Phytochemical, antioxidant and antibacterial activities of two kinds of Sabah Zingiberaceae

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Abstract. Free radical is a byproducts of biochemical processes that happen in human body. In order to overcome this free radicals damage, antioxidant functions as a molecule that would interact safely to free radicals and terminate the current reaction before further damage is happening. Two choices of antioxidants are available which are natural and synthetic but natural are more preferable because synthetic might cause toxicity and higher cost. The common natural source of antioxidants are medicinal plants and herbs. Due to this, two medicinal plants, \textit{Alpinia galanga} and \textit{Kaempferia galanga}, from Zingiberaceae family is chosen to study the phytochemical constituents and antioxidant and antibacterial activities of different crude extracts; hexane, ethyl acetate and methanol, from dried rhizomes. These two species of \textit{Zingiberaceae} were collected specifically in Beaufort, Sabah. The crude extracts were obtained by maceration process by using low polarity to high polarity solvent. Phytochemical studies were done. Antioxidant and antimicrobial activities were determined by using DPPH assay and Agar Disc-Diffusion assay, respectively. Results for phytochemical screening for both plants shows that methanol extracts has the following phytochemical properties; saponins, phenols, flavonoids, tannins, steroid and terpenoid. All of the crude extract tested showed that absorbance increase in accordance with the increasing of sample concentration. Both of the plant shows the antioxidant activity as follow order of crude extract: methanol > ethyl acetate > hexane. Four bacteria strain has been tested for antibacterial activity for both plants which are \textit{S. aureus}, \textit{B. Cereus}, \textit{S. thyphimurium} and \textit{V. Cholerae}. For both plant, the non-polar extracts (hexane) exhibits greater antibacterial activity than that of polar extracts (ethyl acetate). However, there was no antibacterial activity observed in methanol extract for both plant. Overall, \textit{A. galanga} and \textit{K. galanga} could serve as potential sources of antibacterial and antioxidant agents

1. Introduction

Various biochemical processes in human body may produce by-products like free radicals and reactive oxygen species (ROS). The free radicals cause oxidative stress to surrounding living cells. Oxidative stress is among the major causative factors in inducing many chronic and degenerative diseases including atherosclerosis, diabetes mellitus, neurodegenerative diseases, ischemic heart disease, ageing, cancer, immunosuppression and others [39].Antioxidants are those substances that have the capability to break free radical chain reactions. Antioxidants can be grouped into two; natural and synthetic.

Currently, there is a growing interest toward natural antioxidants, especially of plant origin [39]. Plants are potential sources of natural bioactive compounds such as secondary metabolites and antioxidants [42] especially medicinal plants and herbs. Natural antioxidants from medicinal plants and vegetables strongly have supported the idea that antioxidant activity from plant constituents protects against oxidative stress in biological systems [6,7,23,30]. Such biological and pharmacological activities are due to phytochemicals bioactive compounds from medicinal plants [8,26,27,35].

Considering the above, \textit{Alpinia galanga} (\textit{A. galanga}) and \textit{Kaempferia galanga} (\textit{K. galanga}), species belonging to the \textit{Zingiberaceae} family, are chosen in this study. \textit{Zingiberaceae} family is widely distributed in the Southeast Asia region and the plants are characterised based on tuberous or non-tuberous rhizomes and has strong aromatic and medicinal properties [8]. Both the plant,\textit{A. galanga} and
K. galanga, has medicinal properties [3]. Previous studies have proven that both, A. galanga and K. galanga, extracts have also been found to exhibit pharmacological activities like anti-inflammatory and analgesic, antimicrobial, antioxidant and anticancer [9,37].

This study focuses on preliminary phytochemical screening, antioxidant and antibacterial properties of plant of A. galanga and K. galanga using different crude extracts. In the study of antioxidants, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) test was conducted to obtain the scavenging activity percentage of free radicals.

2. Materials and Methods

2.1. Materials
The samples of Alpinia galanga (A. galanga) and Kaempferia galanga (K. galanga) were bought from a local market located in Kota Kinabalu town of Sabah in September 2015. The sample being used is the rhizome of both plants.

