Investigation of the bioactivities of extracts from *Vernonia Amygdalina* Del

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Abstract. The antibacterial, antioxidant activity, and anticancer of the extract from Vernonia Amygdalina Del leaves, was assessed towards 6 selected bacteria, in the DPPH test, as well as on hepatic (HepG2), blood (K562) cancer cell lines. The DPPH assay revealed that the highest DPPH radical scavenging activity with the warm ethanol extract (IC₅₀ = 259.03 ± 5.42 µg/mL). And the ethyl acetate extract showed a high antibacterial effect expressed as minimum inhibitory concentration (MIC = 100 mg/mL) against Gram-positive bacteria. At the concentration of 250 mg/mL, this extract also demonstrated its ability to resist blood cancer cells K562 (with a survival rate of 25%) and liver cancer cells HepG2 (with a survival rate of 0%).

Keywords: Vernonia Amygdalina Del, DPPH, antibacterial, anticancer

1. Introduction

*Vernonia Amygdalina* is shrub, distributed mainly in Africa and Asia [1–3]. In Vietnam, it is usually called as name "bitter leaf plant" because of its bitter taste. The bitter taste is constituted by the presence of saponins, alkaloids, tannins, and glycosides. In many households, *Vernonia Amygdalina* is grown as a spiced vegetable. Besides, the bitter leaf has many uses in folk medicine. Previous studies have shown that *Vernonia Amygdalina* was commonly used in much traditional medicine to treat liver diseases, malaria, cough, high blood pressure... This plant is present in malaria treatment regimens in some parts of Africa [4]. In Ghana, people use *Vernonia Amygdalina* leaves to treat diabetes, fever, constipation, high blood pressure, and as a laxative [4].

Biocompatibility studies of bear bile showed the presence of saponins, flavonoids, alkaloids, terpenes, steroids, coumarins, phenolic acids, lignans, xanthones, anthraquinones, and sesquiterpenes [1–3, 5–6]. These compounds are related to the useful properties of *Vernonia Amygdalina* such as antibacterial, antioxidant, anti-cancer, and some other biological activities. Therefore, in recent years, methods for the extraction of these compounds have attracted the attention of many research groups. In particular, saponins and phenolics are emerging groups of substances with a wide range of biological applications.

And in this study, we present a method for extracting bitter leaf extracts, quantification of saponins, and phenolics. Then, evaluate the antioxidant, antibacterial, as well as anti-cancer ability of this extract.
2. **Experiment**

2.1. **Experiment apparatus**

All experiments were performed in a biochemistry laboratory (Faculty of Chemical Engineering - Ho Chi Minh City University of Technology). The solvents ethanol, ethyl acetate, methanol, gallic acid, H$_2$SO$_4$, diosgenin, gentamicin, and trypan blue were purchased Merk Chemical Co., Inc. Whatman filter paper (110 nm) Merk Chemical Co., Inc) was used to filter the plant extract. This extract was concentrated using a 110-DAVS rotary evaporator. Quantitative analysis of phenolics using Folin-Ciocalteu reagent purchased from the Merk Chemical Co., Inc. The process of culturing and experimenting on microorganisms was carried out with the following equipment: horizontal shaker IKA®HS260 basis, drying oven MOV-212F, incubator Memmert, OD Chromtech CT-2200 Spectrophotometer.

2.2. **Materials and microbial strains**

*Vernonia Amygdalina* was collected at a garden house in Trang Bom district, Dong Nai province, Vietnam. Next, *Vernonia Amygdalina* leaves are dried at 50°C to reduce moisture, then finely grounded to obtain powder form and stored at 0 - 4°C. *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Methicillin-resistant Staphylococcus aureus* (MRSA) ATCC 43300, *Salmonella typhimurium* ATCC 14028.

