# Evaluation of biological activities of *Alpinia mutica* Roxb. and its chemical constituents

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**Abstract**: Phytochemicals investigation on rhizomes of *Alpinia mutica* has afforded five compounds namely 5,6-dehydrokawain **(1)**, flavokawin B **(2)**, pinostrobin **(3)** and pinocembrin **(4)** together with β-sitosterol **(5)**. All crude extracts of the plant demonstrated strong cytotoxicity against CEMss (human T4 lymphoblastoid) cancer cells with $IC_{50}$ values less than 19 µg/mL, while flavokawin B **(2)** was the most cytotoxic isolate with $IC_{50}$ value 1.86±0.37 µg/mL. Most of the crude extracts and isolated compounds showed weak activity in antimicrobial and diphenylpicrylhydrazyl (DPPH) radical scavenging activity tests.

**Keywords**: *Alpinia mutica*, CEMss, flavokawin B, antimicrobial, DPPH.

## INTRODUCTION

Zingiberaceae family or well known as ginger family consists of about 1,200 species of which 1,000 are found in tropical Asia (Larsen *et al*., 1999). Ginger species are basically rhizomatous, aromatic herbs ranging in size from as small as 15 cm to as tall as 5 m. Majority of the species grow in the wild and prefer shaded and moist habitats. Gingers are among major tropical plant families and are characterized by their aromatic parts. They are widely used in folk medicine for the treatment of skin diseases, wounds, stomach disorder and sometimes grown as ornamentals. Many studies have shown that at least more than ten cultivated species of Zingiberaceae have been used frequently in traditional medicine (Larsen *et al*., 1999).

*Alpinia mutica* (syn: *Languas mutica*) is a perennial herb belonging to Zingiberaceae family. This plant which is also known as 'chengkenam' has been cultivated as ornamentals and its rhizomes have been used traditionally by the locals as a stomachic to treat flatulence. Previous phytochemicals study on rhizomes and fruits of *A. mutica* have reported isolation of flavonoid derivatives and kavalactone (Sirat *et al.*, 1996; Jantan *et al*., 2004; 2008 and Malek *et al*., 2011).

Methanol extract and several compounds isolated from fruits of *A. mutica* were reported to have strong antiplatelet aggregation activity (Jantan *et al*., 2004; 2008), while the ethyl acetate extract of rhizomes showed significant anticancer properties towards several cancer cells besides exhibited substantial antioxidant activity (Malek *et al*., 2011; Phang *et al*., 2011). Previous study by Habsah *et al*. (2000) mentioned both dichloromethane and methanol extracts of rhizomes of *A. mutica* displayed significant antioxidant activity while in antimicrobial activity test, the dichloromethane extract showed stronger activity than its methanol extract.

In continuation of our investigation on local traditional plants, we now described phytochemical and biological activity studies on rhizomes of *A. Mutica.* The present study was conducted to isolate and characterize major chemical constituents, assess cytotoxic activity against T-cell acute lymphoblastic leukemic (CEMss) cancer cells, antimicrobial activity against various pathogenic microorganisms as well as antioxidant properties of the rhizomes extracts from the plant.

## MATERIALS AND METHODS

### *Plant material*

*Alpinia mutica* was collected from Forest Research Institute Malaysia (FRIM), Kepong on April 2010. The voucher specimen is kept in the Herbarium of the Institute. The rhizomes were sliced, air-dried at room temperature and ground prior to use.

### *Extraction and isolation*

Air-dried rhizomes of *A. mutica* (699.50 g) was soaked at room temperature for 72 hours with solvents of increasing polarity (hexane, chloroform, ethyl acetate and methanol) sequentially for three times. Each of solution obtained was rotary-evaporated yielded hexane (12.81 g), chloroform (8.36 g), ethyl acetate (2.66 g) and methanol (28.68 g) extracts. Each of these was column-chromatographed employing silica as absorbent using mixtures of hexane, hexane/ethyl acetate and ethyl acetate/methanol as solvent eluents.