2.2. Extraction
Extraction was performed using maceration method at room temperature. Both the plants were macerated with varying polarities such as hexane, ethyl acetate and methanol. Samples were cleaned and air dried in the shade until no moisture left. Then, the dried samples were grounded into a moderately coarse powder using a heavy duty blender. The dried samples were weighed using an electronic balance after being blended to a powder form. Then, the powdered form of samples was kept in a refrigerator at 4°C for further use. As for the extraction, the maceration process was performed using solvents from low polarity to high polarity; Hexane, Ethyl acetate and Methanol. In the ratio of 1:3, about 130 g of the powdered sample of A. galanga was soaked overnight in a beaker with 390 mL of hexane (menstruum). The beaker is covered with aluminium foil to prevent the evaporation of the menstruum during the extraction period [21]. The liquid is then filtered using a filter paper and the solid residue (marc) is pressed using filter paper to recover as much solution as possible. Fresh solvent is replaced to the marc. This step is repeated three times (3 days). Then, the filtrates of all three days were combined and clarified again by means of filtration. The filtrates obtained were concentrated under vacuum on a rotary evaporator at 40°C. Then, the dried crude extracts were removed from the rotary evaporator and transferred to a universal bottle and kept in a refrigerator at 4°C for further use. The leftover marc from hexane is used for the maceration of the following polarity. The same extraction procedure is repeated for the sample of K. galanga. The well-prepared plant extracts were used for phytochemical screening and test on antioxidant and antibacterial activity.

2.3. Phytochemical screening
The test to determine saponins, flavonoids, tannins, phenols, steroids and terpenoids were performed to confirm the presence of secondary metabolites within the crude extracts of A. galanga and K. galanga. About 1 mg/mL of the hexane, ethyl acetate, and methanol crude extracts stock solution were prepared. Phytochemical screening was done based on the method used by [13,15,38].

2.3.1. Test for Saponins.
Foam test were conducted. About 1 mL of stock solution was diluted with 10 mL of distilled water in a test tube. The test tube was covered with a cock and shaken vigorously for 15 min. The formation of foam indicated the presence of saponins in the sample.

2.3.2. Test for Flavonoids.
About 1 mL stock solution was taken in a test tube and 0.5 mL of dilute NaOH solution was added. An intense yellow colour appeared in the test tube. Then, a few drops of diluted hydrochloric acid were added and it became colourless indicating the presence of flavonoids.
2.3.3. Test for Tannins.
About 1 mL stock solution was taken in a test tube and 0.5 mL of 10% FeCl₃ solution was added. A bluish or green black colour appearance indicated the presence of tannins.

2.3.4. Test for Phenols.
Ferric chloride method was used to test for phenols. About 1 mL of stock solution was dissolved in 2 mL of distilled water. Then, 10% FeCl₃ solution were added. Bluish black colour formed indicated the presence of phenol.

2.3.5. Test for Steroids.
About 1 mL stock solution was taken in a test tube and dissolved with 2 mL of chloroform. Then, an equal volume of concentrated sulphuric acid was added slowly by the side of the test tube to the mixture. The upper layer in the test tube turns into red and sulphuric acid layer showed yellow with green fluorescence indicating the presence of steroids.

2.3.6. Terpenoids.
Approximately 0.5 mL of stock solution was dissolved in 0.5 mL of chloroform. Then, about 1 mL of concentrated sulphuric acid was added to the solution. Formation of reddish brown colour shows the presence of terpenoids.

2.4. Antioxidant activity
Antioxidant properties were analyzed using DPPH assay.

2.4.1. DPPH assay.
To prepare a stock solution, each crude extracts of both plant samples (1 mg/mL) was diluted to final concentrations of (0.05, 0.1, 0.2, 0.4, 0.8 and 1) mg/mL. DPPH methanol was prepared where 2.4 mg of DPPH was diluted in 100 mL of methanol. To prepare a test solution, 30 μL of sample stock was diluted with 270 μL of DPPH methanol. They were mixed well and kept in dark for one hour. To prepare for control, 30 μL of distilled water and 270 μL of DPPH methanol were mixed. After one hour, 100 μL of each test solutions and 100 μL of control were filled in microplate and run in spectrophotometer at 517 nm wavelength. The absorbance values measured at 517 nm are then converted into the percentage antioxidant activity using the following equation

\[
\text{Scavenging activity (\%)} = \frac{\text{Abs for control} - \text{Abs for sample}}{\text{Abs for control}} \times 100
\]

The tests were done in triplicate. The concentration of sample required to scavenge 50% of DPPH (IC50) were determined.

