Induction of cytotoxicity by *Bruguiera gymnorrhiza* in human breast carcinoma (MCF-7) cell line via activation of the intrinsic pathway

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**Abstract**

Breast cancer is among the frequently occurring cancer worldwide. The foremost underline aim of this study was to determine the growth inhibitory effect along with mechanistic study of a *Bruguiera gymnorrhiza* extract on MCF-7. The cytotoxicity activity was determined by using the MTS assay. Butanol extract exhibited the maximum cytotoxicity activity against the MCF-7 cells with IC\(_{50}\) of 3.39 \(\mu\)g/mL, followed by diethyl ether and methanol extract (IC\(_{50}\) at 16.22 \(\mu\)g/mL and 37.15 \(\mu\)g/mL, respectively) at 72 h. The DeadEnd\textsuperscript{TM} Colorimetric Apoptosis Detection System confirmed the induction of apoptosis (via DNA fragmentation) in MCF-7 cells. Both butanol and diethyl ether extracts of *B. gymnorrhiza* significantly increase the caspase-3 level. However, the diethyl ether extract induced higher caspase-9 levels compared to caspase-8, suggesting that the intrinsic pathway was the major route in the process of apoptosis. Thin-layer chromatography profiling demonstrated the presence of phenolic, terpene, and alkaloid compounds in crude methanol, diethyl ether, and butanol extracts. The phytochemicals present in the extracts of *B. gymnorrhiza* might have the potential to be a future therapeutic agent against breast cancer.

**Key words:** Apoptosis, *Bruguiera gymnorrhiza*, cancer, caspases, DNA fragmentation, human breast cancer, phytochemicals, TUNEL assay

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**INTRODUCTION**

Breast cancer is among the frequently occurring cancer in men and women.\[1\] In 2018, approximately 15% of all cancer deaths were due to breast cancer among women.\[2\] The most common treatments available for breast cancers are chemotherapy, radiotherapy, and surgical treatment. However, chemotherapy and radiotherapy treatments yield adverse side effects which can be overcome by using targeted drug delivery systems and potential phytochemical-based drugs.\[3-5\]

Based on the World Health Organization report, a major percentage of the world’s population utilizes plant-derived medicines for health care.\[2\] Plant-derived medicines...
or natural drugs, which form the basis of traditional medicine, have been used for centuries by different cultures. The significance of medicines of natural origin has satisfactorily understood in the pharmaceutical industry. Various Mangrove species produce secondary metabolites with various bioactive properties such as insecticidal, antibacterial, antidiarrheal, cytotoxic, and antiviral.[7-14]

The main purpose of a therapeutic drug in cancer treatment is to kill the cancer cell without harming normal cells. In cell death mechanism, apoptosis gains much importance due to safely triggering the suicidal process in cancer cells without affecting normal cells. Apoptosis is an extremely regulated mechanism that shows a distinct role in the process of cell growth, death, and development. Cells undergoing apoptosis possess morphological and biochemical alterations such as condensation of chromatin material and cytoplasm shrinkage, phosphoserine exposure, and DNA fragmentation.[17]

Therefore, in this study, the cytotoxicity effects and cell death mechanisms of the extracts of mangrove plant Bruguiera gymnorrhiza on MCF-7 were investigated. Bioactive compounds present in B. gymnorrhiza show an essential part in inhibiting the cell growth apoptosis mechanism by the activation of caspases, thus potentially developed as one of the candidates of chemotherapeutic agents with minimal side effects.

MATERIALS AND METHODS

Collection of Bruguiera gymnorrhiza and extract preparation

The leaves of B. gymnorrhiza were collected from Umbai, Malacca (N 02° 09’ 330” E 102° 20’ 99”). The preparation of extracts according to our formerly reported methods was done using methanol, diethyl ether, and butanol solvents.[18,19]

Cytotoxicity assay

The cytotoxicity of extracts was determined by using CellTiter 96 AQueous One Solution Proliferation assay (MTS) as a formerly reported method.[20-23] The MCF-7 cell line was cultured in ISO-certified Animal Cell Culture Laboratory, (MS/ISO/IEC 17025:2015 SAMM No. 796), Institute of Marine Biotechnology. The absorbance was observed at 490 nm using a plate reader (enzyme-linked immunosorbent assay Multiskan, Thermo Fisher, USA).

DNA fragmentation through TUNEL system (late apoptosis)

DNA fragmentation study was performed by using the DeadEnd™ Colorimetric Detection System (Promega, Madison US). The MCF-7 cells were cultured in an 8-well slide chamber and then incubated in 5% (v/v) CO2 incubator at 37°C for 24 h. The experiment was performed according to a previously reported method.[15,20] The dark stain of fragmented DNA represents the induction of apoptosis, which was observed by an inverted microscope (Olympus, Selangor Malaysia).

Caspase assay

The activation level of caspases 3/7, Caspase-8 and Caspase-9 was determined by using Caspase-GloTM Assay (Promega, USA). The assay was performed according to a previously described method.[20,23] The breast cancer cell line was treated with active partition extracts of B. gymnorrhiza at a concentration of IC50 at 72 h and incubated for several time points (0 h–36 h) at 37°C. The reading of each sample was measured using a luminometer at Optical density (OD) of 490 nm.

Thin-layer chromatography profiling

Thin-layer chromatography (TLC) analysis was done on TLC silica gel plates (60 F254) to identify the presence of phytochemicals in the active extracts of B. gymnorrhiza. The B. gymnorrhiza extract was diluted in a mandatory amount of solvent using a previously reported method.[18,24] The developed plates were air-dried, heated, and observed under ultraviolet light at both 254 nm and 365 nm as well as anisaldehyde and Dragendorff’s derivatized reagents.