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Column chromatography fractionation of hexane extract gave 5,6-dehydrokawain (**1,** 1.36 g), flavokawin B (**2,** 0.28 g), pinostrobin (**3,** 53 mg) and β-sitosterol (**5,** 36 mg). Similar column fractionation of chloroform extract gave 5,6-dehydrokawain (**1,** 0.70 g), flavokawin B (**2,** 6 mg) and pinocembrin (**4,** 32 mg) while ethyl acetate extract gave only 5,6-dehydrokawain (**1,** 43 mg). Fractionation of methanol extract did not lead to isolation of any pure compounds.

**5,6-*Dehydrokawain (1)*** was isolated as pale yellow crystals, m.p. 135-136 °C (Itokawa *et al*., 1981, m.p. 136.5-138.5 °C), $C_{14}H_{12}O_3$, IR (KBr disc, $\nu_{max}$, $cm^{-1}$): 1714, 1639, 1553, 1451. EIMS *m/z* (%): 228 ($[M]^+$, 100), 200 (58), 157 (39), 125 (30), 69 (44). Proton and carbon NMR spectral data were compared with the published data (Dharmaratne *et al*., 2002).

***Flavokawin B (2)*** was isolated as yellow crystals, m.p. 88.6-89.6 °C (Itokawa *et al*., 1981, m.p. 91.5-92.0 °C), $C_{17}H_{16}O_4$, IR (KBr disc, $\nu_{max}$, $cm^{-1}$): 1622, 1572, 1341, 1215. EIMS *m/z* (%): 284 ($[M]^+$, 99), 207 (100), 181 (64), 103 (45), 77 (38). Proton and carbon NMR spectral data were compared with the published data (Jhoo *et al*., 2006).

***Pinostrobin (3)*** was isolated as white crystals, m.p. 98-100 °C (Yap *et al*., 2007, m.p. 96-98°C), $C_{16}H_{14}O_4$, IR (KBr disc, $\nu_{max}$, $cm^{-1}$): 3262, 1638, 1575, 1298, 1156. EIMS *m/z* (%): 270 ($[M]^+$, 100), 193 (92), 166 (53), 138 (34), 110 (30), 95 (32). Proton and carbon NMR spectral data were compared with the published data (Yap *et al*., 2007).

***Pinocembrin (4)*** was isolated as pale yellow crystals, m.p. 200-201 °C (Sirat *et al*., 1996, m.p. 204-205 °C), $C_{15}H_{12}O_4$, IR (KBr disc, $\nu_{max}$, $cm^{-1}$): 3079, 1634, 1463, 1294, 1163. EIMS *m/z* (%): 256 ($[M]^+$, 100), 179 (96), 152 (87), 124 (54), 96 (24), 78 (32), 69 (38). Proton and carbon NMR spectral data were compared with the published data (Yap *et al*., 2007).

**β-*Sitosterol (5)*** was isolated as white amorphous solids, m.p. 130.5-131.5 °C (Ahmad *et al*., 2010, m.p. 137-138 °C), $C_{29}H_{50}O$, IR (KBr disc, $\nu_{max}$, $cm^{-1}$): 3334, 2937, 2862, 1456, 1372, 1042. EIMS *m/z* (%): 414 ($[M]^+$, 77), 396 (49), 381 (34), 329 (67), 303 (33), 273 (40), 255 (70), 231 (40), 213 (69), 159 (78), 145 (100), 133 (76), 119 (66), 105 (91), 81 (90), 55 (84). Proton and carbon NMR spectral data were compared with the published data (Ahmad *et al*., 2010).

***Cytotoxic assay***

All crude extracts and isolated compounds from rhizomes of *A. mutica* were screened for cytotoxic activity against CEMss (human T4 lymphoblastoid) cancer cells. The cells were obtained from National Institutes of Health (NIH), USA. The assay was carried out according to the methods previously described (Sukari *et al*., 2010). The cytotoxic index used was $IC_{50}$, which is the concentration that yield 50% inhibition of the cell compared with the untreated control. The extracts and isolated compounds which exhibit cytotoxic index $IC_{50}$ less than 10 µg/mL were considered to have significant cytotoxic activity (Mackeen *et al.,* 1997).