2.5. Antibacterial activity
Disc-diffusion method was used for testing the antibacterial activity. Gram positive bacteria, Staphylococcus aureus (S. aureus) and Bacillus cereus (B. cereus) and gram negative bacteria, Vibrio cholerae (V. cholerae) and Salmonella thyphi (S. typhimurium) were obtained from Pascasiaiswazah Laboratory, Faculty of Science & Natural Resources, University Malaysia Sabah. All the test bacteria maintained in glycerol stock at -80°C. Then these were sub-cultured in Luria broth for 18 hrs before use. The rhizome extracts from the A. galanga and K. galanga, each at (10, 20, 30, 40 and 50) mg/mL were prepared using distilled water (1:1 v/v). In vitro antibacterial activity was studied using the disc diffusion method. Briefly, 250 ml of autoclaved Luria agar was cooled down to 50°C and mixed with 2.5 mL of bacterial suspension. This was poured into a sterile Petri dish and allowed to set. After that, sterile filter paper disc (Whatman No.1, 6 mm) was impregnated with 20 μL of each of the extracts of different concentration prepared. Then, about 10 μL per disc of antibiotic Streptomycin (50 mg/mL) was
used as a standard to confirm that the entire microorganism tested was inhibited by the antibiotic and sterile distilled water used as negative control. All the test plates were then inverted and incubated aerobically at 37°C for 24 hrs. The antimicrobial activity was recorded by measuring the clear inhibition zone (in mm) around each disc. Minimum Inhibition Concentration (MIC) was determined as well from the observation of antibacterial activity on each plate to determine the minimum concentration the extracts can inhibit the bacteria. All analyses were carried out in triplicate and the mean±SD was calculated. The activities were categorized as weak (≤ 9.5 mm), moderate (10 to 14.5 mm) and strong (≥ 15 mm) according to [20].

3. Results and Discussion

3.1. Preparation of plant material

In order to prepare the plant extracts for research studies, choice of plant materials is important. In this study, rhizome was selected for both plants; *Alpinia galanga* (*A. galanga*) and *Kaempferia galanga* (*K. galanga*), since the rhizome of these two plants are rich in natural resources and have medicinal properties which have been used traditionally. The dried rhizomes are then powdered to break the structure of the plant material so that the phytochemicals contained in the rhizomes of the plant are exposed to the extraction solvent. In addition, some studies has shown that cultivating and harvesting medicinal plants at different age, climate, geography, and environment affect their chemical compound and bioactivities [18,22,33].

3.2. Yield of crude extract

Results of weight of crude extracts and percentage of crude extracts yield of rhizomes of *Alpinia galanga* (*A. galanga*) and *Kaempferia galanga* (*K. galanga*) is listed in Table 1 and Table 2 respectively. Both the plants samples have higher yield of crude extracts in methanol, 9.08% for *A. galanga* and 5.93% for *K. galanga*. Lowest yield of crude extracts is obtained from hexane extract for *A. galanga* (0.75%) and ethyl acetate extract of *K. galanga* (0.75%).

| Extracts  | Weight of crude extracts (g) | Percentage of crude extracts yield (%) |
|-----------|-----------------------------|---------------------------------------|
| Hexane    | 0.98                        | 0.75                                  |
| Ethyl acetate | 1.87                      | 1.44                                  |
| Methanol  | 11.81                       | 9.08                                  |

Table 1: Yield of *Alpinia galanga* extracts

| Extracts  | Weight of crude extracts (g) | Percentage of crude extracts yield (%) |
|-----------|-----------------------------|---------------------------------------|
| Hexane    | 1.62                        | 1.25                                  |
| Ethyl acetate | 0.98                      | 0.75                                  |
| Methanol  | 7.71                        | 5.93                                  |

Table 2: Yield of *Kaempferia galanga* extracts

In this study, the maceration extraction method had been used with solvent of different polarities; hexane, ethyl acetate and methanol. The results show that most polar solvent (methanol) has the highest composition of the yield extract for both samples and *A. galanga* has the lowest yield in hexane extract. However, the yield obtained was lowest in ethyl acetate extract of the *K. galanga*. Therefore, in this study, methanol solvent would be the best solvent to bring the highest yield of extract for both the plants.
3.3. Photochemical analysis

Table 3 and Table 4 show the qualitative analysis of phytochemical constituents of plant samples, *A. galanga* and *K. galanga*. Phytochemical screening of rhizome extracts of *A. galanga* showed the presence of the following constituents: flavonoids, steroids, and terpenoids in hexane extract, flavonoids, phenols, steroids, and terpenoids in ethyl acetate extract, and all phytochemical in methanol extracts. Phytochemical screening of rhizome extracts of *K. galanga* showed the presence of the following constituents: flavonoids, steroids, and terpenoids in hexane extract, tannins, steroids, and terpenoids in ethyl acetate extracts, and all phytochemical except steroids in methanol extracts.

