Induction of apoptosis by selected *Xylocarpus sp.*, fractions in the human cervical cancer cell line, HeLa

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**ABSTRACT**

Cancer is unchecked growth of normal cells. The screening of new potential agents is vital to develop effective cytotoxic drugs with reduced side effects. The cytotoxic effects, mode of cell death and screening of phytochemicals which induced cell death by the mangrove plant *Xylocarpus granatum* extract on the human hepatocellular carcinoma cell line was studied. The crude methanol extract of *Xylocarpus granatum* (bark) partitioning produced the high yield in diethyl ether (54.08%) followed by butanol (37.12%) and aqueous (3.21%). The *Xylocarpus granatum* bark extract produced a cytotoxic effect with IC₅₀ < 30 μg/ml as compared to leaf and root (IC₅₀ > 100 μg/ml). The DNA fragmentation study done to determine the mode of cell death. The results attained by the colorimetric TUNEL system propose that *Xylocarpus moluccensis* extract induced DNA fragmentation in the HepG2 cell line. The darkly stained nuclei observed in HepG2 cells treated with *Xylocarpus moluccensis* (diethylether) extract for 24 hours. The DNA fragmentation indicating that apoptosis was the mode of cell death on HepG2 cell line. Furthermore, The *Xylocarpus moluccensis* (diethyl ether) extracts phytochemical study confirms the presence of flavonoid, steroid, terpenoid, phenols, tannins, glycoside, and saponin. Thus, the further study of potential phytochemicals need to done in order to confirm these phytochemicals as a future therapeutic agent for the treatment of cancer.

**INTRODUCTION**

Cancers are diseases caused by abnormalities in cells. The main characteristic to distinguish the cancerous cells from normal cells is the ability of the cells to proliferate faster, in an uncontrolled fashion (Kleinsmith, 2006). This highly fatal disease is responsible for 8.8 millions of deaths in 2015 globally. For advanced liver cancer, the treatment may involve chemotherapy, radiation therapy, or both (Kleinsmith, 2006). Most common cancer types worldwide are lung (13%), female breast (12%), bowel (10%), prostate (8%), stomach (7%), liver (6%), cervix (4%), esophagus (3%), bladder (3%) and non-Hodgkin lymphoma (3%) (Ferlay et al., 2015). Generally, cancers are caused by...
by various factors such as cellular genetic mutations, homeostatic failure to eliminate abnormal cells, chemicals change inside the body, unhealthy lifestyles and exposure to carcinogens (Kleinsmith, 2006). These control systems are lost in cancer cells, and make them divide continuously.

Cancer treatment aims to extend and maintain the quality of life by effectively eliminating cancer treatments can involve surgery, chemotherapy, radiation, hormone therapy, immunotherapy, targeted therapy, and transplantation. Tropical medicinal plants are a rich source of pharmacologically important phytochemicals. The bioactive compounds of the plant such as alkaloids, flavonoids, tannins and phenolic compounds were considered to be more significant. The phytochemical research usually has been done based on the ethnopharmacological information forms an effective approach in the discovery of new anti-infective agents from higher plants. Apoptosis extensively described as a significant mechanism of regulated death. Apoptosis tightly regulated by different groups of the executioner and regulatory molecules. Mechanism of action of apoptotic cell death typically characterized by condensation of chromatin material, fragmentation of DNA occurred in the nucleus, cell shrinkage, dynamic membrane blebbing, and loss of adhesion to extracellular matrices. Further, biochemical alterations include; externalization of phosphatidylserine, and the activation of cysteine aspartyl proteases called caspases which lead to cell death (Jan & Chaudhry). The genus Xylocarpus belongs to the mahogany family, Meliaceae, which is distributed in the tidal forests including mangrove habitat from Africa to Australia, Malaysia, and India. Traditionally, the bark of Xylocarpus is used as an astringent, febrifuge and also to treat dysentery and diarrhea. The flowers were used as a cure for elephantiasis and swelling of the breasts, while the seed for treatment of itchiness. The methanolic extract of X. moluccensis shows low cytotoxicity, while diethyl ether, butanol, and aqueous extract used in this study. The dried samples were extracted with methanol to yield crude methanolic extracts. The percentage of crude extracts (yield per 100 g samples) was shown in Table 1. All the crude extracts were then subjected to cytotoxicity assay.