***Antimicrobial assay***

Antimicrobial activity of extracts and obtained products were screened against four pathogenic bacteria and three types of fungi. Bacterial strains used were Methicillin-Resistant *Staphylococcus aureus* (*MRSA*) (gram-positive), *Pseudomonas aeruginosa* (gram-negative), *Salmonella choleraesuis* (gram-negative) and *Bacillus subtilis* (gram-positive) while, the fungi strains used were *Candida albicans*, *Aspergillus ochraceaus* and *Sacchoromyces cerevisiae*. Both bacteria and fungi strains were originally supplied by American Type Cell Culture Collection (ATCC). The assay was carried out according to the methods previously described (Mackeen *et al*., 1997). The isolates diffused from disc into agar, thus inhibiting the growth of the surrounding microorganisms. The antimicrobial activity was recorded as diameter (mm) of the clear inhibition zone surrounding the disc.

***DPPH radical scavenging activity assay***

Assay was carried out following procedures previously described (Saha *et al*., 2004) with trivial changes. Stock solutions (10 mg/ml in dimethyl sulfoxide) of samples were diluted to various concentrations (500 to 7.815 µg/ml in dimethyl sulfoxide) in 96-well plate. After that, each well was added with 5 µL of DPPH solution (10 mg/ml in dimethyl sulfoxide) and the plate was shook tenderly and put aside for 45 minutes at ambient temperature. An absorbance was determined at 517 nm using UV-Vis spectrometer. Radical scavenging activity was estimated as follow:

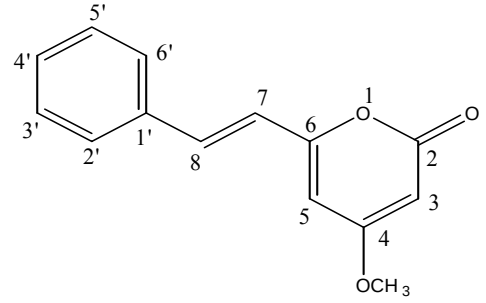
$$\% \text{ Antioxidant Activity} = \frac{\text{OD (DPPH)} - \text{OD (DPPH + Sample)}}{\text{OD (DPPH)}} \times 100$$

*OD = Optical Density

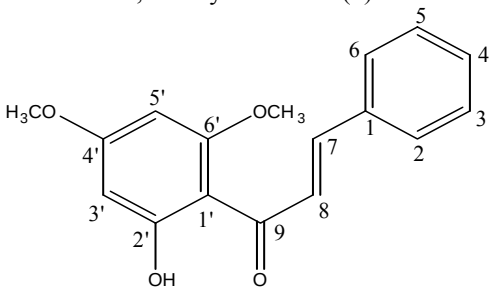
## RESULTS

Isolation work on rhizomes extracts of *Alpinia mutica* has led to the identification and characterization of one kavalactone, 5,6-dehydrokawain **(1)** and flavonoid derivatives, flavokawin B **(2)**, pinostrobin **(3)** and pinocembrin **(4)** together with β-sitosterol **(5)**. Chemical structures of all isolated compounds are showed in fig. 1. All the compounds were identified and characterized using spectroscopic techniques and comparing its spectral and physical data with published values. The major constituent, 5,6-dehydrokawain **(1)** was isolated from all crude extracts except methanol, while flavokawin B **(2)**

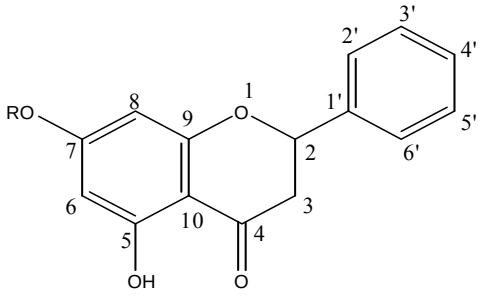
was obtained from hexane and chloroform extracts. Besides, other minor substituents were also isolated from hexane and chloroform extracts.