2.3. **Experimental methods**

**Extraction method.**

To a solution of 250 mL solvent (such as methanol 80%, ethanol 70%, or ethyl acetate) was added 50 g *Vernonia Amygdalina* leaf powder. The mixture was stirred for 24 h at room temperature (80°C in the case of solvent ethanol). The mixture was then extracted three times with respective solvent (20 mL × 3), dried over Na$_2$SO$_4$, and filtered. After evaporation of the solvent, the residue was obtained and stored at 4°C.

**Total phenolics content determination.**

The total phenolic content of the extract was determined using the Folin–Ciocalteu method [7]. The phenolics standard curve was performed by using gallic acid at different concentrations of 10, 20, 40, 60, 80, and 100 μg/mL. Base on the linear equation of this calibration curve, the total phenolics value of the extract was determined.

**Total saponins content determination.**

To 0.25 mL solution of vanillin 8% and H$_2$SO$_4$ 72% has dropped the sample (0.25 mL). The mixture was stirred and incubated in a thermostatic bath at 60°C for 10 min. This mixture was then cooled in iced water for 3-4 min. After the cooling process was completed, the optical absorbance was monitored was measured at wavelength 544 nm.

The standard curve was performed by using diosgenin at different concentrations of 100, 200, 300, 400, 500 μg/mL. According to the linear equation of this calibration curve, the total saponins value of the extract was determined.

**Investigation of antioxidant capacity by DPPH free radical scavenging method.**

In each test tube, 1mL extract sample/or blank sample was mixed to 1mL methanolic DPPH solution (0.2mM). This mixture then was stirred and incubated in dark for 30 min. After incubation finished,
the optical absorbance was monitored was measured at wavelength 517 nm. The DPPH free radical scavenging capacity was determined by the following formula:

\[ S\% = \frac{A_c - A_s}{A_c} \times 100\% \]

where \(A_c\) is the absorbance of the blank sample and \(A_s\) is the absorbance of the extracted sample. Determine the IC50 value - the ability to inhibit 50% of free radical formation in the reaction mixture. Vitamin C at different concentrations (2, 4, 6, 8, 10 µg/mL) was used as a control sample.

**Investigation of antibacterial capacity by agar well diffusion method.**

Bacterial strains were isolated on TSA, incubated for 16-20 hours at 37°C. Then, spread the bacteria evenly over the surface of the petri dish containing the MHA. The wells were formed using a punched tube, diameter = 8mm.

The test sample was produced by mixing the extraction sample solution and DMSO in a ratio of 5:1 respectively.

Add 50 µL of the test sample to the wells, the negative control is the well containing DMSO, the positive control is Gentamicin (500 µg/mL). The Petri dishes were then incubated for 16-20 h at 37°C. After incubation was completed, measured the size of the antibacterial rings achieved was.

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of extract, which can inhibit the visible microbial growth.

**Investigation of anticancer (mitotic resistance)**

Proliferation K562 cells and preserved them in DMSO solvent. This solution was centrifuged to remove DMSO. The then collected cell biomass was inoculated in the RPMI medium of the petri dish containing the 24-well plates. Incubated in a 37°C incubator for 24 hours to stabilize cells. Add 1 µL of the test sample (250 mg/mL EA extract) to the wells, the negative control is the well containing DMSO. The control sample is consisting no cell fluid or extraction solvent. The Petri dishes were then incubated for 48 h at 37°C. After incubation was completed, stained cells with trypan blue, and counted by 16-cell counting chamber under the microscope. Draw the chart and used statistical software Graph Pad Prisme to analyze the data.

**Statistical analysis**

All the experiments were preceded thrice and the average results were collected. The statistical analyses were performed using one-way ANOVA and the results were expressed as mean ± SD.

### 3. Results and discussion

#### 3.1. Extraction yields

In the extraction process, the experimental condition was tested for the solvent system. The results show extract was obtained in moderate yields (5 % - 23 %) for most of the solvents: methanol 80 %, ethanol 70 % (warm), ethanol 70 % (cold), and ethyl acetate. Methanol (80 %) was found as the best solvent for this experiment, with a yield up to 23 %.

