aims to evaluate the antioxidant activity of the *B. motleyana* extracts and their antiproliferative effect on PANC-1 cancer cells. The ethanolic (EBM), ethyl acetate (EABM), and n-hexane (HBM) extracts of *B. motleyana* leaf were evaluated for their in vitro antiproliferative activity on pancreas cancer (PANC-1) cell lines. Mechanisms of extracts through p53 and Bcl-2 proteins expression using immunocytochemistry. Cell viability assays were performed by calculating the percentage of viable cells and used to determine the IC50 of extracts at 72 h. Spectrophotometrically measuring its antioxidant DPPH and ABTS radical scavenging activities. The phytochemicals profile of extracts was carried out using qualitative tests to identify alkaloids, flavonoids, saponins, triterpenoids/steroids, and tannins. Differences were considered as statistically significant at p<0.05 according to one way ANOVA test followed by Tukey’s test. EBM contains more phytochemicals compound than EABM and HBM. The IC50 of EBM, EABM, and HBM were 41.36±1.79 µg/mL, 62.02±2.52 µg/mL, and 124.33±6.04 µg/mL, respectively (DPPH method, p<0.05). In addition, 34.07±2.51 µg/ml, 89.33±4.21 µg/ml, and 202.53±6.81 µg/ml, respectively (ABTS method, p<0.05). The extracts can inhibit the proliferation of PANC-1 cells at 24 h and 72 h. EBM has the strongest IC50 compared to EABM and HBM was 20.75±2.13 µg/mL, 94.63±5.32 µg/mL, and 176±7.10 µg/mL, respectively (p<0.05). Furthermore, EBM could exhibit antiproliferation via modulating the p53 and Bcl-2. EBM, EABM, and HBM have antioxidant and antiproliferative activity against PANC-1 cells.

**Keywords**: *Baccaurea motleyana*, PANC-1 cell, Antioxidant, Antiproliferation, p53, Bcl-2.

**INTRODUCTION**

Pancreatic cancer affects more than 250,000 people worldwide each year. Additionally, the number of people receiving a pancreatic cancer diagnosis is rising steadily.¹ With only a 4% survival rate, it is well known that individuals with pancreatic cancer respond poorly to the majority of traditional chemotherapy treatments.² Despite the fact that several small molecule kinase inhibitors have been created to combat pancreatic cancer, their clinical effectiveness is still constrained, mostly because of their severe side effects.³ Natural remedies to sustain a healthy human life can be found in plants. The culture has been using plants in traditional medicine for a very long period.⁴,⁵ An expansive plant genus is called Baccaurea. This genus contains about 43 recognized species that have spread from India, Indonesia (Borneo, Sumatra, Java), Malaysia, Thailand, the Philippines, and Pacific Islands. One of the species of Baccaurea is *Baccaurea motleyana* Müll. Arg, also known as Rambai locally, is found in the East Kalimantan tropical rain forests.⁶–⁸

*B. motleyana* has historically been used to heal conditions of the stomach and eyes.⁹ According to reports, *B. motleyana*’s fruit peel extract has anticancer activity against the colon cancer cell line (HT-29).¹⁰ Other

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investigations have demonstrated the presence of phenolic chemicals in this plant extract.\textsuperscript{11} There are numerous phenolic compounds from plants that are bioactive and might make a viable therapeutic candidate based on their range of functions.\textsuperscript{12} Phenolic chemicals can cause apoptosis, autophagy, and cell cycle arrest with high specificity by interfering with the many checkpoints used by cancer cells.\textsuperscript{13–15} Through the expression of p53 and Bcl-2, this work intends to provide an overview of the antiproliferative activity of \textit{B. motleyana} leaf extracts against the PANC-1 cell line.

**EXPERIMENTAL**

**Sample Collection and Botanical Identification**

The \textit{B. motleyana} leaf was taken from a farm in Namorambe, Deli Serdang district, North Sumatera, Indonesia. Then, in order to prepare voucher specimens for the herbarium, these samples were sent to the Unit Herbarium at Universitas Sumatera Utara. The final step involves a top taxonomist from Universitas Sumatera Utara identifying plants and herbarium specimens. The entire herbarium was placed along with the voucher specimen and a reference number (number 34012012022).

