

Cytotoxic and Antibacterial Evaluation of Coumarins and Chromanone Acid from *Calophyllum symingtonianum*

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ABSTRACT

Calophyllum symingtonianum is a rare species from the *Calophyllum* genus that belongs to the Guttiferae family. *Calophyllum* has been recognized as a potential medicinal plant due to its many bioactive phytochemicals especially coumarins and chromanone acids. In this study, the cytotoxic activities against MCF-7 and A-549 and antibacterial activities towards *S. aureus*, *B. subtilis*, *P. aeruginosa* and *E. Coli* of three coumarins known as inophyllum D, inophyllum H and calanone as well as chromanone acid identified as isocordato-oblongic acid isolated from this plant were evaluated. Inophyllum H exhibited the highest IC₅₀ values against MCF-7 at 25.56 µg/mL and A-549 at 26.41 µg/mL. Isocordato-oblongic acid showed moderate antibacterial activity against *S. aureus* and *B. subtilis* at 125 µg/mL and 62.5 µg/mL, respectively. This study suggests *C. symingtonianum* as a potential plant for cytotoxic and antibacterial phytochemicals.

INTRODUCTION

The statistics from WHO in 2012 have reported that cancer has become a remarkable cause of non-communicable deaths. The top five causes of cancer deaths include lung cancer for men and breast cancer for women (Global Cancer Facts & Figures 2nd Edition, 2011). The search for new alternative therapeutic approaches on many current dreaded diseases such as cancer has become a topic of prominent study among researchers around the globe. The last three decades have witnessed the development of pharmacological industries to produce new antibiotics. However, the resistance towards those antibiotics also increases over the year (Kader *et al.*, 2013). Such fact is a concern and new antibacterial from natural sources need to be explored. Antibacterial constituents from plants may have a clinical value since they act against bacteria via different mechanisms from currently available antibiotics (Kaikabo and Eloff, 2011). Nature has remained as the most valuable reservoir of therapeutic agents against a wide range of diseases in modern drug development. *Calophyllum* from Guttiferae family is a

pan-tropical genus comprising of approximately 200 species and locally known in Malaysia as “*bintangor*”. The plants are commonly employed in folk medicine to treat diabetes, hypertension, diarrhea, bronchitis, gastric and hepatic disturbances pain, chronic ulcers, inflammation, hemorrhoids, rheumatism varicose and also used in the prevention of wound infection (Alkhamaiseh *et al.*, 2011). *Calophyllum* species has received considerable attention following the discovery of anti-HIV RT-1 from coumarin class of compound from *Calophyllum lanigerum* (Mckee *et al.*, 1996). Apart from anti-HIV, coumarins also have been reported to exhibit cytotoxic activity against a few cell lines such as KB cell lines (Guilet *et al.*, 2001), K562, U251 and PC3 human tumor cell lines (Su *et al.*, 2008). Chromanone acid is a class of compound unique to *Calophyllum* genus especially from the bark resins. A majority of the chromanone acids possesses a phloroglucinol ring fused to the 2,3-dimethylchromanone ring (Ha *et al.*, 2012). Previous report on the isolation of a few chromanone acids from *C. inophyllum* showed good antibacterial activity towards *S. aureus* in disc diffusion assay (Yimdjo *et al.*, 2004). *Calophyllum symingtonianum*, known as ‘*bintangor bukit*’ is one of the *Calophyllum* species found in the Malay Peninsula. It usually grows in forest hills at an altitude of 100-150 m (Kawamura *et al.*, 2012) and have evergreen broad-leaved tree.

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To the best of our knowledge, there is no report on cytotoxic and antibacterial activities of isolated coumarins identified as inophyllum D (1), inophyllum H (2), calanone (3) as well as chromanone acid known as isocordato-oblongic acid (4) (Figure 1) from this plant. Therefore, this study was embarked to evaluate specifically their cytotoxic activities against MCF-7 and A-549 cancer cell lines as well as antibacterial activities against *S. aureus*, *B. subtilis*, *P. aeruginosa* and *E. Coli*.