**MATERIALS AND METHODS**

Materials Human hepatocellular carcinoma cell line (HepG2) purchased from American Type Cell Culture, USA. Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from Nacalai Tesque, Japan. Penicillin-streptomycin solution, Fetal Bovine Serum (FBS), and other cell culture supplies obtained from Gibco, USA. CellTiter 96™ Aqueous Non-Radioactive Cell Proliferation Assay kit (MTS), Calorimetric Apoptosis Detection Kit (FACS), purchased from Promega, USA. All other chemical solvents purchased from Merck, Germany.

**Preparation of extracts and percentage of yield**

The mangrove plants (Xylocarpus granatum) collected from Umbai, Malacca. Xylocarpus granatum leaves collected from Umbai, Malacca GPS coordinates (N 02°09'.330", stem bark and root GPS coordinates (E 102°20'.192°). The specimens deposited in the Institute of Marine Biotechnology, UMT, Malaysia. The leaves were freeze-dried and ground to powdered form by using the mechanical grinder. The crude methanol extract prepared by using the cold extraction technique (Gul-E-Saba et al., 2018). Furthermore, diethyl ether, butanol, and aqueous extract used in this study. The dried samples were extracted with methanol to yield crude methanolic extracts. The percentage of crude extracts (yield per 100 g samples) was shown in Table 1. All the crude extracts were then subjected to cytotoxicity assay.

**Cytotoxicity study by using MTS assay**

CellTiter 96™ Aqueous Non-Radioactive Cell Proliferation Assay (MTS assay) was used to evaluate the cytotoxicity activity of extract on the HepG2 cell line (Chaudhry et al., 2019a; Hudayeh et al., 2017). The human hepatocellular carcinoma cell line, HepG2, was cultured in RPMI, supplemented with fetal bovine serum (FBS) and 1% (v/v) of the antibiotic solution, penicillin-streptomycin. For the negative control, the cells treated with 1% (v/v) DMSO without extracts. Vincristine sulfate (Sigma, USA) used as positive control. After that, CellTiter 96™ Aqueous Solution Reagent (20µl) was added into each well for 3hr at 37°C. Finally, the absorbance recorded using ELISA 96-well plate reader at 490nm.

**Determination of apoptotic cell death (DNA fragmentation) by TUNEL assay**

Apoptosis Detection System, Terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay used to study DNA fragmentation induced apoptosis in HepG2 cells. The cells were cultured in the labtek chamber slides at a cell density of 2 x 10⁴ cells/chamber and incubated at 37°C in a...
### Table 1: The percentage of partitioned extracts compared to crude extract

| Sample                 | Extract         | Weight | Percentage of partitioned extract per crude extract (%) |
|------------------------|-----------------|--------|---------------------------------------------------------|
| Xylocarpus granatum    | Methanol        | 6.29   |                                                         |
|                        | Diethyl ether   | 4.53   | 72.02                                                   |
|                        | Butanol         | 1.12   | 17.81                                                   |
|                        | Aqueous         | 0.37   | 5.88                                                    |
| Xylocarpus molluccensis| Methanol        | 4.66   |                                                         |
| Leaf                   | Diethyl ether   | 2.52   | 54.08                                                   |
|                        | Butanol         | 1.73   | 37.12                                                   |
|                        | Aqueous         | 0.15   | 3.21                                                    |

### Table 2: IC<sub>50</sub> values of *Xylocarpus granatum* extracts.

| Plant species                          | Plant parts | Cytotoxicity effects IC<sub>50</sub> (µg/ml) |
|----------------------------------------|-------------|---------------------------------------------|
| *Xylocarpus granatum*                  | Leaf        | >100                                        |
|                                        | Bark        | 29.51                                       |
|                                        | Root        | >100                                        |
| *Xylocarpus granatum* (bark)           | Leaf-Diethyl-ether | 85.11                       |
|                                        | -Butanol    | 87.10                                       |
|                                        | -Aqueous    | >100                                        |

### Table 3: Alkaloid content scale for extracts

| Sample / Extract                  | Observation                        | Alkaloid content scale |
|-----------------------------------|------------------------------------|------------------------|
| Diethyl ether                     | No Change                          | 0                      |
| Butanol                           | Transparent solution with +1       | +1                     |
| faint trace of precipitate        | Transparent solution +3            | +3                     |
| Aqueous                           |                                    |                        |

### Table 4: Observation of flavonoid content in the extracts

| Sample / Extract | Observation        | Result   |
|------------------|--------------------|----------|
| Diethyl ether    | Blue-green color   | Positive |
| Butanol          | Orange             | Positive |
| Aqueous          | Transparent Solution | Negative |