**Extracts Preparation of \textit{B. motleyana} Leaf**

To ensure thorough extraction, freshly washed \textit{B. motleyana} leaves were cut into small pieces, steeped in ethanol as the solvent, filtered, and continually soaked three times. The three separate soaks took place over the course of 72 hours, with each soak lasting 24 hours. This process was done with n-hexane and ethyl acetate as the additional solvents, respectively. In a vacuum rotary evaporator set at 40°C, each filtrate was collected and concentrated until only about 1/10 of the original volume remained. The EBM, EABM, and HBM were then obtained by freezing the remaining filtrates. Then, until further usage, the crude extracts were maintained in glass bottles with screw caps and chilled at 4°C.\textsuperscript{16}

**Phytochemicals Screening of Extracts**

A phytochemical assay, based on established procedure, was conducted to explore the secondary metabolites, such as alkaloids, flavonoids, saponins, triterpenoids/steroids, and tannins.\textsuperscript{17}

**DPPH Radical Scavenging Activity**

Using the previously published approach, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to determine the extract’s in vitro free radical scavenging activity. 24 mg of DPPH was dissolved in 100 ml of methanol to create the stock solution, which was then kept at 20°C until needed. 100 µl of the samples were combined with a 3 ml aliquot of DPPH solution at different concentrations (10-250 µg/ml). After thoroughly shaking, the reaction mixture was allowed to sit at room temperature for 15 minutes in the dark. Next, the absorbance at 517 nm was measured. As stated previously, the control was created without any sample.\textsuperscript{18}

**ABTS Radical Scavenging Activity**

The cation scavenging activity of 2,20-azinobis (3-ethylbenzthiazoline-6-sulphonic acid), also known as ABTS, was carried out. Briefly, a dark-colored solution containing ABTS radical cations was produced by reacting ABTS solution (7 mM) with potassium persulfate (2.45 mM) solution. The ABTS radical cation was diluted with 50% methanol before being used in the test, resulting in an initial absorbance of about 0.70±0.02 at 745 nm and a temperature control of 30°C. By combining 300 µl of test samples (10-250 µg/ml) with 3.0 ml of the ABTS working standard in a microcuvette, the free radical scavenging activity was evaluated. After thoroughly mixing the solution, the absorbance drop was timed for exactly one minute and up to six minutes.\textsuperscript{19}

**Cell Proliferation Assay**

A 96-well plate with 100 µl of finished growth media and 5x10\textsuperscript{3} PANC-1 cells per well of plating density was used to seed the cells. Before adding extracts, these cells were incubated for 24 hours at 37°C, 5% CO\textsubscript{2}, 95% air, and 100% relative humidity. The extract-treated cells were cultured for anywhere from 24 to 72 hours. Gradually throughout the incubation period, the MTT assay was carried out. The treated cells were then incubated for 30 min at room temperature after being fixed with 50% cold trichloroacetic acid. The plates were then dried and five times washed with distilled water before the cells were stained for 30 minutes at room temperature with 100 µl of 0.4% MTT in 1% acetic acid. The cells were repeatedly rinsed four times.
times with 1% acetic acid. Upon drying, 100 µL of 10 mM Tris buffer was added and the plates were shaken for 5 min. Finally, a microplate reader was used to read the absorbance at a wavelength of 540 nm (BMG LABTECH, Germany). By comparing it to the absorbance value recorded for the control sample, the percentage of absorbance representing the cell growth activity was calculated. The following formula was used to calculate the cell proliferation: (absorbance of sample/absorbance of control) × 100%. To establish the concentration of an inhibitor at which the response is reduced in half, the in vitro study required the determination of the IC\textsubscript{50} value. At the inhibitory concentration, it resulted in the suppression of 50% of cell proliferation.\textsuperscript{20,21}