**Total phenolic and total saponin content**

To get more details of the extraction, the total phenolics (TP) and saponins contents also were investigated. The results are shown in Table 1, the protic solvents such as methanol, ethanol gave a low yield of total phenolics (14.79 ± 0.53; 18.85 ± 0.68; 22.45 ± 2.35 mg/mL). Compare to alcoholic solvents, EA proceeds the extraction process to occur higher concentration of TP (25.2 ± 2.62

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\[ S\% = \frac{A_c - A_s}{A_c} \times 100\% \]

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mg/mL). Due to the low polarity, the non-polar electrostatic interactions of phenolics to solvent EA may be formed and increase the solubility. So, EA is the best solvent to isolate the TP.

### Table 1. Total phenolics and total saponins in *Vernonia Amygdalina* Del extract.

| Solvents            | Total phenolic content (mg GAE/g extract) | Total saponin (mg/g extract) |
|---------------------|------------------------------------------|------------------------------|
| MeOH 80%            | 14.79 ± 0.53a                            | 791.60 ± 58.92a              |
| EtOH 70 % (hot)     | 18.85 ± 0.68b                            | 736.93 ± 68.62a              |
| EtOH 70 % (cold)    | 22.45 ± 2.35a                            | 794.27 ± 83.27b              |
| EA                  | 25.2 ± 2.62a                             | 674.26 ± 42.77b              |

As the results are shown in Table 1, the saponins were obtained in high concentrations for all of the testing organic solvents (674.26 - 794.27). This result demonstrated the high content saponins related to bitter taste in the *Vernonia Amygdalina* Del plant, consistent with previous studies. And the cold ethanol extract sample accommodated the highest saponin content (794.27 ± 83.27 mg/g extract).

### 3.2. The antioxidant capacity

As shown in Figure 1, the DPPH radical scavenging activity of samples is directly proportional to the extract concentration. And the warm ethanol extract was found as the strongest antioxidant result.

![Figure 1. The DPPH radical scavenging activity of extract samples.](image1)

![Figure 2. IC50 values of the extract samples](image2)

In addition, Figure 2 performed the IC50 values of extracts in different solvents methanol, hot ethanol, cold ethanol, EA (290.93, 259.03, 317.46, and 424.16 µg/mL respectively). The IC50 value = 259.03 µg/mL of the warm ethanol extract, is the highest antioxidant capacity which is equal to 1/30 of the antioxidant value of vitamin C. In the previous report, Venskutonis (2013) functional groups hydroxyl in phenolic compounds are the proton source to reduce the free radical [9]. However, this goes against the results of our research. In our study, the hot ethanol extract is the highest DPPH free radical scavenging capacity, and the EA extract obtained the highest TP. It is possibly related to the extract chemical constituents and the quantity of the major single compounds, which depended on the type of solvents in the extraction process.

### 3.3. The antibacterial capacity

### Table 2. Antibacterial spectrum of extract samples

| Extract samples’ diameters of the antibacterial spectrum (mm) | *E. faecalis* | *S. aureus* | *E. coli* | *P. aeruginosa* | MRSA | *S. typhimurium* |
|---------------------------------------------------------------|---------------|-------------|-----------|-----------------|------|-----------------|
| Methanol 80%                                                  | 0             | 10±1.52     | 0         | 0               | 12±0.53 | 0               |
| Ethanol 70% (hot)                                             | 0             | 12±1        | 0         | 0               | 13±1  | 0               |
Ethanol 70% (cold) 0 13±1.52 0 0 16±0.58 0
Ethyl acetate 21±1 24±1.73 11±0.58 11±0.58 24±0.52 11±1
Gentamicin 26±1.52 27±0.58 27±1.52 37±1 27±1 28±1.52

Methanol, ethanol expresses slightly antibacterial capacity on 2 bacteria strains *S. aureus*, *MRSA*, and no effect on *E. faecalis*, *E. coli*, *P. aeruginosa*, *S. typhimurium* (antibacterial spectrum: 10-16mm). While the EA extract sample affected all test bacteria (antibacterial spectrum: 11-24 mm). It pressed stronger antibacterial activity against Gram-positive bacteria (*E. faecalis; S. aureus; MRSA*) than against Gram-negative bacteria. Thus, the EA extract was chosen as the best antibacterial extract. 