### Table 5: Observation of steroid content in the extracts

| Extract      | Observation           | Result   |
|--------------|-----------------------|----------|
| Diethyl ether| Darker green color    | Positive |
| Butanol      | Light yellow          | Negative |
| Aqueous      | No change             | Negative |
Table 6: Observation of terpenoid content in the extracts

| Extract   | Observation            | Result |
|-----------|------------------------|--------|
| Diethyl ether | Dark grey and greenish color | Positive |
| Butanol   | Light brown color      | Negative |
| Aqueous   | No changes             | Negative |

Table 7: Observation of phenol and tannin content in the extracts

| Extract   | Observation  | Result |
|-----------|--------------|--------|
| Diethyl ether | Dark green color | Positive |
| Butanol   | Light brown  | Negative |
| Aqueous   | No changes   | Negative |

Table 8: Observation of glycoside content in the extracts

| Extract   | Observation | Result |
|-----------|-------------|--------|
| Diethyl ether | Green       | Positive |
| Butanol   | Yellow      | Negative |
| Aqueous   | No changes  | Negative |

Figure 1: HepG2 cells treated with (a) DNase as a positive control, (b) 1% DMSO as a negative control, (c) 0.22 pg/ml Xylocarpus moluccensis leaf diethyl ether extract after 24 hours incubation. The cells were observed under a light microscope using 40x magnification.

Phytochemical screening

Phytochemical screening conducted to detect groups of phytochemicals present in the Xylocarpus moluccensis leaf diethyl ether, butanol, and aqueous extracts. Phytochemical screening was conducted to detect groups of phytochemicals present in the extracts. The identification of flavonoid (Shinoda’s Test), glycoside (Liebermann’s Test), phenol, tannin, saponin, steroid, and terpenoid, tests were conducted according to the respective techniques described by Yadav and Agarwala (2011) and alkaloid (Mayer’s Test) was referred to Firdouse and Alam (2011) with slight modifications.

Alkaloid test (Mayer’s test)

The extracts (5mg) were dissolved in 5 ml of ammonia chloroform and the mixture was filtered. Subsequently, 1 ml of sulphuric acid was added into the filtrate and vigorously shaken for 15 seconds. The solution was left undisturbed until two separated layers formed. The upper acidic layer was transferred into a new test tube and tested with 3 drops of Mayer reagent. The formation of white precipitate indicated the presence of alkaloids.

Flavonoid test (Shinoda test)
For flavonoid presence, (5 mg) of the extracts were dissolved in 1 ml methanol and 5 mm magnesium coil. Subsequently, 1-2 drops of concentrated sulfuric acid (H$_2$SO$_4$) was transferred and the magnesium coil was allowed to dissolve in the mixture. The formation of orange, pink scarlet, red or occasionally green to blue colour showed the presence of flavones.

**Steroid test**

The 5 mg of the extracts were dissolved in 1 ml chloroform followed by 1 ml of acetic acid. The development of greenish coloration indicated the presence of steroids.

**Terpenoids test**

5 mg of the extracts were dissolved in 1 ml of chloroform and allowed to evaporate to dryness. Subsequently, 1 ml of concentrated sulfuric acid was added. A grayish color indicated the presence of terpenoid.

**Phenols and tannins test**

The extracts were mixed with 2 ml of 2% (Wv) ferric chloride (FeCl3) solution. A blue-green or black coloration shows the presence of phenols and tannins.

**Glycoside test (Liebermann's test)**

The Glycoside presence was indicated by mixing 5 mg of the extracts with chloroform and acetic acid (2ml each). The mixture was allowed to cool in ice. After then, 2-3 drops of concentrated sulfuric acid were added to the mixture. A violet to blue green coloration indicated the presence of a steroidal nucleus, i.e., glycone portion of glycoside.

**Saponin test**

The saponin confirmation was done by boiling extracts with 3 ml of distilled water in a test tube for 3 minutes. Then, the mixture was shaken vigorously and observed after 10 minutes. The formation of stable foam indicated the presence of saponin. Distilled water was used as a negative control.

**Statistical analysis**

All experiments performed in three replicates, and the results computed as mean ± standard deviation. ANOVA (one-way) and Dunnet post-test performed using SPSS 16.0.

**RESULTS AND DISCUSSION**

Cytotoxicity effects of methanol and partition extracts of Xylocarpus granatum on HepG2 cells.