**Immunocytochemistry**

PANC-1 cells (5x10\textsuperscript{4} cells/well) were placed on coverslips in a 24-well plate and incubated for 24 hours. After that, extracts were applied to the cells, and they were then incubated for 24 hours. After incubation, the cells were washed in PBS before being fixed in cold methanol for 10 minutes. The cells were then washed three times with PBS, blocked in hydrogen peroxide blocking solution for 10 minutes at room temperature, and incubated for 1 hour with primary antibodies against Bcl-2 and p53. Finally, PBS was used to wash the cells before they were incubated for 10 minutes with secondary antibodies. Following a PBS rinse, the cells were incubated in a 3,3-diaminobenzidine (DAB) solution for 10 minutes before being washed with distilled water. After that, the coverslips were taken off and rinsed in distilled water before being dipped into xylol and 70% ethanol. The cells were then counterstained for 5 minutes with Mayer Haematoxylin. Protein expression was viewed using a light microscope (Nikon YS100). The blue color is produced by cells that do not express a particular protein, whereas the brown color is produced by cells that do.\textsuperscript{22–24}

**Statistical Analysis**

Data are expressed as mean ± SD from three separate observations. For antioxidant and antiproliferative activity, one way ANOVA test followed by Tukey’s test (p < 0.05) was used to analyze the differences among IC\textsubscript{50} of various extracts for different antioxidant and antiproliferative activity.

**RESULTS AND DISCUSSION**

**Phytochemicals Screening**

After extraction using the maceration method, the yields from the EBM, EABM, and HBM were 25.73%, 10.11%, and 3.46%, respectively. In comparison to EABM and HBM, EBM had higher secondary metabolite chemicals, according to the phytochemical analysis of extracts. Alkaloid, flavonoid, saponin, triterpenoid/steroid, and tannin were all present in the EBM. Although flavonoid, saponin, triterpenoid/steroid, and tannin were discovered in EABM, alkaloid has not been recognized there. HBM, however, solely contains triterpenoids and steroids (Table-1).

**Table-1: Phytochemical Compounds of B. motleyana Extracts**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dried powder</th>
<th>EBM</th>
<th>EABM</th>
<th>HBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Saponin</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Triterpenoid/steroid</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Tannin</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = detected, - = not detected. EBM (ethanolic extract of B. motleyana leaf), EABM (ethyl acetate extract of B. motleyana leaf), HBM (n-Hexane extract of B. motleyana leaf)

**Antioxidant Activity**

The results indicated that EBM, EABM, and HBM, in that sequence, had scavenging effects on the DPPH radical (Fig.-1). For the EBM, EABM, and HBM, the IC\textsubscript{50} values for scavenging DPPH radicals were 41.36 ± 1.79 µg/mL, 62.02 ± 2.52 µg/mL, and 124.33 ± 6.04 µg/mL, respectively (Table 2). Even though ascorbic acid antioxidant capacity was shown to be higher (p<0.05), the study demonstrated that EBM and EABM exhibit significant antioxidant activity.
ABTS radical was scavenged by all *B. motleyana* preparations in a concentration-dependent manner (10–250 µg/ml) (Fig.-1). The current results demonstrated that the ability of samples to scavenge ABTS radicals can be graded as EBM > EABM > HBM. The IC₅₀ values for EBM and EABM were 34.07 ± 2.51 and 89.33 ± 4.21 µg/ml, respectively, whereas the value for HBM was 202.53 ± 6.81 µg/ml (Table-2). Although it was discovered that extracts have lower (p<0.05) antioxidant capacity than ascorbic acid. Since DPPH and ABTS radical scavenging tests have been widely used to evaluate the antioxidant properties of various extracts, they were used to create a clear profile of the antioxidant capacity of *B. motleyana* preparations. A stable free radical called DPPH is transformed into colorless DPPH when it encounters antioxidants. The ability of each extract to scavenge DPPH improved with concentration, as seen in Fig.-1. Peak DPPH scavenging activity was seen for all four extracts at the highest dose tested (250 mg/ml) (Fig.-1). Antioxidants can transform ABTS into colorless ABTS. All extracts showed increasing ABTS scavenging activity in a concentration-dependent manner, mirroring the findings of the DPPH assay.