![Table 3: The qualitative analysis of *Alpinia galanga* extracts for phytochemical constituents](image)

| Extracts  | Saponins | Flavonoids | Tannins | Phenols | Steroids | Terpenoids |
|-----------|----------|------------|---------|---------|----------|------------|
| Hexane    | -        | +          | -       | -       | +        | +          |
| Ethyl acetate | -   | +          | -       | +       | +        | +          |
| Methanol  | +        | +          | +       | +       | -        | -          |

*Note: + sign represents presence and - sign represents absence of phytochemical, test were done in duplicate*

![Table 4: The qualitative analysis of *Kaempferia galanga* extracts for phytochemical constituents](image)

| Extracts  | Saponins | Flavonoids | Tannins | Phenols | Steroids | Terpenoids |
|-----------|----------|------------|---------|---------|----------|------------|
| Hexane    | -        | +          | -       | -       | +        | +          |
| Ethyl acetate | -   | -          | +       | -       | +        | +          |
| Methanol  | +        | +          | +       | +       | -        | -          |

*Note: + sign represents presence and - sign represents absence of phytochemical, test were done duplicate*

3.4. DPPH Assay

*A. galanga* and *K. galanga* has highest scavenging activity in methanol extract and the lowest scavenging activity in hexane extract. For *A. galanga* methanol extract, the highest antioxidant activity was at 1 mg/mL (82.05%) while the antioxidant activity was highest at 1 mg/mL (83.21%).

![Percentage of scavenging activity of *Alpinia galanga* hexane extract](image)

**Figure 1:** Graph of DPPH radical scavenging activity of *Alpinia galanga* hexane extract
Figure 2: Graph of DPPH radical scavenging activity of *Alpinia galanga* ethyl acetate extract

![Graph of DPPH radical scavenging activity of *Alpinia galanga* ethyl acetate extract](image)

Figure 3: Graph of DPPH radical scavenging activity of *Alpinia galanga* methanol extract

![Graph of DPPH radical scavenging activity of *Alpinia galanga* methanol extract](image)

Figure 4: Graph of DPPH radical scavenging activity of *Kaempferia galanga* hexane extract

![Graph of DPPH radical scavenging activity of *Kaempferia galanga* hexane extract](image)
Figure 5: Graph of DPPH radical scavenging activity of *Kaempferia galanga* ethyl acetate extract

Figure 6: Graph of DPPH radical scavenging activity of *Kaempferia galanga* methanol extract

Table 5: The IC\textsubscript{50} value of plant extracts

| Sample           | Crude Extract | IC\textsubscript{50}(\mu g/mL) |
|------------------|---------------|-------------------------------|
| *Alpinia galanga*| Hexane        | 800.52                        |
|                  | Ethyl Acetate | 475.87                        |
|                  | Methanol      | 364.31                        |
| *Kaempferia galanga* | Hexane     | 831.82                        |
|                  | Ethyl Acetate | 492.75                        |
|                  | Methanol      | 424.44                        |

To further analyse, among the three crude extracts; hexane, ethyl acetate and methanol, both plant samples, *A. galanga* and *K. galanga*, has highest scavenging activity in methanol extract and the lowest scavenging activity in hexane extract. Hence, it can be concluded that methanol extracts exhibit the best antioxidant activity and this might due to the presence of higher number of phytochemicals.
3.5. Antibacterial activity

The crude extracts were tested against four bacteria; two gram positive bacteria: *Bacillus cereus* (*B. cereus*) and *Staphylococcus aureus* (*S. aureus*) and two gram negative bacteria: *Salmonella thyphimurium* (*S. thyphimurium*) and *Vibrio cholerae* (*V. cholerae*).