In this study, in vitro cytotoxicity effects of the methanol extracts prepared from the *Xylocarpus granatum* was conducted on human hepatocellular carcinoma cell line (HepG2) as shown in Table 2. The Xylocarpus granatum extracts, only bark extract produced a cytotoxic effect with IC$_{50}$ <30 μg/ml as compared to leaf and root (IC$_{50}$ > 100 μg/ml). Similarly, the other species of mangrove *Xylocarpus moluccensis* also produced an effective cytotoxic effect on hepatocellular carcinoma cell line (data not shown). Therefore, *Xylocarpus moluccensis* methanol extract and Xylocarpus granatum bark used for further apoptosis study.

As shown in Table 1, partitioning of crude methanol extract from *X* moluccensis leaf produced the highest yield with 72.02% than to that of butanol (17.81%) and aqueous fractions (5.88%). Similarly, the crude methanol extract of *X* granatum bark produced the highest yield when subjected to diethyl ether partitioning (54.08%) followed by butanol (37.12%) and aqueous (3.21%). Moreover, a comparative study of Xylocarpus granatum (bark) and *Xylocarpus moluccensis* (leaf) partitioned cytotoxicity study showed that *Xylocarpus moluccensis* (leaf) diethyl ether produced more cytotoxic effect (data not shown) as compare to Xylocarpus granatum (bark) IC$_{50}$ = 85.11 μg/ml. Therefore, *Xylocarpus moluccensis* (leaf) diethyl ether extract used to study DNA fragmentation.

**Induction of Apoptosis in HepG2 cells via DNA fragmentation (Hallmark of apoptosis)**

As explained above, the diethyl ether extracts of *Xylocarpus moluccensis* produced a significant cytotoxic effect as compared to Xylocarpus granatum bark. Therefore, the *Xylocarpus moluccensis* diethyl ether extract mode of cell death study was conducted. Interestingly, darkly stained nuclei also observed in cells treated with *Xylocarpus moluccensis* diethyl ether extract for 24 hours (Figure 1), indicating that apoptosis was the mode of cell death on HepG2 cell line.

**Phytochemical screening tests**

The *Xylocarpus moluccensis* diethyl ether extracts subsequently subjected to a phytochemical screening, which confirms the presence of Flavonoid, steroid, terpenoid, phenols, tannins, glycoside, and saponin.

**Alkaloid test (Mayer’s test)**

The extracts were tested with Mayer reagent, and the formation of a white precipitate indicates the presence of alkaloids (Figure not shown). Table 3 shows the ranking of alkaloid content which correlated to the degree of precipitation occurred. The aqueous extract showed the highest amount of alkaloid with +3 scales, followed by butanol extract with a smaller amount (+1 scale), while, diethyl ether
extract, on the other hand, exhibited no presence of alkaloid (Table 3).

**Flavonoid test (Shinoda test)**
The extract was also tested for flavonoid content. The formation of orange, pink scarlet, red color or green to blue color indicates the presence of flavones. Based on the Shinoda test, flavonoid compounds were found to be present in diethyl ether and butanol extracts but not in aqueous extract (Table 4).

**Steroid Test**
In this test, the development of greenish coloration indicates the presence of steroids. It was found that steroid was present in diethyl ether extract, and absent in butanol and aqueous extracts (Table 5).

**Terpenoid test**
The presence of terpenoid compounds in the extracts was detected with a grayish color of extract-test reagent mixture. As shown in Table 6, terpenoid was only found in diethyl ether extract and was absent in butanol and aqueous extracts.

**Phenols and tannins test**
Phenols and tannins content in the extract was detected based on the presence of a blue-green or black coloration in the test mixture. Thus, based on this test, only diethyl ether extract showed the presence of flavonoid, but not butanol and aqueous extracts (Table 7).

**Glycoside test (Liebermann’s test)**
Liebermann’s test demonstrated a green coloration was observed in diethyl ether extract indicating the presence of glycoside. However, yellow and no change in coloration was observed when the test was carried out on butanol and aqueous extracts, respectively, indicating the absence of glycoside (Table 8).

**Saponin test**
Saponin content level was the highest in aqueous extract, while diethyl ether and butanol extracts contained a smaller amount. Therefore, based on the phytochemical tests carried out on all three extracts, it was found that diethyl ether extract contained flavonoid, steroid, terpenoid, phenols, tannins, glycoside and low amount of saponin. Butanol and aqueous extracts contained alkaloid and saponin, although the levels of both types of compounds were low in butanol extract and high in the aqueous extract.