Table-2: IC₅₀ (µg/mL) of *B. motleyana* Extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPPH*</td>
</tr>
<tr>
<td>EBM</td>
<td>41.36 ± 1.79a</td>
</tr>
<tr>
<td>EABM</td>
<td>62.02 ± 2.52b</td>
</tr>
<tr>
<td>HBM</td>
<td>124.33 ± 6.04c</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>6.61 ± 0.18</td>
</tr>
</tbody>
</table>

Antioxidant and antiproliferative activity of *B. motleyana* extracts. Each value represents a mean±SD (n=3). EBM (Ethanolic extract of *B. motleyana* leaf), EABM (Ethyl acetate extract of *B. motleyana* leaf), HBM (n-Hexane extract of *B. motleyana* leaf). *The IC₅₀ of samples was significantly different for DPPH, ABTS, and Cytotoxic (p<0.05), †IC₅₀ of samples was significantly different with ascorbic acid (p<0.05), ‡IC₅₀ of samples was significantly different with ascorbic acid (p<0.05), §IC₅₀ of samples was significantly different with DPPH, ABTS, and Cytotoxic (p<0.05), †The IC₅₀ of samples was significantly different with ascorbic acid (p<0.05), ‡IC₅₀ of samples was significantly different with ABTS (p<0.05), †IC₅₀ of samples was significantly different with HBM (p<0.05), ‡IC₅₀ of samples was significantly different with HBM (p<0.05)

*B. motleyana* extracts exhibit novel antioxidant action. According to studies like our own, other Baccaurea species' antioxidant capacities can be compared to those of *B. motleyana*. Using the DPPH method, the IC₅₀ of the leaf and bark of *B. racemosa* has been recorded. These values, which are 4.298 ± 0.306 µg/ml and 10.627 ± 0.996 µg/ml, respectively, are highly positive. According to a different investigation, the optimum IC₅₀ values for *B. racemosa* leaf extract were found with ethanol and ethyl acetate solvents and were 2.42 ± 0.08 µg/ml and 3.24 ± 0.01 µg/ml, respectively. Additionally, the DPPH technique was used...
to report the antioxidant activity of *B. macrocarpa* extracts. Compared to ethyl acetate and n-hexane extracts, methanolic extract has the highest antioxidant activity.\textsuperscript{33,34} Of course, the extraction conditions, solvents, and assay techniques have a significant impact on its antioxidant activity.\textsuperscript{35,36}

**B. motleyana** Extracts Inhibit the Growth of PANC-1 cells

The anti-proliferation effects of *B. motleyana* extracts were conducted in PANC-1 cells using MTT assay for 24 h and 72 h. After treatment with EBM, EABM, and HBM the % viability cell was a significantly different decrease (p<0.05) (Fig.-2). The incubation time of EBM, EABM, and HBM also gave impacted to % viability of PANC-1 cells.\textsuperscript{37} Based on the result, the % viability of PANC-1 cells is lower at 72 h than at 24 h (p<0.05). The IC\textsubscript{50} of EBM, EABM, and HBM have been conducted at 72 h were 20.75 ± 2.13 μg/mL, 94.63 ± 5.32 μg/mL, and 176 ± 7.10 μg/mL, respectively (Table-2). The EBM is the strongest extract against PANC-1 cells compared to EABM and HBM (p<0.05).\textsuperscript{38} The n-hexane and dichloromethane extracts of *B. motleyana* fruits and peel showed significant activity on human colon cancer cell lines (HT-29) with IC\textsubscript{50} values ranging from 43.6 ± 0.3 to 82.4 ± 2.4 μg/ml.\textsuperscript{10} Until now, there have been no additional reports regarding the cytotoxic activity of *B. motleyana*. PANC-1 cells were treated with the EBM, EABM, and HBM. Immunocytochemistry was performed to examine the expressions of p53 and Bcl-2 proteins that participate in cell proliferation (Fig.-3). When treated with all the extracts, the expression of p53 increased slightly in PANC-1 cells (p<0.05). As well as the expression of Bcl-2 was slightly decreased when treated with all extracts in PANC-1 (p<0.05) (Fig.-3).