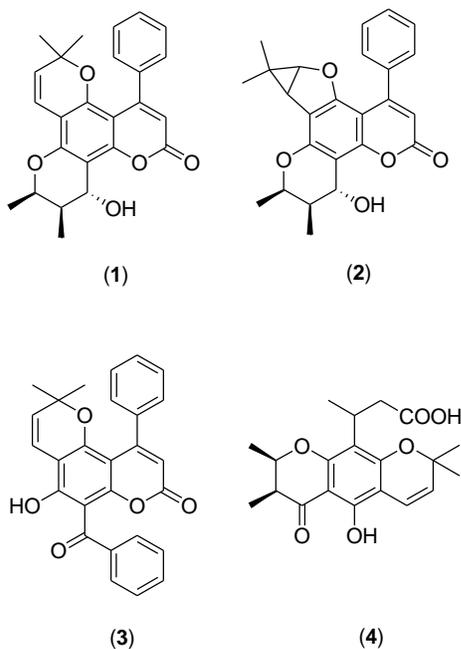


Fig. 1: Chemical Structure of (1-4).

MATERIALS AND METHODS

Chemicals and Reagents

Antibacterial Assay: Nutrient agar (NA) and nutrient broth (NB) were purchased from Merck while streptomycin sulphate (SS) and 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium (INT) were obtained from Sigma. Sodium chloride (NaCl), tryptone, glycerol and H₂SO₄ (98%) were supplied by Qrec while yeast extract was obtained from Scharlau. Tween 80 was purchased from Fischerbrand. Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), TrypLE™ Express and Penicillin-streptomycin were obtained from Gibco. Phosphate buffer saline (PBS) and trypan blue stain 0.4% were purchased from Sigma while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was supplied by Molecular Probes.

Plant Materials

Sample of *C. symingtonianum* was collected from Kuantan, Pahang in February 2012 and deposited in Herbarium, Kulliyah of Pharmacy, International Islamic University Malaysia, Pahang. Inophyllum D (1), inophyllum H (2), calanone (3) and isocordato-oblongic acid (4) were isolated from the barks and leaves of *C. symingtonianum* (Aminudin *et al.*, 2015) and were

characterized by 1D and 2D NMR spectroscopy techniques as well as compared to published literatures.

Cytotoxic Assay

The cytotoxic activity was evaluated by MTT colorimetric assay (Mosmann, 1983; Taher *et al.*, 2012) on human breast cancer (MCF-7) and human lung carcinoma (A-549) cell lines. The sample stock solution (100 µg/mL) was dissolved in 1% (v/v) DMSO in phosphate buffered saline (PBS). The samples were further diluted with Dulbecco's modified Eagle's Medium (DMEM) to afford concentration ranging from 100 – 3.13 µg/mL obtained from twofold dilution. The cells were cultured in DMEM media supplemented with 10% fetal bovine serum and 2% penicillin-streptomycin. In brief, 90 µL of cell suspension in DMEM were seeded in 96-well microplate and was counted directly by using trypan blue dye. The cells were treated with samples after reaching confluence (2 x 10⁵ cell/mL) and were pre-incubated at 37°C in humidified atmosphere with 5% CO₂ for 24 hours. 20 µL of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT (5 mg/mL in PBS) was added to all well in dark condition and pre-incubated for another 4 hours. 100 µL of DMSO was added to all well to solubilize the water-insoluble purple formazan crystal formed and pre-incubated in dark condition at room temperature. The absorbance was read after 1 hour at 570 nm and 630 nm as the reference wavelength. Untreated cells served as control group and were considered as 100% viable cells. Results were expressed as percentage of cell viability of samples relative to the untreated control cell following the formula below:

$$\% \text{ Inhibition Concentration (\%IC)} = \frac{[(A_{\text{sample}} - A_{\text{MTT blank}}) / (A_{\text{control}} - A_{\text{MTT blank}})] \times 100\%}{}$$

where A_{sample} is the absorbance of cells treated with samples, $A_{\text{MTT blank}}$ is the absorbance of MTT reagent with DMSO only and A_{control} is the absorbance of untreated control cells.