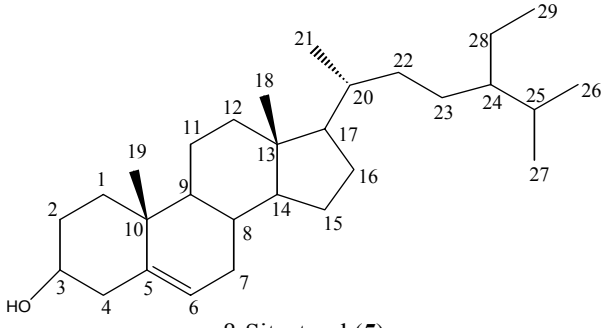

5,6-Dehydrokawain (**1**)




Flavokawin B (**2**)




Pinostrobin (**3**) R= $CH_3$, Pinocembrin (**4**) R= H




β-Sitosterol (**5**)

**Fig. 1**: Chemical structures of isolated compounds from rhizomes of *A. mutica*

All rhizomes extracts and isolated compounds were subjected to cytotoxic activity screening against CEMss (human T4 lymphoblastoid) cancer cells. Cytotoxic activities of the samples are displayed in table 1. All the crude extracts demonstrated significant cytotoxicity against CEMss cancer cells with $IC_{50}$ values less than 19 µg/mL. Flavokawin B **(2)** was the most cytotoxic isolate against CEMss cancer cell line tested with $IC_{50}$ value 1.86±0.37 µg/mL which was comparable to the standard drug used. All other compounds also showed strong activity with $IC_{50}$ values ranging from 2-5 µg/mL. All crude extracts and isolated compounds were also screened against seven pathogenic microorganisms; consist of two gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*), two gram-negative bacteria (*Salmonella choleraesuis* and *Pseudomonas aeruginosa*) and three fungi (*Candida albicans*, *Aspergillus ochraceaus* and *Sacchoromyces cerevisiae)*. The antimicrobial screening activity results are summarized in Table 1. A study on antioxidant capacity of rhizomes extracts of *A. mutica* was also conducted. Antioxidant activity of the isolates were evaluated using DPPH free radical scavenging activity and the results is summarized in Figure 2.

## DISCUSSION

Previous cytotoxic investigations on the isolates of the plant have revealed different ranges of activity against several cancer cell lines. A study on the rhizomes of *A. mutica* has shown that the ethyl acetate extract exhibits significant cytotoxicity against several cancer cell lines (Malek *et al.*, 2011). In related investigations, flavokawin B **(2)** was implicated as the compound which displayed strong cytotoxicity effect on human liver cancer cells (HepG2) and (Hepa1c1c7) (Jhoo *et al*., 2006; Shaik *et al*., 2009) also against human pharyngeal epidermoid cancer (KB) and colon cancer (HCT 116) cell lines (Malek *et al*., 2011). At the same time, 5,6-dehydrokawain **(1)** showed mild cytotoxicity against liver cells (Hepa1c1c7) (Shaik *et al*., 2009) and was found to be inactive against some other panel cells (Win *et al*., 2007; Malek *et al*., 2011).

Flavonoid pinostrobin **(3)** was found to be inactive and showed insignificant cytotoxicity against various cancer cell lines (Pouget *et al*., 2001; Usia *et al*., 2002; Win *et al*., 2007; Li *et al*., 2010; Chou *et al*., 2010). In contrast, pinocembrin **(4)** showed moderate cytotoxicity towards human pancreatic (Win *et al*., 2007; Awale *et al*., 2009; Li *et al*., 2010) and colon (HT-1080 and colon 26-L5) tumour cells (Usia *et al*., 2002; Awale *et al*., 2009), while inactive against other panel cell lines (Usia *et al*., 2002; Awale *et al*., 2009; Umehara *et al*., 2009; Kurniadewi *et al*., 2010). In our present study, *A. mutica* appears to be a promising plant exhibiting substantial anticancer effect shown by its rhizomes extracts and chemical constituents and hence, warrant further investigation. In all cases, the rhizomes extracts tested were more cytotoxic than the isolated compounds. These results suggested the synergistic effects shown by the isolated compounds towards the cytotoxic properties of the crude extracts. To our knowledge, the cytotoxic activity against CEMss cancer cells of rhizomes extracts and compounds isolated from *A. mutica* has never been reported previously. In