Momod Johnson [10], reported that bitter leaf extract has good resistance against Gram-positive bacteria. Thus, the *Vernonia Amygdalina* Del extract showed impressive antibacterial activity against Gram-positive bacterial strains. However, it is not effective against Gram-negative bacteria.

### Table 3. MIC50 values of the EA extract.

| Concentrations | *E. faecalis* | *S. aureus* | *E. coli* | *P. aeruginosa* | *MRSA* | *S. typhimurium* |
|----------------|--------------|-------------|-----------|-----------------|--------|-----------------|
| L1 (200 mg/mL) | -            | -           | +         | +               | -      | +               |
| L2 (100 mg/mL) | -            | -           | +         | +               | -      | +               |
| L3 (50 mg/mL)  | +            | +           | +         | +               | +      | +               |
| L4 (25 mg/mL)  | +            | +           | +         | +               | +      | +               |
| L5 (12.5 mg/mL)| +            | +           | +         | +               | +      | +               |
| C              | +            | +           | +         | +               | +      | +               |
| DMSO           | +            | +           | +         | +               | +      | +               |

+: Bacterial colonies appear
_: No bacterial colonies appear
C: MHA
DMSO: MHA + DMSO

According to the results of EA extract's antibacterial spectrum, we screened the minimum inhibitory concentration (MIC) value on bacterial strains. The results in Table 3 show that the EA extract can inhibit the growth of *E. faecalis; S. aureus* and *MRSA*, with MIC = 100 mg/mL. This result is consistent with the data in Table 2, the antibacterial spectrum of the EA extract is superior to other extracts. However, this extract did not yield MIC results for *E. coli; P. aeruginosa*, and *S. typhimurium*. This can be explained because the test MIC concentration is not high enough that the sample does not capture the MIC of these bacteria.

The EA extraction gave the best antibacterial results which are related to high total phenol content. Besides, the EA extract also contains other substances such as vernodal in and vernolide, which are reported to be active against Gram-positive bacteria [7].

### 3.4. The anticancer capacity

At the outset of this investigation, blood cancer cells K562 and liver cancer cells HepG2 hepatocellular carcinoma were selected as starting material to determine the EA extract's anticancer ability (Table 4).

### Table 4. The survival rate of cancer cells treated by EA extract (250mg/mL)

| Cells                 | The percentage of viable cells (%) |
|-----------------------|-----------------------------------|
| Blood cancer cells K562 | 25                                |
| Liver cancer cells HepG2 | 0                                 |

Extensive studies on the experiment indicated that using EA extract 250 mg/mL, blood cancer cells K562 with a survival rate of 25 %. While in similar conditions, the HepG2 cells couldn’t survive in...
the concentration of 250 mg/mL of extract. This data means the ability to resist cancer cells is high, especially against HepG2 hepatocellular carcinoma cells. According to this result, *Vernonia Amygdalina* Del extract needs to be studied more carefully and in detail to bring effective applications in the synthesis of drugs against liver cancer.

4. **Conclusion**

The mechanism of antibacterial activity, antioxidant, anti-cancer is dependent on the constituents of extract. And the components are related to the types of extraction solvents. In our research, we found that *Vernonia Amygdalina* Del extract contains high total phenolics (25.2 ± 2.62 g/mL), saponins content (794.27 ± 83.27 mg/g extract), which joined in the free radical scavenging activities (IC50 = 259.03 µg/mL), MICs for *Enterococcus faecalis; Staphylococcus aureus* and *MRSA* were 100 mg/mL. And the survival ratios of K562 and HepG2 were 25 % and 0 % respectively (extract concentration = 250 mg/mL). It suggests that *Vernonia Amygdalina* Del extract may be considered for further development as a safe and effective anti-cancer product.

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