Antibacterial Assay

The antibacterial activity of all compounds was tested quantitatively by evaluating their minimum inhibition concentration (MIC). Two *Gram* positive strains of *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29737) and two *Gram* negative bacterial strains of *Escheria coli* (ATCC 10536) and *Pseudomonas aeruginosa* (ATCC 9027) were chosen. The MIC was carried out by serial broth microdilution with slight modification (Kaikabo and Eloff, 2011; Ahmed *et al.*, 2012; Perumal *et al.*, 2012; Sarikurkcü *et al.*, 2015; Sufian *et al.*, 2013). The sample stock solution (1000 µg/mL) was prepared in 5% DMSO in nutrient broth (NB) supplemented with 0.02% (v/v) Tween 80. Further twofold dilution with NB was performed to afford concentration of samples from 1000-7.81 µg/mL. The bacteria were thawed and streaked on agar plates (90 x 15 mm) by using quadrant streak technique. The agar plates were incubated for 16 hours to promote the growth of bacteria. Several single colonies of the bacteria were streaked out and mixed in sterilized nutrient broth (NB) and incubated about two to three hours with

swirling. The turbidity of the bacteria was examined by measuring the optical density (OD) using UV spectrophotometer at 600 nm. The optimum OD for the bacteria is in the range 0.1 – 0.2, which is equivalent to the 0.5 Mc Farland standard solution (10^6 CFU/mL).

50 μ L of bacteria inocula was dispensed in the 96-well microplate followed by 50 μ L of sample solution. Streptomycin sulphate was employed as positive control in this assay. The microplates were pre-incubated for 24 hours at 37°C for *S. aureus*, *E. coli* and *P. aeruginosa* and 30 °C for *B. subtilis*. 25 μ L of *p*-iodonitrotetrazolium (INT) (0.2 mg/mL in sterile distilled water) solution was added to all wells and were pre-incubated for at least 30 minutes. Bacterial growth in the wells was indicated by the formation of reddish-pink color while clear well indicates inhibition of bacterial growth by the sample.

Statistical Analysis

Three replicates of each sample were used for statistical analysis with values reported as mean \pm SD. Standard curves were generated and calculation of the 50% inhibitory concentration (IC₅₀) values was done using GraphPad Prism for Windows (version 5.02) software. The Student's *t*-test was carried out using SPSS (version 22) software for comparison between treatment of samples and positive controls. A value of *p* < 0.05 was considered significantly different.

RESULTS AND DISCUSSION

Cytotoxic Activity

The evaluation of the cytotoxic effects of all compounds towards human breast cancer cell line (MCF-7) and human lung carcinoma (A-549) was done by using MTT assay. The percentage of cell viability for both cell lines after treatment with all compounds for 24 hours compared to untreated control cell was determined. The IC₅₀ values obtained act as parameter for cytotoxic activity where it refers to 50% cell inhibition by the compounds. The cytotoxicity of inophyllum H (2) against MCF-7

and A-549 cell lines at six different concentrations is illustrated in **Figure 2**. From the graph, inophyllum H (2) demonstrated cytotoxic effects to both cell lines with obvious dose-dependent manner. Inophyllum H (2) gives significant IC₅₀ values at 25.56 μ g/mL and 26.41 μ g/mL against MCF-7 and A-549, respectively. Meanwhile, inophyllum D (1) and calanone (3) showed no cytotoxicity towards both cell lines with IC₅₀ values of more than 100 μ g/mL (**Table 1**). The presence of *gem*-dimethylcyclopropane fused to pyran ring as in inophyllum H (2) structure may contribute to the cytotoxic activity towards both cell lines. Isocordato-oblongic acid (4) also was found to be devoid to the cytotoxic assay with 71.99% and 71.29% cell viability at the highest concentration of 100 μ g/mL.

Antibacterial Activity

The antibacterial activity of all compounds was determined by minimum inhibition concentration (MIC) in serial broth microdilution. Tetrazolium microplate assay by employing INT was chosen as it enhances sensitivity and accuracy of MIC determination since the formation of violet formazan derivatives by bacteria can be quantified and yield greater reproducible result (Masoko *et al.*, 2007). Based on the result tabulated in Table 2, isocordato-oblongic acid (4) showed moderate inhibition towards *S. aureus* and *B. subtilis* bacteria at 125 μ g/mL and 62.5 μ g/mL respectively. No inhibition was observed for *Gram* negative bacteria. It indicates isocordato-oblongic acid (4) was more selective towards *Gram* positive bacteria. The presence of pentanoic acid moiety in the structure increases the lipophilic character, thus enabling the penetration of low lipid content of cell wall *Gram* positive bacteria and reaching its active site (Biagi *et al.*, 1970). Previous report on the isolation of six chromanone acids from *C. brasiliense* demonstrated moderate-to-strong antibacterial activity especially to *Gram* positive bacteria (Cottiglia *et al.*, 2004) thus supports the findings. Meanwhile, all coumarins are found to be inactive towards all bacteria tested, except for inophyllum H (2) that exhibited weak activity against *B. subtilis* at 250 μ g/mL.