Statistical analysis

GraphPad Prism software (GraphPad Software, Inc San Diego, CA) was used to calculate the growth inhibition (IC50). Significant differences were expressed by using analysis of variance and (Dunnett’s) test using SPSS software version 20.0 (IBM SPSS, USA, New York, US).

RESULTS AND DISCUSSION

Cytotoxicity effect of the Bruguiera gymnorrhiza extracts on MCF-7

In this study, the cytotoxicity effects of the B. gymnorrhiza methanol, diethyl ether, and butanol extracts on the MCF-7 cell line were investigated. This study demonstrated that B. gymnorrhiza methanol, diethyl ether, and butanol extracts of B. gymnorrhiza produced time-dependent increased inhibitory effects on MCF-7 cells. Interestingly, the significant inhibitory effect was observed when cells were treated with methanol, diethyl ether, and butanol extracts at concentrations of 12.50 μg/mL and 6.25 μg/mL. [Figure 1a-c]. However, diethyl ether and butanol extracts only showed a significant cytotoxicity effect at concentrations of 3.12 μg/mL and lower concentrations. B. gymnorrhiza butanol extract showed the highest cytotoxicity effects with the IC50 value of 3.39 μg/mL, followed by diethyl ether extract (16.22 μg/mL). However, B. gymnorrhiza methanol extract showed an inhibitory effect above 30 μg/mL. (37.15 μg/mL). The diethyl ether and butanol extracts of B. gymnorrhiza produce cytotoxicity similar to previous studies.[25-27] Therefore, diethyl ether and butanol extracts were selected for further mode of cell death investigation.
The apoptotic effects of *Bruguiera gymnorrhiza* extracts on MCF-7 cell line

The DNA fragmentation in MCF-7 was observed by the dark stain in the nucleus of the cancer cell. 1% (v/v) dimethyl sulfoxide was considered negative control and DNAsase as positive, according to data shown in our previous study.\(^{[19]}\) Interestingly, dark brown stained nuclei were observed in cells treated with the diethyl ether and butanol extracts over the 36-h treatment period, as revealed in Figure 2a-f. These results strongly denote that both extracts of *B. gymnorrhiza* killed MCF-7 cell line through apoptosis. The induction of apoptosis is the foremost active strategy to inhibit cell growth and acts as an important effective therapeutic target in drug development.\(^{[17,28-31]}\)

The effects of *Bruguiera gymnorrhiza* extracts on caspase-3, 8, and 9 protein levels in MCF-7 cells

The significant pathways responsible for the activation of apoptosis involve activation in caspase expression and activation.\(^{[17]}\) The expression level of caspase-3 [Figure 3a] significantly increased in cells that were treated with diethyl ether extracts at various treatment periods (9, 12, 16, and 20 h) and reached its peak at 24 h (2.75 folds) and with butanol extract at 16, 20, and 24 h, with the highest level at 16 h (3.25 folds). Interestingly, the level of caspase-8 [Figure 3b] increased at 12 h by 2.7 folds, reaching its peak at 24 h by 5.92 folds in diethyl ether extract-treated cells. The significant increase in the protein level of caspase-9 [Figure 3c] was also noticed, with the highest activity produced when the cells were treated at 1 h (10 folds).

Apoptosis is chiefly controlled by caspases; aspartate-specific cysteine proteases involve in the process of apoptosis (function as both initiators and executioners).\(^{[17,32]}\) Caspase activation is triggered by two major signaling routes: (i) the extrinsic-death receptor and the (ii) intrinsic-mitochondrial pathways.\(^{[33]}\) The induction of apoptosis by anticancer agent might be possible by extrinsic pathway and intrinsic pathway or by both pathways activation.\(^{[34]}\)

Thin-layer chromatography

The results showed the separation of compounds spotted on the TLC plate in different solvent system ratios [Figure 4a and b]. The results indicate that the extracts contained conjugated carbon double bonds (C = C) and alkaloid. The presence of brown spots on a yellow background indicates the presence of organic compounds. The dark orange, violet, and gray spots represent alkaloids, phenol, and terpenes,
Figure 3: The expression of (a) caspase-3, (b) caspase-8, and (c) caspase-9 in MCF-7 cells treated with *Bruguiera gymnorrhiza* diethyl ether and butanol extracts

In our previous study, Xylocarpus moluccensis (mangrove species) successfully induces the process of apoptosis in HeLa cells. Xylocarpus moluccensis induces apoptosis through activation of the extrinsic pathway in HepG2 cell lines. However, in the present study, the major activation route was intrinsic along with the extrinsic pathway, which suggested that mangrove species have the potential to induce apoptosis with both extrinsic and intrinsic pathways. The difference might be due to a particular mechanism activated in specific cell lines. The phytochemical study further confirms the presence of potential phenolic, terpene, and alkaloid compounds in crude methanol, diethyl ether, and butanol extracts. The active compounds present in *B. gymnorrhiza* might have the potential to be a future therapeutic agent. Further study of isolation of pure compounds from *B. gymnorrhiza* will be our next priority to study the underlying clear mechanism.

**CONCLUSION**

One of the significant discoveries in cancer research was investigating the cell death mechanism exerted by anticancer chemotherapy. In this study, the extracts of mangrove plant *B. gymnorrhiza* showed significant cytotoxicity effects with IC$_{50}$ values at 72 h – the induction of apoptosis was confirmed by DNA fragmentation with the underlying mechanism, mainly due to intrinsic pathways. TLC profiling showed that the extracts contain alkaloid, phenolic, and terpenoid compounds. Thus, from this study, it is understood that there is enormous potential for mangrove extracts prepared from *B. gymnorrhiza* to develop as chemotherapeutic agents in cancer treatment.

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Conflicts of interest
There are no conflicts of interest.

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