| Table 6: Antibacterial activity of *Alpinia galanga* extract against selected bacteria |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|
| Extract                     | Bacteria        | Concentration  | Inhibition | Diameter (mm) |
| Hexane                      | Staphylococcus aureus | 10             | √           | 16.67±1.15     |
|                             | Salmonella thyphimurium | 10             | √           | 20.67±0.58     |
| Ethyl acetate               | Staphylococcus aureus | 10             | √           | 11.00±0.00     |
|                             |                  | 20             | √           | 11.67±0.29     |
|                             |                  | 30             | √           | 12.83±0.29     |
|                             | *Bacillus cereus* | 10             | √           | 11.00±0.00     |
|                             |                  | 20             | √           | 11.60±0.17     |
|                             | *Salmonella thyphimurium* | 10             | √           | 11.00±0.00     |
|                             |                  | 20             | √           | 18.67±2.31     |

*Note: Each value is expressed as mean±standard deviation (n=3), √ Sign represents presence of inhibition zone of crude extracts, The diameter of inhibition zone is including the size of disc which is 6 mm.

| Table 7: Antibacterial activity of *Kaempferia galanga* extract against selected bacteria |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|
| Extract                     | Bacteria        | Concentration  | Inhibition | Diameter (mm) |
| Hexane                      | *Bacillus cereus* | 10             | √           | 12.33±0.58     |

*Note: Each value is expressed as mean±standard deviation (n=3), √ Sign represents presence of inhibition zone of crude extracts, The diameter of inhibition zone is including the size of disc which is 6 mm.

Frequently it would be expected that gram positive bacteria would be more active than gram negative bacteria. This is because of the structure of gram negative bacteria which possess an outer membrane composed of a lipopolysaccharide monolayer surrounding their cell wall which act as barrier to the penetration of active molecules and the periplasmic space contains enzymes that able to degrade exogenous molecules [32,41]. This theory is proven through this study by which the positive bacteria, *S. aureus* and *B. cereus*, were more susceptible to the extracts of *A. galanga* and *K. galanga* than that of gram negative bacteria, *S. thyphimurium* and *V. Cholerae*. Based on that, the hexane extract of *A. galanga* showed strong inhibitory activity against *S. aureus* and *S. thyphimurium*, with inhibition zones of 16.67 mm and 20.67 mm respectively at 10 mg/mL whereas the ethyl acetate extract of *A. galanga* showed moderate inhibitory activity against *S. aureus* and *B. cereus*, with inhibition zones of 11.00 mm at 10mg/mL, 11.67 mm at 20 mg/mL and 12.83 mm at 30 mg/mL for *S. aureus* and 11.00 mm at 10 mg/mL and 11.60 mm at 20 mg/mL for *B. cereus*.

4. Conclusion

Medicinal plants play a vital role in overcoming the oxidative stress that causes chronic and degenerative diseases. Hence, two medicinal plants from Zingiberaceae family, *Alpinia galanga* (*A. galanga*) and *Kaempferia galanga* (*K. galanga*) are chosen in this study to screen for their phytochemical constituents, antioxidant and antibacterial potential. Three extracts with different polarities were obtained from both plants using dried rhizomes of the plant; hexane, ethyl acetate, and methanol. Methanol extracts give the highest yield of crude extracts for both plant samples. *A. galanga* and *K. galanga* are the sources of
secondary metabolites; saponin, phenols, flavonoids, tannins, steroids and terpenoids, that contributes to the antioxidant and antibacterial property. Methanol extracts give highest number of those phytochemical constituents for both plant samples. In conjunction, methanol extracts give the highest antioxidant activity for both plant samples followed by ethyl acetate and then hexane. For A. galanga methanol extract, the highest antioxidant activity was at 1 mg/mL (82.05%) with IC₅₀ value of 0.364 mg/mL meanwhile for K. galanga methanol extract the highest antioxidant activity was at 1 mg/mL (83.21%) with IC₅₀ value of 0.424 mg/mL. Non-polar extracts (hexane) of both plant samples exhibits greater antibacterial activity compared to the polar extracts (ethyl acetate). Methanol extracts do not show any antibacterial activity. Overall, A. galanga and K. galanga could serve as potential sources of antimicrobial and antioxidant agents. Further research is needed towards isolation of pure compounds from the crude extracts and to better understand the mechanism of such actions scientifically.

5. References

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