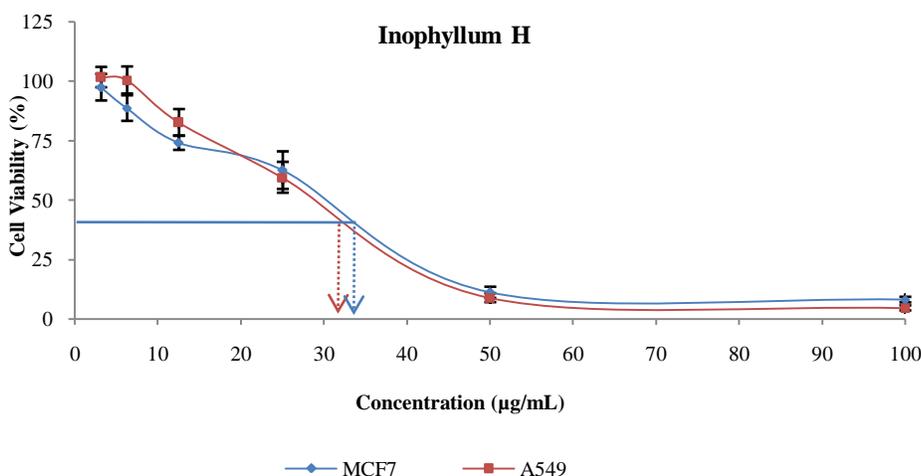


Fig. 2: Graph of percentage cell viability of MCF-7 and A-549 against six different concentrations of inophyllum H (2).

Table 1: Cytotoxic activity of tested samples.

| Compounds | MCF-7 | | A-549 | |
|------------------------------|---------------------|--------------------------|---------------------|--------------------------|
| | IC (%) ^a | IC ₅₀ (µg/mL) | IC (%) ^a | IC ₅₀ (µg/mL) |
| Inophyllum D (1) | 64.24 ± 3.56* | >100 | 76.43 ± 1.67*** | >100 |
| Inophyllum H (2) | 7.96 ± 1.17*** | 25.56 ± 3.01 | 4.46 ± 1.06*** | 26.41 ± 2.38 |
| Calanone (3) | 74.52 ± 1.87*** | >100 | 63.64 ± 1.03*** | >100 |
| Isocordato-oblongic acid (4) | 71.99 ± 6.31* | >100 | 71.29 ± 0.96** | >100 |

^a Cell viability at 100 µg/mL as mean ± SD of triplicate experiments; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with control; IC₅₀ inhibition: very strong < 5 µg/mL; strong < 5 – 10 µg/mL; moderate: 10 – 20 µg/mL; weak: 20 – 100 µg/mL and inactive > 100 µg/mL (Wibowo *et al.*, 2011).

Table 2: Minimum inhibition concentration (MIC)^a of tested samples.

| Compounds | Gram positive | | Gram negative | |
|------------------------------------|------------------|--------------------|----------------------|----------------|
| | <i>S. aureus</i> | <i>B. subtilis</i> | <i>P. aeruginosa</i> | <i>E. coli</i> |
| Inophyllum D (1) | >1000 | >1000 | >1000 | >1000 |
| Inophyllum H (2) | >1000 | 250 | >1000 | >1000 |
| Calanone (3) | >1000 | >1000 | >1000 | >1000 |
| Isocordato-oblongic acid (4) | 125 | 62.5 | >1000 | >1000 |
| Streptomycin sulphate ^b | 12.5 | 1.56 | 50 | 0.78 |

^aMinimum inhibition concentration (µg/mL) in triplicate, ^bStandard control, MIC determination; high activity < 10 µg/mL (Ríos and Recio, 2005).

CONCLUSION

In conclusion, inophyllum H (2) demonstrated the highest cytotoxic activity towards MCF-7 and A-549 cancer cell lines among all tested compounds. Further study on its mechanism and potential to be developed as anticancer agents needs to be explored. The significant antibacterial activity showed by isocordato-oblongic acid (4) towards Gram positive bacteria further confirmed the potential of chromanone acid derivatives that is unique to *Calophyllum* to be developed as antibacterial agents in the future.

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