The screening and development of potential bioactive agents are essential for future effective chemotherapeutics. A bioactive compound causing an effect on, causing a biological reaction which triggers a biological response in the living tissue. The activation of apoptosis as a response of bioactive compounds could play a significant role in the significant killing of cancer cells. Apoptosis is the crucial mechanism to regulate the survival and death process of cells in cancer disease. Deregulation in the process of apoptosis stimulates cellular multiplication, which leads to the tumor and eventually metastasis and is a significant hurdle to effective cancer treatment. (Jan R and Chaudhry G.S., 2019; Onyeagucha et al., 2017; Hassan et al., 2014). The plant-derived anticancer compounds are very effective in clinical studies ongoing (Cragg and Newman, 2005). Mangrove plants are rich in secondary metabolites also possess other benefits to treat a variety of inflammatory diseases (Das et al., 2015; Bandaranayake, 2002). To screen the potential phytochemicals from mangroves against liver cancer treatment, this study was performed to investigate the cytotoxicity effects of the extracts prepared from mangrove species, Xylocarpus-granatum and Xylococcus-granatum data not shown).

The methanol extract of Xylocarpus moluccensis fruit husk shows antibacterial activity against Staphylococcus aureus. Limonoids and tirucallane are two types of terpenoids that were isolated from X. moluccensis seeds (Wu et al., 2010). Some of the limonoids isolated from genus Xylocarpus plants were found to exhibit significant cytotoxicity against different types of cancer (Shen et al., 2009; Uddin et al., 2007) reported, gedunin extracted from X. granatum possessed cytotoxicity activity on colon cancer cell line (CaCo-2). Limonoids isolated from this species were found to be cytotoxic towards human colon carcinoma (HCT-0), human hepatocellular carcinoma (Bel-7402), human gastric cancer (BGC-823) and ovarian cancer cell lines (A2780). Various studies also demonstrated the cytotoxicity effects against human breast ductal carcinoma cells (MDA-MB-453S), and human gastric adenocarcinoma cells (AGS cell line) (Mondal et al., 2008; Roy and Simlai, 2013). Similarly, Isoazadironolide and turbapubesin E compounds isolated from this plant showed cytotoxic activity against lymphocytic leukemia cell line (P388) (Tan and Luo, 2011). Interestingly, Xylocarpus granatumcrude bark extract showed higher cytotoxicity than to that of the partitioned extracts. However, certain compounds that produced synergistic interaction (anti-proliferative) may be responsible for exhibiting lower cytotoxicity effects as compared to partition extract.

Induction of apoptosis is a safe to approach towards the killing of cancer without harming normal cells.
and prevent inflammation (Kasibhatla and Tseng, 2003). In this study, the cell death mode induced by the prepared extracts was detected by DNA fragmentation using the TUNEL assay. In the TUNEL assay, the nicked DNA ends labeled with stained observable by dark coloration inside the nucleus of cancer cells (Kyrilikova et al., 2012). The dark-stained nuclei were obviously observed in HepG2 cells that treated with the extracts. Thus, it strongly suggests that the cytotoxicity effects via DNA fragmentation (apoptosis) on HepG2 cells. Previous studies also support the induction of apoptosis by Xylocarpus genus due to the presence of potential secondary metabolites. A tetranor-triterpenoid and Tirucallane terpenoids induce apoptosis in a cervical cancer cell line (HeLa-PRB) and gastric cancer cell line (MKN-28) respectively (Patwardhan et al., 2013; Lange et al., 2016). The phytochemicals such as steroids, phenols, and glycosides successfully induce apoptosis in various cancer cells. The steroids isolated from the Aglaia argentea (bark) found to be cytotoxic against murine leukemia cells (P-388) Besides, phenol-rich mangrove plant, Avicennia marina extract induced apoptosis in AU565 and BT483 (breast cancer) and HepG2 and Huh7-liver cancer cells (Farabi et al., 2017; Huang et al., 2016). From this study, the methanol extract prepared from the bark of Xylocarpus granatum owned the potential to be developed as chemotherapeutic agents.

CONCLUSIONS

This study performed to investigate the cytotoxicity effects of the extracts of mangrove species Xylocarpus granatum and to determine the mode of cell death induced by the extracts on human hepatocellular carcinoma cell line (HepG2). The methanol extract of Xylocarpus granatum (bark) found to be cytotoxic against HepG2. The extracts were confirmed to induce DNA fragmentation as apoptotic cell death. Moreover, the solvent partition extract of Xylocarpus granatum showed cytotoxicity <100µg/ml. The presence of bioactive compounds, flavonoids, steroids, terpenoids, and other compounds present in the extract prepared from Xylocarpus moluccensis diethyl ether extract possess the potential to be future therapeutics.

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Conflict of interest

The authors declare that there is no conflict of interest associated with the manuscript.

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