The growth and spread of cancer depend heavily on proliferation. This is demonstrated by altered cell cycle-related protein expression and/or activity.\textsuperscript{39,40} Cell development is also induced by the constitutive activation of several signal transduction pathways.\textsuperscript{41} In this study, determine p53 and Bcl-2 expression using immunohistochemistry in PANC-1 cells. Genetic alterations in cancer cause p53 wild concentrations to decline, making wild p53 protein one of the prognostic indicators of the occurrence of malignancy. While the Bcl-2 protein will grow and influence p53’s biological functions.\textsuperscript{42–44} This study reported that p53 and Bcl-2 expressions were significantly different from the control group after being treated with EBM and

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**Fig.-2:** Effect of *B. motleyana* Extracts on the Viability of PANC-1 cells, Each Value Represents a Mean ± SD (n=3, *p<0.05). (A) EBM (Ethanolic Extract of *B. motleyana* Leaf), EABM (Ethyl Acetate Extract of *B. motleyana* Leaf), HBM (n-Hexane Extract of *B. motleyana* Leaf)
EABM (p<0.05) (Fig.-3). The effect of this protein expression has an impact on % PANC-1 cell viability (Figure 2). This has also been reported where 3-deoxyanthocyanidins inhibit MCF-7 cell proliferation by stimulation of the p53 gene and downregulation of the Bcl-2 gene.\textsuperscript{45} The same thing also shows that \textit{Lycium barbarum} and \textit{Lycium ruthenicum} showed stronger antiproliferation activity by activating p53 signaling pathways and downregulating the Bcl-2 level.\textsuperscript{46}

![Fig.-3: Expression Protein of p53 and Bcl-2 After Treated with \textit{B. motleyana} extracts compared to the control cell, Each Data Represents a Mean ± SD (n=3, p<0.05). (A-1) p53 Expression After Treatment with Ethanolic extract of \textit{B. motleyana} leaf, (A-2) p53 expression after treatment with ethyl acetate extract of \textit{B. motleyana} leaf, (A-3) p53 Expression After Treatment with n-Hexane Extract of \textit{B. motleyana} Leaf, (A-4) p53 Expression of Control Cell. (B-1) Bcl-2 Expression After Treatment with Ethanolic Extract of \textit{B. motleyana} leaf, (A-2) Bcl-2 Expression After Treatment with Ethyl Acetate Extract of \textit{B. motleyana} Leaf, (A-3) Bcl-2 Expression After Treatment with n-Hexane Extract of \textit{B. motleyana} leaf, (A-4) Bcl-2 Expression of Control Cell *Significantly Different Between Extracts with Control (p<0.05)](image)

Extracts' antioxidant and antiproliferation efficacy has been assessed using a variety of in vitro methods. Extracts' effects are influenced by their secondary metabolites.\textsuperscript{47} According to this inquiry, EBM is an extract that contains a variety of secondary metabolites, including tannin, alkaloid, flavonoid, saponin, and triterpenoid/steroid (Table-1). While only triterpenoid/steroid has been identified in HBM, alkaloid has not been found in EABM. These secondary metabolite profiles provide an explanation for the extracts' action.\textsuperscript{48}

**CONCLUSION**

In this study, \textit{B. motleyana} extracts' phytochemical profiles, antioxidant activity, and antiproliferative mechanism were all assessed for the first time. Overall, EBM exhibited higher antioxidant capacity measured by DPPH and ABTS compared with EABM and HBM because of the significantly higher phytochemical compounds in EBM. In addition, EBM showed stronger antiproliferative activity towards PANC-1 cells with lower IC\textsubscript{50} at 72 h. Furthermore, EBM could exhibit antiproliferation via modulating the p53 and Bcl-2. Finally, the search for other mechanisms for EBM as an anticancer need to be continued.
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CONFLICT OF INTERESTS
The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS
All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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