**Table 1**: Cytotoxic and antimicrobial activity screening of rhizomes extracts and isolated compounds from *A. mutica*

| Extracts/ Isolated compounds/ standard drugs | *$IC_{50}$ values (µg/mL) | Diameter of inhibition zone (mm) | | | |
|---|---|---|---|---|---|
| | | Methicillin-Resistant *Staphylococcus aureus* (*MRSA*) | *Salmonella choleraesuis* | *Bacillus subtilis* | *Aspergillus ochraceaus* |
| Hexane | 12.70±0.26 | - | 10±0.02 | - | - |
| Chloroform | 11.40±0.20 | - | 10±0.03 | 9±0.06 | 7±0.05 |
| Ethyl acetate | 13.30±0.25 | 7±0.05 | 9±0.03 | - | 7±0.02 |
| Methanol | 18.20±0.81 | - | - | - | - |
| 5,6-Dehydrokawain **(1)** | 5.42±0.49 | - | - | - | - |
| Flavokawin B **(2)** | 1.86±0.37 | - | - | - | - |
| Pinostrobin **(3)** | 5.12±0.07 | 9±0.04 | - | - | - |
| Pinocembrin **(4)** | 2.21±0.57 | - | - | 7±0.03 | - |
| 5-fluorouracil | 1.43±0.06 | - | - | - | - |
| Streptomycin (10 µg) | - | 26±0.03 | 24±0.02 | 28±0.01 | - |
| Nystatin (0.5 mg) | - | - | - | - | 21±0.01 |

*Values were means ± standard deviation of triplicate analyses

antimicrobial screening activity, all crude extracts except methanol showed weak activity against *S. choleraesuis*. Moreover, the semi-polar of chloroform and ethyl acetate extracts also showed weak activity against *B. subtilis* and *MRSA*, respectively. As for isolated compounds, only pinostrobin **(3)** and pinocembrin **(4)** showed weak activity against *MRSA* and *B. subtilis*, respectively. In the antifungal screening activity, all the rhizomes extracts and isolated compounds were inactive against all the fungi tested except for chloroform and ethyl acetate extracts which demonstrated a weak activity against *Aspergillus ochraceaus*. Previous study by Habsah *et al*. (2000) indicated the methanol and dichloromethane extracts of *A. mutica* rhizomes showed significant antimicrobial activity towards several pathogenic microorganisms.

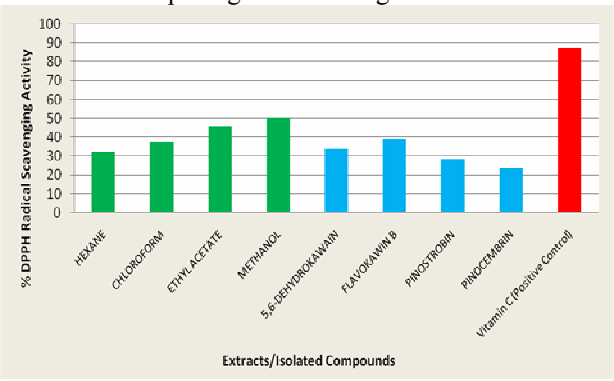



**Fig. 2**: Percentage of DPPH Radical Scavenging activity of the isolates of *A. mutica* at 500 µg/mL.

In addition, all rhizomes extracts and isolated compounds showed moderate to weak antioxidant properties in comparison with Vitamin C (Positive Control) with methanol extract and flavokawin B **(2)** demonstrated the highest percentage. Previous study on DPPH radical scavenging activity of rhizomes of *A. mutica* showed that the ethyl acetate extract demonstrated the highest percentage (Phang *et al*., 2011) while 5,6-Dehydrokawain **(1)**, flavokawin B **(2)**, pinostrobin **(3)** and pinocembrin **(4)** were inactive and showed insignificant activity (Habsah *et al*., 2003; 2004).

## CONCLUSION

Phytochemicals investigation on rhizomes of *Alpinia mutica* has afforded 5,6-dehydrokawain as the major constituent, together with flavokawin B, pinostrobin, pinocembrin and β-sitosterol. All crude extracts and isolated compounds exhibited strong cytotoxic activity against CEMss cancer cell lines, while showed moderate antioxidant activity in DPPH radical scavenging activity assay. Hence, this study reveals that rhizomes of the plant are a potential source of natural anticancer agent.

## ACKNOWLEDGEMENTS

We wish to express our gratitude to Ministry of Science, Technology and Innovation (MOSTI) for financial assistance under Research University Grant Scheme (RUGS) funded to one of the authors (N. A. Mustahil) also Department of Chemistry, Faculty of Science, Universiti Putra Malaysia for all the facilities provided.

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